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Digitonin perfusion in the study of metabolic zonation of the rat liver: potassium as an intracellular concentration reference

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The mammalian liver microcirculation seems to be uniquely organized in order to create maximum difference of the biochemical milieu to which the upstream (periportal) and downstream (perivenous) parenchymal cells are exposed. Indeed the existence of a subdivision of hepatocytes in zones of different metabolic function, long predicted by enzyme zonation studies (for a review, see Jungermann & Katz, 1982), has now been documented in direct studies for gluconeogenesis, urea synthesis and glutamine synthesis (Häusinger, 1983; Quistorff, 1985; Pösö *et al.* 1986; Quistorff *et al.*, 1986). However, an understanding of the physiological implications of this functional subdivision is still lacking.

Since such zonation can be expected to be closely related to the perfusion pattern of the liver, a technique which utilizes the direction of liver perfusion as the discriminatory principle in the selective study of periportal and perivenous cells would appear to be optimal. The digitonin perfusion technique is based on this principle (Quistorff *et al.*, 1985). The technique has developed in two directions: either for direct use of the eluate in the study of enzyme and metabolite contents of the periportal and the perivenous zone (Quistorff & Grunnet, 1987) or using the digitonin perfusion for selective destruction of part of the microcirculation, followed by isolation of hepatocytes from the non-affected part by standard collagenase perfusion techniques (Lindros & Penttilä, 1985; Quistorff, 1985; Quistorff *et al.*, 1986).

This paper discusses the mode of action of the digitonin perfusion and also introduces K^+ as a reference substance, allowing estimation of enzyme activity distribution within the microcirculatory unit of the liver as well as intracellular concentrations of cytosolic metabolites.

Methods

The data presented were obtained with the dual-digitonin-pulse perfusion technique, described in detail in Quistorff & Grunnet (1987). The technique involves a brief digitonin pulse (10 s of digitonin, 4 mg/ml in Krebs-Henseliet buffer, at 12 ml/min) followed by an immediate switching of flow direction and washout without digitonin at 20 ml/min, sampling the eluate for enzyme and for metabolite measurements. The same procedure was repeated with the opposite flow direction after an interval of 30 s, obtaining thereby both periportal and perivenous eluate of the same liver. Routinely a liver biopsy was taken before the digitonin treatment (right lower lobe; Quistorff, 1985). K^+ and Na^+ were measured by flame photometry with the samples diluted in LiCl. Protein was measured according to Lowry *et al.* (1951).

Results

Fig. 1 shows a simplified model of the liver microcirculation, with only two populations of hepatocytes and no zone of mixed cells. While such a model assuming a step-gradient is appropriate for the distribution of some enzymes like glutamine synthase (Gebhardt & Mecke, 1983) and in the sense that the dual-digitonin-pulse technique samples eluate from only inlet and outlet cells, it may not be adequate for enzymes or metabolites which are not distributed according to a discontinuous periportal-perivenous activity or concentration gradient. In Fig. 1(b) a brief periportal digitonin pulse has been applied with the result of partial destruction of the periportal area and elution of cytosolic

material from the destroyed cells. If, at this stage, collagenase isolation of the remaining hepatocytes were carried out (Quistorff, 1985), the resultant cell preparation would be enriched in perivenous cells, with two-thirds perivenous cells and one-third periportal cells, as demonstrated by the example in the Figure. Conversely, with a perivenous digitonin pulse to the liver (Fig. 1c) followed by collagenase cell isolation, a cell preparation enriched in periportal cells would be obtained.

Thus while the digitonin cell isolation technique can only produce either periportal or perivenous cell preparations from one liver in addition to the initial periportal or perivenous eluate, respectively, the dual-digitonin-pulse technique gives perivenous as well as periportal eluate from the same liver, but no cells.

By measuring the activity of the marker enzymes glutamate dehydrogenase, glucose-6-phosphatase and amyloglucosidase in the eluate, we know that under the present experimental conditions the contamination with mitochondrial, endoplasmic reticulum and lysosomal material is insignificant (Quistorff *et al.*, 1985; Quistorff & Grunnet, 1987).

The eluted cytosolic material is diluted by the perfusate. Assuming a cytosolic K^+ concentration of 160 $\mu\text{mol/ml}$ (Williams & Woodbury, 1971; Christensen & Folke, 1984) the actual dilution may be calculated as shown in Table 1. Under the present experimental conditions the most concentrated samples of cytosolic material were eluted in the fractions 10–25 s at approx. 10-fold dilution. Note that perivenous eluates were significantly more diluted than periportal eluates. Similar dilution factors may be obtained by a different method (Quistorff & Grunnet, 1987).

Table 2 shows data on protein, K^+ and Na^+ concentration in the eluate from both the periportal and the perivenous part of the liver. K^+ and protein concentration in the eluate increased with a broad maximum in the 10–15 s, 15–20 s and 20–25 s fractions for both periportal and perivenous eluate. In Table 2 these three fractions are therefore combined. Note that both K^+ and protein were significantly lower in perivenous eluate. Parallel with the K^+ release there

Fig. 1. Model of liver microcirculation during digitonin perfusion

The model assumes two populations of hepatocytes, periportal (PP) and perivenous (PV), each of which is homogeneous with respect to the parameter in question, but not necessarily of the same size. The Figure shows an example of hepatocyte isolation from the liver after a pulse of digitonin was given to partially destruct either the PP area (b) or the PV area (c) (Quistorff, 1985). The calculations below demonstrate the 'purity' of the cell preparation obtained in the two cases.

Table 1. *Dilution of the periportal and perivenous cytosol eluted with the dual-digtonin-pulse perfusion technique*

Livers of fed, female Wistar rats were perfused by the dual-digtonin-pulse technique (perfusion scheme B in Fig. 1 of Quistorff & Grunnet, 1987) with digtonin (4 mg/ml). Eight periportal and eight perivenous eluate fractions were collected over the time intervals indicated after digtonin treatment. The dilution factors of the eluted cytosol given for each fraction were calculated from the K^+ concentration measured in the eluate, assuming an intracellular K^+ of 160 $\mu\text{mol/ml}$ of cytosol, and using as blank the K^+ concentration in the Krebs-Henseleit perfusate. Results are means $\pm$ S.D. ($n = 8$). Statistical significance (paired t -test): * $P < 0.01$ periportal eluate versus perivenous eluate.

Elution fraction . . .	Dilution factor						
	0-5s	5-10s	10-15s	15-20s	20-25s	25-30s	30-35s
Periportal cytosol	209 ± 75.4	24.8 ± 24.6	8.92 ± 1.58	8.41 ± 3.42	11.2 ± 1.22	14.6 ± 1.35	18.6 ± 1.81
Perivenous cytosol	143 ± 147	30.6 ± 9.45	12.8* ± 2.40	15.0* ± 1.57	20.7* ± 2.81	29.1* ± 5.58	38.3* ± 10.2

* $P < 0.01$ periportal eluate versus perivenous eluate.

was an uptake of Na^+ by the liver, peaking also in the 10-25s fraction and consistently approx. 2-3 $\mu\text{mol/ml}$ larger than the K^+ release. Under the digtonin treatment the cells are losing anions in the form of metabolites, notably ATP, ADP and P_i (Quistorff & Grunnet, 1987), and it is not known at present which anions compensate for the 'extra' Na^+ uptake observed. Interestingly, the ratio between protein and K^+ in the eluate (Table 2, column 4), was nearly constant, approx. 0.6 mg/ μmol of K^+ during the elution without statistically significant difference between periportal and perivenous eluate.

Discussion

Mode of action of the digtonin perfusion technique. The following observations have been made. Continuous infusion of digtonin caused complete decolorization of the liver under appropriate conditions. The decolorization process may be followed by visual inspection of the surface of the liver while slowly progressing from periportal areas towards perivenous outlets. The decolorization pattern thus formed on the liver surface unequivocally reflects whether digtonin perfusion was started with normal perfusion direction or with retrograde perfusion (Quistorff *et al.*, 1985; Lindros & Penttilä, 1985). It was further shown that at 20 ml of digtonin/min (6 mg/ml) the liver almost completely cleared the perfusate of digtonin for an interval of about 50 s after which, however, the digtonin concentration in the effluent sharply increased towards 6 mg/ml. The amount of digtonin delivered during the time interval of near-complete clearance correlated well with the total amount of cholesterol in the liver (Quistorff *et al.*, 1985). Finally, histological examination of sections of liver, stained with the chromotrope, Aniline Blue, (Lillie, 1940) and fixed in phosphate-buffered formaldehyde (Poulsen & Christoffersen, 1979) at various stages during digtonin (2 mg/ml) perfusion, showed that the area of destruction was always associated with the direction of entry of the digtonin (periportal or perivenous region) and that initially the degree of destruction was

evenly distributed over the section. However, when more than about one-quarter of the total liver was affected, desynchronization of flow in different microcirculatory units was apparent (B. Quistorff, N. Grunnet & O. Albrechtsen, unpublished work).

These facts taken together strongly suggest that the mode of action of the digtonin perfusion is a titration of the cholesterol content of the hepatocytes along the sinusoid, in such a way that the 'digtonin front' moves through the sinusoids at a speed determined by the delivery of digtonin relative to the amount of cholesterol present. Since 70% of total cholesterol is in the plasma membrane (de Duve, 1971) this is likely to be the prime target of the digtonin action, leaving the hepatocytes 'nude', stripped of the plasma membrane (Cook *et al.*, 1983). This notion is supported by the fact that even the completely decolorized liver, having lost >80% of its protein and soluble metabolite content, has entirely maintained its macroscopic appearance (Quistorff *et al.*, 1985).

K^+ as an endogenous concentration reference substance. In a previous paper we discussed that the evaluation of a periportal/perivenous gradient of an enzyme based on comparison of specific activities will rely on whether or not total protein is zonated (Quistorff & Grunnet, 1987). The data in that work as well as the data reported here (Table 2) indeed suggest a higher periportal protein concentration. However, since the absolute concentrations developed in the eluate to some extent depend upon the sequence of perfusion (porta $\rightarrow$ cava followed by cava $\rightarrow$ porta or vice versa: Quistorff & Grunnet, 1987), and since also significant inter-animal variation is seen in this respect, it becomes important to solve the reference problem, e.g. by finding a substance for which the absolute zonal distribution is known. No such substance is yet known; however, K^+ might be a good candidate, primarily because of its very high intracellular concentration, which makes significant changes (on a percentage scale) unlikely under physiological conditions. Furthermore, K^+ is for the same reason unlikely to be

Table 2. *K^+ , Na^+ and protein elution with the dual-digtonin-pulse perfusion technique*

Liver perfusion and fraction collection were as described in Table 1. The combined data of the fractions 10-15s, 15-20s and 20-25s are given as means $\pm$ S.D. ($n = 8$). Na^+ and K^+ values are corrected for the content of these ions in the Krebs-Henseleit perfusion medium. Statistical significance: (paired t -test): * $P < 0.05$ periportal eluate versus perivenous eluate.

	K^+ ($\mu\text{mol/ml}$)	Na^+ ($\mu\text{mol/ml}$)	Protein (mg/ml)	Protein: K^+ (mg/ μmol of K^+)
Periportal eluate	14.9 $\pm$ 1.72	-17.8 $\pm$ 3.19	9.51 $\pm$ 1.70	0.62 $\pm$ 0.11
Perivenous eluate	10.3 $\pm$ 1.50*	-12.6 $\pm$ 2.85*	5.62 $\pm$ 0.72*	0.52 $\pm$ 0.06

* $P < 0.05$ periportal eluate versus perivenous eluate.

significantly zoned, although this is not known at present.

Accepting K^+ as a non-zoned concentration reference, the data in Table 2 indicate that the cytosolic protein concentration is about 95 mg/ml of cytosol, indicating that there is no significant difference in cytosolic protein concentration between periportal and perivenous cells.

Estimation of enzyme distribution in the liver microcirculation. The activity of a cytosolic enzyme such as alanine aminotransferase in the initial biopsy will be the weighted sum of the activity in periportal and perivenous cells. In an experiment with the dnal-digtonin-pulse technique we usually have data for the specific activity of a particular enzyme both from the initial biopsy and from the periportal and perivenous eluates. However, since the periportal and perivenous activity measured in the eluates is expressed per mg of cytosolic protein, while the activity in the biopsy is expressed per mg of total protein, a conversion factor is needed. From the cytosolic eluates we obtain the average value of mg of cytosolic protein/ μ mol of K^+ . This value may be converted to mg of cytosolic protein/ml of cytosol, assuming a cytosolic K^+ concentration of 160 μ mol/ml (Williams & Woodbury, 1971; Christensen & Folke, 1984). According to Weibel *et al.* (1969) the rat liver contains 0.444 ml of cytosolic groundsubstance/g of liver wet wt. Thus the conversion factor is:

$$\frac{\text{mg of total protein/g of liver biopsy wet. wt}}{\text{mg of eluate protein}/\mu\text{mol of } K^+ \times 160 \times 0.44}$$

According to the model of the liver microcirculation shown in Fig. 1, we may now write:

$$A_{bi} = vA_{pp} + (1 - v)A_{pv} \quad (1)$$

where v is the volume fraction of the hepatocytes where the specific activity of the enzyme in question is A_{pp} (i.e. the specific activity found in periportal (eluate), and conversely $(1 - v)$ the volume fraction where the activity is A_{pv} . A_{bi} is the specific activity in the biopsy expressed as units/mg of cytosolic protein, calculated as explained above. Applying eqn. (1) to the two highly zoned enzymes, alanine aminotransferase and glutamine synthase (data from Table 4, Quistorff & Grunnet, 1987), v may be calculated to be 0.85 ± 0.04 and 0.52 ± 0.12 , respectively. Thus for gluta-

mine synthase 15% of the hepatocytes contain > 99% of the activity. Gebhardt & Mecke (1983) found this fraction to be approx. 7%, using immunohistochemical techniques. For alanine aminotransferase the calculation indicates that the high and low activity of the enzyme is distributed in cell populations of almost equal size. However, redefining the model of Fig. 1 and allowing for a continuous enzyme activity gradient, the result would be compatible with a linear periportal-perivenous gradient of alanine aminotransferase, as was indeed suggested by the results of Morrison *et al.* (1965) in microdissection studies.

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Oxygen gradients: the problem of hypoxia

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Oxygen gradients in liver

Due to O_2 consumption inter- and intra-cellular O_2 gradients are formed in tissues. In liver there are steep inter-cellular O_2 gradients along the sinusoids from the portal to the central regions of the liver lobules (Sies, 1977; Ji *et al.*, 1982). As demonstrated by use of micro-needle electrodes and multiwire surface electrodes, the actual pO_2 in liver ranges between 1 and 60 mmHg with mean values around 22 mmHg (Fig. 1).

From comparison of pO_2 of half-maximal changes (p_{50} values) of oxidation-reduction states of cytochrome c in isolated liver cells and mitochondria, Jones (1984) estimated an intracellular O_2 gradient of about 4 mmHg from the extracellular medium towards the outer mitochondrial membrane under O_2 -limiting conditions. From the O_2 dependence of the hepatocellular O_2 uptake (Fig. 1) an O_2 gradient of maximally 5 mmHg from the extracellular space towards

the mitochondrial membrane can be concluded. Comparison of p_{50} values of the metabolism of two substrates of cytochrome $P-450$ by isolated hepatocytes and isolated liver microsomes (Jones & Mason, 1978) as well as the comparison of the optimum pO_2 for the induction of lipid peroxidation by carbon tetrachloride (CCl_4), again in isolated hepatocytes and isolated microsomes (de Groot & Noll, 1986; Noll & de Groot, 1984), reveal that O_2 gradients from the extracellular medium to cellular compartments other than mitochondria appear to be below the detection limit. Thus within the liver cell a significant O_2 gradient appears to exist only towards the mitochondrial compartment and, furthermore, this O_2 gradient must be predominantly at the regions immediately surrounding the mitochondria.

Oxystat system

As pointed out, steep intercellular O_2 gradients exist between the various cells of the intact liver. Therefore, isolated liver cells and subcellular fractions are the preferred biological systems for the study of the response of hepatocellular functions to different O_2 conditions. We have developed a special incubation apparatus for isolated cells and subcellular fractions, the oxystat system, which com-

Fig. 1. O_2 dependence of hepatocellular O_2 uptake and CCl_4 -induced lipid peroxidation

O_2 uptake and lipid peroxidation in hepatocytes were determined as previously described (de Groot & Noll, 1984; de Groot *et al.*, 1985). For comparison, the pO_2 frequency distribution curve of the liver *in situ* is included (Kessler *et al.*, 1973).

binates the advantage to maintain steady state pO_2 with high precision from 300 mmHg down to 0.01 mmHg with the capability to monitor O_2 uptake simultaneously (Noll *et al.*, 1986, 1987). In contrast to the more commonly used manometric technique, the oxystat system maintains O_2 supply by injection of O_2 dissolved in aqueous solution. The actual pO_2 in the incubation chamber is continuously monitored by either a Clark-type electrode (at $pO_2 \geq 0.2$ mmHg) or by an O_2 sensor based on the O_2 dependence of bacterial luminescence (at $pO_2 < 0.2$ mmHg). The oxystat system represents a feedback control circuit supported by a computer which reads the O_2 sensor signals, compares them with a preselected value, and triggers a motor-driven piston burette to deliver O_2 -saturated medium into the reaction chamber until the setpoint is reached. Further, it calculates the O_2 uptake from the amounts of O_2 -saturated medium added.

Oxygen dependence of liver cell functions

The study of the O_2 dependence of mammalian cell functions usually reveals that most of them only respond to decreases of the pO_2 when a characteristic threshold value has been passed. A typical example is the O_2 uptake of the isolated liver cell (Fig. 1). Its rate is almost constant within a pO_2 range of 5–100 mmHg. Below the critical pO_2 of 5 mmHg, however, the rate of O_2 uptake starts to decrease and it is half-maximal at a pO_2 of 0.5 mmHg. Since hepatocellular O_2 uptake reflects more than 90% of the electron flow through the mitochondrial respiratory chain (de Groot *et al.*, 1985) and in that way is a direct function of the energy (ATP) turnover of the cell, the decrease of the O_2 uptake rate indicates an equivalent decrease of the energy-consuming functions of the cell.

It is known that a decreased pO_2 is paralleled by decreases in the ATP/ADP and NAD/NADH concentration ratios and by an increase in the reduction state of components of the mitochondrial respiratory chain such as cytochrome *c* and cytochrome *aa₃* (e.g. Oshino *et al.*, 1974; Wilson *et al.*, 1979). Experiments in isolated liver cells, performed under steady state pO_2 in the oxystat system, reveal that the decrease in the ATP/ADP concentration ratio is especially prominent at pO_2 below 5 mmHg in line with the impairment of energy supply at pO_2 below 5 mmHg, as described above. The ATP/ADP concentration ratio started from

Fig. 2. Possible mechanisms of cell death caused by O_2 limitation of cytochrome oxidase function and by an increased reductive activation of haloalkanes, such as CCl_4 and halothane, under hypoxia

values above 10 at a pO_2 of 100 mmHg, decreased slowly to values of 7–8 at pO_2 of 5 mmHg, but then abruptly dropped to values of about 1 under anaerobic conditions (data not shown).

Decreasing pO_2 are not necessarily combined with decreasing cell functions. In fact, certain functions increase with decreasing pO_2 or even become feasible only at low pO_2 . One remarkable example is the reductive metabolism of halogenated alkanes such as CCl_4 and the anaesthetic halothane ($CF_3CHBrCl$) (de Groot & Noll, 1983, 1986). The metabolism of these well known hepatotoxins is