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THE EFFECT OF HEPATOCYTE CULTURE CONDITIONS ON CYTOCHROME P-450 LINKED DRUG METABOLISING ENZYMES

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Rat hepatocytes, in culture, are a popular model for the study of cytochrome P-450 mediated drug metabolism (for reviews see 1,2). However a limitation of cultured hepatocytes in these studies is the rapid loss of cytochromeP-450 (3,4). Recently two culture systems that prevent this loss have been devised, these are: (a) the addition of any one of a number of substituted pyridines (5), the most potent of which is metyrapone (6), to the culture medium and (b) the use of a medium, "P-450 medium", without cystine and cysteine and supplemented with 100 μM 5-aminolevulinic acid (7). The present work shows that while "P-450 medium" maintains cytochrome P-450 at the same concentration as found in the intact liver the cytochrome has a low activity towards four commonly used drug substrates. In contrast culture medium containing metyrapone maintains the activity of drug metabolising enzymes generally considered to be associated with cytochrome P-450 at the same activity as found in the initial cell preparation and induces those activities associated with cytochrome P-448.

METHODS

Isolation and culture of hepatocytes: was as previously described (8).
Biochemical assays: these were done on cell homogenates (20 secs Ultra Turrax) in 0.15 M KCl-50 mM Tris-HCl pH 7.4. Cytochrome P-450 and protein were assayed as previously described (9). Ethylmorphine N-demethylase (10), Benzphetamine N-demethylase (10), 7-Ethoxycoumarin O-deethylase (11) and 7-ethoxyresorufin O-deethylase (12) were assayed by modifications of the reference techniques. 7-Ethoxycoumarin O-deethylase was measured in hepatocyte monolayers by the method of Fry & Bridges (13).

RESULTS AND DISCUSSION

Cytochrome P-450 is a collective term for a group of microsomal haemoproteins which exhibit different substrate specificities (14). Although multiple forms of cytochrome P-450 exist in rat hepatocytes only a few have been studied in detail. Two of these are cytochrome P-450 LM2 and cytochrome P-448 (also known as P-450LM4)(15). Of the substrates studied in the present work, 7-ethoxycoumarin is metabolised by both forms, ethylmorphine and benzphetamine are metabolised preferentially by P-450 LM2, while 7-ethoxyresorufin is metabolised preferentially by cytochrome P-448 (12,14,15).

Table 1 shows that when rat hepatocytes are cultured in a commonly used medium, such as Williams' E, they lose 60% of their cytochrome P-450 content during the first 24 hours and thereafter there is a further loss of the cytochrome. This loss of cytochrome P-450 is paralleled by a loss of ethylmorphine and benzphetamine N-demethylases as well as 7-ethoxycoumarin O-deethylase. In contrast 7-ethoxyresorufin-O-deethylase, an activity associated

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Table 1. Effect of hepatocyte culture medium on cytochrome P-450 concentration and four cytochrome P-450 mediated enzyme activities.

Culture Medium	Culture Time (hours)	% Initial concn. or % initial activity				
		Cytochrome P-450	Ethylmorphine N-demethylase	Benzphetamine N-demethylase	7-Ethoxycoumarin O-deethylase	7-Ethoxyresorufin O-deethylase
	24	48 ± 5	36 ± 2	38 ± 4	33 ± 1	31 ± 6
Williams'E	48	32 ± 2	21 ± 3	36 ± 11	25 ± 4	101
	72	23 ± 5	26 ± 5	11 ± 4	22 ± 4	111 ± 5
	96	22 ± 4	21 ± 2	17 ± 4	13 ± 2	75 ± 13
	24	95 ± 3	86 ± 7	57 ± 8	107 ± 15	986 ± 37
Williams'E	48	102 ± 4	95 ± 8	51 ± 2	201 ± 18	2950 ± 391
+ 0.5 mM	72	101 ± 6	123 ± 10	77 ± 13	301 ± 26	3337 ± 397
Metyrapone	96	110 ± 6	109 ± 14	84 ± 6	188 ± 24	1995 ± 275
	24	96 ± 6	18 ± 5	12 ± 3	28 ± 4	6 ± 1
P-450	48	68 ± 9	34 ± 15	22 ± 9	14 ± 2	23 ± 8
Medium	72	44 ± 6	19 ± 2	15 ± 2	17 ± 2	60 ± 11
	96	33 ± 8	13 ± 2	12 ± 5	6 ± 1	56 ± 7

Rat hepatocytes were cultured in the respective medium, which was changed every 24 hours, for the times shown. The results are given as the mean ± S.E.M. of individual values found in preparations derived from three separate rat livers (i.e. N=3). The initial cytochrome P-450 concentration of isolated cells was 166 ± 17 pmoles/mg protein. The initial enzyme activities (nmoles product formed/hour/mg protein) were ethylmorphine 177 ± 6, benzphetamine 136 ± 14, 7-ethoxycoumarin 28.7 ± 2, 7-ethoxyresorufin 0.842 ± 0.02.

with cytochrome P-448, only parallels the loss of the cytochrome during the first 24 hours of culture and thereafter increases to the same level as found in the initial cell preparation. These observations are compatible with the concept that the culture of rat hepatocytes in a standard medium results in a change in the proportions of cytochrome P-450 and P-448 (16). However the culture of rat hepatocytes in "P-450 medium" maintains high concentrations of cytochrome P-450 but the species of cytochrome maintained has a low activity towards all four substrates studied (Table 1). The addition of 0.5 mM metyrapone to Williams medium E prevents the loss of cytochrome P-450 over a 96 hour culture period and maintains ethylmorphine and benzphetamine demethylase activities, which are predominantely associated with cytochrome P-450 LM2 (Table 1). In contrast the activities associated with cytochrome P-448 are increased. This is most dramatically shown by the 30-fold increase, observed after 72 hours of culture, in the activity of 7-ethoxyresorufin-O-deethylase (Table 1). These increases, produced by metyrapone can be prevented by incorporating 2.5 μM cycloheximide, which inhibits protein synthesis by 85% (5), into the culture medium (Table 2). Thus the results with cultured hepatocytes suggest that metyrapone, which is generally considered to be an inhibitor of cytochrome P-450 mediated drug metabolism (17), can also be an inducer. The induction of cytochrome P-448 associated activities by metyrapone can be demonstrated either with homogenates of cultured cells (Table 1) or intact cells in monolayer culture (Table 3).

Metyrapone is one of a number of substituted pyridines that prevent the loss of cytochrome P-450 during the first 24 hours of hepatocyte culture (5,6). The results in Table 4 compare the relative efficiency of these pyridines at maintaining the cytochrome with their effect on cytochrome P-450 mediated drug metabolism after 72 hour of culture. Only metyrapone and pyridine are able to prevent the loss of the cytochrome after 72 hours of culture. However all of the pyridines studied induce 7-ethoxyresorifin O-deethylase activity (Table 4).

In conclusion the present work shows that while it is possible to maintain cytochrome P-450 in hepatocyte culture at *in vivo* concentrations by using "P-450 medium" or pyridines neither of these culture systems maintain P-450 mediated drug metabolism at the same activity as present in the initial cell preparation or intact liver.

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Table 2. Effect of cycloheximide on the induction of 7-ethoxyresorufin and 7-ethoxycoumarin-O-deethylases by metyrapone

Additions to Williams medium E		% Activity of untreated cells	
0.5mM Metyrapone	2.5μM Cycloheximide	7-Ethoxyresorufin O-deethylase	7-Ethoxycoumarin O-deethylase
-	-	100	100
-	+	33	123
+	-	2040	255
+	+	238	138

Hepatocytes were cultured in Williams' E ± Metyrapone ± Cycloheximide for 24 hours. The results are given as the average of individual values found in preparations derived from two separate rat livers. The activity (nmoles product formed/hour/mg protein) of 7-Ethoxyresorufin and 7-Ethoxycoumarin O-deethylases found in untreated hepatocytes was 0.247 and 8.9 respectively.

Table 3. Induction, by metyrapone, of 7-ethoxycoumarin O-deethylase, in hepatocyte monolayers.

Culture medium	7-Ethoxycoumarin O-deethylase activity (nmoles/hr/mg protein) after culture (hours) for:			
	24	48	72	96
Williams' E	4.15 ± 0.45	3.25 ± 0.14	2.5 ± 0.7	2.1 ±0.25
Williams'E + 0.5 mM Metyrapone	10.45 ± 0.55	15.5 ± 0.9	19.3 ± 1.8	17.0 ±1.9

Hepatocytes were cultured in the respective medium for the time stated. The medium was then changed to Williams' E containing 70 μM 7-ethoxycoumarin and 7-ethoxycoumarin O-deethylase activity assayed as described by Fry & Bridges (13). The results are given as the mean ± S.E.M. for individual values found in preparations derived from 3 separate rat livers (i.e.N=3). The initial 7-ethoxycoumarin O-deethylase activity of freshly isolated hepatocytes was 4.7 ± 0.1 nmoles/hr/mg protein.

Table 4. Effect of culturing hepatocytes with pyridines for 72 hours on cytochrome P-450 concentration and associated enzyme activities.

Addition to Williams' medium E	% Initial concentration or % initial activity		
	Cytochrome P-450	Ethylmorphine N-demethylase	7-Ethoxyresorufin O-deethylase
0.5 mM Metyrapone	97	92	3562
25 mM Pyridine	108	175	4108
10 mM Isonicotinamide	45	39	1971
10 mM 3-Aminopyridine	57	47	3027
10 mM 3-Acetylpyridine	64	69	3150
5 mM 3-Hydroxypyridine	77	104	3680

The initial cytochrome P-450 concentration was 220 pmoles/mg protein and the activities of ethylmorphine-N-demethylase and 7-ethoxyresorufin O-deethylase were 177 and 0.84 nmoles product formed/hour/mg protein respectively.