Periportal- and perivenous-enriched hepatocyte couplets: differences in canalicular activity and in response to oxidative stress

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Unlike isolated single hepatocytes, hepatocyte couplets retain their apical polarity, and, during short-term culture form an enclosed canalicular space or vacuole between the two adjacent cells into which biliary secretion is initiated. Hepatocyte couplets were prepared after partial collagenase perfusion of rat liver. Centrifugal elutriation was used to fractionate the preparation into six couplet-containing suspensions. Image analysis was used to determine the size of cultured couplets. The size of the couplets ranged from 34.1 ± 0.76 μm and 684 ± 24.1 μm^2 (mean length and area respectively $\pm$ S.E.M.) in Fraction 2, to 43.7 ± 0.57 μm and 1033 ± 33.8 μm^2 length and area respectively in Fraction 7. Glutamine synthetase activity was assessed in each freshly eluted fraction and was shown to be predominant in Fractions 6 and 7. Pretreatment of rats with CCl_4, which selectively destroys perivenous hepatocytes, decreased the proportion of couplets in these fractions by over 67%, and their glutamine synthetase activity by over 97%. It was concluded that Fractions 2 and 3 contained predominantly couplets of Zone 1 (periportal) origin, Fractions 4 and 5 those from Zone 2, and Fractions 6 and 7 predominantly couplets of Zone 3 (perivenous) origin. The development of canalicular secretory activity was assessed in the couplets after a 15 min incubation with a fluorescent bile acid, cholyl-lysyl-fluorescein (CLF). This was sigmoidal in all fractions, but slower in the periportal couplets, taking 5.1 h for 50% to show secretory activity in Fraction 2, compared with 2.7 h for Fraction 7. Incubation of hepatocyte couplets with 1 or 10 μM taurodehydrocholate, a non-toxic bile acid analogue, did not influence the rate of development of accumulation of CLF by the couplets or the area of the canalicular vacuole in any fraction. However, it did decrease the CLF content of couplets incubated with CLF for 15 min to a greater extent in those of perivenous origin. After subjecting the couplets to oxidative stress by incubation with 20 μM menadione (2-methyl-1,4-naphthoquinone), it was evident that periportal couplets were less able to maintain canalicular secretory activity than perivenous couplets.

INTRODUCTION

The functional unit of the liver is the acinus, which extends from the portal venule, along sinusoids to the terminal hepatic venule. The liver acinus extends for approximately 20 hepatocytes (Loud, 1968): periportal hepatocytes (zone 1) near the portal venule, perivenous (zone 3) near the terminal hepatic vein, and those in zone 2 are mid-zonal. There is a large body of evidence to support the concept of morphological, biochemical and metabolic heterogeneity of these cells, and various functions have been ascribed to specific zones of the acinus. Oxidative metabolism, gluconeogenesis, urea synthesis and bile formation are predominantly periportal activities, whereas glutamine synthesis, xenobiotic metabolism and ketogenesis are particularly prevalent in the perivenous hepatocytes (Jungermann and Katz, 1989; Traber et al., 1988), with glutamine synthesis being an exclusively perivenous function (Gebhardt and Mecke, 1983, 1984).

Various techniques have been employed to investigate the significance of this functional heterogeneity. Orthograde and retrograde liver perfusion (Haussinger, 1983) and the use of miniature electrodes (Matsumura et al., 1992) represent successful non-invasive techniques. Separating isolated hepatocytes into periportal and perivenous fractions has been achieved by zone-selective damage to hepatocytes using bromobenzene, CCl_4 or allyl alcohol (James et al., 1981; Gebhardt et al., 1988; Sesardic et al., 1989). The digitonin/collagenase technique described by Lindros and Penttila (1985) and Quistorff (1985) appears to be the most successful, although these methods generally result in

preparations of low initial viability (Burger et al., 1989). Isolation of periportal and perivenous hepatocytes has also been achieved by microdissection (Misra et al., 1988), but this is tedious and generates low yields.

Based on the finding that periportal hepatocytes are consistently smaller than perivenous cells (Schmucker et al., 1978), centrifugal elutriation has been used successfully to isolate hepatocyte sub-populations (Seibert et al., 1989; Gumucio et al., 1986; Willson et al., 1985), with a high viability and yield. Our previous studies have shown (Wilton et al., 1991) that hepatocyte couplets can be separated from singlets by centrifugal elutriation, and this yields highly enriched preparations of hepatocyte couplets (> 80% purity). Hepatocyte couplets comprise two adjacent hepatocytes which retain between them a bile canaliculus as a closed space. Unlike single cells, hepatocyte couplets retain their apical polarity with tight junctions dividing the canalicular and basolateral domains. The mitochondria, smooth and rough endoplasmic reticulum and Golgi apparatus of these couplets retain normal ultrastructural appearances (Gautam et al., 1987; Nikola and Frimmer, 1986). Functional gap junctions (Reverdin and Weingart, 1988; Spray et al., 1986) as well as tight junctions (Gautum et al., 1987) are maintained between the adjacent cells. Hepatocyte couplets maintained *in vitro* have been shown to accumulate biliary components in the canalicular vacuole within 2 h of incubation at 37 °C (Wilton et al., 1993); this is maintained for up to 30 h (Oshio and Phillips, 1981). Hepatocyte couplets can therefore be considered a primary secretory unit which offers a worthwhile model to study the processes of canalicular bile

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formation at the site of origin. The ability to separate couplets from different regions of the liver would thus enable the heterogeneity of hepatobiliary function to be studied.

Here centrifugal elutriation has now been used to fractionate hepatocyte couplets into six sub-populations. The activity of glutamine synthetase was determined in each of the fractions to identify the couplets on a basis of their zonal heterogeneity within the liver acinus. The separated fractions were used to investigate whether functional hepatobiliary differences could be identified between fractions enriched in either periportal or perivenous hepatocytes.

MATERIALS AND METHODS

Materials

Cholyl-lysyl-fluorescein (CLF) was synthesized and its purity checked as previously described (Mills et al., 1991). Type A collagenase and ATP were obtained from Boehringer Mannheim (Lewes, East Sussex, U.K.). Sodium pentobarbital (Sagatal) was obtained from RMB Animal Health Ltd. (Dagenham, Essex, U.K.). Leibowitz 15 (L-15) tissue-culture medium supplemented with glutamine (2 mM) was obtained from Gibco (Paisley, Scotland, U.K.). BSA (fraction V) was purchased from Winlab (Maidenhead, Berks., U.K.). Peanut oil, menadione (2-methyl-1,4-naphthoquinone), Triton-X-100 and glutamine synthetase (EC 6.3.1.2) were purchased from Sigma (Poole, Dorset, U.K.). CCl_4 was purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Other fine-grade chemicals were obtained from Sigma or BDH (Poole, Dorset, U.K.).

Animals

Male Wistar rats (230–250 g) were allowed free access to food (Standard Laboratories Ltd.) and water before surgery, which for untreated rats was performed between 08:00 and 09:00 h. The rats treated with CCl_4 were starved for 24 h before surgery, which was performed at approx. 15:00 h. All the rats were housed in an environment of constant temperature and humidity with alternating 12 h light and dark cycles.

Isolation of hepatocyte couplets

Rat hepatocyte couplets were isolated by the method described by Phillips et al. (1982) and Gautam et al. (1987) with

Table 1   Elutriation flow rate, fraction number and cell yield for fractions 1–8 inclusive

Each value is the mean ± S.E.M. ($n = 4$).

Elutriation flow rate (ml/min)	Fraction	$10^{-6} \times$ Cell population	S.E.M.
22.3–25.2	1	2.63	0.14
25.2–28.1	2	7.68	0.74
28.1–31.0	3	8.20	0.70
31.0–33.9	4	9.30	0.62
33.9–36.8	5	9.35	0.38
36.8–39.7	6	7.77	1.04
39.7–42.6	7	5.85	0.32
42.6–45.5	8	2.79	0.46

modifications (Wilton et al., 1993). Elutriation was carried out with a JE-5.0 rotor head mounted in a JE6-B centrifuge (Beckman Instruments). The rotor and elutriation buffer [Krebs–Henseleit solution containing 1 % (w/v) glucose and BSA, pH 7.4] were maintained at room temperature (21 °C). The rotor speed was maintained at 850 rev./min, and the different cell populations were separated by sequentially increasing the flow of elutriation buffer through the chamber. Cells were loaded into the elutriator at a buffer flow rate of 10 ml/min, the flow rate was then increased to 20 ml/min, and the first 150 ml of elutriated medium was wasted. Fractions (100 ml) were then collected at flow rates of 25.2–45.5 ml/min in steps of approx. 2.9 ml/min. A description of elutriation flow rate, fraction number and yield per fraction (cells/fraction) is provided in Table 1.

After collection, fractions were kept on ice and then centrifuged at 500 rev./min for 5 min, and the pellets were resuspended in L-15 medium (4 ml). Quantification of cell population is described in terms of units. A unit is defined as a singlet (one cell), couplet (two attached cells) or triplet (three attached cells). The number of units in each fraction was counted by using an improved Neubauer haemocytometer. Viability was determined by Trypan Blue exclusion (Moldeus et al., 1978).

Perivenous-specific injury was induced in rats by treatment with CCl_4 (Sesardic et al., 1989; Kuo and Darnell, 1991). After starvation for 18 h, the rats were treated with CCl_4 as a 10 % (v/v) solution in peanut oil (800 mg of CCl_4/kg body wt.) given by intraperitoneal injection. After 6 h, hepatocyte couplets were prepared as described above.

Glutamine synthetase activity

After elutriation, cells were centrifuged at 500 rev./min for 5 min. Cell suspensions from each of the fractions were then resuspended in Tris buffer (10 mM, pH 7.4). The cell number and viability count in each fraction were determined, and fractions were then sonicated for 3 × 6 s (Dawe Sonicator). Cell debris was removed by centrifugation at 13000 rev/min for 2 min. The resultant supernatant was then used for determination of glutamine synthetase activity by the method described by Rowe et al. (1970). Briefly, 100 μl of the sample was added to 800 μl of buffer mixture. The buffer mixture contained imidazole hydrochloride (56 mM), $MgCl_2$ (22 mM), 2-mercaptoethanol (28 mM), sodium L-glutamate (56 mM) and hydroxylamine (11 mM), at pH 7.2. The mixture was incubated in a water bath at 37 °C for 5 min before starting the reaction by addition of 100 μl of ATP (100 mM). After 15 min the reaction was stopped by addition of 1.5 ml of a solution containing $FeCl_3$ (0.37 M), HCl (0.67 M) and trichloroacetic acid (0.2 M). The A_{535} was measured at room temperature, against a reagent blank. Glutamine synthetase standards were used in the range 0–10 units; one unit is defined as that which reacts with 1 μmol of glutamate in 5 min at pH 7.1.

Culture of hepatocyte couplets and assessment of canalicular secretory activity

Cells used in the assessment of canalicular activity were plated in 35 mm-diameter tissue-culture dishes (Cel-cult; Sterilin, Harlow, Essex, U.K.) at a concentration of approx. 1×10^5 units/plate in 2 ml of L-15 medium. The cells were then incubated at 37 °C for the appropriate time.

Canalicular vacuoles were revealed after incubation with a fluorescent cholephile, CLF. CLF stock solution (2 μl; 2.1 mM in 0.9 % NaCl) was added to the samples and incubated for 15 min. The couplets were then washed with L-15 medium

(37 °C) twice, before examination with an inverted fluorescence microscope (Olympus IM2-RFL) with a stage incubator, enabling long-term maintenance at 37 °C. Monochrome images of the couplets were recorded (Hitachi KP-116 camera) and contrast-enhanced (CE-2 Contrast Enhancer; Brian Reece Scientific Ltd., Newbury, Berks., U.K.). The length and area of the couplets and their canalicular vacuoles were determined with an image analysis system (Mini-Magiscan; Applied Imaging, Sunderland, U.K.).

The development of canalicular secretory activity was assessed in randomly chosen fields of view. All the couplets in a given field were counted ($n > 50$). Couplets exhibiting active canalicular secretion and accumulation of the CLF into a sealed canalicular vacuole were measured as a proportion of the total number of couplets present.

Uptake of CLF

Cultures of couplets were incubated with CLF for 15 min, as described above. The cultures were then washed twice with 2.0 ml of phosphate-buffered saline, pH 7.4 (PBS), followed by a 10 min incubation with 2% (v/v) Triton X-100 in PBS. The fluorescence present in the detergent solution was measured with a fluorimeter (Spex Fluorolog; Stanmore, Middx., U.K.), by using an excitation wavelength of 495 nm and an emission wavelength of 525 nm. The sample fluorescence values were compared with that of the standard CLF. The total CLF content of the fractions was expressed in terms of pmol of CLF/10^6 cells.

Incubation with taurodehydrocholate

The influence of the non-toxic bile salt taurodehydrocholate (TDHC) on the development of canalicular secretory activity and cellular uptake of CLF was investigated in couplets from various fractions. Couplets were prepared in L-15 medium as described above, but plated in L-15 containing 0, 1 or 10 μM TDHC. Stock solutions of 1 mM and 10 mM TDHC were prepared in physiological saline, and added to the L-15 at a dilution of 1/1000 to produce the required volume. Control preparations had the same volume of saline added to the medium.

Sensitivity to oxidative insult

Previous studies (Wilton et al., 1993) have shown that canalicular secretory function of hepatocyte couplets is impaired by the addition of the hepatotoxicant menadione. To investigate the influence of acinar origin on the sensitivity of canalicular activity, menadione in dimethyl sulphoxide (10 μl) was added to the couplets to give a final concentration of 20 μM, a concentration previously shown to decrease canalicular secretion in mixed couplet preparations by approx. 65 % of control levels (Wilton et al., 1993). At the same time, 2 μl of 2.1 mM CLF was added. The cells were re-incubated at 37 °C for 15 min. Canalicular secretory function and CLF content were determined as described above.

RESULTS

Hepatocytes were isolated from rats by a restricted collagenase perfusion as a mixture of singlets, couplets, triplets and other small multiples. Dead cells and the majority of viable single cells were eluted before fraction collection. The remaining preparation was separated by centrifugal elutriation into six subpopulations enriched with couplets, Fractions 2–7 inclusive (see Figure 1).

The viability of eluted fractions has been shown to be higher than that of the suspension loaded on to the elutriator (Wilton et al., 1991), and in these experiments the cell viability was > 97 % in all these fractions. The consequence of pre-treatment of rats with CCl_4 was to decrease dramatically the proportion of couplets present in Fractions 5, 6 and 7, by 35, 67 and 70 % respectively (see Figure 1), whereas the proportion of couplets in Fractions 2 and 3 was increased. The viability of hepatocytes in fractions isolated from CCl_4-treated rats was > 85 %.

The length (i.e. the longest dimension of the adjacent cells) and area of the couplets was investigated by image analysis. The mean area of the couplets within each fraction increased as the flow rate of the elutriation buffer increased (Figure 2). Couplets in Fraction 2 were the smallest (34.1 ± 0.76 μm length, mean $\pm$ S.E.M.), and those in Fraction 7 the largest
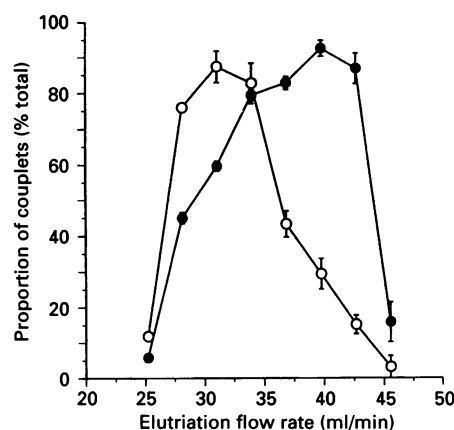
Figure 1   Separation of hepatocyte populations by centrifugal elutriation

The figure shows the proportion of cell units in each fraction present as hepatocyte couplets after centrifugal elutriation of a mixed population of hepatocyte multiples. The census was made in cells prepared from untreated (●) and CCl_4-pretreated (○) rats. Rotor speed was 850 rev./min, and elutriation flow rates are as shown. Each value is the mean $\pm$ S.E.M. ($n = 4$ for untreated, $n = 3$ for CCl_4-pretreated animals).
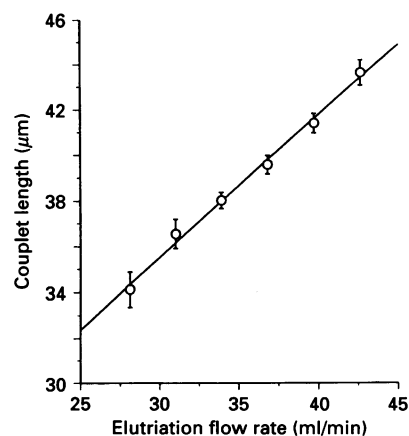
Figure 2   Absolute length (μm) of couplets in each couplet-enriched elutriated fraction

Each value is the mean $\pm$ S.E.M. of 40 individual measurements taken between 2 and 7 h of incubation at 37 °C. Couplets were prepared from four rats. The correlation coefficient of the line was 0.987.

Figure 3   Absolute area (μm²) of couplets in each couplet-enriched elutriated fraction

Each value is the mean ± S.E.M. of 40 individual measurements taken between 2 and 7 h of incubation at 37 °C. Couplets were prepared from four rats. The correlation coefficient of the line was 0.992.
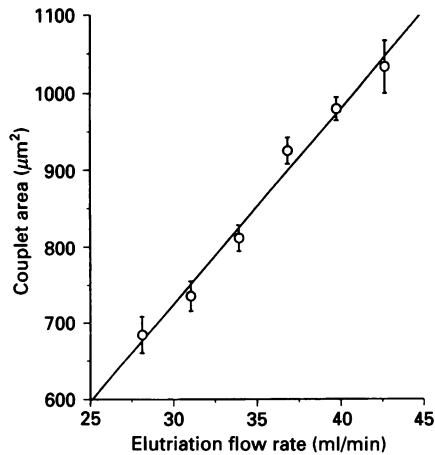
Table 2   Glutamine synthetase activity in each eluted fraction

One unit is defined as that amount which reacts with 1 μmol of glutamate in 15 min at pH 7.1. Each value is the mean ± S.E.M. ($n = 5$).

Fraction	Glutamine synthetase activity (units/10⁶ cells)	S.E.M.
2	11.01	1.72
3	8.95	1.41
4	10.96	2.00
5	9.79	1.51
6	27.73	0.58
7	30.20	1.67
8	12.05	1.21

(43.7 ± 0.57 μm). There was a linear relationship between size and fraction number, and regression analysis gave a correlation coefficient of 0.994. A similar relationship arose when the area of the couplets within each fraction was determined (Figure 3); again the relationship was linear, with a correlation coefficient of 0.986. The mean area of couplets present in Fraction 7 was 50 % greater than that of couplets in Fraction 2.

To establish further whether the separation of couplets by virtue of their size reflected differences in their lobular origin, glutamine synthetase activity was determined. The results show in cells isolated from untreated rats that there is a 2–3-fold greater activity of glutamine synthetase in cells in Fractions 6 and 7, relative to those of all other fractions assessed (Table 2). However, CCl_4 administered intraperitoneally 6 h before cell isolation decreased the measured glutamine synthetase activity in Fractions 6 and 7 by over 97 %, from 27.73 to 0.69 units/ 10⁶ cells in Fraction 6 and from 30.20 to 0.71 units/10⁶ cells in Fraction 7.

In hepatocyte couplets, CLF secreted across the canalicular pole enters the canalicular vacuole, where it accumulates before the vacuole contracts, expelling the contents into the surrounding medium (Wilton et al., 1993). The vacuole then reseals, and refills, thereby constituting a dynamic process (Gautam et al.,
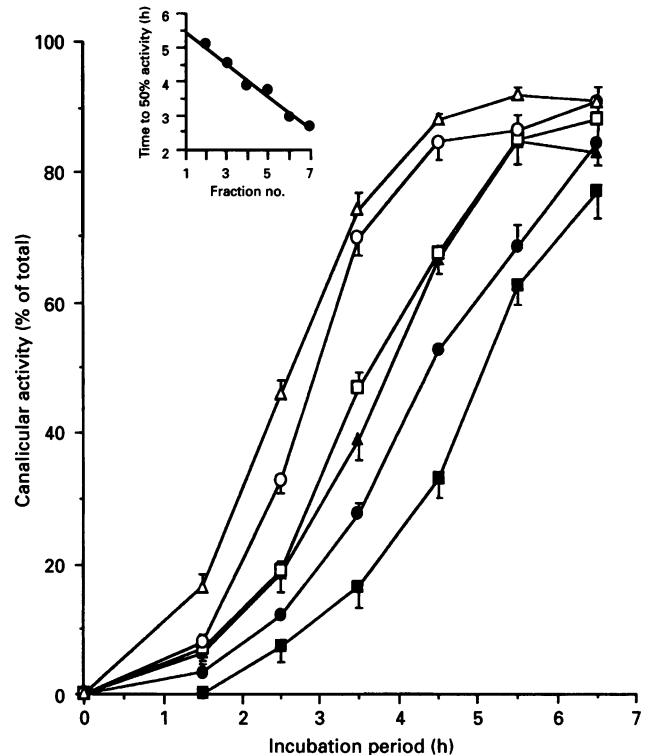

Figure 4   Canalicular secretory activity in hepatocyte fractions prepared by elutriation

Secretory activity was assessed in Fraction 2 (■), Fraction 3 (●), Fraction 4 (▲), Fraction 5 (□), Fraction 6 (○) and Fraction 7 (△) as a percentage of the total number of couplets present exhibiting canalicular accumulation of the fluorescent cholephile CLF after incubation (15 min) with 2 μM CLF. Each value is the mean ± S.E.M. ($n = 4$). The time taken to reach 50% activity in each fraction was calculated over the linear portion of the curve, and these values are plotted in the insert. The regression analysis of the line was time to 50% activity (h) = (−0.48 × Fraction number) + 5.95, and gave a correlation coefficient of 0.978.

1989). The rate of development of canalicular accumulation of a fluorescent cholephile was assessed in each of the couplet-enriched fractions.

The data indicate that the initiation of secretory function and canalicular accumulation was sigmoidal in all fractions, but occurred earlier in the perivenous hepatocyte couplets, and was progressively delayed in smaller couplets. This point is illustrated by calculating the time at which 50 % of the couplets present are exhibiting canalicular accumulation of CLF. This was calculated over the linear portion of the curve, and the values are plotted in the inset of Figure 4: 50% activity occurred after 2.66 h in Fraction 7, but after 5.05 h in Fraction 2 (correlation coefficient 0.987). Image analysis also showed that the size of the canaliculi increased with time; at the earlier time points the larger couplets (Fractions 6 and 7) possessed the largest canalicular vacuoles, but by 5.5 h canalicular size was essentially similar in all fractions (Table 3, controls).

In a seperate series of experiments, the influence of a non-toxic bile acid analogue, TDHC, on development of canalicular secretory function was assessed. Addition of 1 or 10 μM TDHC to the incubation medium at the time of plating had no significant influence on either the time of initial accumulation of CLF or the slope in the sigmoidal development of canalicular secretory activity in any of the fractions at any of the time points studied (results not shown).

Previous studies have shown that, in a heterogenous population

Table 3   Canalicular area (μm^2) of couplets after 2.5, 3.5, 4.5 or 5.5 h of incubation at 37 °C

Couplets were incubated in L-15 medium, and canalicular vacuoles were detected after a 15 min incubation with CLF and 0 or 20 μM menadione as described in the Materials and methods section. Couplets were incubated with 0 or 1 μM TDHC throughout ($n = 10$). Abbreviation: n.f., non-functional couplets. Superscripts a and b refer to significant differences between control and treatment values: [a]$P < 0.01$; [b]$P < 0.001$.

Time	Fraction...	2	3	4	5	6	7
2.5 h	Control	n.f.	n.f.	n.f.	38.06 ± 8.92	25.16 ± 4.33	50.91 ± 4.11
	TDHC	n.f.	n.f.	n.f.	58.15 ± 11.55	61.01 + 7.89[a]	65.86 ± 16.65
3.5 h	Control	41.73 ± 11.12	44.32 ± 10.44	65.1 ± 7.45	64.19 ± 19.33	93.28 ± 14.46	82.79 ± 42.44
	TDHC	29.31 ± 6.43	33.11 ± 30.33	60.73 ± 12.21	72.28 ± 6.35	90.66 ± 13.05	66.11 ± 11.29
4.5 h	Control	75.25 ± 7.85	70.86 ± 7.62	80.13 ± 5.98	101.43 ± 7.79	108.64 ± 16.17	92.45 ± 10.13
	TDHC	43.24 ± 14.71	83.31 ± 26.91	104.21 ± 7.32	77.86 ± 11.37	51.11 ± 8.73	109.35 ± 14.67
	Menadione	24.24 ± 2.92	25.12 ± 7.79	24.02 ± 4.36	26.95 ± 5.32	72.26 ± 13.21	77.90 ± 9.45
5.5 h	Control	136.69 ± 17.77	110.89 ± 9.32	128.62 ± 16.49	125.32 ± 5.97	134.36 ± 11.84	117.73 ± 18.81
	TDHC	102.94 ± 26.55	96.91 ± 6.08	99.84 ± 18.12	120.63 ± 9.59	85.77 ± 14.39	105.92 ± 21.94
	Menadione	24.36 ± 1.15[b]	24.44 ± 3.05[b]	39.22 ± 2.77[b]	37.52 ± 6.78[b]	98.03 ± 10.23	71.26 ± 22.26
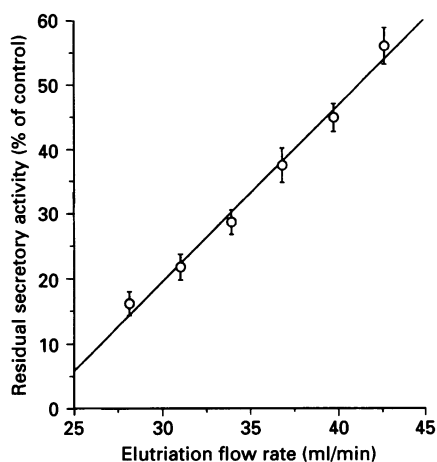
Figure 5   Effect of 20 μM menadione on canalicular secretory activity in fractions 2–7

Couplets were incubated with 2 μM CLF and 20 μM menadione for 15 min. Canalicular secretory activity was assessed as discussed in the Materials and methods section, and is expressed in terms of the control value for each fraction. Each value is the mean ± S.E.M. ($n = 9$).

of hepatocyte couplets, a 15 min incubation with 20 μM menadione inhibited canalicular secretory function by approx. 65% (Wilton et al., 1993). The influence of acinar origin on

sensitivity of canalicular function to oxidative toxic insult was therefore assessed. The canalicular accumulation of CLF in the menadione-treated cells was described as a proportion of the cells showing fluorescent canaliculi compared with control cells. The data presented therefore take into account the different rates of canalicular activity in the various fractions at these time points (see Figure 4). The effect of menadione on canalicular accumulation of CLF differs markedly in the different fractions (Figure 5). Inhibition is most evident in the smaller, periportal-enriched couplets. A 15 min incubation with 20 μM menadione decreased canalicular accumulation of CLF to 16.1 ± 1.84% of the control ($n = 12$), whereas in Fraction 7 the value was 55.9 ± 2.86% ($n = 12$). The extent of inhibition showed a linear relationship with elutriation flow rate ($r^2 = 0.997$), indicating a strong correlation with couplet size.

Fluorescence microscopy was able not only to document the proportion of couplets accumulating CLF but also to indicate qualitatively that TDHC and menadione might influence the cellular content of CLF and the intensity of fluorescence in the canalicular vacuole at a given time point. This could not be investigated by image-analysis techniques, owing to bleaching. A measure of total uptake and retention of CLF could be determined by permeabilizing cells with Triton X-100 and subsequent assay of the released materials in the fluorimeter. In controls this showed (Table 4) that, after a 16 min incubation with CLF, the larger couplets (Fractions 6 and 7) contained more CLF than the smaller couplets (Fractions 2 and 3) but, in relation to cell volume, the overall content of CLF was essentially

Table 4   Total content of CLF in cultured primary hepatocyte couplets after 5 h incubation at 37 °C with 0, 1 or 10 μM TDHC

Cells were incubated (15 min) with 2 μM CLF and 0 or 20 μM menadione, washed twice, and fluorescence was determined as described in the Materials and methods section. Content is expressed in terms of pmol of CLF/10^6 cells and values are expressed as means ± S.E.M. ($n = 6$). The superscripts a and b refer to significant differences between control and treatment values: [a]$P < 0.01$; [b]$P < 0.001$.

Fraction	Control	1 μM TDHC	10 μM TDHC	20 μM Menadione	+1 μM TDHC	+10 μM TDHC
2	490.9 ± 17.9	443.8 ± 17.1	372.6 ± 19.2[a]	236.5 ± 29.3[b]	232.9 ± 22.7[b]	227.0 ± 23.3[b]
3	515.2 ± 22.4	461.6 ± 19.6	380.8 ± 16.1[a]	242.2 ± 35.6[b]	232.2 ± 21.9[b]	217.3 ± 19.7[b]
4	555.5 ± 16.0	506.1 ± 18.2	416.1 ± 16.9[b]	235.4 ± 14.8[b]	232.8 ± 12.8[b]	232.3 ± 12.0[b]
5	556.6 ± 21.7	529.7 ± 18.9	313.2 ± 16.8[b]	286.3 ± 14.6[b]	266.8 ± 11.4[b]	262.8 ± 12.1[b]
6	688.4 ± 12.9	562.4 ± 18.0[a]	353.8 ± 16.9[b]	519.9 ± 10.8[a]	485.6 ± 26.9[a]	481.4 ± 28.3[a]
7	829.9 ± 50.2	646.5 ± 22.2[a]	392.5 ± 27.1[b]	706.2 ± 17.8	683.6 ± 22.6[a]	683.6 ± 19.3[a]

the same. When TDHC was present in the incubation medium, it caused a small decrease in the total content of CLF which was greater in Fraction 7 (22% and 53%) than in Fraction 2 (10% and 34%) at 1 μM and 10 μM TDHC respectively. Since TDHC had little influence on canalicular area (Table 3), the decrease in cellular content was probably due to a decrease in cellular uptake and retention of CLF rather than to a decrease in canalicular secretion.

When couplets were incubated with 20 μM menadione, the cellular content of CLF was decreased substantially by approx. 50% in Fractions 2 and 3, but by only 25–30% in Fractions 6 and 7 (Table 4). In distinction from TDHC, menadione decreased the average size of the canalicular lumen (Table 3), this effect was seen dramatically in the smaller (periportal-enriched) couplets (Fraction 2), where the mean canalicular area was decreased by 80%, whereas in Fraction 7 it was decreased by only 40%. The inclusion of TDHC before and during exposure to menadione did not affect the response to menadione.

DISCUSSION

We have shown previously that centrifugal elutriation provides a reproducible method of separating hepatocyte couplets from single cells and larger multiples (Wilton et al., 1991). Here we have demonstrated that these couplets can be further fractionated into sub-populations.

Hepatocytes are known to be smaller within the periportal region relative to those from the perivenous region of the liver acinus (Schmucker et al., 1978; Sigal et al., 1992). Separation of isolated single cells into different sub-populations by centrifugal elutriation has been achieved by a number of groups (Willson et al., 1985; Gumucio et al., 1986; Seibert et al., 1989), but has not been described for hepatocyte couplets. Seibert et al. (1989) found the mean diameter of parenchymal cells from untreated rats to range from 19.6 μm (periportal cells) to 23.5 μm (perivenous cells). We found the mean length of couplets present in Fraction 2 was 34.1 μm, and that in Fraction 7 43.7 μm, and this supports the conclusion that the small couplets eluted in Fractions 2 and 3 are predominantly periportal, those in Fractions 4 and 5 mid-zonal, and the large-diameter couplets eluted in Fractions 6 and 7 are predominantly of perivenous origin. These values, which correspond to the two hepatocytes which comprise a couplet, are approximately twice those found by Seibert et al. (1989).

Hepatocytes display a marked heterogeneity of function with regard to metabolic and transport processes (Traber et al., 1988; Jungermann and Katz, 1989). The physiological significance of the heterogeneous location of enzymes and transport systems involved in ammonia and glutamine metabolism has been well elucidated (Haussinger, 1990). Glutamine synthetase in particular has a specific localization within the acinus, being limited to the last three cells surrounding the terminal hepatic venule (Gebhardt and Mecke, 1983, 1984), which have been shown to be consistently larger than the periportal hepatocytes (Schmucker et al., 1978). We found that most of the total glutamine synthetase activity in untreated rat hepatocytes was present in the relatively large couplets of Fractions 6 and 7, but there was also some enzyme activity in all the remaining fractions. The activity of glutamine synthetase in couplet fractions will be influenced by minor cross-contamination with large singlet hepatocytes (Fractions 2, 3 and 4) and triplets and quadruplets made up of small sized hepatocytes (Fractions 6 and 7). This results in a masking of the true separation of hepatocyte couplets. A similar degree of enrichment has been observed by other workers investigating glutamine synthetase activity in single hepatocytes

(Burger et al., 1989). Further confirmation of this conclusion came from a 6 h pre-treatment of the animals with CCl_4, which is known to result in zone-selective killing of perivenous hepatocytes (Gebhardt et al., 1988; Sesardic et al., 1989; Kuo and Darnell, 1991). This resulted in a very different population profile from that of hepatocytes from untreated rats, with proportionally more couplets eluted in Fractions 2 and 3 (owing to decrease in perivenous single cells) and very few couplets eluted in Fractions 6 and 7. The activity of glutamine synthetase was almost entirely eliminated in cells isolated from CCl_4-pretreated rats. Based on cell size, selective killing and the distribution of glutamine synthetase activity in the elutriated hepatocyte sub-populations, we conclude that the couplets in Fractions 2 and 3 are predominantly of periportal origin and those of Fractions 6 and 7 of perivenous origin.

The digitonin/collagenase perfusion method used suffers the major disadvantage of being destructive of one zone of the acinus, and producing cells with low viability ($< 80\%$; Burger et al., 1989). We also found evidence of periportal-cell damage in producing couplets from CCl_4-treated rats, and viability in all fractions so produced was decreased.

Functional differences in periportal- and perivenous-enriched sub-populations

The development of hepatobiliary secretory function in the various fractions showed marked differences. Once accumulation of CLF was initiated, this activity increased in a sigmoidal manner and at a greater rate in Fraction 7 than in Fraction 2. The temporal differences in the development of canalicular accumulation of CLF may be due to a number of factors. Secretion and accumulation of primary biliary components into the canalicular vacuole of two adjacent hepatocytes does not occur until there is sufficient canalicular membrane present and the tight junctions surrounding the canalicular pole are resealed (Gautam et al., 1987). It has also been suggested that the integrity of the cytoskeleton in the pericanalicular region is also necessary for bile secretion to occur (Kawahara et al., 1989). It is possible that these processes occur at a faster rate in the larger perivenous couplets.

Dynamic changes in the size of the canalicular vacuole make it difficult to assess changes in vacuole area over time or between fractions. Table 3 does indicate that canalicular-vacuole area was larger after incubation for 5.5 h relative to the earlier incubation periods, and that this was true within each fraction considered. This could be due to stretching of the canalicular membrane, which had re-formed after 2.5 or 3.5 h, but is more likely to be due to the recruitment of new canalicular membrane from the dissociated apical pole remnants from other regions of the cell (Gautam et al., 1989), thereby providing more material for the expansion. After incubation periods of 2.5 or 3.5 h, canalicular vacuoles were smaller in the periportal-enriched fractions than in the perivenous-enriched couplets. By 5.5 h, however, this difference was lost, with the vacuoles in all the fractions having a similar size. Thus the ratio of canalicular-vacuole to couplet area is much larger in the predominantly periportal hepatocytes than in the perivenous cells, a situation analagous to that found *in situ* (Jones et al., 1978), and correlates with the greater activity of the periportal cells for bile acid secretion in the animal (Jungermann and Katz, 1989).

THDC decreased the total uptake of CLF by couplets in all the fractions (Table 4), although it had no effects upon canalicular size. The action is therefore presumably due to competition with CLF for transport across both the plasma membrane and the canalicular membrane.

Menadione has been shown to decrease canalicular secretory activity in hepatocyte couplets in a dose-dependent manner (results not shown); the present work assesses the relative sensitivity of the sub-populations to a menadione insult. A 15 min incubation with 20 μM menadione decreased the canalicular secretory activity of couplets in all the fractions. Of the canalicular vacuoles remaining, vacuole size was decreased relative to control couplets, and these two observations taken together explain the decrease in couplet content of CLF on incubation with menadione (Table 2).

Menadione has been shown to induce depletion of glutathione and NADPH (Di Monte et al., 1984; Ross et al., 1985) and NAD$^+$ (Stubberfield and Cohen, 1988), the elevation of free cytosolic Ca (Orrenius et al., 1989) and ATP depletion (Gores et al., 1988) in hepatocytes. Any of these can be considered individually to be cytotoxic; which event is most important is not yet clear. What is particularly interesting, however, is the differential effect of 20 μM menadione on canalicular secretory activity in the different couplet sub-populations. It has been suggested by Matsumura et al. (1992) that intracellular Ca^{2+} is not uniformly distributed across the liver acinus. The concentration of cytosolic Ca^{2+} is thought to be higher in the periportal cells, whereas the perivenous hepatocytes, which have a greater area of endoplasmic reticulum (Kanai et al., 1986; Loud, 1968), would have a higher capacity for sequestered Ca^{2+} stores. Hepatic heterogeneity extends to glutathione metabolism. Glutathione peroxidase activity (Kera et al., 1987) and cellular glutathione (Smith et al., 1979) have been shown to be present at higher levels in periportal cells, whereas the activity of the glutathione S-transferases is higher in the perivenous hepatocytes (El Mouelhi and Kauffman, 1986; Redick et al., 1982). These parameters may contribute to the smaller decrease by menadione in both canalicular-vacuole area (and therefore volume) and canalicular accumulation of CLF in the periportal hepatocytes seen in this experiment. Menadione rapidly depletes GSH in the hepatocyte, forming GSSG (Di Monte et al., 1984), and this leads to protein thiol oxidation and inhibition of Ca^{2+}-ATPase (Orrenius et al., 1989). Menadione also forms glutathione conjugates (Ross et al., 1985). Further, Ca^{2+} has been shown to induce contraction of the canalicular vacuole (Kitamura et al., 1991; Watanabe et al., 1991; Watanabe and Phillips, 1984). Gores et al. (1988) showed that menadione might induce cell death by inhibiting ATP production in hepatocytes. Oxidative energy metabolism has been shown to occur at a far higher rate in periportal than in perivenous hepatocytes (Jungermann and Katz, 1986) and may also be an important factor in determining the relatively high sensitivity of canalicular function in periportal hepatocyte couplets.

In conclusion, the studies enable a novel approach to the investigation of heterogeneity of liver cells in their biliary secretory function.

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