Distribution of albumin, α_1 -inhibitor 3 and their respective mRNAs in periportal and perivenous rat hepatocytes isolated by the digitonin–collagenase technique

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The expression of albumin and α_1 -inhibitor 3 genes was investigated in rat cell suspensions enriched in periportal ($n = 10$) and perivenous ($n = 10$) hepatocytes obtained by the digitonin–collagenase technique. The degree of enrichment of the cell suspensions was assessed: (1) by enzymic assays for the periportal marker alanine aminotransferase and for the perivenous marker glutamine synthetase; and (2) by their content of mRNAs for the periportal marker hepatic glutaminase and for glutamine synthetase. The existence of an antegrade intra-lobular gradient for albumin and α_1 -inhibitor 3 mRNAs was demonstrated, with periportal:perivenous ratios of 2.33 and 3.80, respectively. However, no gradient was demonstrated for the respective protein contents with corresponding ratios of 0.98 and 1.21. A

certain degree of overlap existed between periportal and perivenous suspensions for their content in albumin and α_1 -inhibitor 3 mRNAs. A morphometrical analysis of the surface of digitonin-permeabilized hepatic tissue revealed that this overlap could be explained by a variable extent of permeabilization of the mediolobular zone from one rat to another and from one lobule to another in a given animal. These results suggest that while the digitonin–collagenase technique is well suited for studies *in vitro* of proteins expressed in sharp intra-lobular gradients or restricted to an intra-lobular compartment, it is not completely reliable for proteins distributed in continuous moderate intra-lobular gradients, such as albumin and α_1 -inhibitor 3.

INTRODUCTION

The functional unit of the mammalian liver is the hepatic lobule, or acinus, according to Rappaport's concept of liver architecture. Each lobule, containing one central vein and limited by several portal spaces, is composed of three distinct zones, the periportal, the mediolobular and the perivenous (respectively named zones 1, 2 and 3 in Rappaport's concept). Many metabolic and functional differences between periportal and perivenous hepatocytes of the same lobule have been described, and the concept of metabolic zonation, proposed by Jungermann and co-workers [1,2], has now gained wide acceptance.

Recently, zonal hepatocellular heterogeneity of the expression of several plasma proteins and of their respective mRNAs has been described in the rat hepatic lobule (for review, see [3]). Most of these proteins, like albumin [4–12], α_1 -inhibitor 3 [13], α_2 -macroglobulin [13], fibrinogen [4] and haptoglobin [14], are distributed in continuous antegrade gradients. A few plasma proteins are distributed along a retrograde gradient, such as α -fetoprotein until the fourth postnatal week [6,10,15–17], α_1 -antitrypsin [13,18] and angiotensinogen [19]. α_{2u} -globulin presents an age- and androgen-dependent distribution. Its expression is restricted to the perivenous lobular compartment in young male rats, presents no intra-lobular zonation in adult rats and is distributed along an antegrade gradient in ageing animals [20–23]. All these results have been obtained using morphological methods on intact liver tissue, and are based on immunohistochemistry and *in situ* hybridization techniques. They have not been verified in zonally enriched hepatocyte populations. This step is necessary: (1) to exclude possible artifacts resulting from the fixation and conservation of liver tissue inherent to morphological investigations, and (2) to allow the study of the regulation of the expression of plasma proteins in the various zones of the

hepatic lobule. The digitonin–collagenase perfusion technique [24,25] is now widely used for isolation of cell populations enriched in periportal or perivenous hepatocytes. It is based on the selective antegrade or retrograde permeabilization of one zone of the hepatic acinus, the hepatocytes of the intact residual zone being isolated by a subsequent collagenase perfusion. This technique has proved of value for the investigation of several heterogeneously distributed enzymes [24–27].

The aim of the present work was therefore to: (a) verify the heterogeneous intra-lobular distribution of albumin and α_1 -inhibitor 3, two of the main plasma proteins synthesized by normal rat hepatocytes, and of their respective mRNAs, in periportal and perivenous hepatocytes isolated by the digitonin–collagenase technique, and (b) to evaluate the validity of this technique for the isolation of zonally enriched hepatocyte suspensions suitable for the study of plasma protein heterogeneity *in vitro*.

Experimental

Cell suspensions selectively enriched in either periportal or perivenous hepatocytes were obtained by the digitonin–collagenase technique and were characterized as follows. Their degree of enrichment was assessed by the determination of the zonal enzyme activities of alanine aminotransferase (ALAT) and glutamine synthetase, and by the quantification of glutamine synthetase and hepatic glutaminase mRNAs by Northern-blot analysis. Lobular distribution of the marker enzymes is as follows: ALAT [24,28,29] and hepatic glutaminase [30] are predominantly periportal and glutamine synthetase is restricted to a small perivenous compartment [31]. In these well-characterized cell suspensions, we determined the distribution of

Abbreviations used: ALAT, alanine aminotransferase; PP:PV, periportal:perivenous ratio.

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the plasma proteins albumin and α_1 -inhibitor 3 and of their respective mRNAs. In addition, inter- and intra-individual variability in the extent of tissue damage caused by digitonin perfusion of the liver was determined by morphometry.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats (180–200 g), fed *ad libitum*, were used throughout the study. Twenty animals were submitted to the digitonin-collagenase technique to obtain periportal- ($n = 10$) or perivenous- ($n = 10$) enriched hepatocyte suspensions. Twenty other animals were subjected to antegrade ($n = 10$) or retrograde ($n = 10$) digitonin perfusion alone and were used for morphometrical analysis.

Digitonin perfusion and tissue processing

Digitonin perfusion was performed essentially as described by Quistorff and Grunnet [32]. Briefly, the liver was perfused for 10 min at 35 °C with Krebs-Henseleit bicarbonate buffer (equilibrated with 95%/5% O_2/CO_2). A solution of 0.4% (w/v) digitonin (Prolabo, Paris, France) in Krebs-Henseleit bicarbonate buffer [33] was then infused at a rate of 10 ml/min for 20 s through either the portal vein or via the caudal vena cava. The flow direction was then reversed and increased to 20 ml/min to eliminate residual digitonin. The liver was then fixed *in situ* by perfusing, for 5 min, a solution of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Tissue samples were dehydrated and embedded in paraffin for histological examination and morphometrical analysis.

Isolation of periportal and perivenous hepatocytes

To obtain periportal and perivenous cells, digitonin perfusion was followed by 25 ml/min perfusion of 13 i.u./300 ml collagenase (Boehringer, Mannheim, Germany) [24,25]. Cells were purified using a Percoll gradient [Percoll/1.5 M NaCl (9:1, v/v)] (Pharmacia, Uppsala, Sweden) [34]. The average viability of the final suspensions assessed by Trypan Blue exclusion was $85.7\% \pm 11.2$. Cell pellet was used for total mRNA extraction, total protein extraction and enzyme determination.

Enzymic assay

The activities of ALAT and glutamine synthetase were assayed on cell homogenates and on the caudal lobe of the liver removed before digitonin perfusion. Standard biochemical methods, as described respectively by Bergmeyer and Bernt [35] and Pamijsjans et al. [36], were used. Total protein content was measured according to Lowry et al. [37].

Molecular probes

All the probes used were cDNA fragments. The probe for glutamine synthetase mRNA was a 715 bp *EcoRI*-fragment of clone cGS4 [38] kindly provided by A. F. M. Moorman (Department of Anatomy and Embryology, Academic Medical Center, Amsterdam, The Netherlands). The probe for hepatic glutaminase mRNA, generously given by M. Watford (Rutgers University, New Brunswick, New Jersey, U.S.A.), was a 1 kb *EcoRI*-insert [39]. Albumin mRNA was detected using a 932 bp *PstI*-fragment of clone pRSA 13 [40]. The most abundant α_1 -inhibitor 3 mRNA was detected using a 0.7 kb fragment of clone pRLA₁I_{23J} [41], generously provided by G. Fey (Institute of Microbiology, Biochemistry and Genetics, University of Erlangen, Erlangen, Germany).

Northern-blot analysis

Total RNAs, extracted according to Chomczynski and Sacchi [42], were electrophoresed and transferred onto Hybond C super nylon membrane (Amersham, Les Ulis, France) as indicated by Sambrook et al. [43]. RNA loading was controlled by ethidium bromide staining (0.5 mg/ml ethidium bromide in gel loading buffer). Probes were labelled with [α - ^{32}P]-dCTP by random priming using a multi-prime DNA labelling kit (Amersham). Hybridization conditions were as follows: 50% formamide, $5 \times$ SSC (0.75 M NaCl/0.75 M trisodium citrate, pH 7), 0.05 M phosphate buffer (pH 7), 0.1% SDS, $5 \times$ Denhardt's solution (0.1% Ficoll 400/0.1% polyvinylpyrrolidone/0.1% BSA), 0.25 mg/ml sonicated salmon sperm DNA, 3.5×10^6 – 5×10^6 c.p.m. of ^{32}P -labelled probe per cm^2 of membrane. Hybridization was performed at 42 °C. The hybridization conditions used for α_1 -inhibitor 3 mRNA hybridization were more stringent ($1 \times$ SSC, 55 °C) because of its reported sequence similarity with α_2 -macroglobulin mRNA [41,44].

Blots were washed at room temperature using $2 \times$ SSC ($1 \times$ SSC for α_1 -inhibitor 3)/0.1% SDS (w/v) solution and then at 65 °C using $0.1 \times$ SSC/0.1% SDS. Blots were exposed to Kodak X-OMAT films (Kodak, Paris, France). Membranes were then dehydrated for 2 h at 68 °C (2.5 mM Tris/HCl, pH 7.4/ 10^{-3} mM EDTA/0.005% SDS/0.25 $\times$ Denhardt) to allow subsequent hybridizations. Autoradiograms were quantified by densitometry with a dual-wavelength thin-layer chromato-Scanner (model CS-930, Shimadzu Corporation, Kyoto, Japan).

Antibodies

Rabbit anti-(rat albumin) was purchased from Nordic (Tilburg, The Netherlands). Rabbit anti-(rat α_1 -inhibitor 3) was kindly provided by F. Gautier (Laboratoire des protéines et des liquides biologiques, Faculté de Médecine, Tours, France). Rabbit anti-glutamine synthetase was a gift from C. Bréchet (INSERM U370, Hôpital Necker, Paris, France). Species-specific peroxidase-labelled polyclonal goat anti-(rabbit immunoglobulins) antibody was from Immunotech (Marseille, France).

Western-blot analysis and immunoblotting

Cellular samples were homogenized in a 25 mM Tris, pH 8/50 mM NaCl buffer, containing protease inhibitors (1 μ g/ml antipain, 1 μ g/ml leupeptin, 100 ki.u. Trasylol, 1 mM benzamidin and 1 mM phenylmethanesulphonyl fluoride). Membranes were solubilized for 30 min on ice, vortexed every 5 min, in the same buffer containing 10% Triton-X 100 and 10% sodium deoxycholate. Homogenates were centrifuged at 18000 g for 10 min at 4 °C. After protein determination by Lowry assay [37], equal amounts of proteins were diluted with Laemmli electrophoresis sample buffer (1:5, v/v) in the presence of β -mercaptoethanol, and separated by SDS/PAGE in 7–9% (w/v) polyacrylamide gel [45]. Proteins were transferred from gels onto Hybond-ECL nitrocellulose membranes (Amersham) overnight at 150 mA. After blocking for 1 h at room temperature in PBS-Tween buffer (0.1 M PBS/0.1% Tween 20) containing 5% (w/v) dried milk, nitrocellulose membranes were incubated for 1 h with the specific antibodies in PBS-Tween buffer. After washing, incubation with peroxidase-labelled goat anti-(rabbit immunoglobulins) antibody was performed for 1 h. Immuno-reactive bands were identified by chemoluminescence (Amersham, ECL reagent) on exposure to Kodak X-OMAT films.

Morphometry

Tissue samples were processed for histological examination according to standard techniques. Tissue sections were stained with haematoxylin and eosin. Morphometrical analysis was performed using a Biocom 200 image analyser (Biocom, Les Ulis, France). In each animal, we evaluated: (a) the percentage of tissue permeabilized by digitonin in the whole liver, and (b) the mean surface of the digitonin-permeabilized tissue in each hepatic lobule. To determine the percentage of tissue permeabilized by digitonin in the whole liver, semi-automatic image analysis was performed on one tissue section of one liver fragment for each animal. We measured: (a) the total surface (μm^2) of the section, and (b) the percentage of digitonin-permeabilized surface in the section. To evaluate the mean surface of tissue permeabilized by digitonin in each hepatic lobule, we measured the surface (μm^2) of the digitonin-permeabilized tissue in 20 consecutive, unselected lobules for each animal.

RESULTS

Characterization of the cell suspensions

Enzymic assays

Hepatocyte preparations obtained by the digitonin–collagenase technique were characterized by enzymic activities of the periportal marker ALAT and of the perivenous marker glutamine synthetase. We then compared the activities in each isolated periportal and perivenous population with the total activity measured in the corresponding intact liver tissue, represented by the caudal lobe removed before digitonin perfusion. We established a cell:tissue ratio which reflected the individual enrichment for the zonal markers in each population (see Table 1). All the periportal preparations showed a higher ALAT activity (mean cell:tissue ratio 1.7) and a lower glutamine synthetase activity (mean cell:tissue ratio 0.03) than the intact liver. Conversely, all the perivenous preparations were poor in ALAT (mean cell:tissue ratio 0.8) and enriched in glutamine synthetase (mean cell:tissue ratio 2.73). The periportal:perivenous (PP:PV) ratio determined from the mean activities in each group was 2.46 for ALAT and 0.01 for glutamine synthetase, which was in accordance with the literature [46].

Distribution of mRNAs for zonal enzyme markers

The periportal predominance in the distribution of hepatic glutaminase mRNAs, as reported by Watford and Smith [30], was verified in our samples (PP:PV ratio 7.61). In the same way, glutamine synthetase mRNAs predominated in our perivenous preparations (PP:PV ratio 0.004) as reported by Gebhardt et al.

Table 2 Relative abundance of hepatic glutaminase, glutamine synthetase, albumin and α_1 -inhibitor 3 mRNAs in perivenous and periportal hepatocytes isolated by the digitonin–collagenase technique

Autoradiograms of different time exposure were quantified by densitometry. Results are expressed as densitometry units representing cDNA hybridized per 20 μg of total RNA and are means $\pm$ SEM for ten preparations in each group. * $P < 0.05$, *** $P < 0.0001$ for differences between periportal and perivenous preparations (Student's *t*-test).

mRNA	Periportal	Perivenous	PP:PV
Hepatic glutaminase	16630 $\pm$ 4302*	2185 $\pm$ 529	7.61
Glutamine synthetase	326 $\pm$ 95***	75782 $\pm$ 29181	0.004
Albumin	15575 $\pm$ 2656*	6674 $\pm$ 1075	2.33
α_1 -inhibitor 3	23645 $\pm$ 6815*	6229 $\pm$ 2770	3.80

Figure 1 Northern-blot analysis of albumin mRNA in periportal and perivenous hepatocytes isolated by the digitonin–collagenase technique

(a) Autoradiogram of ten periportal samples (left) versus ten perivenous samples (right). (b) Densitometric quantification of the hybridization signal. PP, periportal; PV, perivenous.

[47] and Watford and Smith [30]. There was no overlap in the hepatic glutaminase and glutamine synthetase mRNA content between samples considered either as periportal or as perivenous. Results are shown in Table 2.

Distribution of albumin, α_1 -inhibitor 3 and their respective mRNAs in zonally enriched hepatocyte suspensions

Distribution of specific mRNAs for albumin and α_1 -inhibitor 3

Northern-blot analysis of albumin (Figure 1) and α_1 -inhibitor 3 (Figure 2) mRNAs showed a periportal predominance. The PP:PV ratios between the mean values in each group were 2.33 and 3.80 respectively. However, there was a degree of overlap

Table 1 Activities of ALAT and glutamine synthetase in perivenous and periportal hepatocytes isolated by the digitonin–collagenase technique

Results are expressed as $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of proteins and are the mean $\pm$ SEM of ten different cell preparations in each group of animals. The cell:tissue ratio denotes the cell activity relative to that observed in the corresponding caudal lobe of the liver. PP:PV was determined from mean activities in each group. *** $P < 0.0001$ for differences in activities and cell:tissue ratio between periportal and perivenous preparations (Student's *t*-test).

Enzyme	Periportal		Perivenous		PP:PV
	Activity	Cell:tissue	Activity	Cell:tissue	
Alanine aminotransferase	552 $\pm$ 63***	1.7 $\pm$ 0.1***	212 $\pm$ 20	0.8 $\pm$ 0.1	2.46
Glutamine synthetase	0.17 $\pm$ 0.07***	0.03 $\pm$ 0.01***	15.31 $\pm$ 1.64	2.73 $\pm$ 0.26	0.01

Figure 2 Northern blot analysis of α_1 -inhibitor 3 mRNA in periportal and perivenous hepatocytes isolated by the digitonin–collagenase technique

(a) Autoradiogram of ten periportal samples (left) versus ten perivenous samples (right). (b) Densitometric quantification of the hybridization signal. PP, periportal; PV, perivenous.

Table 3 Relative abundance of albumin, α_1 -inhibitor 3 and glutamine synthetase proteins in perivenous and periportal hepatocytes isolated by the digitonin–collagenase technique

Autoradiograms of different time exposure were quantified by densitometry. Results are expressed as densitometry units and are means $\pm$ SEM for ten preparations tested in each group. *** $P < 0.0001$ for differences between periportal and perivenous preparations; NS: not statistically significant (Student's *t*-test).

Protein	Periportal	Perivenous	PP:PV
Albumin	36108 $\pm$ 3604 (NS)	36813 $\pm$ 3539	0.98
α_1 -inhibitor 3	39300 $\pm$ 8999 (NS)	32296 $\pm$ 5866	1.21
Glutamine synthetase	891 $\pm$ 163***	19162 $\pm$ 2018	0.05

between the individual values measured in each group (Figures 1 and 2), due to an important inter-individual variability, as attested to by the variation listed in Table 2.

Table 4 Morphometrical analysis

Semi-automatic image analysis was performed on one tissue section of one liver fragment for each animal in each group ($n = 10$). The total surface of the section and the sum of the surfaces of all the digitonin-permeabilized areas visible on the section were measured to determine the percentage of digitonin-permeabilized surface (μm^2) in the section. The surface of digitonin-permeabilized tissue in each hepatic lobule (μm^2) was measured in 20 consecutive, unselected lobules for each animal.

Antegrade perfusion			Retrograde perfusion		
Permeabilized tissue/liver (%)	Permeabilized tissue/lobule (μm^2)		Permeabilized tissue/liver (%)	Permeabilized tissue/lobule (μm^2)	
	Mean	Range ($n = 20$)		Mean	Range ($n = 20$)
45.9	183 790	130 900–228 500	27.7	155 185	56 000–351 000
24.3	135 874	45 500–272 000	21.6	134 767	60 800–347 000
19.3	130 370	55 500–256 500	17.5	116 531	33 150–309 200
50.1	195 513	13 900–246 000	16.3	64 475	36 050–95 600
18.7	121 752	61 000–219 500	17.4	117 001	36 200–275 100
32	166 572	89 000–210 000	31.4	163 449	79 000–250 000
24.9	139 584	47 000–225 000	18.9	121 085	640 000–215 000
28	151 356	92 000–235 000	21.9	135 920	77 000–230 000
21.5	133 438	56 000–180 000	21.2	136 274	51 000–180 000
26.8	144 870	70 000–205 000	17.4	118 442	46 000–230 000

Distribution of albumin and α_1 -inhibitor 3 proteins

In order to assess the sensitivity of the Western-blot analysis, we first verified that, under our experimental conditions, glutamine synthetase protein was heterogeneously distributed in the zonally enriched hepatocyte population, with a PP:PV ratio of 0.05. Under the same experimental conditions, Western-blot analysis revealed no clear zonal predominance for albumin and α_1 -inhibitor 3 proteins. The PP:PV ratios for the mean values in each group were respectively 0.98 and 1.21; the results are given in Table 3.

Morphometrical results

The percentage of digitonin-permeabilized tissue in the whole liver was variable from one animal to another. After antegrade perfusion, the percentage of digitonin-permeabilized tissue ranged from 18.7% to 50.1%, while after retrograde perfusion, it ranged from 16.3% to 27.7% (see Table 4). These results suggest that, in most instances, the mediolobular zone was usually not damaged or was only mildly damaged after digitonin perfusion.

The measure of the mean surface of digitonin-permeabilized tissue in each hepatic lobule confirmed the inter-individual variation in the extent of digitonin permeabilization. Furthermore, this measure showed the existence of a significant intra-individual variation in the extent of the digitonin permeabilization from one lobule to another, as attested to by the large ranges observed for each animal (Table 4). This suggests that cell sampling is different from one lobule to another.

DISCUSSION

The cell populations that we obtained using the digitonin–collagenase technique are correctly enriched in periportal or perivenous hepatocytes according to the literature standards. Cell:tissue ratios were used to assess the good individual enrichment of each sample for the enzymes ALAT and glutamine synthetase. The quantification of hepatic glutaminase and

glutamine synthetase mRNAs by Northern-blot analysis also validates the quality of our isolations. The PP:PV ratios measured in our samples are even greater than those obtained using the same technical approach by Watford and Smith (2.33 and 0.16 for hepatic glutaminase and glutamine synthetase mRNAs, respectively [30], versus 7.61 and 0.004 in our study) and by Gebhardt et al. (0.02 for glutamine synthetase mRNA) [47].

Under these conditions, we observed significant PP:PV ratios for albumin and α_1 -inhibitor 3 at the mRNA level. The corresponding PP:PV ratios were respectively 2.33 and 3.80. However, it is likely that these gradients might be slightly overestimated, in particular for α_1 -inhibitor 3 mRNA, as a result of the high inter-individual variation observed between samples. In any case, they are of the same order of magnitude as those determined using the same technique for several key enzyme mRNAs, such as phosphoenolpyruvate-carboxykinase [30]. Combined with our previous *in situ* hybridization observations [6,13], the results obtained in isolated zonal populations show that albumin and α_1 -inhibitor 3 mRNAs are distributed along a periportal gradient. Their pattern of expression is therefore different from that of compartmentalized proteins, such as glutamine synthetase, restricted to a very limited perivenous compartment [31], or carbamoylphosphate synthetase, present in a large periportal and mediolobular compartment [48].

In our preparations, we could not verify that the intra-lobular gradient observed for albumin and α_1 -inhibitor 3 at the mRNA level was retained at the protein level. This apparent discrepancy might be artifactual. Indeed, using the same experimental conditions, the PV:PP ratio for glutamine synthetase is also lower at the protein level than at the mRNA level. This suggests a lower sensitivity of the Western-blot technique used for protein analysis as compared with the Northern-blot technique used for mRNA analysis. In addition, in contrast to glutamine synthetase, located in the cytosol [49], albumin and α_1 -inhibitor 3 are secreted proteins located in the lumen of intracellular organelles. Despite the refined technique of membrane permeabilization used in our study, this might result in a lower extraction of albumin and α_1 -inhibitor 3 proteins. However, we cannot exclude the possibility that the apparent dissociation between the protein and the mRNA gradients might correspond to a dissociation between mRNA and protein expression, as previously described by morphological methods for several enzymes, such as pyruvate kinase L [50] and glucokinase [51] and for the plasma protein α_1 -antitrypsin [13]. The periportal predominance of certain plasma proteins mRNAs, exemplified by albumin and α_1 -inhibitor 3, might be interpreted as the consequence of the higher blood concentrations of amino-acids, hormones and regulating peptides in the periportal and mediolobular zones. However, this cannot explain the permanent or transient perivenous predominance of other plasma proteins, such as α -fetoprotein, α_1 -antitrypsin and α_2 -globulin, as suggested by previous morphological investigations [3]. A critical step for the understanding of the intra-lobular regulation of the expression of plasma proteins is the design of detailed experiments *in vitro* using isolated periportal and perivenous hepatocytes. With the digitonin-collagenase technique, in accordance with previously published results [30,47] we obtained cell suspensions presenting clear-cut differences in the distribution of certain periportal markers, such as hepatic glutaminase, and of certain perivenous markers, such as glutamine synthetase. However, the same cell suspensions presented a certain degree of overlap in the distribution of albumin and α_1 -inhibitor 3 mRNAs. Consequently, some individual preparations could not be considered as reliable representatives of periportal or perivenous hepatocytes, with respect to plasma protein

expression. This inter-individual variability might be due to a variable degree of contamination of the final cell suspensions by mediolobular hepatocytes and to a variable degree of cross-contamination of the final cell suspensions by digitonin-permeabilized hepatocytes originating from the opposite zone. The extent of mediolobular contamination cannot be reliably assessed in the final cell suspensions because of the lack of markers specific for this zone of the lobule. We therefore addressed this problem by a morphometrical study of digitonin-perfused liver, which shows that in most cases only 20–30% of the liver tissue was actually permeabilized by digitonin perfusion. This clearly indicates that the so-called 'periportal' and 'perivenous' cell suspensions are of mixed topographical origin and that a certain degree of mediolobular contamination is always present. Moreover, the morphometrical results show that the amount of contamination differs from one animal to another and from one lobule to another, resulting in variable cell sampling. The possible cross-contamination of final cell suspensions in some digitonin-permeabilized hepatocytes has been noted by Watford and Smith, who reported high glutamine synthetase mRNA expression in one of their periportal samples [30]. In the case of plasma proteins, this artifact is of particular relevance since we have previously shown that significant intracellular levels of plasma proteins and of their specific mRNAs are retained after digitonin permeabilization [52]. The combination of mediolobular contamination and cross-contamination might result in significant inter-individual variations in the degree of enrichment of the final cell suspensions. Moreover, such artifacts might explain previous observations showing that the intralobular gradients measured in cell suspensions obtained through the digitonin-collagenase technique are underestimated when compared with the pre-existing gradients measured in intact liver tissue obtained by microdissection, or in eluates provided by the dual-pulse digitonin perfusion [28,29,53–55].

The possible pitfalls of the digitonin-collagenase technique are of limited relevance for proteins distributed in restricted intra-lobular compartments, such as glutamine synthetase, or in sharp intra-lobular gradients, such as hepatic glutaminase. However, for proteins such as albumin and α_1 -inhibitor 3, which demonstrate moderate intra-lobular continuous gradients, the sensitivity of the digitonin-collagenase technique is not sufficient to ensure a reliable enrichment in every case. This problem can be overlooked when one considers the mean values obtained after pooling cell suspensions prepared from several animals. However, it is of particular relevance for studies *in vitro*, for which it is necessary to work on individual preparations.

In conclusion, the existence of an antegrade intra-lobular gradient for albumin and α_1 -inhibitor 3 has been demonstrated at the mRNA level in isolated periportal and perivenous hepatocytes obtained by the digitonin-collagenase technique. However, while this technique is well adapted for the study of molecules distributed in compartments or in sharp intra-lobular gradients, its reproducibility remains insufficient to provide reliably enriched zonal populations suitable for the study *in vitro* of plasma proteins, which are distributed in moderate continuous intra-lobular gradients.

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