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EVIDENCE THAT THE LOSS OF RAT LIVER CYTOCHROME P450 *IN VITRO* IS NOT SOLELY ASSOCIATED WITH THE USE OF COLLAGENASE, THE LOSS OF CELL–CELL CONTACTS AND/OR THE ABSENCE OF AN EXTRACELLULAR MATRIX

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Abstract—Two methods avoiding the widespread technique of collagenase perfusion have been employed to study the regulation of total cytochrome P450 content in rat hepatocyte culture. One technique required the perfusion of the liver with the chelating agent EDTA to dissociate the parenchymal cells prior to culture. Over a period of 48 hr, cultured hepatocytes isolated by EDTA perfusion showed comparable losses of cytochrome P450 as cells isolated by perfusion with collagenase. The second technique involved the culture of 210–240 μm thick "precision cut" liver slices. The results presented here indicate that the liver slices remain viable for 24 hr of culture, but that liver slices also lose their cytochrome P450 content at a comparable rate to collagenase prepared cells in culture. Collectively the results suggest that there is not a direct causal relationship between the loss of cytochrome P450 and one or a combination of the use of collagenase; the loss of cell–cell contacts and the absence of an extracellular matrix.

One problem associated with the use of liver cell cultures in biochemical pharmacology has been the loss of liver-specific functions such as the high level of expression of cytochromes P450 [1]. An understanding of the reasons for this loss may provide insight into the mechanisms which regulate this gene superfamily [2].

Cytochromes P450 are spectrophotometrically detectable in a restricted number of tissues and so mechanisms which regulate tissue differentiation may exert control in their expression. It is generally accepted that cell–cell interactions play a role in tissue growth and differentiation [3, 4] and it has been reported that contacts between hepatocytes and a liver-derived cell line prevents the loss of cytochrome P450 [5], although others "using the same protocol" have been unable to reproduce this effect [6].

It has been reported that a reciprocal relationship may be observed between activities related to growth and those related to differentiation in rat hepatocytes cultured at different densities [7]. Thus at high densities, differentiated functions are expressed by the cells and at low densities growth functions are expressed. In this respect the density of liver cells *in vivo* has been calculated to be twice as great as the maximum density achieved in conventional hepatocyte culture systems [8]. However, differentiated functions may be expressed in hepatocytes cultured at low densities if adult rat liver plasma membranes containing a trypsin-sensitive factor are also added to the culture dish [7]. Other work has shown that hepatocytes cultured on an extracellular matrix retain their differentiated functions [9, 10].

The widespread use of collagenase preparations in the isolation of liver cells may therefore be responsible for the loss of a surface factor(s) which plays an important role in the expression of cytochromes P450. Therefore, the present work evaluates the levels of cytochrome P450 in hepatocyte cultures prepared with the chelating agent EDTA, to avoid proteolysis, as well as in "precision cut" liver slices where in addition cell density, cell–cell contacts and interactions with the extracellular matrix should not have been disrupted.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA†), insulin, gentamycin, hydrocortisone 21-hemisuccinate, dexamethasone, Percoll, collagen type I and fibronectin were obtained from the Sigma Chemical Co. (Poole, U.K.). Glycerol was obtained from BDH (Poole, U.K.). Renex 690 was a gift from ICI/Atlas Chemicals (Leatherhead, U.K.). Collagenase H was obtained from Boehringer (Lewes, U.K.). Sodium pentobarbitone was obtained from May and Baker Ltd (Dagenham, U.K.). William's Medium E (WME) and foetal calf serum were obtained from Flow Laboratories (Irvine, U.K.). Enzyme assays were performed on a Roche "Cobas Bio" centrifugal analyser using kits purchased from Roche

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† Abbreviations: BSA, bovine serum albumin; HBSS, Hank's balanced salt solution; EBSS, Earle's balanced salt solution; LDH, L-lactate:NAD oxidoreductase (EC 1.1.1.27); GOT, L-aspartate:2 oxoglutarate aminotransferase (EC 2.6.1.1.); GPT, L-alanine:2-oxoglutamate aminotransferase (EC 2.6.1.2); TCA, trichloroacetic acid.

Ltd (Welwyn Garden City, U.K.). All other chemicals were of the highest purity available from commercial sources.

Animals. Liver slices and hepatocytes were prepared from 250–300 g adult male CD rats supplied by Charles River Ltd (Margate, U.K.).

Isolation of hepatocytes with EDTA. This technique was performed as reported by Meredith [11]. Briefly, the animal was anaesthetized by i.p. injection of 60 mg sodium pentobarbitone per kg body weight and a heparin-filled 16G cannula was inserted into the portal vein. After securing the cannula by ligation, a cut was made in the posterior vena cava. The perfusion buffer (0.14 M NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1.6 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ and 2 mM EDTA) was pumped through the liver at a rate of 50 mL/min. The perfusate was gassed with 5% CO_2/95% O_2 before passing through the liver at a pH of 7.4 and a temperature of 36–37°. The perfusate was run to waste from the liver. After 30 min the liver was excised from the body cavity and the cells dispersed by combing the liver with a pair of curved forceps in perfusion buffer without EDTA. The resultant suspension was filtered through 120 μm nylon mesh (Nybolt No. 10.5, John Stanair and Co., Whitefield, Manchester, U.K.) and centrifuged at 50 *g* for 3 min. The pellet was resuspended in culture medium. A further three centrifugation steps were performed before the cells were centrifuged through a solution of Percoll. One volume of cell suspension was mixed with two volumes of Percoll buffer which comprised 13 volumes of 1.4 M NaCl, 50 mM KCl, 8 mM $MgCl_2$, 16 mM Na_2HPO_4 and 4 mM KH_2PO_4 added to 87 volumes of Percoll. Centrifugation at 300 *g* for 3 min separated viable and non-viable cells (as determined by trypan blue exclusion). The viable cells were washed in medium a further two times by centrifugation at 50 *g* to remove the Percoll [11].

Isolation of hepatocytes with collagenase. The animal was treated in the same way as for an EDTA perfusion except that the perfusion buffer was Hank's balanced salt solution (HBSS) (0.14 M NaCl, 5.4 mM KCl, 0.34 mM $Na_2HPO_4 \cdot 7H_2O$, 0.44 mM KH_2PO_4, 5.6 mM D-glucose and 15.7 mM $NaHCO_3$). After clearing the liver of blood and excision from the body cavity, the liver was perfused with HBSS containing 0.025% (w/v) collagenase H. This buffer was recirculated for 30 min [12]. The cells were dispersed in HBSS containing 2% (w/v) BSA, filtered through 120 μm mesh and washed in medium as outlined above. Since the viability of hepatocytes prepared with collagenase was routinely greater than 85%, the Percoll separation and clean-up was not employed.

Culture of hepatocytes. Cells were counted in a haemocytometer, suspended in culture medium and 20 mL of suspension containing 18 million cells was added to 150 mm diameter Lux™ plastic Petri dishes (Flow Laboratories). The plates were incubated in a humidified atmosphere of 5% CO_2 in air and maintained at a temperature of 36–37°. The medium was renewed after 2 hr to remove any unattached cells and thereafter every 24 hr. Where cells were cultured in the absence of serum, the plates were coated with 300 μg of collagen type I per plate and the medium supplemented with 1 μg fibronectin/mL for the first 2 hr of culture to promote attachment [13, 14].

Liver slices. Blood was cleared from the liver with HBSS, the liver excised and circular columns of tissue cut using a 10 mm diameter (No. 6) cork borer (Fisons Scientific Equipment, Loughborough, U.K.). The thinnest, most reproducible tissue slices (233 ± 17 μm) were then cut at room temperature (20 ± 2°) in Earle's balanced salt solution (EBSS) (121 mM NaCl, 5.4 mM KCl, 1 mM $NaH_2PO_4 \cdot H_2O$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1.8 mM $CaCl_2$, 5.6 mM D-glucose and 26 mM $NaHCO_3$, pH 7.4) using an autoclavable Krumdieck tissue slicer [15, 16]. The slices were floated onto stainless steel wire mesh (Locker Wire Weavers Ltd, Warrington, U.K.) and placed in plastic circular 15 mm diameter by 41 mm vials (Metal Box Ltd, U.K.) containing 1 mL of culture medium. The vial was closed with a plastic cap which had a centrally located 3 mm diameter hole to contain the medium but still permit the exchange of gases. The vials were then placed on a roller system housed in a humidified incubator under the same conditions used for hepatocyte culture. The vials were rotated at approximately 9 rpm and the medium renewed after the first 2 hr of culture.

Assays. At the specified time-points cells and slices were homogenized in ice-cold phosphate buffer (11 mM $NaH_2PO_4 \cdot 2H_2O$, 53.5 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.2) using an Ultra Turrax blender type T25 (20,500 rpm, 10 sec).

Cytochrome P450. This was measured by the method of Omura and Sato [17] following a 1:1 dilution of homogenates in solubilizing buffer [0.2% (v/v) Renex 690, 20% (v/v) glycerol, 50 μM EDTA and 50 mM Tris–HCl, pH 7.2].

Enzyme leakage. LDH, GOT and GPT were determined by measuring activities in cell homogenates and corresponding culture media. Homogenates were routinely diluted up to 25 times with 50 mM Tris–HCl buffer, pH 7.5, to give activities within the linear range of the assay method. LDH, GOT and GTP assays were performed as previously described [18, 19] except that the LDH assay was performed at 30°.

Potassium content. One volume of ice-cold 15% (w/v) trichloroacetic acid (TCA) was added to 8 volumes of homogenate and the resultant precipitate centrifuged at 1000 *g* for 5 min at 4°. The supernatant was analysed for potassium using an ion-selective electrode incorporated in a Beckman Electrolyte 2 analyser system (Beckman Instruments Ltd, High Wycombe, U.K.). The potassium concentration measured in 1 volume of TCA added to 8 volumes of phosphate buffer as a blank was subtracted from that measured in tissue containing supernatants to determine the amount of potassium associated with the tissue.

Protein. Protein was determined by the method of Lowry *et al.* [20] using BSA as the standard.

Histology. Freshly cut liver slices were fixed in 10% (v/v) buffered formalin and then embedded in paraffin wax blocks. Sections of 4 μm thickness were cut on a microtome, mounted on glass slides and stained with hematoxylin and eosin. Microscopic examination using an eyepiece graticule calibrated

Table 1. Comparison of the effect of liver cell isolation technique and subsequent culture with steroids on the loss of cytochrome P450 in cultured rat hepatocytes

Hours of culture	Cytochrome P450 content (as % initial value) in: EDTA prepared hepatocytes +DEX	+HC	Collagenase prepared hepatocytes +HC
12	ND	ND	76 ± 9.6 (4)
24	45 ± 5.0 (3)	61 ± 5.3 (3)	49 ± 5.7 (7)
48	26 ± 4.3 (3)	24 ± 5.9 (3)	24 ± 6.0 (3)

Hepatocytes were isolated as described in Materials and Methods. The results are presented as the mean ± SD of the percentage of cytochrome P450 remaining in the cultures with the number of separate experiments (N) given in parentheses. Initial cytochrome P450 concentrations: EDTA prepared hepatocytes 190 ± 12 pmol/mg cell protein, collagenase prepared cells 220 ± 20 pmol/mg cell protein. Hepatocytes were cultured in WME supplemented with 1 μM insulin, 5% (v/v) foetal calf serum, 50 μg gentamycin/mL medium and the following additions where indicated; DEX, 0.1 μM dexamethsone; HC, 100 μM hydrocortisone.
ND, not determined.

with a 1 mm stage micrometer was employed to determine slice thickness.

RESULTS AND DISCUSSION

Hepatocytes isolated by perfusion with EDTA had an initial viability, as determined by trypan blue exclusion, of 50–70% before centrifugation through Percoll and, in agreement with Meredith [11], a viability of greater than 99% after centrifugation through Percoll. Hepatocytes isolated by perfusion with collagenase had viabilities of 85% or more and hence the Percoll "clean up" was considered unnecessary.

In both cases culture of EDTA or collagenase-prepared hepatocytes resulted in the loss of cytochrome P450 (Table 1). Substituting 100 μM hydrocortisone for 0.1 μM dexamethasone [11] did not affect the loss of cytochrome P450 in cells isolated by EDTA. Furthermore, culturing EDTA-prepared hepatocytes in medium containing 0.1 μM dexamethasone, 0.5 mM methionine and on a collagen substratum was, in contrast to Meredith [11], without effect on cytochrome P450 levels (data not shown for brevity). This finding is similar to our previous experience on the lack of effect of methionine on the cytochrome P450 content of cultures prepared using collagenase [21]. Exchanging EDTA for collagenase may be a superior method for isolating liver cells because the collagenase used by most investigators is a crude extract from *Clostridium histolyticum* which can mediate the proteolysis of hepatocyte proteins [22]. Meredith [11] reported that hepatocytes isolated by EDTA maintained their levels of cytochrome P450 for 8 days of culture, but we clearly have been unable to repeat this result.

The preparation of hepatocyte cultures with either collagenase or EDTA relies on the dissociation of cell–cell contacts as well as their removal from the extracellular matrix. In order to assess the role these phenomena may play in the regulation of cytochrome P450 we examined the levels of cytochrome P450 in "precision cut" liver slices. The results presented in Table 2 show that the viability of liver slices on isolation, based on the specific activities of LDH, GOT and GPT, are equivalent to the viability of collagenase-prepared hepatocytes (as assessed by trypan blue exclusion). In contrast to the enzyme activities retained by the slices, the results presented in Table 2 show that there is a disproportionately greater loss of potassium from the liver slices which is in agreement with other workers [23]. Isolated hepatocytes also show a marked decrease in potassium levels (Fig. 1). Cutting the slices a 4° or 37° did not significantly alter this phenomenon (data not shown for brevity) suggesting that the loss of potassium is not due to an effect on ion pump activity. In addition, the absence of Ca^{2+} and Mg^{2+} in the cutting buffer did not prevent the loss of potassium (data not shown for brevity). The differences in molecular dimensions between potassium and proteins may account for the loss in slice- and isolated cell-potassium suggesting widespread but discrete loss of membrane integrity. However, potassium may be lost in slices from mechanically undamaged to damaged cells by way of gap junctions. The loss of potassium is reversible since liver slices and cultured hepatocytes re-establish their intracellular potassium content within a few hours of incubation (Fig. 1).

Previous work has shown that liver slices of around 250 μm thickness showed signs of deterioration after 20 hr of incubation in a static system but negligible signs of degeneration when cultured on a roller system [24]. The current work employing 210–250 μm thick liver slices (Table 3), which are approximately 10 cells thick (Fig. 2), confirms that the slices remain viable for 24 hr as judged by their potassium content (Fig. 1) and leakage of LDH and GOT (Fig. 3). Indeed, after 24 hr of culture the leakage of enzymes from the liver slices is less than that observed from cells cultured in the same medium formulation (Fig. 3). The medium used for slice

Table 2. The effect of slicing rat liver on the specific activities of LDH, GOT, GPT and potassium content

Parameter	Whole liver	Slices	% Specific activity in slices versus whole liver
LDH	3.4 ± 0.28	3.1 ± 0.43	89 ± 6
GOT	0.41 ± 0.084	0.45 ± 0.080	114 ± 26
GPT	0.20 ± 0.050	0.21 ± 0.032	108 ± 12
K^+	410 ± 52	160 ± 55	39 ± 15

Enzyme activities are expressed as μmoles NADH oxidized/min/mg homogenate protein. Potassium (K^+) content is expressed as nmoles potassium/mg homogenate protein. Activities determined in homogenates prepared from slices were determined by first pooling at least five slices. Results are presented as the mean ± SD of individual values determined in three separate experiments (N = 3).

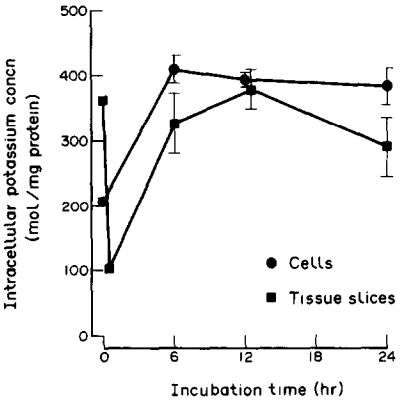

Fig. 1. Potassium content in cultured liver slices and cultured liver parenchymal cells. See legend of Fig. 3 for experimental details.

Table 3. The protein content and histological thickness of liver slices

Expt. No.	Mean protein (mg/slice)	Mean histological thickness (μm)
1	1.97	210 ± 18
2	1.92	250 ± 16
3	2.01	240 ± 12

Tabulation of data from three separate experiments. Protein content was determined by pooling at least five slices. The histological thickness was determined by measuring the thickness of fixed and stained slices at three separate positions and calculating the mean. Between three and five separate slices were examined in each experiment.

culture was serum-free because serum is employed to promote cell attachment to a substratum [13, 14]. The cells within the liver slice are already in contact with an extracellular matrix and so it was considered that serum was an unnecessary addition to the medium. The viability and levels of cytochrome P450 in liver slices is therefore compared to cultured cells in serum-free medium. Between 12 and 24 hr it was observed that a significant number of cells had detached from the plate surface. Presumably this accounts for the high leakage of LDH and GOT seen at 24 hr as enzyme leakage (Table 4) and detachment was counteracted by the inclusion of hydrocortisone to the medium. The addition of adrenal corticosteroids has become a common supplement to hepatocyte culture media and is considered essential in long-term serum-free cultures [25]. Although hydrocortisone enhances the attachment of cells to a substratum, it can be seen that it is without any significant effect on the fall of cytochrome P450 seen in cultures absent in hydrocortisone (Table 5). Furthermore, Table 5 shows that the levels of cytochrome P450 in liver slices falls at a comparable rate to cytochrome P450 in cultured cells.

The loss of cytochrome P450 in cultured hepatocytes can be prevented by the addition of 0.5 mM metyrapone to the culture medium [1]. In preliminary experiments metyrapone containing medium was also found to affect the loss of cytochrome P450 in liver slices such that they contained 70% of their initial P450 content after 20 hr of culture in serum-free and hormone-free medium.

The term "cytochrome P450" describes a gene superfamily of proteins [2] and the relative contribution that each isozyme makes to the total amount can only reliably be quantitated by immunochemical methods [12, 26]. Thus, in the uninduced adult male rat liver the major forms comprising cytochrome P450 content are IIA1, IIC6, IIC11 and IIIA2. Together IIC6 and IIC11 comprise over half of "cytochrome P450" constitutively expressed in normal adult male rat liver [26] and so the observed losses of cytochrome P450 in hepatocyte cultures and liver slices probably result from the loss of these isozymes. It has been reported that only the IIIA2 isozyme level falls to any great extent in cultured hepatocytes and that IIC11 actually increases [26]. Steward *et al.* [26] report that the decrease in spectrophotometrically determined levels of cytochrome P450 is not mirrored by a decrease in the total immunodetectable levels of cytochrome P450 and conclude that primarily the loss of

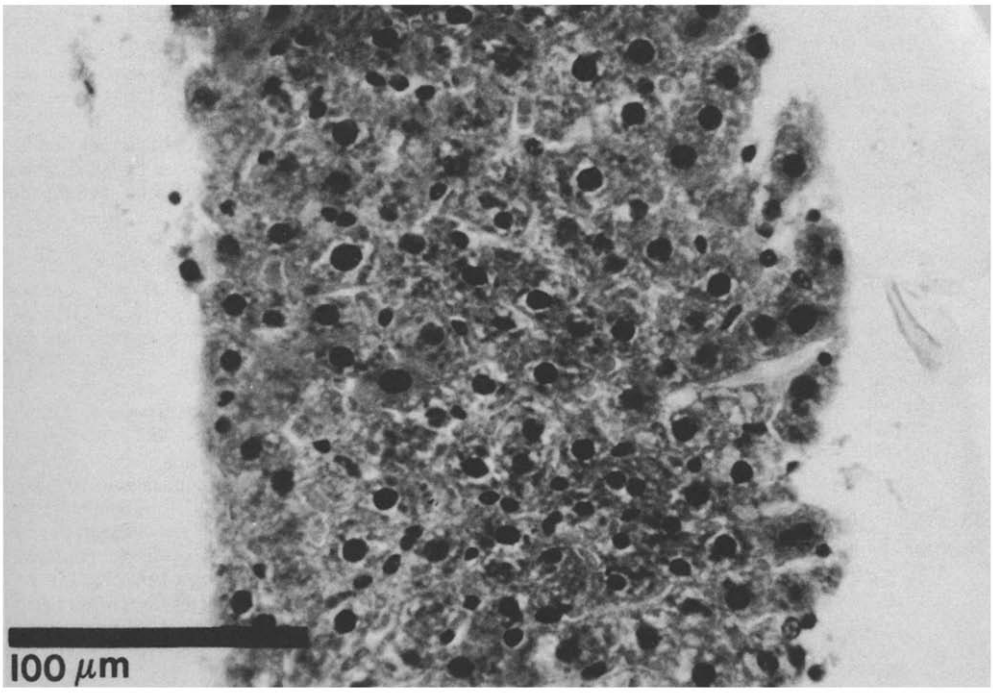

Fig. 2. Section of a freshly cut liver slice demonstrating the slice thickness. Tissue was stained with haemotoxylin and eosin.

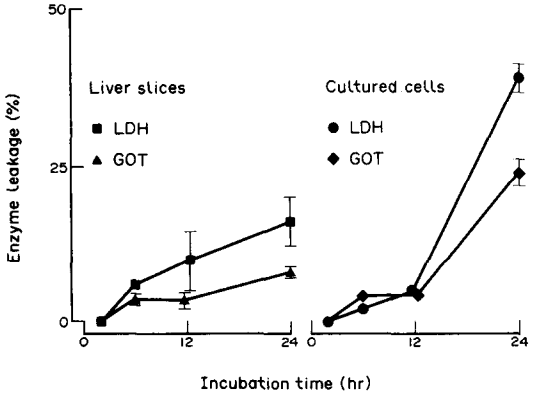

Fig. 3. Leakage of LDH and GOT from cultured liver slices and cultured liver parenchymal cells. The cells were isolated by collagenase perfusion and cultured serum-free as outlined in Materials and Methods. The cells and slices were cultured in WME supplemented with 1 μM insulin and 50 μg/mL gentamycin and after 2 hr the medium was renewed for both cells and slices. Data given are the mean ± SD of three separate plates (cells) or three separate vials (slices) from single experiments. Data are typical of at least three separate experiments.

Table 4. Effect of hydrocortisone (HC) on enzyme leakage from rat hepatocytes cultured for 24 hr in serum free media

Enzyme	Addition to culture medium None	10^{-4} M HC
LDH	32 ± 9	11 ± 3*
GOT	25 ± 2	9 ± 1*

Cells were isolated by collagenase perfusion and cultured serum-free in WME supplemented with 1 μM insulin, 50 μg/mL gentamycin and where indicated 100 μM hydrocortisone (+HC). After the first 2 hr of culture the medium was renewed. Data are the mean % leakage ± SD from three separate experiments.

* Indicates significantly different ($P < 0.05$) from value in hepatocytes cultured without HC.

cytochrome P450 is associated with a lack of haem prosthetic group incorporation into the apo-cytochrome. The reported maintenance of the IIC11 isozyme in cultured hepatocytes by this group is in contrast with other workers who report that this isozyme rapidly falls in cultured hepatocytes [27, 28].

The IIC11 isozyme is ultimately regulated by growth hormone *in vivo* [29], but the addition of growth hormone to the culture medium of adult male hepatocytes does not prevent the loss of the IIC11 isozyme rather the induction of the female specific isozyme IIC12 [28]. The non-cyclical variation of growth hormone concentration may account for the loss of the cytochrome P450 IIC11 isozyme and the induction of the IIC12 isozyme.

Whether the loss of cytochrome P450 in cultured hepatocytes is primarily due to a lack of haem prosthetic group incorporation into the cytochrome or to the lack of apoprotein synthesis, the data presented here suggest that this phenomenon is not solely dependent on the use of collagenase; the loss

Table 5. A comparison of loss of cytochrome P450 in rat hepatocytes and rat liver slices in culture

Hours of culture	Cultured hepatocytes +HC	Cultured hepatocytes NA	Liver slices NA
6	75 ± 12 (4)	75 ± 8 (3)	60 ± 2.9 (3)
12	56 ± 7 (4)	46 ± 6 (3)	49 ± 9.5 (3)
24	39 ± 12 (5)	35 ± 8 (6)	26 ± 11 (3)

Cells were isolated using collagenase and cultured serum-free as described in Materials and Methods. Both cells and slices were cultured in WME supplemented with 1 μM insulin, 50 μg gentamycin/mL medium and where indicated 100 μM hydrocortisone (HC). Results are presented as the mean ± SD percentage P450 remaining. The number of separate experiments is given in brackets. Initial cytochrome P450 concentration: hepatocytes 210 ± 37 pmol/mg cell protein; liver slices 250 ± 28 pmol/mg whole liver protein.
NA, no further additions to medium.

of cell–cell contact and/or the absence of an extracellular matrix. The absence of an endogenous inducer(s) not present in culture may, therefore, be the cause of the fall of cytochrome P450 in cultured rat liver slices. An endogenous inducer may still require the presence of other factors such as cell–cell contact to have its effect and so precision-cut liver slices may prove to be a useful system in which to identify such complex interactions.

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REFERENCES

1. Paine AJ, The maintenance of cytochrome P450 in rat hepatocyte culture: some applications of liver cell cultures to the study of drug metabolism, toxicity and the induction of the P450 system. *Chem Biol Interact* **74**: 1–31, 1990.
2. Paine AJ, The cytochrome P450 gene superfamily. *Int J Exp Pathol* **72**: 349–363, 1991.
3. Reid LM, Stem cell biology, hormone/matrix synergies and liver differentiation. *Curr Opin Cell Biol* **2**: 121–130, 1990.
4. Fraslin J, Kneip B, Vaulont S, Glaise D, Munnich A and Guguen-Guillouzo C, Dependence of hepatocyte-specific gene expression on cell–cell interactions in culture. *EMBO J* **4**: 2487–2491, 1985.
5. Begue J, Guguen-Guillouzo C, Pasdeloup N and Guillouzo A, Prolonged maintenance of active cytochrome P450 in adult rat hepatocytes co-cultured with another liver cell type. *Hepatology* **4**: 839–842, 1984.
6. Rogiers V, Vandenberghe Y, Callaerts A, Verleye G, Cornet M, Mertens K, Sonick W and Vercruysse A, Phase I and Phase II xenobiotic biotransformation in cultures and co-cultures of adult rat hepatocytes. *Biochem Pharmacol* **40**: 1701–1706, 1990.
7. Nakamura T, Yoshimoto K, Nakayama Y, Tomita Y and Ichihara A, Reciprocal modulation of growth and differentiated functions of mature rat hepatocytes in primary culture by cell–cell contact and cell membranes. *Proc Natl Acad Sci USA* **80**: 7229–7233, 1983.
8. Ichihara A, Nakamura T, Noda C and Tanaka K, Control of enzyme expression deduced from studies on primary cultures of hepatocytes. In: *Isolated and Cultured Hepatocytes* (Eds. Guillouzo A and Guguen-Guillouzo C), pp. 187–208. Libby Eurotext Ltd/ INSERM, London, 1986.
9. Bissell DM, Arenseon DM, Maher JJ and Roll FJ, Support of cultured hepatocytes by a laminin-rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver. *J Clin Invest* **79**: 801–812, 1987.
10. Schuetz EG, Li D, Omiecinski CJ, Muller-Eberhard U, Kleinman HK, Elswick B and Guzelian PS, Regulation of gene expression in adult rat hepatocytes cultured on a basement membrane matrix. *J Cell Physiol* **134**: 309–323, 1988.
11. Meredith MJ, Rat hepatocytes prepared without collagenase: prolonged retention of differentiated characteristics in culture. *Cell Biol Toxicol* **4**: 405–425, 1988.
12. Shean K and Paine AJ, Immunochemical quantification of cytochrome P450IA and IIB subfamilies in the livers of metyrapone-treated rats: relevance to the ability of metyrapone to prevent the loss of cytochrome P450 in rat hepatocyte culture. *Biochem J* **267**: 715–719, 1990.
13. Seglen PO and Fossa J, Attachment of rat hepatocytes *in vitro* to substrata of serum protein, collagen, or concanavalin A. *Exp Cell Res* **116**: 199–206, 1978.
14. Blaauboer BJ and Paine AJ, Attachment of rat hepatocytes to plastic substrata in the absence of serum requires protein synthesis. *Biochem Biophys Res Commun* **90**: 368–374, 1979.
15. Krumdieck CL, Dos Santos JE and Ho K, A new instrument for the rapid preparation of tissue slices. *Anal Biochem* **104**: 118–123, 1980.
16. Brendel K, Gandolfi AJ, Krumdieck CL and Smith PF, Tissue slicing and culturing revisited. *Trends Pharmacol Sci* **8**: 11–15, 1987.
17. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. *J Biol Chem* **239**: 2379–2385, 1964.
18. Recommendations of the German Society for Clinical Chemistry. *Z Klin Chem Klin Biochem* **10**: 281–291, 1972.
19. Bergmeyer HU, Horder M and Rej R, IFCC method for alanine aminotransferase (L-anine: 2-oxoglutarate aminotransferase, EC 2.6.1.2). *J Clin Chem Clin Biochem* **24**: 481–495, 1986.
20. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
21. Allen CM, Hockin LJ and Paine AJ, The control of glutathione and cytochrome P450 concentrations of primary cultures of rat hepatocytes. *Biochem Pharmacol* **30**: 2739–2742, 1981.
22. Capuzzi DM, Sparks CE and DeHoff JL, Effects of residual enzymes on degradation of radioiodinated VLDL by collagenase-dispersed hepatocytes. *Biochem Biophys Res Commun* **90**: 587–595, 1979.
23. Smith PF, Fisher RL, Shubat PJ, Gandolfi AJ, Krumdieck CL and Brendel K, *In vitro* cytotoxicity of allyl alcohol and bromobenzene in a novel organ culture system. *Toxicol Appl Pharmacol* **87**: 509–522, 1987.
24. Smith PF, Gandolfi AJ, Krumdieck CL, Putnam CW, Zukoski CF, Davis WM and Brendel K, Dynamic organ culture of precision liver slices for *in vitro* toxicology. *Life Sci* **36**: 1367–1375, 1985.
25. Bissel DM and Guzelian PS, Phenotypic stability of adult rat hepatocytes in primary monolayer culture. *Ann NY Acad Sci* **349**: 85–98, 1980.
26. Steward AR, Dannan GA, Guzelian PS and Guengerich FP, Changes in the concentration of seven forms of

cytochrome P450 in primary cultures of adult rat hepatocytes. *Mol Pharmacol* **27**: 125–132, 1985.
27. Waxman DJ, Morrissey JJ, Naik S and Jauregui HO, Phenobarbital induction of cytochromes P450. High level, long term responsiveness of primary rat hepatocyte cultures to drug induction and glucocorticoid dependence of the phenobarbital response. *Biochem J* **271**: 113–119, 1990.
28. Guzelian PS, Li D, Scheutz EG, Thomas P, Levin W, Mode A and Gustafsson J-A, Sex change in cytochrome P450 phenotype by growth hormone treatment of adult rat hepatocytes maintained in a culture system on matrigel. *Proc Natl Acad Sci USA* **85**: 9783–9787, 1988.
29. Waxman DJ, Interactions of hepatic cytochromes P-450 with steroid hormones. Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem Pharmacol* **37**: 71–84, 1988.