

# Studies on the maintenance of cytochromes P-450 and $b_5$ , monooxygenases and cytochrome reductases in primary cultures of rat hepatocytes

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The cytochrome P-450 content of rat hepatocytes declined rapidly over 72 h in culture, due primarily to denaturation to cytochrome P-420. Six different media were investigated for their ability to conserve cytochrome P-450 during culture, and the most successful was a modified Earle's medium. After 72 h culture in this medium, cytochromes P-450 and  $b_5$ , NADH-cytochrome  $b_5$ - and NADPH-cytochrome  $c$ -reductases were maintained at 40, 100, 35 and 52% of fresh cell values, respectively. Cytochrome P-450 showed differential functional stability during culture with ethoxyresorufin  $O$ -deethylation being more stable than either pentoxyphenoxazone  $O$ -depytylation or biphenyl 4-hydroxylation. Monooxygenase activities declined faster than did cytochrome P-450 content. This discrepancy was not explained by loss of the flavin nucleotides, FMN or FAD.

<i>Cultured hepatocyte</i>	<i>Cytochrome P-450</i>	<i>Mixed-function oxidase</i>	<i>NADPH-cytochrome <math>c</math> reductase</i>
		<i>NADH-cytochrome <math>b_5</math> reductase</i>	

## 1. INTRODUCTION

Primary cultures of hepatic parenchymal cells have become an established method for studying mechanisms of hepatotoxicity and carcinogenesis under defined conditions in vitro [1,2]. However, the toxicity and carcinogenicity of many chemicals depend upon activation and detoxification by the hepatic microsomal cytochrome P-450 monooxygenase system, the activity of which declines to low levels within the first 24–48 h of culture [3]. For this reason it is difficult to interpret experimental data on cellular mechanisms of hepatotoxicity and carcinogenesis obtained using cultured hepatocytes.

There have been many attempts to maintain the levels of cytochrome P-450 and its monooxygenase activities in culture by adding hormones and/or inducers to the media [4–7]. Recently, prolonged maintenance of active cytochrome P-450 has been achieved in rat hepatocytes co-cultured with another liver epithelial cell type [8]. To achieve maximum cytochrome P-450 maintenance during both conventional cell culture and co-culture it will be necessary to determine which of all the media available for cell culture is the most efficient at maintaining the concentration and activity of the cytochrome. We have studied the effect of 6 different media on the level of cytochrome P-450 in rat hepatocytes in culture.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Flow Laboratories supplied all the media used,

**Abbreviations:** EROD,  $O$ -deethylation of ethoxyresorufin; PPOD,  $O$ -depytylation of pentoxyphenoxazone

the foetal calf serum, BME vitamin solution and fungizone. The hormones, amino acids, bovine serum albumin (Fraction V), oleic acid, linoleic acid, NADH, NADPH, isocitric acid, isocitrate dehydrogenase, cytochrome *c*, Lubrol PX, FAD and FMN were all supplied by Sigma. Collagenase was obtained from Boehringer Mannheim, and biphenyl and 4-hydroxybiphenyl from BDH. Resorufin, ethoxyresorufin and pentoxyphenoxazone were synthesized as described by Burke and Mayer [9].

## 2.2. Preparation of media

William's E, Waymouth's, Dulbecco's, Eagle's and Ham F12 media were supplemented with 5% foetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone. Waymouth's medium was also supplemented with the hormone mixture described by Decad et al. [10] instead of serum and the composition of the modified Earle's medium was as described [11].

## 2.3. Primary culture of rat hepatocytes

Hepatocytes from male Hooded Lister rats (180–220 g) were prepared (≥90% viable by trypan blue exclusion) and cultured on collagen coated petri dishes as described [11,12].

## 2.4. Preparation of whole cell homogenate

All enzymic measurements of fresh and cultured cells were carried out using cell homogenates. Cultured hepatocytes were washed with 0.1 M sodium phosphate buffer, pH 7.6, scraped off the plates using a 'rubber policeman' and homogenised using a Teflon-glass Potter-Elvehjem type homogeniser. Freshly isolated cells were homogenised at a concentration of  $10^6$  cells/ml. For cytochrome P-450 measurement homogenates were prepared in 2 ml of 0.1 M sodium phosphate buffer, pH 7.6, containing 1 mM dithiothreitol, 1 mM EDTA, 15% (v/v) glycerol and 0.2% (v/v) Lubrol PX. This buffer inhibited the *O*-deethylation of ethoxyresorufin, so the cells used for all determinations other than those of cytochrome P-450 content were homogenised in 0.1 M sodium phosphate buffer, pH 7.6. Homogenates were stored at  $-80^{\circ}\text{C}$  until analysis.

## 2.5. Analytical methods

The concentration of cytochromes P-450 and

P-420 were measured as described by Omura and Sato [13]. Cytochrome *b*<sub>5</sub> content and the activities of NADPH-cytochrome *c*- and NADH-cytochrome *b*<sub>5</sub>-reductases were measured as described by Falzon and co-workers [14].

Biphenyl hydroxylation was measured by the fluorimetric method of Burke and Prough [15] at 70 µM substrate concentration, using an NADPH generating system consisting of 0.25 mM NADP, 2.5 mM DL-isocitric acid, 0.6 U isocitrate dehydrogenase and 5 mM magnesium sulphate. Incubations containing 0.5 ml cell homogenate were carried out in air in round bottomed tubes in a shaking water bath at 37°C.

The *O*-deethylation of 5 µM ethoxyresorufin (EROD reaction) and *O*-dealkylation of 5 µM pentoxyphenoxazone (PPOD reaction) were measured by direct fluorimetric detection of the resorufin formed [9]. Reactions containing 0.5 ml of cell homogenate and an NADPH-generating system as above were carried out in air in round bottomed tubes at 37°C under yellow light. The fluorescence intensity of the reaction mixture was read at timed intervals at an emission wavelength of 585 nm and an excitation wavelength of 530 nm.

Protein was measured by the method of Lowry et al. [16] using crystalline bovine serum albumin as standard. Results are expressed in terms of total cellular protein.

## 3. RESULTS

Table 1 shows the ability of the 6 different culture media to maintain the content of cytochrome P-450 and P-420 in cultured hepatocytes over a 48 h period. There was a marked denaturation of cytochrome P-450 to cytochrome P-420, which increased with time in culture in all media. The media varied substantially in terms of cytochrome P-450 maintenance. Waymouth's medium supplemented with a hormone mixture [9] was better for cytochrome P-450 maintenance than Waymouth's medium with serum supplement. The best medium in terms of P-450 maintenance was the modified Earle's medium and when the cytochrome P-450 content of the cultured cells was taken into account, maintenance of cytochromes P-450 plus P-420 was approx. 90% even after 72 h (table 2). The addition of glycerol, antioxidants (5 mg/l ascorbate,

Table 1

Maintenance of cytochrome P-450 in hepatocytes cultured in different media for 24–48 h

Medium	Cytochrome P-450 (% fresh cell levels)		Total cytochromes P-450 + P-420 (% fresh cell values)		Cytochrome P-450/ P-420 ratio	
	24 h	48 h	24 h	48 h	24 h	48 h
Modified Earle's	74 (4)	63 (4)	93 (4)	86 (4)	2.2 (4)	1.0 (4)
William's E	45 (4)	44 (4)	68 (4)	56 (4)	1.2 (4)	0.6 (4)
Waymouth's	38 (2)	26 (2)	55 (2)	42 (2)	1.7 (2)	1.2 (2)
Dulbecco's	32 (2)	24 (2)	56 (2)	52 (2)	1.1 (2)	0.7 (2)
Eagle's	32 (2)	7 (2)	54 (2)	31 (2)	1.2 (2)	0.3 (2)
Ham's F12	10 (2)	0 (2)	45 (2)	64 (2)	0.3 (2)	—
Waymouth's + hormones	74 (2)	32 (2)	80 (2)	40 (2)	2.6 (2)	1.2 (2)

The values are means, with the number of experiments given in parentheses: the variation between experiments was less than 10%. Initial fresh cell values (expressed per mg total cell protein) were: cytochrome P-450, 0.43 nmol/mg protein; total cytochrome P-450 + P-420, 0.47 nmol/mg protein; cytochrome P-450/P-420 ratio 9.3

Table 2

Maintenance of enzyme activities in hepatocytes cultured in modified Earle's medium

Time	Cytochrome P-450	Total cytochrome P-450 + P-420	Cytochrome <i>b</i> <sub>5</sub>	NADH cytochrome <i>b</i> <sub>5</sub> reductase	NADPH cytochrome <i>c</i> reductase	EROD	PPOD	Biphenyl 4-OH
24 h	85 ± 6 (7)	91 ± 5 (7)	100 (2)	68 ± 6 (3)	98 ± 9 (3)	45 (2)	22 (2)	48 (2)
48 h	55 ± 6 (9)	85 ± 6 (9)	100 (2)	66 ± 12 (5)	49 ± 16 (5)	12 (2)	6 (2)	11 (2)
72 h	40 ± 4 (4)	86 ± 8 (4)	100 (2)	35 ± 2 (4)	52 ± 1 (3)	5 (2)	7 (2)	0 (2)

EROD, *O*-deethylation of ethoxyresorufin; PPOD, *O*-depentylation of pentoxyphenoxazone. Results of more than 3 experiments are presented as means ± SE, with the number of experiments given in parentheses. For duplicate experiments only means are shown and the variation was less than 5%. All data expressed as % initial values in fresh cells. Fresh cell values (expressed per mg total cell protein) are: cytochrome P-450, 0.45 nmol/mg protein; total cytochrome P-450 + P-420, 0.47 nmol/mg protein; cytochrome *b*<sub>5</sub>, 0.28 nmol/mg protein; NADH-cytochrome *b*<sub>5</sub> reductase, 0.65 μmol/min per mg protein; NADPH cytochrome *c* reductase, 21.9 nmol/min per mg protein; EROD, 440 pmol/min per mg protein; PPOD, 126 pmol/min per mg protein; and biphenyl 4-hydroxylation, 68 pmol/min per mg protein

25 μg/l vitamin E) or inhibitors of protein degradation (10 mM NH<sub>4</sub>Cl) to the modified Earle's medium did not prevent the conversion of cytochrome P-450 to P-420 (not shown).

Further investigation using modified Earle's medium showed that cytochrome *b*<sub>5</sub> content was fully conserved in the cells for 72 h, while the activities of both NADH-cytochrome *b*<sub>5</sub>- and

NADPH-cytochrome *c*-reductases declined slowly, at approximately the same rate as cytochrome P-450 (table 2). The activity of EROD and PPOD and the 4-hydroxylation of biphenyl were not well maintained even in the 24 h cultures as shown in table 2. The *O*-deethylation of ethoxycoumarin declines at approximately the same rate as EROD and the decline in the *N*-demethylation of

aminopyrine is similar to that of PPOD (not shown). In contrast, the metabolism of benzo(a)pyrene is maintained at fresh cell levels for at least 24 h in hepatocytes cultured in this medium (H. Morrison and B. Jernström, unpublished). The flavin nucleotides, FMN and FAD, are prosthetic groups of NADPH-cytochrome *c* reductase. Their addition to EROD incubations and to biphenyl 4-hydroxylation reactions at concentrations of 5 µg/ml incubate (corresponding to concentrations in the liver [17]), failed to improve the activity of these reactions in 24 and 48 h cultured hepatocytes (not shown).

#### 4. DISCUSSION

The data presented here demonstrate that culture media vary a great deal in their ability to maintain cytochrome P-450 content in cultured hepatocytes. Ham's F12, Eagle's and Dulbecco's media are not suitable for studies of cytochrome P-450 using cultured rat hepatocytes. Waymouth's medium is suitable for short term studies provided it is supplemented with a hormone mixture [10] instead of serum. The most efficient medium tested for conserving cytochrome P-450 content was the modified Earle's medium and this may be due to prevention of cytochrome P-450 breakdown by the high concentration of hydrocortisone (A. Guillouzo, personal communication). Modified Earle's medium is therefore the most suitable of the 6 tested for short term studies of metabolism and toxicity since 40% of the cytochrome P-450 content was maintained even after 72 h of culture. In longer term studies, however,  $10^{-4}$  M hydrocortisone may prove detrimental to the cells after 7–10 days exposure [18].

It has been reported that the antigenic apoprotein of cytochrome P-450 does not decline rapidly in culture, in contrast to the spectrophotometrically determined levels of the CO-binding holoprotein [19]. Our results may explain this by indicating that the decline in cytochrome P-450 is primarily due to denaturation to P-420, and not to loss of apoprotein. Despite the maintenance of high levels of cytochrome P-450 in the cells cultured in modified Earle's medium several monooxygenase activities declined rapidly over the first 24 h of culture. Recently, the measurement of cytochrome P-450 in cultured rat hepatocytes using specific an-

tibodies has revealed that the concentrations of different isozymes are maintained to different extents in culture [19]. This variation in the behaviour of cytochrome P-450 isozymes is reflected here in the extents to which different monooxygenases are maintained by Earle's medium.

Our observations that monooxygenase activities declined faster than cytochromes P-450 or *b*<sub>5</sub> or cytochrome reductases suggest that the loss or inactivation of some other component (but not FMN or FAD) is contributing to the decrease in monooxygenation.

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