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CYTOCHROME P450 MAINTENANCE AND DIAZEPAM METABOLISM IN CULTURED RAT HEPATOCYTES

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Abstract—Diazepam metabolism has been investigated in rat hepatocytes cultured for 3, 24, 48 and 72 hr under five different conditions. Although four of the treatments studied reduced markedly the spontaneous loss of cytochrome P450, they had different effects on the metabolism of diazepam (DZ) presumably by affecting the relative proportions of cytochrome P450 isozymes during the period of culture. Thus P450 medium or dimethyl sulphoxide-supplemented medium maintained the rate of disappearance of DZ from the culture medium and metabolite profile in 24 hr cultures at the initial levels found in 3 hr cultures, while culture at 30° or in metyrapone-containing medium resulted in the production of oxazepam, a metabolite normally only produced by dog, monkey and human hepatocytes. These findings indicate that the well recognized phenotypic alteration of cytochrome P450-dependent mono-oxygenase activities that occurs when rat hepatocytes are cultured in different media can result in a range of metabolic options that are normally only available in other animal species.

The prediction of routes by which a new chemical entity may be metabolized from knowledge of its chemical structure alone is still in its infancy and so, in practice, a multiplicity of tissue samples/incubations are often required to identify all of the metabolites produced when a xenobiotic undergoes biotransformation. With certain well studied compounds, it has been demonstrated that the types of metabolites produced may be attributed to the heterogeneous nature of the drug metabolizing enzyme systems. For example, the cytochrome P450 mono-oxygenase family involved in the metabolism of a wide range of endogenous and xenobiotic compounds has been extensively characterized and multiple forms of the haemoprotein have been demonstrated [1].

A promising method for reducing the large number of systems required is to incubate drugs with primary cultures of rat hepatocytes [2–4]. However a limitation to their use in the identification of pathways of metabolism is their rapid loss of cytochrome P450 during culture. This loss has been shown to be prevented by culturing rat hepatocytes in medium containing 0.5 mM metyrapone [2] or 2% (v/v) dimethyl sulphoxide [5] or by formulating a special 'P450-medium' [2].

In order to determine the relative merits of these different culture systems the present work evaluates their effects on the metabolism of diazepam. Diazepam (DZ‡) is used clinically as an anxiolytic and muscle relaxant and is a useful model substrate as it is metabolized by several pathways and as such is often used for kinetic studies in many species including man [6–8].

MATERIALS AND METHODS

Preparation of hepatocytes. Livers were obtained from male CD rats (230–250 g) obtained from Charles River, Margate, Kent, which were allowed food and water *ad lib.* prior to death.

Hepatocytes were isolated from the rat livers by end of lobe perfusion using the technique described by Chenery *et al.* [8]. The isolated cells were filtered through 65 micron nylon mesh (Henry Simon Ltd, Stockport, Cheshire, U.K.) and washed three times in Earles balanced salt solution without Ca^{2+} and Mg^{2+} (Gibco Europe Ltd, Cowley, Middx, U.K.) containing 1% bovine serum albumin (Sigma Chemical Co., Poole, U.K.) by centrifugation at 50 *g* for 3 min followed after the final wash by resuspension in the respective culture medium. Hepatocytes (viability by trypan blue exclusion >85%) were plated onto 35 mm six-well Nunc™ plates after each well was coated with 0.85 mg soluble calf skin collagen (Lorne Diagnostics, Bury St Edmunds, U.K.), at a density of 0.7×10^6 cells/well/2 mL medium. Cells adhered to the substrata within 2 hr, when the culture medium was changed. The monolayer became confluent within the subsequent 24 hr. The cells were cultured either at 30° or 37° under a humidified atmosphere of 5% CO_2 in air.

WME culture medium was purchased from Flow Labs (Irvine, U.K.) while P450 medium was prepared in our laboratory as described by Paine and Hockin [9]. All media contained bovine insulin (1 μM), hydrocortisone hemisuccinate (100 μM), penicillin (10^5 I.U./L), streptomycin (100 mg/L), neomycin (100 mg/L) and 10% (v/v) newborn calf serum.

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‡ Abbreviations: DZ, diazepam; NOR, *N*-desmethyldiazepam; TEM, temazepam; OXA, oxazepam; 4OHNOR, 4′-hydroxy-*N*-desmethyldiazepam; 4OHDZ, 4′-hydroxydiazepam; WME, Williams medium E; P450 medium, RPMI 1640 medium without cyst(e)ine supplemented with 100 μM 5-aminolaevulinic acid; DMSO, dimethyl sulphoxide.

Metyrapone (0.5 mM) and 2% (v/v) DMSO were added to the medium before the addition of cells.

Cytochrome P450 *and protein.* Cytochrome P450 and protein were determined as described previously [8, 9].

Incubation with diazepam. Metabolism studies were performed with hepatocytes cultured for 3, 24, 48 and 72 hr. Diazepam (20 μM) was prepared in the same medium as used to culture the hepatocytes but without phenol red and newborn calf serum. The cell monolayers were washed with isotonic saline at 37° and 2 mL of the drug incubation medium warmed to the same temperature was added to each well. The plates were then held at 37° in the CO_2 incubator for 180 min and 1 mL samples were taken from successive wells at 15 min intervals between zero time and 180 min in order to calculate the initial rate of disappearance of DZ as described by Seddon *et al.* [10]. These samples were placed in capped polystyrene tubes and stored at −80° until analysis. In another experiment hepatocytes were incubated with [2-^{14}C]diazepam (Amersham International, Aylesbury, U.K.) at a final specific radioactivity of 5 μCi/μmol.

Diazepam and metabolites. Diazepam and metabolites were determined by HPLC as described previously [8]. Prior to analysis, 30 units of crude β-glucuronidase containing sulphatase activity (Sigma grade HA-4) was added per mL medium and incubated at 37° for 30 min. Hydrolysis of DZ conjugates has been reported to be complete within 2 to 3 min with this method [11]. Routinely 5–25 μL of deconjugated medium was injected directly onto a Waters C-18 microbondapak (30 cm × 46 mm) column with a prefit filter. The separation of DZ and metabolites employed a gradient of methanol (65%–78%, v/v) in 10 mM ammonium acetate (adjusted to pH 6 with CF_3COOH). Oven temperature was held at 50° [8]. Retention time for DZ standard as the least polar compound was 9.2 min and accordingly total analysis time was 10 min. The retention times of authentic standards of NOR, TEM, OXA, 4OHDZ and 4OHNOR (generous gifts from Roche Products Ltd, Welwyn Garden City, U.K.) were 8.3, 7.8, 7.2, 6.9 and 6.5 min, respectively. All samples were analysed within 7 days of completing the incubation with cultured hepatocytes, although DZ and its metabolites are reported to be stable at −80° for at least 90 days [10]. The amounts of DZ lost and metabolites formed were normalized with respect to the amount of cellular protein contained in each well of the culture plate.

RESULTS

Cytochrome P450 *maintenance studies*

The effect of the different conditions on cytochrome P450 content of adult rat hepatocytes during culture is shown in Table 1. Cytochrome P450 content of hepatocytes cultured in WME at 37° declined by half during the first 24 hr of culture, and decreased further to 35% and 22% after 48 and 72 hr of culture, respectively. In contrast, culture of hepatocytes in WME at 30° retarded the loss of total cytochrome P450 such that the mean cellular content was 89% of the initial value after 24 hr of culture, but thereafter the decline was similar to that in hepatocytes cultured in WME at 37°. The culture of hepatocytes at 37° in WME containing 2% (v/v) DMSO or the special 'P450 medium' also retarded the decline of cytochrome P450 content during the first 24 hr of culture. However, beyond a day of culture, incorporation of DMSO into the medium or culture in P450 medium had little effect on the loss of cytochrome P450 content. The most effective method of maintaining cytochrome P450 content at initial levels for longer than 24 hr was culture of the hepatocytes at 37° in Williams medium E containing 0.5 mM metyrapone (Table 1). Nevertheless, it was observed that metyrapone was unable to prevent completely the loss of cytochrome P450 in hepatocytes cultured for more than 24 hr unless 10^{-4} M hydrocortisone was present in the culture medium (Table 2).

Table 1. Comparison of total cytochrome P450 content in adult rat hepatocytes cultured in different media

Culture medium	Cytochrome P450 (% initial content) after culture for: 24 hr	48 hr	72 hr
WME	48 ± 12	35 ± 7	22 ± 11
WME at 30°	89 ± 11	50 ± 14	39 ± 5
WME + 0.5 mM metyrapone	99 ± 6	113 ± 21	125 ± 14
WME + 2% DMSO	70 ± 8	54 ± 6	31 ± 3
P450 medium	80 ± 9	59 ± 6	56 ± 13

Hepatocytes were cultured, as described in Materials and Methods, in the respective medium at 37° unless otherwise indicated. All media contained 10^{-4} M hydrocortisone, 10^{-6} M insulin and 10% newborn calf serum. Cytochrome P450 was determined at the times shown and is expressed as the mean ± SD of the initial values found in three separate hepatocyte preparations (N = 3) which were 193 ± 15 pmol/mg protein.

Table 2. Effect of hydrocortisone (HC) on the ability of metyrapone to maintain the cytochrome P450 content of hepatocytes cultured in Williams medium E (WME)

Culture medium	HC (10^{-4} M)	Cytochrome P450 (% initial content) after culture medium for: 24 hr	48 hr	72 hr
WME	−	39 ± 6	26 ± 6	17 ± 8
	+	48 ± 12	35 ± 7	22 ± 11
WME + 0.5 mM metyrapone	−	75 ± 16	47 ± 8	30 ± 5
	+	99 ± 6	113 ± 21	125 ± 14

Hepatocytes were cultured at 37°, as described in Materials and Methods, in WME containing 10% newborn calf serum and 10^{-6} M insulin with and without 10^{-4} M hydrocortisone and 0.5 mM metyrapone. Cytochrome P450 was determined at the times shown and is expressed as the mean ± SD of the initial values found in three separate hepatocyte preparations (N = 3) which were 185 ± 21 pmol/mg protein.

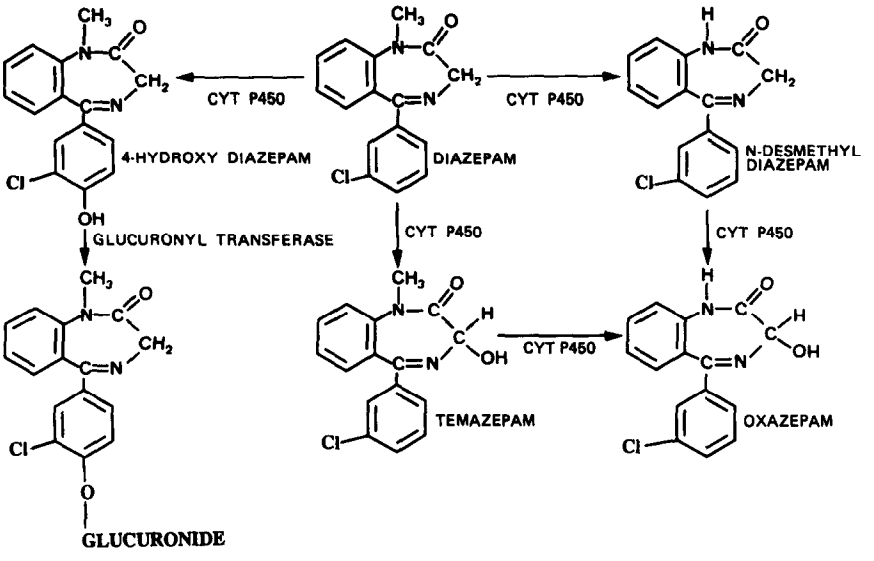

Fig. 1. Scheme of hepatic DZ metabolism in the rat (from Seddon *et al.* [10]).

Diazepam metabolism studies

The established pathways of DZ metabolism are shown in Fig. 1. The metabolism of diazepam was investigated in the five separate culture systems. In all systems the disappearance of DZ closely approximated a mono-exponential decline, as exemplified by Fig. 2, with 90% of radiolabelled [^{14}C]DZ and its metabolites being found in the cell culture medium. Addition of DZ-containing media to collagen-coated plates and incubation in the absence of hepatocytes did not lead to the disappearance of the drug, and neither was there the production of metabolites in the different media over the 180 min incubation period in the absence of hepatocytes.

After 3 hr of culture the rate of disappearance of DZ was essentially the same under all culture conditions (11.2–11.6 nmol/hr/mg cell protein). However, in hepatocytes cultured in Williams medium E at 37° for 24 hr the rate of disappearance of DZ from the incubation medium, concomitant with the loss of cytochrome P450, declined to 47% of the value measured in the 3 hr cultures (cf. Tables 1 and 3). Culture conditions that prevented or retarded the loss of the hepatocytes' cytochrome P450 content were found to maintain the rate of removal of DZ from the medium at levels closer to the initial value. Thus WME supplemented with 2% DMSO actually maintained the rate of DZ removal at the initial levels after 24 hr culture. Thereafter the rate of DZ removal declined, albeit by differing degrees, in all the culture systems studied (Table 3). The results presented in Table 4 show that hepatocytes cultured for 3 hr in WME alone at 37° metabolized DZ into three of its major metabolites: 4'-hydroxydiazepam, desmethyldiazepam and temazepam, but together these accounted for only 34% of the DZ metabolized. 4OHNOR or OXA were not detected in hepatocytes cultured in WME at 37° so more DZ was metabolized than could be

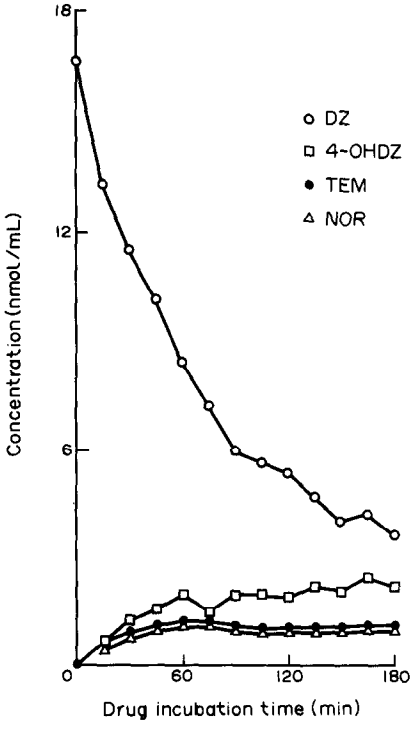

Fig. 2. Removal of diazepam and production of metabolites by rat hepatocytes cultured for 3 hr at 37° in Williams medium E. Rat hepatocytes were cultured for 3 hr in WME containing 10% (v/v) newborn calf serum, 10^{-6} M insulin and 10^{-4} M hydrocortisone and then incubated for 180 min with diazepam as described in Materials and Methods.

Table 3. Effect of culture conditions on the initial rate of removal of diazepam from the incubation medium concomitant with the age of hepatocyte culture

Culture medium	% DZ removed after culture for: 24 hr	48 hr	72 hr
WME	47	18	6
WME at 30°	64	50	27
WME + 0.5 mM metyrapone	73	43	26
WME + 2% DMSO	100	70	37
P450 medium	87	41	26

The results are the averages of two separate experiments which did not vary by more than 10% and are expressed as the percentage of the initial rate of removal, calculated as described by Chenery *et al.* [8], determined in 3 hr cultures. The initial rates of diazepam removal from the culture medium were 11.2 and 11.6 nmol/hr/mg cell protein.

accounted for by established metabolites. Similarly incubation of rat hepatocytes under the other culture conditions shown in Table 4 did not result in the detection of 4OHNOR, and disproportionate amounts of DZ removed and metabolites formed also resulted except for hepatocytes cultured for 72 hr in WME at 37° or in WME containing 0.5 mM metyrapone.

In cells cultured in WME at 37° or in medium containing DMSO 4OHDZ was the major metabolite detected at all culture ages. In contrast, although 4OHDZ was the major metabolite at early time points in the other culture systems, longer periods of culture, especially in WME containing 0.5 mM metyrapone or in P450 medium, resulted in TEM becoming the most prominent metabolite detected. However, under all culture conditions there was a reduction in the amount of DZ metabolized with the increasing age of the cultures (Table 4).

The results presented in Table 4 also show that oxazepam (OXA) was never observed in hepatocytes cultured in WME at 37°. In contrast, the culture of hepatocytes in WME containing 0.5 mM metyrapone resulted in the appearance of OXA after 24 hr of culture, as did their culture at 30° for 48 hr. Under both these culture conditions OXA constituted approximately 10% of the DZ metabolized by the cells after 72 hr of culture. The appearance of OXA coincided with an increase in the amount of TEM produced, which apparently was at the expense of 4OHDZ such that the latter metabolite was no longer detected in the incubation medium of hepatocytes cultured for 72 hr with metyrapone. OXA was not produced by hepatocytes cultured in the special P450 medium or in WME containing DMSO, in which the metabolite profile resembled the initial pattern, albeit at reduced levels, throughout the 72 hr culture period (Table 4).

DISCUSSION

The use of primary cultures of rat hepatocytes for the prediction of pathways of metabolism is gaining popularity [4, 11]. In this respect, DZ is considered to be metabolized in the rat primarily to 4OHDZ

Table 4. Effect of hepatocyte culture conditions on diazepam (DZ) metabolism

	Age of culture/culture medium																			
	3 hr					24 hr					48 hr					72 hr				
	W	30	M	D	P	W	30	M	D	P	W	30	M	D	P	W	30	M	D	P
% DZ removed from medium at 180 min	71	55	61	65	61	53	43	44	69	58	38	35	37	56	37	12	38	22	43	22
Metabolites in medium at 180 min as a % DZ removed																				
NOR	8	13	10	7	8	10	18	16	8	10	17	14	19	13	17	24	16	22	16	18
4OHNOR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
TEM	9	14	11	9	10	10	21	38	8	13	14	14	50	12	16	27	33	68	13	30
4'-OHDZ	17	25	19	16	14	16	22	7	17	16	27	18	6	26	25	52	12	—	41	17
OXA	—	—	—	—	—	—	—	8	—	—	—	1	10	—	—	—	7	11	—	—
Total metabolites as % DZ removed	34	52	40	32	32	36	61	69	33	39	58	47	85	51	58	103	68	101	70	65

A single preparation of hepatocytes were cultured in the respective medium containing 10% newborn calf serum 10^{-4} M hydrocortisone and 10^{-6} M insulin for the times shown and then incubated with 20 μM DZ for 180 min as described in Materials and Methods. The results are the mean values of triplicate incubations.

Abbreviations used for culture conditions: W = Williams medium E (WME) at 37°, 30 = WME at 30°, M = WME plus 0.5 mM metyrapone at 37°, D = WME plus 2% DMSO, P = P450 medium.

Abbreviations used for metabolites: DZ = diazepam, NOR = *N*-desmethyldiazepam, 4OHNOR = 4-hydroxy-*N*-desmethyldiazepam, TEM = temazepam, 4OHDZ = 4-hydroxydiazepam, OXA = oxazepam.

and its subsequent glucuronide [6, 7] and the present study readily demonstrates that this pathway occurs in rat hepatocyte culture, as well as the N-demethylation of DZ to NOR and its C3-hydroxylation to TEM, both of which can be metabolized in other species of experimental animals to OXA (Fig. 1). Accordingly oxazepam is not normally produced by rats but the present work demonstrates that rat hepatocytes can acquire this pathway when cultured in WME at 30° or in medium containing metyrapone. It is possible that metyrapone, as an inhibitor of cytochrome P450-mediated metabolism [12] may inhibit the conversion of DZ into 4OHDZ and so favour the formation of TEM from DZ which in turn leads to the production of OXA. This mechanism, however, would seem unlikely since reduced formation of 4OHDZ and the production of OXA was also observed when rat hepatocytes were cultured at 30°. Thus it is more likely that the hepatocyte culture conditions studied produced profound changes in the levels of P450 isozymes and this possibility merits a more detailed analysis on a form by form basis. However, it is interesting to note that the ability of metyrapone to prevent the loss of the total amount of cytochrome P450 is dependent on the presence of hydrocortisone in the culture medium (Table 2). Although the mechanism underlying this effect is unknown it is noteworthy that the ability of nicotinamide, another pyridinic compound which like metyrapone is able to prevent the loss of cytochrome P450 content in rat hepatocyte culture, is also dependent upon culture with glucocorticoids [13]. Finally it has previously been demonstrated in hepatocyte culture that metyrapone or P450 medium do not maintain the relative patterns of some P450 isozymes or linked enzyme activities at *in vivo* levels [14, 15]. Thus P450 medium maintains isozymes of cytochrome P450 with little or no activity towards ethoxycoumarin, ethoxyresorufin, benzphetamine or ethylmorphine while culture of hepatocytes with metyrapone maintains or elevates these enzyme activities [15–17]. More recently, culture of hepatocytes with DMSO has been shown to elevate the P450IIE subfamily [18]. Collectively these observations suggest that hepatocytes cultured under different conditions may represent useful model systems to identify most pathways of metabolism and hence reduce the number of experiments performed on living animals that are currently required to identify the metabolites produced when a drug such as Diazepam undergoes extensive metabolism. Furthermore the observation that culture of hepatocytes in P450 medium or DMSO supplemented WME permitted more DZ to be metabolized by the cells but did not result in a corresponding increase in known metabolites formed must suggest that there are as yet unidentified metabolites of DZ, and hence the current work clearly demonstrates the potential as well as limitations of hepatocyte culture in the identification of pathways of metabolism.

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