Dual-digitonin-pulse perfusion

Concurrent sampling of periportal and perivenous cytosol of rat liver for determination of metabolites and enzyme activities

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A previously described digitonin-perfusion technique [Quistorff, Grunnet & Cornell (1985) *Biochem. J.* **226**, 289–297], by which intracellular material of rat liver could be liberated, has been refined, now allowing release of cytosol of high purity from both periportal and perivenous parts of the same liver. The cytosolic fractions are obtained by perfusing the liver for short intervals (10–20 s) with digitonin (4–5 mg/ml), first in the normal perfusion direction and then, after an interval of 1–2 min, in the retrograde direction, the eluate being collected during and after both intervals. The technique is termed ‘dual-digitonin-pulse perfusion’. The eluate fractions showed a peak specific activity of the cytosolic enzymes alanine aminotransferase (ALAT), lactate dehydrogenase (LDH) and pyruvate kinase (PK) of 3–5-fold higher than obtained in a biopsy from the same liver. For glutamine synthetase (GS) a 10-fold higher specific activity was obtained. Zonation, defined as the ratio of the specific activities in periportal and perivenous eluates, of ALAT, LDH and PK was 10, 1.7 and 0.70 respectively. Zonation of GS was < 0.01. These factors may be modified by a slight zonation of cytosolic protein of 1.2–1.3. Peak concentrations in the eluate of ATP, ADP, P_i , NAD^+ and glycerol 3-phosphate were 32.5 ± 11.4 , 19.9 ± 4.3 , 71.9 ± 25.4 , 2.41 ± 0.83 and 6.84 ± 2.74 nmol/mg of protein for periportal eluates. There was no difference between periportal and perivenous eluates except for glycerol 3-phosphate, which was significantly higher in perivenous eluates, 12.8 ± 4.5 nmol/mg of protein.

INTRODUCTION

We have previously shown that digitonin perfusion of rat liver enabled release and sampling of intracellular material from different compartments of either the periportal or the perivenous zone of the liver microcirculation (Quistorff *et al.*, 1985; Quistorff, 1985). The results suggested that the effect of digitonin perfusion was actually a titration of cholesterol by the digitonin along the sinusoids, with the transit time of the ‘digitonin front’ being defined by the amount of digitonin delivered relative to the cholesterol content of the liver. It was clear, however, from these studies that the specificity of the technique in terms of zonal release was highly dependent on an equal perfusion of all sinusoids. Thus a complete zonal separation would require the digitonin front to move in concert in all sinusoids. Consequently, in the practical experiment the zonal specificity was high only for the initial eluate, and the technique therefore required sampling of perivenous and periportal material to be done from two different livers.

The method has now been improved, allowing separate sampling of cytosolic eluate from both the periportal and the perivenous area of the same liver within a 3–4 min experiment. We report here results obtained with two versions of this new technique on the concentration of some metabolites, total protein and enzyme activities of cytoplasmic samples from the periportal and the perivenous part of the microcirculation of the same liver.

MATERIALS AND METHODS

Reagents

All chemicals were of analytical grade. Enzymes and coenzymes were from Boehringer, Mannheim, Germany. Digitonin was from Sigma Chemical Co., St. Louis, MO, U.S.A., and was purified as previously described (Quistorff *et al.*, 1985).

Animals

Wistar female albino rats weighing 170–190 g were used. Animals had free access to water and food pellets and were kept on a 12 h-dark 12 h-light cycle, changing from dark to light at 09:00 h. Experiments were carried out between 10:00 h and 12:00 h. The animals were anaesthetized with sodium pentobarbital (400 μ l, 50 mg/ml) given as an intraperitoneal injection about 30 min before the experiment. At 5–10 min before surgery the animals started breathing O_2/CO_2 (19:1).

Perfusion system

Essentially the perfusion system described in Quistorff (1985) was used. However, instead of the manually operated valves, a system consisting of a number of miniature three-way electromagnetic valves were used (model S305-03; Sirai Inc., Milan, Italy). In this way the switching of perfusate composition, flow rate and flow direction could be performed faster and more precisely, and also the dead space of the tubing system could be

Abbreviations used: ALAT, alanine aminotransferase (EC 2.6.1.2); PK, pyruvate kinase (EC 2.7.1.40); LDH, lactate dehydrogenase (EC 1.1.1.27); GS, glutamine synthetase (EC 6.3.1.2); GlDH, glutamate dehydrogenase (EC 1.4.1.3).

decreased to about 0.45 ml. A fraction collector for sampling of eluate was operated in concert with the electromagnetic valves. The perfusion and fraction-collection system may be operated either semi-automatically or fully computerized. Oxygen electrodes (Clark-type; type 5331 Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) were inserted in the perfusion circuitry close to the inlet and outlet of the liver. The electrode chambers were thermostatically controlled and magnetically stirred, and had a dead space of about 250 μ l. Inlet oxygen partial pressure was 60–73 kPa (450–550 Torr), and flow was adjusted between 20 and 26 ml/min to give an outlet oxygen partial pressure of 2.7–9.3 kPa (20–70 Torr). The perfusate was Krebs–Henseleit (1932) bicarbonate buffer with or without 10 mM-glucose and digitonin (4–5 mg/ml). Ca^{2+} was omitted from the perfusate during and after the digitonin treatment (cf. perfusion schemes B and C, Fig. 1), since it had been observed in other experiments that Ca^{2+} promotes the release of mitochondrial enzymes (Cornell *et al.*, 1985). The buffer was equilibrated with O_2/CO_2 (19:1) at 36–37 °C.

Perfusion schemes

The liver remained *in situ* during the perfusion. Three different perfusion schemes were followed; see Figs. 1(a)–1(c). With all schemes the initial perfusion interval lasted about 10 min, including the sampling of a biopsy of the liver, as previously described (Quistorff, 1985). Scheme A was a one-way perfusion with a constant continuous load of digitonin, essentially as described previously (Quistorff *et al.*, 1985). Schemes B and C, however, applied changes of flow direction with two short digitonin pulses, the ‘dual-digitonin-pulse technique’. In scheme B, flow rate was decreased to 12 ml/min after the initial perfusion interval. The system was allowed to stabilize for 10 s, and the perfusate was switched to the digitonin-containing perfusate for 8–10 s, and then back to Krebs–Henseleit buffer without digitonin for an additional 5 s in order to clear the tubing of digitonin. Then simultaneously flow rate was increased to 20 ml/min, flow direction was switched to cava $\rightarrow$ porta and sampling of eluate started. The sampling was continued for 20–40 s, depending on the number of samples needed. After an additional 30 s of washout, the above procedure was repeated, but with opposite direction of flow and with 15 s of digitonin perfusion, as explained in Fig. 1(b). After the sampling of the last eluate fraction, the whole liver was removed and homogenized as described below. Perfusion scheme C is similar to B, but somewhat simpler: flow was not reversed before sampling, and flow rate was kept at 20 ml/min after the initial perfusion interval (see Fig. 1c). For both perfusion schemes B and C the sequence I $\rightarrow$ II (see Fig. 1) was inverted in half of the experiments.

Measurements of enzyme activity

The biopsies were homogenized in 10–20 vol. of ice-cold buffer containing 25 mM-glycylglycine, pH 7.4, 150 mM-KCl, 5 mM- MgSO_4 , 5 mM- Na_2EDTA , 10 mM-mercaptoethanol and 0.2% defatted bovine serum albumin in a Potter–Elvehjem homogenizer, followed by sonication for 10 s at 30 W (Sonifier B-12; Branson, Dansbury, CT, U.S.A.). The homogenate was centrifuged for 15 min at 16000 g at 4 °C and the supernatant used for enzyme assays and for total protein measure-

ment. Eluate samples in which enzyme activity was measured were collected as 3–5 s fractions (1–1.7 ml of eluate) in 200 μ l of 40 mM-dithiothreitol, and measurements were performed on supernatants after centrifugation as above. Activities of ALAT, LDH and GIDH were measured by standard techniques as described in Bergmeyer (1974). Total PK was measured at pH 7.6 in an assay buffer containing 0.1 M-triethanolamine, 0.1 M-KCl, 2.5 mM- MgSO_4 , 1.3 mM-ADP, 0.17 mM-NADH, 0.2 mg of defatted bovine serum albumin/ml and 9 units of LDH/ml. The reaction was initiated by addition of phosphoenolpyruvate to 4 mM. GS was measured as described by Schousboe (1982), protein as described by Lowry *et al.* (1951), and glucose-6-phosphatase as described by Hers & Van Hoof (1966), with the modifications introduced by Gennser *et al.* (1971). Amyloglucosidase was measured as described by Lundquist (1971).

Measurements of metabolites

Eluate fractions for metabolite measurements were collected as 1–1.7 ml fractions in 200 μ l of ice-cold 2 M- HClO_4 or, for triacylglycerol determination, in 200 μ l of 2 M-KOH. After centrifugation of the fractions (1000 g for 10 min), supernatants were neutralized with 4 M-KOH/0.2 M-triethanolamine buffer to pH 6.5–7.0. Glucose and glycerol 3-phosphate were measured spectrophotometrically, ATP and ADP fluorimetrically as described by Lowry & Passonneau (1972) and glycogen as described by Roehrig & Allred (1974). NAD^+ was measured fluorimetrically by the method of Cornell (1983), and triacylglycerols as glycerol after alkaline hydrolysis (Bergmeyer, 1974). P_i was measured as described by Penney (1976). Results are given as means $\pm$ s.d. Enzyme-activity units are given as μ mol/min.

RESULTS

Elution of protein

With perfusion scheme A (Fig. 1) a constant continuous load of digitonin was applied to the liver. Table 1 shows total protein-elution data with this technique for porta $\rightarrow$ cava and cava $\rightarrow$ porta perfusions. Perfusion for 120 s with digitonin (5 mg/ml) eluted about 75% of the total protein. The maximum concentration reached for porta $\rightarrow$ cava and cava $\rightarrow$ porta elution was 35.0 ± 3.6 and 34.5 ± 4.0 mg/ml, at 40.1 ± 4 and 42.3 ± 7.5 s respectively. Compared with the biopsy, this maximum concentration (mg/ml) amounts to 17% of the protein content (mg/g wet wt.) in the intact liver, assuming a liver weight of 6.5 g, i.e. 3.5% of body weight (Weibel *et al.*, 1969). The amount of eluted protein/ml was somewhat lower in all fractions with cava $\rightarrow$ porta than with porta $\rightarrow$ cava perfusion, although not statistically different.

Table 2 shows the concentration profiles for protein elution with the dual-digitonin-pulse perfusion technique (schemes B and C, Fig. 1), in which the periportal and perivenous samples were obtained from the same liver, as opposed to scheme A. Compared with perfusion scheme A, a somewhat lower peak concentration was obtained, owing to the much smaller amount of digitonin applied in these experiments. As with scheme A, the protein concentration was higher with periportal elution, giving

Fig. 1. Perfusion schemes for digitonin treatment of rat liver

Livers of fed female rats were perfused *in situ* at 37 °C with Krebs–Henseleit bicarbonate buffer equilibrated with O₂/CO₂ (19:1). The initial biopsy was the small lower lobe, above the right kidney, whereas for the ‘end biopsy’ the whole liver was removed. In the interval labelled ‘Digitonin’ or ‘D’, digitonin, 5 mg/ml for schemes A and C, and 4 mg/ml for scheme B, was included in the perfusate. The order of the perfusion sequence I→II was reversed in half of the experiments with schemes B and C. Note the different time scale in scheme C. Abbreviations: P→C, porta→cava; C→P, cava→porta.

an average periportal/perivenous concentration ratio of about 1.3 for both schemes B and C; however, this was statistically significant only for one sampling interval (see Table 2). Whether this apparent periportal zonation of cytosolic protein is real will require measurement against some other cytosolic reference substance of known zonal distribution.

The amount of protein eluted with schemes B and C was 101.1 ± 16.2 and 79.0 ± 22.7 mg/g wet wt., corresponding to 60% and 44%, respectively, of the total protein, calculated from the measured biopsy value. Furthermore, we found that these eluates contained less than 2% of the total activity of the mitochondrial marker enzyme GIDH (results not shown). The eluate may therefore be considered ‘pure cytosol’, diluted by the elution buffer, and zonation of cytosolic enzymes or

other cytosolic constituents may be quantified as the ratio of the specific activity of the particular enzyme of periportal and perivenous eluate fractions.

Elution of enzymes

Table 3 shows measurements of ALAT, LDH and PK activities in the eluate for 120 s porta→cava and cava→porta perfusions according to scheme A, Fig. 1. Three characteristic features may be observed from these data. (1) The highest specific activity was obtained initially for ALAT and LDH with porta→cava elution and for PK with cava→porta elution. (2) The maximum specific activity of the three enzymes was 4–5-fold higher than the value found in the homologous liver biopsy. (3) The zonation of ALAT, LDH and PK was 11, 1.5 and 0.75, respectively, calculated as the ratio of the specific

Table 1. Elution of protein from rat liver with continuous digitonin perfusion

Livers were perfused by scheme A (Fig. 1a) with digitonin (5 mg/ml) with either normal flow direction (porta→cava) or reverse flow (cava→porta). A biopsy was taken before and after the digitonin perfusion. Eluate fractions of 5 s were collected and the integrals given were obtained by manual integration of the elution profiles. Results are means ± s.d. ($n = 3$).

	Protein in eluate (mg/fraction)						Protein in biopsy		
	0–30 s	30–45 s	45–60 s	60–90 s	90–120 s	0–120 s total eluate	(mg/g)		Difference (× 6.5) (mg/liver)
							Start	End	
Porta→cava	93.4 ± 3.6	164 ± 15	161 ± 13	267 ± 12	183 ± 19	869 ± 37	190 ± 14	52.6 ± 4.1	891 ± 117
Cava→porta	72.2 ± 3.0	155 ± 18	152 ± 27	255 ± 63	179 ± 49	813 ± 102	209 ± 7.0	52.5 ± 12	1014 ± 52

Table 2. Protein concentration of cytosolic eluate of periportal and perivenous parts of the microcirculation of perfused rat liver

Livers were perfused by scheme B or C (Fig. 1) with digitonin, 5 mg/ml with scheme C and 4 mg/ml with scheme B. A biopsy was taken before and after the digitonin perfusion. Eluate fractions of 4–5 s were collected. The results are means ± s.d., expressed as mg of protein/ml of eluate ($n = 7$ and 6 for scheme B and C respectively). * $P < 0.05$, evaluated with paired-data t test, periportal eluate versus perivenous eluate for the same liver.

Perfusion scheme	Eluate (mg/ml)						Biopsy (mg/g wet wt.)	
B	Periportal			Perivenous			Start	End
	4–8 s	12–16 s	20–24 s	4–8 s	12–16 s	20–24 s	176.3 ± 27	96.8 ± 16
	4.63 ± 2.39	20.1 ± 7.65	13.2 ± 2.93*	3.69 ± 2.17	16.1 ± 1.64	8.49 ± 1.64		
C	Periportal			Perivenous			Start	End
	10–15 s	20–25 s	30–35 s	10–15 s	20–25 s	30–35 s	166.7 ± 24.2	66.4 ± 8.5
	6.37 ± 2.12	17.9 ± 3.75	14.2 ± 3.6	4.33 ± 2.39	17.0 ± 4.49	11.9 ± 2.21		

Table 3. Specific activity of ALAT, LDH and PK in eluates from rat liver perfused continuously with digitonin

Livers were perfused according to scheme A (Fig. 1) with digitonin (5 mg/ml). Eluate fractions of 5 s were collected, and the integrals given in the table were obtained by manual integration of the elution profiles. The column 'Max. value' represents the maximum activity measured in the eluate. Results are means ± s.d. for three experiments in each flow direction [porta→cava (P→C) and cava→porta (C→P)], expressed as munits/mg of protein. * $P < 0.05$ and † $P < 0.01$, evaluated with Student's t test, for P→C versus C→P. Same experiments as in Table 1.

		Specific activity in:							
		Eluate						Biopsy	
		0–30 s	30–45 s	45–60 s	60–90 s	90–120 s	Max. value	Start	End
ALAT	P→C	496 ± 53†	595 ± 115*	344 ± 94	203 ± 62	103 ± 33	912 ± 180†	206 ± 28	24 ± 19
	C→P	45 ± 15	258 ± 135	381 ± 125	381 ± 41†	274 ± 75†	408 ± 64	188 ± 11	73 ± 5
LDH	P→C	4081 ± 351	6214 ± 494†	4608 ± 437	3297 ± 442	1870 ± 500	8065 ± 1055†	2374 ± 218	152 ± 47
	C→P	3155 ± 899	4486 ± 636	4560 ± 558	4144 ± 1021	2988 ± 956	4847 ± 589	2322 ± 63	213 ± 96
PK	P→C	316 ± 84	581 ± 117	468 ± 64	322 ± 59	180 ± 65	632 ± 172	239 ± 29	13 ± 10
	C→P	466 ± 61*	699 ± 116	506 ± 130	320 ± 148	164 ± 145	952 ± 44†	221 ± 98	16 ± 6

Table 4. Enzyme activity in cytosolic eluates of the periportal (PP) and perivenous (PV) parts of the liver microcirculation

Livers were perfused by scheme C (Fig. 1) and eight 5 s fractions sampled. Even-numbered fractions were collected in 200 μ l of 40 mM-dithiothreitol for measurement of enzyme activity and total protein, and odd-numbered in 200 μ l of 2 M-HClO₄ for metabolite measurements (cf. Table 5). In three experiments perfusion was with the sequence I $\rightarrow$ II and in three the opposite sequence, II $\rightarrow$ I (see Fig. 1c). The results are means $\pm$ s.d. for all six experiments, given as munits/mg of protein. * P < 0.05 and † P < 0.001, evaluated with paired-data t test for PP versus PV eluate from the same liver.

		Specific activity in:					
		Eluate				Biopsy	
		15–20 s	25–30 s	35–40 s	45–50 s	Start	End
ALAT	PP	926 $\pm$ 145	888 $\pm$ 203	742 $\pm$ 281	578 $\pm$ 257	183 $\pm$ 32.0	108 $\pm$ 39.0
	PV	78.6 $\pm$ 51.5†	121 $\pm$ 70.8†	156 $\pm$ 97.1	148 $\pm$ 87.2†	–	–
LDH	PP	6360 $\pm$ 1396	7523 $\pm$ 1313	6410 $\pm$ 923	4970 $\pm$ 779	2360 $\pm$ 500	1120 $\pm$ 320
	PV	2943 $\pm$ 1104†	4613 $\pm$ 704†	4855 $\pm$ 811	3821 $\pm$ 522†	–	–
PK	PP	909 $\pm$ 98.4	871 $\pm$ 65.1	810 $\pm$ 108	709 $\pm$ 111	339 $\pm$ 51.0	208 $\pm$ 71.0
	PV	1222 $\pm$ 406*	1221 $\pm$ 443	1032 $\pm$ 32*	872 $\pm$ 123*	–	–
GS	PP	4.78 $\pm$ 2.06	2.94 $\pm$ 0.79	3.78 $\pm$ 0.56	3.84 $\pm$ 1.74	30.7 $\pm$ 4.10	2.67 $\pm$ 0.68
	PV	529 $\pm$ 170†	239 $\pm$ 59.6†	156 $\pm$ 36.4†	124 $\pm$ 23.6†	–	–

Table 5. Metabolite concentrations of cytosolic eluates of periportal and perivenous parts of liver microcirculation

Eluate fractions of 4–5 s were sampled in HClO₄ after digonin perfusion (4 mg/ml) by perfusion scheme B or C (Fig. 1). Results are means $\pm$ s.d., expressed as nmol/mg of protein in the fraction of maximum protein concentration, for porta $\rightarrow$ cava and cava $\rightarrow$ porta elution (cf. Table 2) (n = 7 and 6 for perfusion schemes B and C respectively). * P < 0.05, evaluated with paired-data t test, for periportal versus perivenous eluate. † P < 0.05, evaluated for perfusion scheme B versus C.

Perfusion scheme . . .	Metabolite concentration in eluate			
	Periportal		Perivenous	
	B	C	B	C
ATP	32.5 $\pm$ 11.4	24.3 $\pm$ 14.5	44.1 $\pm$ 22.0†	19.5 $\pm$ 13.5
ADP	19.9 $\pm$ 4.31	16.8 $\pm$ 4.29	16.3 $\pm$ 2.74†	23.6 $\pm$ 9.67
P _i	71.9 $\pm$ 25.4	61.4 $\pm$ 13.7	66.6 $\pm$ 25.7	54.7 $\pm$ 17.8
ATP $\times$ 10 ³	1764 $\pm$ 876	1396 $\pm$ 729	2885 $\pm$ 2294	1585 $\pm$ 483
ADP $\times$ P _i	1.63 $\pm$ 0.36	1.34 $\pm$ 0.67	1.84 $\pm$ 0.60†	1.03 $\pm$ 0.69
ATP/ADP	2.41 $\pm$ 0.83	–	2.45 $\pm$ 1.26	–
NAD ⁺	6.84 $\pm$ 2.73	–	12.8 $\pm$ 4.51*	–
Glycerol 3-phosphate				

activity of the 0–30 s fraction for porta $\rightarrow$ cava and cava $\rightarrow$ porta perfusion. These data are in agreement with results of micro-dissection studies (Welsh, 1972; Shank *et al.*, 1959; Guder & Schmidt, 1976).

In experiments with perfusion schemes B and C (cf. Fig. 1), cytosol of the periportal and perivenous part of the same liver was eluted separately. With perfusion scheme B the digonin is 'installed' in the periportal or perivenous part of the liver microcirculation, and then flushed back out again. One would therefore not expect major differences in enzyme specific activities between different fractions of this washout, as was indeed observed for ALAT, LDH and PK, which maintained an unchanged specific activity within the first 24 s of washout (results not shown). The zonation of these three enzymes with perfusion scheme B was 9.8, 1.8 and 0.72 respectively. With scheme C, where sampling is begun simultaneously with the digonin pulse without flow

reversal after the pulse, the values for zonation of ALAT, LDH and PK for the initial eluate were not significantly different from those found with scheme B. The zonation declined, however, to 3.9, 1.31 and 0.81 respectively for the 45–50 s fraction (Table 4).

In these experiments, GS was also measured, and the biopsy value was similar to previous findings (Tate *et al.*, 1972). We observed a striking zonation of approx. 0.01 for this enzyme (Table 4), in agreement with immunofluorescence studies by Gebhardt & Mecke (1983), who demonstrated that GS is located exclusively in a few rows of cells adjacent to the terminal hepatic vein, and with studies by Häussinger (1983) on glutamine and ammonia metabolism. Furthermore, it may be observed that, although only about 40% of the total ALAT and PK activities (difference between start and end of biopsy) were eluted, this value was close to 100% for GS. Our proposed model of action of the digonin perfusion as

Fig. 2. Metabolites and enzyme elution pattern of rat liver during continuous digitonin perfusion

The liver of a fed rat was perfused with normal direction of flow as described in Fig. 1(a) with digitonin (5 mg/ml); 1–1.5 ml fractions were sampled in 200 μ l of 40 mM-dithiothreitol or 500 μ l of 2 M-HClO₄ for enzyme-activity analysis or metabolite measurements respectively. (a) Enzyme activities: $\circ$, PK (units/ml $\times 10$); $\triangle$, amyloglycosidase (munits/ml); ∇ , glucose-6-phosphatase (units/ml $\times 10$); $\blacksquare$, glutamate dehydrogenase (munits/ml $\times 10^{-1}$). (b) Metabolite concentrations: $\circ$, glycogen (mM glycosyl units); $\triangle$, glucose (mM); $\blacksquare$, triacylglycerol (μ M-glycerol $\times 10^{-1}$). This is a typical experiment from a series of six.

an actual titration of cholesterol along the sinusoid (Quistorff *et al.*, 1985) would in fact predict such a result for an enzyme with exclusive perivenous location.

For total protein and enzymes, the zonation observed with the dual-digitonin-pulse technique (schemes B and C, Fig. 1) was not affected significantly by the perfusion sequence (porta $\rightarrow$ cava followed by cava $\rightarrow$ porta, or vice versa).

Elution of metabolites

In addition to enzymes, it is also possible to obtain other high- M_r constituents of the cytosolic compartment

of the liver as well as low- M_r metabolites in good yield. Fig. 2 shows the elution of glycogen and triacylglycerol (panel b) compared with enzyme elution (panel a) measured in the same experiment, for which perfusion scheme A was used. Glycogen was eluted as a broad peak, somewhat delayed compared with PK, but comparable with the elution of amyloglycosidase and glucose-6-phosphatase. It may be noted that the mitochondrial enzyme GIDH was eluted considerably more slowly than glycogen. The peak concentration of glycogen ranged from 20 to 100 mM, with an average of 42.9 ± 40 ($n = 6$). There may be some glycogen breakdown during elution, since the glucose elution profile (Fig. 2b) seemed to follow that of glycogen, apart from a small initial rise. However, the peak concentration of glucose was less than 2 mM in all experiments. Glycogen was not zoned.

Triacylglycerol was eluted faster than glycogen and displayed a reproducible biphasic elution profile, with a very rapid initial peak after 16 ± 5 s at a peak value of 85 ± 50 μ M. The main fraction was eluted as a broad peak between 50 and 120 s, with a peak concentration of 133 ± 45 μ M ($n = 6$). The biphasic elution of triacylglycerol may be the result of release of lipases together with the triacylglycerol and/or it may reflect release of various triacylglycerol pools. Triacylglycerol showed no zonation.

Low- M_r metabolites

Fig. 3 shows the concentration profiles for some low- M_r metabolites obtained with the dual-digitonin-pulse technique (perfusion scheme B, Fig. 1). With this technique there was a time lapse from the start of digitonin treatment to sampling in HClO₄ of the first fraction of 15 s and 20 s for periportal and perivenous eluate, respectively. The fractions were collected over 4 s intervals. Under these conditions the peak concentration of NAD⁺, ADP, ADP and protein (Fig. 3) occurred in the fraction 8–12 s, whereas P_i and glycerol 3-phosphate continued to increase, with peaks in the 16–24 s and 16–20 s fractions respectively. When expressed per mg of protein, the values tended towards a plateau at the maximum value, coinciding with the maximum protein concentration, except for P_i, which continued to increase with time of elution. This suggests that a hydrolytic activity in the cells may be activated by the digitonin treatment, as was indeed observed by Katz & Wals (1985). This hydrolytic process does not take place to any significant degree after the liberation of the eluate from the liver, since keeping the eluates for an additional 60 s at 22 °C before quenching in HClO₄ (see the Materials and methods section) does not change ATP, ADP, glycerol 3-phosphate or P_i.

Table 5 shows the metabolite concentrations in the eluates obtained with both perfusion schemes B and C. The values in the fraction of maximum protein concentration are given. The only significant difference observed between perivenous and periportal eluate was glycerol 3-phosphate, which showed a 2-fold perivenous zonation.

Perfusion scheme B gave higher ATP and lower ADP values than did scheme C, but without any difference in P_i. Another important difference between schemes B and C was that the results obtained with scheme C were clearly dependent on the perfusion sequence, i.e. whether the periportal zone was treated with digitonin before the

Fig. 3. Elution profiles for some metabolites and total protein after short periportal and perivenous pulses of digitonin in rat liver

The liver was perfused as described in Fig. 1(b) with pulses of digitonin (4 mg/ml). First a porta→cava (p→c) pulse of 10 s was given, then flow was reversed and eight 3 s eluate fractions were sampled in 200 μ l of 2 M-HClO₄. Then after a 60 s interval a 15 s digitonin pulse was given, flow was reversed (c→p) and another eight fractions were sampled as before. This is a typical experiment out of seven.

perivenous zone, or vice versa (the I→II or II→I sequences in Fig. 1). With scheme C (sequence I→II) the ATP/ADP ratio was 1.84 ± 0.41 and 0.54 ± 0.19 for periportal and perivenous eluates respectively, whereas with the reverse sequence, i.e. II→I, the two zones gave 1.03 ± 0.69 and 1.34 ± 0.67 respectively, which is statistically different from the former. This dependence is not seen with scheme B.

DISCUSSION

Comparison of the three perfusion schemes

This paper presents a further development of the digitonin perfusion technique (Quistorff *et al.*, 1985), now allowing sampling of cytosolic enzymes and metabolites from the periportal as well as the perivenous zone of the same liver in a 3 min experiment. Data with

three perfusion schemes are presented (see Fig. 1). Perfusion scheme A is the original technique, and schemes B and C represent two versions of a new dual-digitonin-pulse perfusion technique, where C is technically simpler than B. The aim of techniques B and C is to give amounts of digitonin small enough to destroy the plasma membrane of cells only at the entrance of the microcirculation (periportal or perivenous region), without affecting the remaining part of the liver. The principal difference between schemes B and C is that with scheme C the intracellular material liberated from the upstream region passes through the downstream region, which is thus exposed, albeit briefly, to a number of metabolites and enzymes normally not encountered extracellularly, before the subsequent elution of this region after inversion of the flow. This is not the case with perfusion scheme B, where flow is reversed immediately after the digitonin pulse. This important methodological difference does not seem to play any role in the study of enzyme and total protein zonation; it may, however, be the cause of the lower ATP, ADP and ATP/ADP ratio observed with scheme C than with scheme B (Table 5). We also observed that these parameters with scheme C were significantly higher in the region eluted first, independently of whether it was the periportal or the perivenous region. P_i showed the same pattern, although not statistically significant. This dependence of elution sequence (sequence I $\rightarrow$ II or II $\rightarrow$ I; see Fig. 1) was not seen with perfusion scheme B for any of the parameters measured. It is recommended that, when using the dual-pulse-digitonin perfusion technique, experiments with both elution sequences (I $\rightarrow$ II and II $\rightarrow$ I) should be carried out.

Protein and enzyme elution

According to Mahler & Cordes (1966), a total of 200 mg of protein/g wet wt. in rat liver is composed of 60, 50, 40, 30 and 10 mg as cytosolic, mitochondrial, microsomal, nuclear and lysosomal protein respectively. Thus if 'pure cytosolic protein' was eluted, one would expect an approx. 3-fold higher specific activity for a cytosolic, non-zonated, enzyme compared with the biopsy value. For zonated enzymes the specific activity would be expected to be even higher in the high-activity zone, depending on the degree of zonation. In the present experiments the specific activity of the cytosolic enzymes ALAT, PK and LDH reached a peak activity approx. 4–5-fold higher than the value found in the 'start' biopsy for the same liver. For the very highly zonated enzyme GS, this factor was about 10 (Tables 3 and 4). These results, taken together with the fact that less than 2% of the mitochondrial marker enzyme GIDH is eluted, seem to indicate that the initial eluates (0–30 s) with schemes A and C and the total eluate with B are in fact an almost pure cytosolic fraction.

Defining zonation as the ratio between the specific activity measured in these initial periportal and perivenous eluate fractions, we find zonation of ALAT, PK, LDH and GS of 11, 0.75, 1.8 and 0.01 respectively, which is in agreement with the findings by other, more laborious, methods involving micro-dissection and micro-analysis or immunofluorescence (Jungermann & Katz, 1982; Gebhardt & Mecke, 1983). The fact that the zonation of GS may be measured as 0.009 (Table 4) indicates that the zonal specificity of the elution

technique is indeed better than 1% for the initial fraction, since the value measured in the periportal eluate, 4.78 munits/mg of protein, is less than 1% of the perivenous value measured.

Estimate of cytosolic metabolite concentrations

The eluted intracellular material will be diluted by the perfusate. An estimate of this dilution factor may be obtained as follows: PK is a cytosolic enzyme with little zonation. In the present experiments PK activity per g wet wt. was measured in a biopsy and in the eluates from the same liver. The volume of the cytosolic ground substance was estimated by Weibel *et al.* (1969) as 0.444 ml/g wet wt. of liver, allowing calculation of the ratio between the PK activity per ml of ground substance in the biopsy and PK activity per ml of eluate. This value represents the dilution factor for cytosolic constituents. Applying the dilution factor for the individual fractions to the data of Table 5, the concentration of cytosolic ATP may be calculated as 3.54 ± 1.58 and 4.26 ± 0.79 μ mol/ml of cytosolic ground substance for periportal and perivenous eluate respectively, and similarly for ADP as 2.53 ± 1.79 and 2.26 ± 0.91 respectively.

All in all, the dual-digitonin-pulse perfusion technique, preferably scheme B, described in the present paper seems to be a very competitive alternative for many applications, in particular the study of the zonation pattern of cytoplasmic enzymes and metabolites of liver.

We thank Lissi Immerdal and Marte Nord for skilful technical assistance. Part of this investigation was supported by T. Holms Fond.

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Received 5 August 1986/20 October 1986; accepted 27 November 1986