Gluconeogenesis in periportal and perivenous hepatocytes of rat liver, isolated by a new high-yield digitonin/collagenase perfusion technique

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A technique is described which allows preparations of hepatocytes, enriched in either periportal or perivenous hepatocytes ('PP-cells' and 'PV-cells' respectively), in a yield of about 30–50% compared with control cell preparations. The liver is first perfused for 40–60 s with digitonin (4 mg/ml) to destroy selectively either the periportal or the perivenous part of the microcirculatory unit, and then the remaining hepatocytes are isolated by the ordinary collagenase perfusion technique. In periportal cells the activities of alanine aminotransferase and pyruvate kinase were 29.4 and 18.7 μmol/min per mg of DNA respectively. The rate of gluconeogenesis was 0.402 μmol/min per mg of DNA. In perivenous cells the corresponding values were 9.55, 22.1 and 0.244 μmol/min per mg of DNA respectively. These data support the concept of a zonation of glucose metabolism within the microcirculatory unit of the liver, with the afferent part (periportal zone) having a 2-fold more active gluconeogenesis than the efferent part (perivenous zone).

Evidence has accumulated suggesting a functional, dynamic, metabolic heterogeneity of hepatocytes of the afferent and efferent zones, i.e. periportal and perivenous zones, in the microcirculatory unit of the liver (Sasse *et al.*, 1975; Rappaport, 1980; Jungermann *et al.*, 1982; Gumucio, 1983; Matsumura & Thurman, 1984; Häussinger *et al.*, 1984; Quistorff & Chance, 1985). Consequently, there has been a need to develop methods which allow high-yield preparation of hepatocytes of known specific origin within the microcirculatory unit of the liver. Several attempts to separate isolated hepatocytes according to various physical and chemical properties related to their origin within the acinus have been reported (for review, see Seglen, 1979). However, only one study aimed at the direct isolation of either periportal or perivenous hepatocytes has appeared (Väänänen *et al.*, 1983). Judged by the activity of the periportal marker enzyme ALAT, this technique provides good separation of periportal and perivenous cells; however, the cell yield is rather low (3–10%), and for that reason the method is unsatisfactory for most metabolic studies.

Quistorff *et al.* (1984, 1985) demonstrated that

Abbreviations used: ALAT, alanine aminotransferase (EC 2.6.1.2); PK, pyruvate kinase (EC 2.7.1.40); PP-cells, PV-cells, hepatocyte preparations enriched in either periportal or perivenous cells respectively.

digitonin perfusion of rat liver allowed selective destruction of the periportal or the perivenous zone with release of cytoplasmic marker enzymes from the particular zone with a selectivity approaching that obtained by microdissection (Jungermann & Katz, 1982). In the present study the digitonin method was used to destroy cells of either the afferent or the efferent part of the microcirculatory unit. Then the remaining hepatocytes, of the other type, were isolated by the conventional collagenase perfusion technique (Seglen, 1976).

Materials and methods

Reagents

All chemicals were of analytical grade. Digitonin was obtained from ICN and purified as described by Quistorff *et al.* (1985). Enzymes were from Boehringer and collagenase from Sigma.

Liver perfusion

Starved (24 h) female Wistar rats, weighing 180–200 g, were used. For the isolation of the liver, rats were anaesthetized with pentobarbital (400 μl; 50 mg/ml), given as an intraperitoneal injection. During perfusion the liver remained *in situ*, connected to the perfusion system via the vena porta and vena cava superior. The vena cava inferior was ligated. The perfusate was Krebs–

Henseleit (1932) bicarbonate buffer with or without 4 mg of digitonin/ml, equilibrated with O_2/CO_2 (19:1). Perfusate temperature was 36–37°C and flow, delivered by a peristaltic pump (LKB 2115), was between 5 and 23 ml/min (see below).

Since the direction of perfusion, either porta→cava or cava→porta, as well as the perfusate medium, had to be changed very quickly in these experiments, two special four-way valves were constructed. The principle of the perfusion set-up appears schematically in Fig. 1. The function of valve A is to switch the direction of flow in the liver, and the function of valve B is to change the perfusate medium. Valve B allows the perfusate that is not being infused into the liver to be recirculated. The dead space in the valve and in the tubing from point 6 or 7 (see Fig. 1) to the liver amounts to 1.3 ml for both cava→porta and porta→cava perfusions. The time involved in change of perfusate and/or direction of flow is less than 0.5 s.

Selective destruction of periportal or perivenous cells

The selective destruction of the perivenous part of the microcirculatory unit, required for preparation of periportal cells, is described first. When connected to the perfusion system the liver was perfused in a flow-through porta→cava perfusion

Fig. 1. *Perfusion set-up for preparation of periportal or perivenous hepatocytes from rat liver*
The liver is connected to the perfusion system via the portal vein (PP) and the hepatic vein via the vena cava superior (PV). Valve A and valve B switch the flow direction through the liver and the type of perfusate, respectively. In the position indicated, flow through the liver is porta→cava and perfusate I is being infused, while perfusate II is recirculated. Turning of valve A through 90° switches flow through the liver to cava→porta, now connecting position 1 with 4 and 2 with 3. Turning valve B switches from perfusate I to II.

without digitonin at a rate of 23 ml/min (see Scheme 1). After 10 min pre-perfusion, the small caudate lobe (next to the right kidney) was ligated and cut off (approx. 0.35 g of tissue); 1–2 min later the direction of flow was changed to cava→porta, and after an additional 2 min the rate was decreased to 7 ml/min. Flow was allowed to stabilize at this lower rate for 30 s before switching to the digitonin-containing perfusate. The exact time of digitonin treatment was guided by the appearance of perivenous decoloration, clearly seen on the surface of the liver (Quistorff *et al.*, 1985). Under the present conditions digitonin perfusion was continued for 40–60 s. Perfusate was then switched to Krebs–Henseleit (1932) buffer without digitonin, and 1–2 s later the flow direction was switched to porta→cava and the rate increased to 23 ml/min (see Scheme 1), the intention being to prevent digitonin from reaching the periportal part of the sinusoid and to clear the liver of any remaining digitonin. During the first 60 s of this period, which lasted 2 min, eluate from the liver was collected in three 20 s intervals for enzyme-activity measurements. Standard hepatocyte preparation by collagenase perfusion was then initiated as described below.

The procedure for selective destruction of periportal cells (for preparation of perivenous cells) was as described above, except that direction of flow was the opposite and the flow rate during digitonin perfusion was 5 ml/min (see Scheme 1).

Control cells were prepared with a pre-perfusion period of 21 min with porta→cava flow of 23 ml/min without digitonin in the medium. In all groups a biopsy was taken during pre-perfusion as described above.

Cell preparation

After the selective cell destruction, hepatocytes from the remaining part of the liver were prepared by collagenase perfusion (Seglen, 1976), leading to cell preparations enriched in either periportal cells ('PP-cells') or perivenous cells ('PV-cells'). Essential details of the cell preparation were as follows: perfusion with Ca^{2+}-free Krebs–Henseleit (1932) solution lasted 12 min in a flow-through perfusion at a rate of 35 ml/min. Then Ca^{2+} and collagenase were added to 100 ml of the perfusate (final concns. 1.2 mM and 0.15 mg/ml respectively). The perfusate was now recirculated and equilibrated with O_2/CO_2 (19:1) in a glass oxygenator. Perfusate leaking out of the liver was pumped back into the reservoir. The collagenase perfusion was continued for 15–25 min at a flow rate of 25–30 ml/min. The disintegrated liver was filtered through a 100-mesh nylon net, flushed with the remainder of the perfusate. The filtrate was incubated at room temperature for 10 min, and
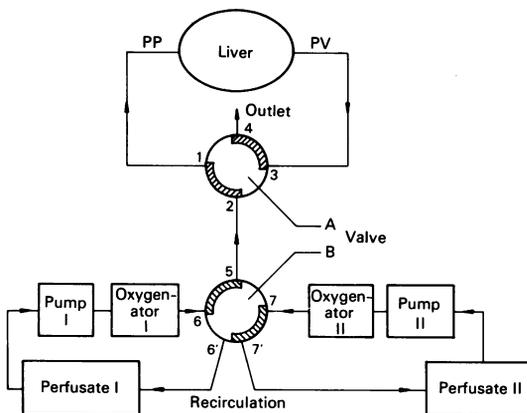
Scheme 1. *Sequence of events during preparation of periportal or perivenous hepatocytes*
P–C indicates porta→cava flow direction and C–P cava→porta flow direction.

Time (min)....	0–10	10–15	15–17	17.0–17.5	17.5–18.33	18.33–19.33	19.33–21	21–33	33–53
Perfusate additions....					Digitonin			Ca^{2+}-free	Collagenase
Samples....		Biopsy				Eluate			
PP-cells Flow direction	P–C			C–P			P–C		
PP-cells Flow rate (ml/min)		23		7		23		30–35	
PV-cells Flow direction			P–C			C–P		P–C	
PV-cells Flow rate (ml/min)		23		5		23			30–35

hepatocytes were isolated by differential centrifugation ($30g$ for 2min), including two washings with Krebs–Henseleit (1932) medium containing 0.2% defatted bovine serum albumin.

All cell preparations were tested for their ability to exclude 0.5% Trypan Blue.

Measurement of enzyme activity

Biopsy. The biopsy from the liver was weighed and homogenized in 20vol. of ice-cold buffer containing 25mM-glycylglycine, pH7.4, 150mM-KCl, 5mM-$MgSO_4$, 5mM-Na_2EDTA, 10mM-2-mercaptoethanol, 0.2% defatted serum albumin and 0.5% Triton X-100. Potter–Elvehjem homogenization was followed by sonication for 10s at 30W. The homogenate was centrifuged for 30min at $33000g$, and the supernatant used for the enzyme assays.

Cell suspension. A 1ml portion of a 10% (w/v) cell suspension was centrifuged briefly ($9000g$ for 5s) and the cells were resuspended in 3ml of ice-cold homogenization buffer. After sonication (10s at 30W), the extract was centrifuged as above and the supernatant used for enzyme-activity measurements.

Eluate collected after digitonin treatment. Eluate (3ml) and homogenization buffer (1ml) were mixed and centrifuged for 10min at $16000g$. Enzyme activity was measured on the supernatants.

The activities of ALAT and PK were measured as described by Bergmeyer (1974), except that PK was measured in the presence of 3.6mM-fructose 1,6-bisphosphate. DNA and protein were measured in the homogenates before centrifugation, with appropriate correction for added protein (Kissane & Robins, 1958; Lowry et al., 1951) respectively. Some 98 ± 11mg (S.D.) of protein/mg of DNA ($n = 16$) was found in the liver biopsy. This value was not statistically different from that found in control cells, PV-cells or PP-cells.

Cell incubations

PP-cells or PV-cells were incubated in 25ml Erlenmeyer flasks at 37°C in Krebs–Henseleit (1932) bicarbonate buffer equilibrated with O_2/CO_2 (19:1), containing 0.2% albumin, 5mM-lactate, 1mM-pyruvate, 10mM-glutamine and, in the vessels for measurement of glycogen synthesis, also 25mM-glucose. The vessels contained about 2×10^7 cells in 2ml. Samples were taken for glucose and glycogen measurements after 40 and 80min of incubation. The samples for glucose measurements were precipitated in 1M-$HClO_4$ and the sample for glycogen in 2M-KOH/0.2M-EDTA. Glucose and glycogen contents was measured as described by Lowry & Passonneau (1972) and Roehrig & Allred (1974) respectively.

Results

Cell yield

In order to obtain high yield of hepatocytes after digitonin treatment of the liver, it is important that the digitonin is allowed to affect only the afferent or the efferent part of the microcirculatory unit of the liver. It was previously shown that for the first 50s the liver cleared the perfusate almost completely of digitonin at a concentration of 6mg/ml and a flow rate of 23ml/min (Quistorff et al., 1985). In the present study, where the goal was to destroy selectively only half of the microcirculatory unit, it was therefore attempted initially to apply similar conditions, but with the digitonin perfusion discontinued after 20–30s. However, with the high flow rate cell yield of more than 2×10^7–5×10^7 cells could not be obtained (1 g of liver corresponds to approx. 10^8 hepatocytes; Weibel et al., 1969), even with short exposure times (10s). The flow rate was then decreased to 5–7ml/min, which made it possible to control the action of digitonin by varying the exposure time. The cell yield obtained under these conditions (see Scheme 1) was $2.02 \times 10^8 \pm 0.23 \times 10^8$ and $1.22 \times 10^8 \pm 0.51 \times 10^8$ cells for PP-cells and PV-cells respectively (means $\pm$ S.D., $n = 5$). Control cell preparations gave $3.8 \times 10^8 \pm 1.1 \times 10^8$ cells. Preparations of PP-cells and PV-cells showed the same viability as control cells, judged by Trypan Blue uptake, i.e. the viability counts were within the range 89–95% in 80% of the preparations. Only such preparations have been included in the study.

Enzyme release during digitonin perfusion

The eluate containing intracellular material liberated by the action of digitonin was collected in three 20s intervals after the digitonin perfusion (see Scheme 1). Table 1 shows the activities of ALAT and PK in the eluate fractions. In all experiments the major part (>75%) of the enzyme activity liberated was found in the 0–20s fraction,

and only 5–8% appeared in the 40–60s fraction. In agreement with previous observations, the PK activity in the eluate was the same with both forward and retrograde digitonin perfusion, suggesting that this enzyme is not significantly zonated in the microcirculatory unit (Quistorff et al., 1985). Microdissection studies, however, show a preferential perivenous location of this enzyme, i.e. a perivenous/periportal ratio of 2.3 in the fed state and 1.3 in the starved state (Guder & Schmidt, 1976; Zierz et al., 1983). The activity of ALAT is some 5-fold higher when liberated from the periportal part of the microcirculatory unit compared with the perivenous part, in agreement with microdissection studies (Morrison et al., 1965; Welsh, 1972).

Activity of cytoplasmic marker enzymes in PP-cells and PV-cells

In all experiments a biopsy was taken during preperfusion. PK and ALAT, as well as protein and DNA concentrations, were measured in the biopsy and in the hepatocytes prepared from the remaining part of the liver. These data are shown in Table 2. PP-cells and PV-cells were prepared after cava→porta and porta→cava digitonin perfusion respectively (see Scheme 1), whereas control cells were prepared without digitonin treatment. Compared with the value found in the biopsy of the same liver, the PV-cells showed a 20% higher PK activity, whereas values for PP-cells and control cells were not significantly different from the biopsy value. ALAT shows a 3-fold higher activity in the periportal region, in agreement with the data obtained with the 'collagenase gradient technique' (Väänänen et al., 1983). The difference between PP-cells and PV-cells with respect to these two enzymes is further demonstrated by the highly significant difference in the ALAT/PK ratio, given as a percentage of the value found in the homologous liver biopsy.

Table 1. *Activities of ALAT and PK in eluate after short-term digitonin perfusion*

The livers of 24h-starved rats were perfused with Krebs–Henseleit (1932) buffer containing 4mg of digitonin/ml for 40–60s in the cava→porta or porta→cava flow direction at rates of 7 and 5ml/min respectively. After reversal of flow direction and increase of flow rate to 23ml/min, the eluate was collected in three 20s fractions. Enzyme activity was measured in these fractions; results are means $\pm$ S.D. for five experiments.

Enzyme	Flow-direction	Fraction . . .	Activity (μmol/min per fraction)		
			0–20 s	20–40 s	40–60 s
ALAT	Porta→cava		28.8 ± 8.9	6.72 ± 2.6	2.12 ± 0.69
	Cava→porta		5.38 ± 0.77	1.89 ± 0.67	0.635 ± 0.16
PK	Porta→cava		21.3 ± 6.11	4.90 ± 1.96	1.71 ± 0.16
	Cava→porta		23.2 ± 4.0	8.54 ± 3.0	2.33 ± 0.33
ALAT/PK	Porta→cava		1.35 ± 0.22	1.38 ± 0.25	1.24 ± 0.18
	Cava→porta		0.230 ± 0.022	0.235 ± 0.061	0.282 ± 0.072

Table 2. *Activities of ALAT and PK in periportal and perivenous hepatocytes*
PP-cells or PV-cells were isolated from the liver of 24 h-starved rats by the standard collagenase perfusion technique (Seglen, 1976) after initial selective destruction of the PV or PP part of the microcirculatory unit, respectively, by short-term perfusion with 4 mg of digitonin/ml. Values are means ± S.D. for the numbers of experiments in parentheses. *$P < 0.05$, **$P < 0.01$ (paired data t-test) for significance of difference with respect to the homologous liver biopsy.

	Activity (μmol/min per mg of DNA)				$\dfrac{\text{ALAT}}{\text{PK}}$	
	PK		ALAT			Cells (% of biopsy value)
	Biopsy	Cells	Biopsy	Cells	Biopsy	
Control cells (6)	19.2 ± 2.8	17.4 ± 1.8	15.0 ± 3.7	12.6 ± 4.2	0.779 ± 0.20 ($\sim$100%)	103 ± 17
PP-cells (5)	—	18.7 ± 4.4	—	29.4 ± 5.0**	—	148 ± 26*
PV-cells (5)	—	22.1 ± 1.9*	—	9.55 ± 2.0*	—	57 ± 2.0**

Table 3. *Glucose and glycogen synthesis in periportal and perivenous hepatocytes*
PP- and PV-cells were isolated as described in Table 2. Values are given as means ± S.D., with the numbers of experiments in parentheses: *$P < 0.05$, **$P < 0.01$.

			Activity (μmol/min mg of DNA)	
	Time (min) . . .		0–40	40–80
Glucose	PP-cells (6)		0.402 ± 0.075*	0.359 ± 0.032**
	PV-cells (5)		0.244 ± 0.061	0.207 ± 0.023
Glycogen	PP-cells (6)		0.057 ± 0.031	0.241 ± 0.14
	PV-cells (5)		0.060 ± 0.023	0.112 ± 0.094

Gluconeogenesis and glycogen synthesis

Incubations of PP-cells and PV-cells were carried out, and gluconeogenesis and glycogen synthesis were measured as shown in Table 3. The results support the concept of metabolic zonation for carbohydrate metabolism, with a preferential location of the gluconeogenic pathway in periportal hepatocytes and, conversely, a preferential location of glycolysis in perivenous hepatocytes (Sasse *et al.*, 1975), since the rate of glucose synthesis in PP-cells, corresponding to about 0.8 μmol of glucose/min per g of liver, was 2-fold higher than in PV-cells.

Glutamine was added to the incubations in order to obtain physiological rates of glycogen synthesis (Katz *et al.*, 1979) and, as shown in Table 3, glycogen synthesis increased to rates approaching those of gluconeogenesis after a characteristic lag phase. It appears that the rate is somewhat larger in PP-cells than in PV-cells; however, owing to large inter-experiment variability the difference is not statistically significant. This rather large variability in glycogen synthesis between cell preparations (Quistorff, 1983), without a similar variation in other biosynthetic pathways such as gluconeogenesis, as reported in the present study, or fatty

acid synthesis (B. Quistorff, unpublished work) is not understood.

Discussion

The combination in the present study of digitonin and collagenase perfusion allows isolation of hepatocytes originating preferentially from either the afferent or the efferent part of the microcirculatory unit of the liver, in high yield. The selectivity of the method in terms of cell origin depends on the uniformity of flow distribution during the short digitonin-perfusion period (see Scheme 1). Judged by the distribution of cytoplasmic marker enzymes in PP-cells and PV-cells, a high degree of selectivity may be achieved by the present technique. PP/PV enzyme-activity ratios for ALAT and PK were 3.1 and 0.85 respectively, to be compared with microdissection studies, where the same ratio for ALAT was reported as 2.2–2.5 with the liver acinus divided in two parts and as 3.0–4.5, excluding the mid-zone (Morrison *et al.*, 1965; Welsh, 1972). For PK the values reported were 0.72–0.79 (Guder & Schmidt, 1976; Zierz *et al.*, 1983). The fact that the PK activity profile in the eluate during digitonin perfusion does not indicate a zonation of PK, in spite of its presence in the

isolated hepatocytes, is not understood. A contributing factor might be that the M-type PK, which in the starved state contributes 20–25% of the total PK activity of the liver, is not zonated, and is located exclusively in non-parenchymal cells (Zierz et al., 1983).

Occasionally (10–15%) a PP-cell preparation does not differ significantly from the homologous biopsy with respect to the ALAT/PK ratio, and thus appears like a control preparation. This is not seen for PV-cell preparations. One would expect such cases to be caused by unequal flow distribution during the digitonin perfusion. However, this could not be observed as differences in decoloration pattern on the liver surface. In careful X-ray angiographic studies on rats in vivo, Daniel & Prichard (1951) observed an apparent shunting of major parts of the blood flow through central parts of the liver in about 30% of the rats investigated. If such phenomena, for which no explanation has been offered so far, also occur in the perfused liver, it could explain the occasional failure of selective cell destruction during the digitonin perfusion.

It is well established that a number of enzymes of metabolic pathways are unequally distributed within the microcirculatory unit of the liver (for reviews, see Rappaport, 1980; Jungermann & Katz, 1982). The functional implication of such enzyme heterogeneity has been demonstrated for ammonia metabolism (Häussinger et al., 1984) and for carbohydrate metabolism (Jungermann et al., 1982; Matsumura & Thurman, 1984), and also indirectly in a study on primary cultures of rat liver hepatocytes (Probst et al., 1982). The present results confirm and extend these studies by directly demonstrating a 2-fold greater rate of gluconeogenesis in hepatocyte preparations enriched in PP-cells as compared with PV-cells.

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