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

BJÖRN QUISTORFF
Department of Biochemistry, University of Copenhagen,
The Panum Institute, Blegdamsvej 3, 2200 Denmark

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 (Hausinger, 1983; Quistorff, 1985; Pöösö 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 upon 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-Henseleit 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 (for 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 μmol/ml (Williams & Woodbury, 1971; Christensen & Folkle, 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⁺ concentrations in the eluate from both the periportal and the perivenous part of the liver. K⁺ and Na⁺ concentrations 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

Vol. 15
was an uptake of Na⁺ by the liver, peaking also in the 10–25s fraction and consistently approx. 2.3 μmol/ml larger than the K⁺ release. Under the digitonin treatment the cells are losing anions in the form of metabolites, notably ATP, ADP and P, (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 digitonin perfusion technique. The following observations have been made. Continuous infusion of digitonin 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 digitonin perfusion was started with normal perfusion direction or with retrograde perfusion (Quistorff et al., 1985). This notion is supported by the fact that even the completely decolorized liver, having lost more than 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 → cava followed by cava → porta or vice versa; Quistorff & Grunnet, 1987), and since also significant interanimal 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 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).

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

Livers of fed, female Wistar rats were perfused by the dual-digitonin-pulse technique (perfusion scheme B in Fig. 1 of Quistorff & Grunnet, 1987) 8 with digitonin (4 mg/ml). Eight periportal and eight perivenous eluate fractions were collected over the time intervals indicated after digitonin 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 μmol/ml of cytosol, and using as blank the K⁺ concentration in the Krebs-Henseleit perfusate. Results are means ± S.D. (n = 8). Statistical significance (paired t-test): *P < 0.01 periportal eluate versus perivenous eluate.

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>Elution fraction . . .</th>
<th>0 5s</th>
<th>5 10s</th>
<th>10 15s</th>
<th>15 20s</th>
<th>20 25s</th>
<th>25 30s</th>
<th>30 35s</th>
<th>35 40s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periportal cytosol</td>
<td>209 ± 75.4</td>
<td>24.8 ± 24.6</td>
<td>8.92 ± 1.58</td>
<td>8.41 ± 3.42</td>
<td>11.2 ± 1.22</td>
<td>14.6 ± 1.35</td>
<td>18.6 ± 1.81</td>
<td>22.9 ± 3.51</td>
<td></td>
</tr>
<tr>
<td>Perivenous cytosol</td>
<td>143 ± 147</td>
<td>30.6 ± 9.45</td>
<td>12.8* ± 2.40</td>
<td>15.0* ± 1.57</td>
<td>20.7* ± 2.81</td>
<td>29.1* ± 5.58</td>
<td>38.3* ± 10.2</td>
<td>47.7* ± 12.3</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.01 periportal eluate versus perivenous eluate.

Table 2. K⁺, Na⁺ and protein elution with the dual-digitonin-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 ± 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.

<table>
<thead>
<tr>
<th></th>
<th>K⁺ (μmol/ml)</th>
<th>Na⁺ (μmol/ml)</th>
<th>Protein (mg/ml)</th>
<th>Protein:K⁺ (mg/μmol of K⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periportal eluate</td>
<td>14.9 ± 1.72</td>
<td>-17.8 ± 3.19</td>
<td>9.51 ± 1.70</td>
<td>0.62 ± 0.11</td>
</tr>
<tr>
<td>Perivenous eluate</td>
<td>10.3 ± 1.50*</td>
<td>-12.6 ± 2.85*</td>
<td>5.62 ± 0.72*</td>
<td>0.52 ± 0.06</td>
</tr>
</tbody>
</table>

*P < 0.05 periportal eluate versus perivenous eluate.
significantly zonated, although this is not known at present. Accepting K⁺ as a non-zonated 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 dual-digitonin-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/μmol of K⁺. This value may be converted to mg of cytosolic protein/ml of cytosol, assuming a cytosolic K⁺ concentration of 160 μmol/ml (Williams & Woodbury, 1971; Christensen & Folkke, 1984). According to Weibel et al. (1969) the rat liver contains 0.444 ml of cytosolic ground substance/mL of liver wet wt. Thus the conversion factor is:

\[
\frac{\text{mg of total protein/g of liver biopsy wet wt.}}{\text{mg of eluate protein/μmol of K⁺} \times 160 \times 0.44}
\]

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

\[
A_{bi} = vA_{po} + (1 - v)A_{pw}
\]

where v is the volume fraction of the hepatocytes where the specific activity of the enzyme in question is \(A_{po}\) (i.e. the specific activity found in periportal (eluate), and conversely (1 - v) the volume fraction where the activity is \(A_{pw}\). \(A_{bi}\) 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 zonated 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 glutamine 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.

I thank Liss Immerdal for skillful technical assistance. Part of this investigation was supported by Statens Lægevidenskabelige Forskningsråd and T. Holms Fond.

Christensen, L. 0. (1971) J. Cell. Biol. 50, 20D-55D
Lille, R. D. (1940) Stain Technol. 15, 11-22

Received 29 December 1986

Oxygen gradients: the problem of hypoxia

HERBERT DE GROOT and THOMAS NOLL
Institut für Physiologische Chemie I der Universität Düsseldorf, Moorstrasse 5, D-4000 Düsseldorf, Federal Republic of Germany

Oxygen gradients in liver

Due to O₂ consumption inter- and intra-cellular O₂ gradients are formed in tissues. In liver there are steep intercellular O₂ gradients along the sinusoids from the portal to the central regions of the liver lobules (Sies, J. 1977; Ji et al., 1982). As demonstrated by use of micro-needle electrodes and multwire surface electrodes, the actual pO₂ in liver ranges between 1 and 60 mmHg with mean values around 22 mmHg (Fig. 1).

From comparison of pO₂ of half-maximal changes (pO₂ values) of oxidation-reduction states of cytochrome c in isolated liver cells and mitochondria, Jones (1984) estimated an intracellular O₂ gradient of about 4 mmHg from the extracellular medium towards the outer mitochondrial membrane under O₂-limiting conditions. From the O₂ dependence of the hepatocellular O₂ uptake (Fig. 1) an O₂ gradient of maximally 5 mmHg from the extracellular space towards the mitochondrial membrane can be concluded. Comparison of pO₂ 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 pO₂ for the induction of lipid peroxidation by carbon tetrachloride (CCl₄) again in isolated hepatocytes and isolated microsomes (de Groot & Noll, 1986; Noll & de Groot, 1984), reveal that O₂ 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 O₂ gradient appears to exist only towards the mitochondrial compartment and, furthermore, this O₂ gradient must be predominantly at the regions immediately surrounding the mitochondria.

Oxystat system

As pointed out, steep intracellular O₂ 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 O₂ conditions. We have developed a special incubation apparatus for isolated cells and subcellular fractions, the oxystat system, which com-