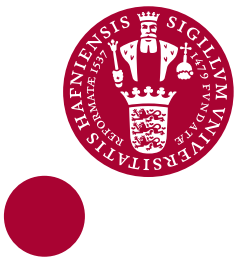

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THE USE OF A HEPATOCYTE COLUMN IN THE STUDY OF METABOLIC ZONATION IN THE LIVER

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INTRODUCTION

The microcirculation of the mamalian liver is unique in the sense that the capilaries (sinusoids) form functional units in which flow in adjacent sinusoids is concurrent and has adjacent exit points. Since there is no portal-venous shunting, the liver parenchyma is zonated by the portal-venous gradient of oxygen, substrates, hormones etc. The possibility that metabolic processes could therefore be similarly zonated has attracted much interest, primarily as a means of understanding various pathological manifestations of the liver[1]. The concept of zonation has found support in numerous reports based on histochemical staining techniques demonstrating heterogeneous distribution of many enzymes within the microcirculatory unit. More recently new techniques have brought convincing quantitative evidence of dynamic zonation, notably the microdissection-microanalysis technique[2]. Other techniques include micro-lightguide surface fluorescence[3] and low temperature redox-ratio-scanning[4].

In this report a novel approach in the study of metabolic zonation is described. Since a major difficulty in the study of metabolic zonation of the liver lies in obtaining a spatially precise sampling of tissue or signal along the portal-venous gradient, we have adapted a hepatocyte column as a large scale model of the sinusoid. The model essentially transforms the liver from a multi-capillary organ into one macro-capillary, in which the portal-venous distance is scaled to 40-70 mm. A preliminary report has appeared[5].

METHODS AND MATERIALS

Hepatocytes were prepared by collagenase perfusion of livers from 24h starved, female Wistar rats, weighing 180-200 g[6]. The hepatocyte column, shown in Fig.1, was packed with 4.5 g (dry wt.) Biogel P-2, 2-400 mesh (Bio-Rad Corp.). The hydrated, washed beads were suspended in 30 ml Krebs-Ringer-bicarbonate buffer, equilibrated with either O_2/CO_2:19%/5% or 95%/5% and mixed with 5 ml hepatocyte suspension (about 0.5 g of liver). This suspension was compressed on the column to a final volume of about 15 ml. It is important that the column is properly compressed, in order to obtain good mechanical trapping of the cells between the beads. On the other hand, too much compression will affect the cells by dehydra-

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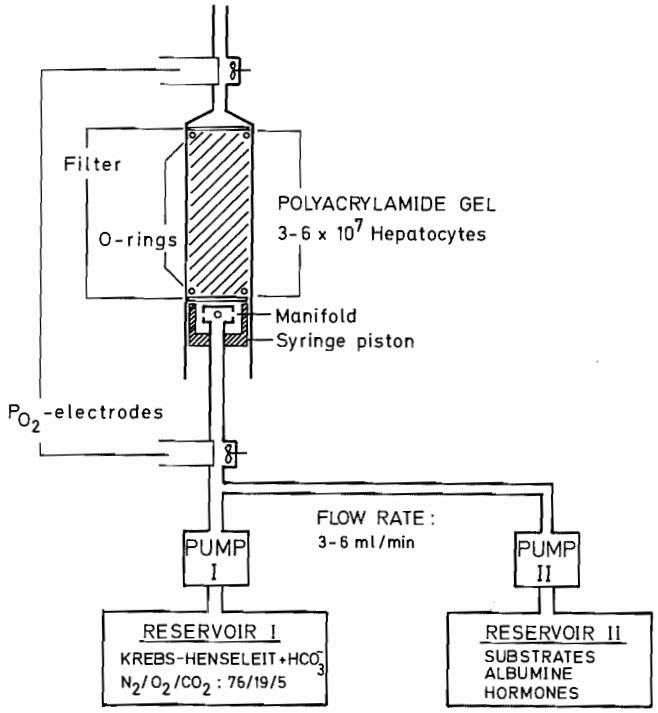

Fig.1. Large-scale model of liver sinusoid. The column is a disposable 35 ml PVC syringe with a manifold inserted in the inverted piston. The entire perfusion set up is enclosed in a thermostatted chamber at 37° C.

tion of the column. The column was perfused in a non-recirculation system at a rate of 3-6 ml/min, depending on the inflow oxygen tension and the rate of oxygen consumption. The standard perfusion medium was Krebs-Ringer-bicarbonate, 5 mM lactate, 0.5 mM pyruvate, 20 mM glucose, 0.2% serum albumin and 50 μunits/ml rat insulin (generously provided by NOVO Inc.). Oxygen electrodes (Clark type) at the afferent and efferent ends of the column allowed monitoring of oxygen consumption. At the conclusion of the experiment the column was exprimated and cut in 4 slices of equal size, each of which were suspended in 2 ml ice cold buffer (50 mM Tris/HCl, pH 7.2, 5 mM dithiothreitol, 4 mM EDTA, 50 μM pyridoxalphosphate, 0.2% defatted, boyine serum albumin). In experiments where glycogen synthase and glycogen phosphorylase were measured, the buffer included 50 mM NaF. Cells and polyacrylamide beads were separated by low speed centrifugation. The yield was 25-30%, which could be increased to 50% by an additional wash. With the suspension obtained from each of the four sections, cells were reisolated by

TABLE I

DNA CONTENT IN DIFFERENT PARTS OF THE HEPATOCYTE COLUMN.

perfusion time (min)		60-90	120-150	160-210	240-260
efferent part	1	32.2 (6)	27.8 (6)	29.8 (8)	24.9 (13)
	2	26.2 (6)	26.5 (4)	31.5 (3)	22.7 (6)
	3	27.8 (5)	28.0 (11)	31.1 (6)	23.0 (7)
afferent part	4	34.1 (13)	34.2 (13)	32.7 (11)	28.0 (8)

The column was packed with about 5o x 10^6 cells from 24h fasted rats and perfused at a rate of 5 ml/min with standard medium equilibrated with 19% $O_2$and 5% CO_2. The column was devided into 4 sections of equal size at the end of the perfusion. Cells were separated from the polyacrylamide gel and DNA measured on the cell suspension. Figures are ug DNA/g packed, hydrated beads, with SD in parenthesis. n = 3-5.

brief centrifugation and either homogenized by sonication in the appropriate buffer for DNA and enzyme activity measurements or deproteinized in 0.6 N PCA for measurement of glycogen. DNA and glycogen were measured according to[7] and[8], respectively. LDH, PK and GPT were measured as described in[9]. Glycogen phosphorylase and glycogen synthase were assayed as described in[10] and[11], respectively. Results are expressed as µmoles or units (µmoles/min) per 2 mg of DNA. We find 2.05 mg of DNA/g liver wet wt.

RESULTS AND DISCUSSION

Although the morphological definition of the functional unit of the mamalian liver is still a matter of dispute, it is generally agreed that there is no heterogeneity among the functional units in terms of metabolic competence and pathological vulnerability. The microvascular bed of the liver may therefore be viewed as a large number of functionally identical sinusoids in parallel coupling. Consequently, for the purpose of the study of metabolic zonation, the liver may be simulated by a column in terms of hepatocyte function, provided a representative sample of hepatocytes from one liver is packed on the column and provided the following criteria are fulfilled: 1) Cells on the same horizontal level in the column should experience the same concentrations of substrates,oxygen, hormones etc., i.e. the perfusion front should be plane and perpendicular

to the length axis of the column. 2) The cells must be evenly distributed on the column. 3) The single hepatocyte should remain immobilized during perfusion. 4) The linear perfusion speed should be comparable to value *in vivo*. 5) Cells should remain viable and metabolically active for a sufficient period of time when packed on the column.

Criterion 1) was tested by perfusing the column with Eosine (1% in standard medium) at a number of different perfusion rates between 3 and 6 ml/min. In all cases a plane dye front moving evenly through the column was observed. A flow distributing device in the afferent end of the column was necessary to obtain a plane front (see fig.1). Criterion 2) was checked by light microscopy of cross-sections of the column after Eosine perfusion. Sections about 1 mm thick were made with a razor blade and the surface was examined under transillumination. We find cells evenly distributed among beads. A further indication of an even distribution of cells is provided by the fact that DNA content of different sections of the column is the same, Table I. It is also seen that the DNA content does not change with perfusion time, which together with the observation (not shown) that the yields of three cytosolic enzymes GPT, LDH and PK are equal to that of DNA in all four sections, indicates that cells does not move during perfusion, criterion 3. LDH and GPT have been followed in the outflow from from the column, and in keeping with the above conclusion, less than 5% was found (not shown), even in perfusions which lasted more than 3h. Criterion 4), Goresky[12] finds a mean transit time for erythrocytes in the sinusoid of dog liver of 5.35 sec and calculates a linear speed of perfusion of 93 μ/sec. In the choice of column dimensions for these experiments we have aimed at a similar value of linear perfusion speed, however, having to consider the amount of tissue needed for the analysis as well as the maximum amount of oxygen which can be delivered with the saline medium. The compromise we have arrived at (see fig.1) requires flow rates of 3-6 ml/min, depending on the input oxygen tension, i.e. linear speed of 130-260 μ/sec. Within this range the hydrostatic pressure difference over the column varies linearly between 70 and 170 cm over water. Criterion 5), Trypan Blue dye exclusion test performed on samples from different parts of the column at the conclusion of the experiment showed 5-15% stained cells, apparently independent of location in the column.

The metabolic competence of the cells has been assayed by their ability to synthesize glycogen. It is clear from histological and microdissection studies that all hepatocytes are capable of glycogen synthesis[13, 14]. On the other hand, time course studies of glycogen synthesis and breakdown points to a significant lower rate of synthesis in perivenous cells[14]. As shown in Table II, we find a similar gradient of glycogen in the column after 2 hours of perfusion. A gradi-

TABLE II

GLYCOGEN SYNTHESIS IN DIFFERENT PARTS OF THE HEPATOCYTE COLUMN

Oxygen gradient (Torr)	Glutamine	Ethanol	n	Efferent 1	Efferent 2	Afferent 3	Afferent 4	Start
137-10	-	-	(6)	8.2 (4.4-23)	15 (8-33)	16 (7-34)	16 (8-30)	0.16
137-10	+	-	(4)	19 (8-30)	40 (16-63)	41 (29-51)	55 (46-65)	0.22
137-10	+	+	(2)	2.5	7.0	10	8.5	0.19
350-70	+	-	(4)	49 (28-68)	57 (27-80)	54 (27-59)	52 (23-77)	0.35

Experimental conditions were as in Table I, except that the oxygen gradient was varied as indicated. The column was perfused for 90-120 min before sampling of 4 sections. 10 mM glutamine and/or 13 mM ethanol was included in the medium as indicated. Figures are μmoles of glucose per 2 mg DNA. The range is given in parenthesis.

ent is actually detectable already after 30-40 min of perfusion (not shown). In agreement with Katz et al.[15] we find the rate of glycogen synthesis markedly increased by glutamine. With glutamine + ethanol there is a pronounced inhibition. In all cases,however, the afferent-efferent gradient of glycogen is present. These experiments were run with an oxygen gradient of 137-10 Torr, i.e. 93% extraction of oxygen. We changed the gradient to 350-70 Torr, i.e. 80% extraction, and observed that this eliminated the glycogen gradient, Table II, last line. In order to elucidate the mechanism of this heterogeneity of glycogen metabolism which was apparently caused by the lower oxygen gradient, the activities of glycogen synthase and glycogen phosphorylase were measured, as shown in Table III. The results, however, do not indicate any activity gradient and could not explain the glycogen zonation. It should be remembered, however, that while the figures given for synthase and phosphorylase indicate the activity at the end of the experiment, the glycogen concentration represents the integrated activity over the total perfusion period. A time course study of the enzyme activities is needed to clarify this point. It should be noted in Table III that we find rather high phosphorylase activity with glycogen synthesis going on, although there is a twofold decrease in the ratio between phosphorylase 'a' and synthase 'a' when compared with the value in the cell suspension, from which the

TABLE III

ACTIVITY OF GLYCOGEN SYNTHASE AND PHOSPHORYLASE

		Efferent		Afferent		Start
		1	2	3	4	
Phosphorylase	'a + b'	1.3 (0.54)	1.4 (0.76)	1.2 (0.36)	1.5 (0.64)	2.1 (1.0)
	'a'	0.86 (0.46)	0.95 (0.56)	0.86 (0.28)	1.1 (0.47)	1.3 (0.60)
	'a' / 'a + b'	0.65 (0.06)	0.66 (0.06)	0.72 (0.03)	0.73 (0.02)	0.65 (0.09)
Synthase	'a + b'	0.56 (0.29)	0.50 (0.18)	0.57 (0.25)	0.68 (0.25)	0.34 (0.12)
	'a'	0.17 (0.08)	0.17 (0.07)	0.17 (0.09)	0.19 (0.08)	0.12 (0.03)
	'a' / 'a + b'	0.31 (0.06)	0.28 (0.03)	0.30 (0.04)	0.27 (0.03)	0.38 (0.05)
	P 'a' / S 'a'	6.1 (3.8)	6.4 (4.3)	6.1 (3.0)	6.4 (2.7)	11 (5.1)

Experimental conditions were as in Table I. The perfusion medium included 10 mM glutamine. Synthase and phosphorylase activities were assayed at 37°C and 25°C respectively. Figures are umoles/min per 2 mg DNA, with SD in parenthesis. n = 4.

column was packed (the 'Start' value). This seems at variance with the findings in[16] but is in agreement with[15, 17].

There is rather large unexplained inter-experiment variation for some of the parameters measured. In contrast, the pattern observed in the column shows much less variation. If, for example, the data on glycogen concentration (Table II) were expressed as % of average glycogen concentration in the column for the particular experiment, the inter-experiment variation would be reduced to about 10%.

CONCLUSION

The observed dependence of glycogen metabolism on oxygen tension is unexplained at present. It is suggested that the hepatocyte column may become a useful tool in the study of metabolic zonation or other aspects of liver metabolism

where the effect of gradients of oxygen, hormones or drugs are studied. In some respects the column technique may also be a replacement of the perfused liver as discussed recently by Crisp et al.[18]

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