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7-ETHOXYCOUMARIN DEETHYLASE ACTIVITY AS A CONVENIENT MEASURE OF LIVER DRUG METABOLIZING ENZYMES: REGULATION IN CULTURED RAT HEPATOCYTES

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Abstract—Assays of 7-ethoxycoumarin *O*-deethylase (ECD) activity in intact cells were used as a sensitive and convenient measure of the drug-metabolizing activity of rat hepatocytes maintained for up to 4 days in primary culture. A combination of nicotinamide or other pyridines with dexamethasone was shown to maintain ECD at or above the activity of untreated livers *in vivo* and to potentiate induction by xenobiotics. Inductions *in vivo* and in culture were quantitatively similar but differed qualitatively as judged by the proportion of ECD activity inhibitable by metyrapone. A survey of possible endogenous regulators of liver monooxygenases established that: dexamethasone and other glucocorticoids induced ECD and potentiated induction by xenobiotics, particularly phenobarbitone; other steroids including testosterone, 17β-estradiol and pregnenolone 16α-carbonitrile caused small inductions; insulin lowered both ECD activity and the proportion of activity inhibitable by metyrapone; dibutyryl cyclic AMP or glucagon lowered ECD; and high concentrations of aminolevulinate partly repressed induction by xenobiotics. Based on these findings, hepatocyte culture conditions which maintain ECD activity and inducibility at or above *in vivo* levels are defined.

The liver microsomal drug-metabolizing or monooxygenase system is active with a wide range of substrates and is induced by a diverse group of foreign chemicals [2, 3]. Detailed characterization of the control of the monooxygenase system in primary cultures of adult mammalian hepatocytes is desirable, not only to clarify the nature of regulatory factors and their mechanisms but also to provide a basis for studies on the monooxygenase-dependent metabolism and subsequent effects of various drugs, toxins and carcinogens in culture (e.g. refs. [4, 5]). The approach in this study has been to devise a sensitive and convenient assay of drug-metabolizing activity in intact cells using a substrate with which a variety of the multiple forms of cytochrome P-450 are active. Based on previous work by Fry and Bridges [6, 7], assays of 7-ethoxycoumarin *O*-deethylase (ECD)† activity in intact rat hepatocytes have been used to survey both xenobiotic and endogenous agents for their effects on monooxygenase activity.

From previous studies with adult rat hepatocytes, two general problems have emerged. The first problem, and the main focus of this report, is that with unsupplemented media, the cytochromes P-450 and many associated monooxygenase activities decline rapidly in the first hours in culture ([4, 5]; cf. ref. [8] for other species). It seems likely that under simple culture conditions requirements for specific regulation of monooxygenase activities or possibly for preserving the normal differentiated state of adult hepatocytes are lacking. Previous observations with rat or chick hepatocyte cultures have suggested that endogenous or related agents which may increase monooxygenase activities include nicotinamide [9–13] and related pyridines [10, 12, 14, 15], glucocorticoids [16–24], the sex steroids 17β-estradiol and testosterone [16, 17, 20, 25], other steroids including PCN [26], thyroid hormones [16, 17, 20, 23], serum [7], glucagon or cyclic AMP [16, 17, 20, 27, 28], insulin ([16, 17, 20, 23] but cf. [27]) and the heme precursor ALA [29, 30]. One aim of this study was to test a broad range of such factors to clarify with a single experimental system the nature and relative importance of endogenous controls on monooxygenase activity.

The second general problem in studies with adult rat hepatocytes is that although the levels of monooxygenase components are increased by known xenobiotic inducers, the spectrum of the cytochrome P-450 forms induced is, for some drugs such as phenobarbitone (PB), different from that induced *in vivo* [4, 25, 31]. In the present study the qualitative aspects of induction have been examined in a preliminary way by measuring the sensitivity of monooxygenase activities in culture to metyrapone, an inhibitor of a specific subset of P-450 forms [32].

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† Abbreviations used: ALA, 5-aminolevulinate; DEX, dexamethasone; dibutyryl cyclic AMP, N^6,$O^{2'}$-dibutyryl adenosine-3′,5′-monophosphate; ECD, 7-ethoxycoumarin *O*-deethylase; 3-MC, 3-methylcholanthrene; PB, sodium phenobarbitone; PCN, pregnenolone 16α-carbonitrile. Cytochrome P-450 isozymes are named according to the nomenclature of Lu and West [1].

A preliminary report of this work has appeared [33].

MATERIALS AND METHODS

Chemicals, enzymes and media. All biochemicals used were obtained from Sigma Chemical Co. (St. Louis, MO) except for sodium phenobarbitone which was purchased from Prosana Laboratories (Sydney, Australia) and collagenase which was from Boehringer Mannheim (Australia). Sources and formulations of media were as previously described [34].

Hepatocyte isolation and culture. Porton-strain Wistar rats allowed free access to laboratory chow were used for all studies. Hepatocytes were isolated by a two-step collagenase perfusion procedure [35], modified to yield sterile preparations [34]. The procedures and media for cell culture were as in ref. [34] except that cultures were established by allowing freshly isolated hepatocytes to attach to collagen-coated, 60 mm diameter culture dishes (Disposable Products, Adelaide, South Australia) in modified Waymouth medium [34] with 10 mM nicotinamide and 3% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia). After allowing 3 hr for cell attachment, the medium was changed to a standard defined "culture medium" which, unless otherwise specified, contained a modification of Waymouth medium MB 752/1 incorporating alanine, serine, asparagine, oleic and linoleic acids, insulin and antibiotics as defined in ref. [34]. In most experiments, the standard culture medium was further supplemented with 10 mM nicotinamide.

The ECD assay was based on previously published procedures of Fry and Bridges [6] and Greenlee and Poland [36]. After experimental pretreatments, hepatocyte monolayers were washed twice with warm 0.15 M NaCl. ECD assays were initiated by adding warm Ca^{2+}-free Krebs–Henseleit buffer containing 7-ethoxycoumarin (final concentration 100 μM) to washed monolayers. The concentration of ethoxycoumarin was chosen to facilitate comparison with previous studies [7, 37] but was slightly suboptimal; 150 μM was required for maximal activity. Assays were either stopped immediately (zero-time blank) or after a further 30 min incubation at 37° by rapidly scraping cells from the plastic and freezing cells plus supernatant medium in liquid N_2. Hydroxycoumarin formation was linear for at least 1 hr and linear with respect to cell concentration over the range 0.3–3.0 $\times$ 10^6 cells/ml in assays with hepatocyte suspensions. Samples containing hydroxycoumarin, free or as glucuronide or sulphate conjugates, could be stored frozen as required. Thawed samples were homogenized for 10 sec with an Ultraturrax homogeniser at 2/3 full speed. A sample of homogenate was saved for protein estimation and duplicate 0.5 ml samples of homogenate were transferred to centrifuge tubes containing 0.7 ml crude β-glucuronidase/sulphatase (Sigma type H-1 β-glucuronidase, 1.4 mg/ml in 60 mM Na acetate buffer, pH 4.5, containing 0.11 M NaCl) and incubated in a shaking water bath at 37° for 2 hr to allow conversion of hydroxycoumarin conjugates to free hydroxycoumarin. Incubation for 2 hr (or as little as 45 min) was sufficient for complete hydrolysis of conjugates and gave values identical with the procedure of Fry and Bridges [6] which involved a 17 hr incubation. Alternative approaches [37] employing salicylamide to inhibit conjugation in the original cell incubation gave much lower values.

Hydroxycoumarin formation was estimated as described by Greenlee and Poland [36] with modifications as follows. After the β-glucuronidase treatment, the product was extracted into 2 ml chloroform by mixing for 20 sec on a vortex mixer. Tubes were centrifuged at 2000 *g* for 7 min and then a 1 ml sample of the chloroform phase was mixed with 2.5 ml 0.01 M NaOH/1 M NaCl for 20 sec on a vortex mixer. After centrifuging tubes for 3 min at 2000 *g*, the fluorescence of hydroxycoumarin in the aqueous phase was estimated using an Aminco Bowman spectrofluorometer with excitation and emission wavelengths of 368 and 456 nm, respectively. Sample fluorescence was compared with that of a freshly prepared standard solution of 5 μM hydroxycoumarin in 0.01 M NaOH/1 M NaCl. Values for hydroxycoumarin formation during a 30 min incubation were calculated after subtracting the fluorescence of zero-time blank incubations. No correction was made for the recovery of hydroxycoumarin through the extraction procedure; this was consistently 80%. A unit of ECD activity is defined as the amount catalysing the formation of 1 pmole hydroxycoumarin per min.

In general, the results shown in the figures and tables were obtained with a single liver cell preparation, but in every case comparable results were obtained in replicate experiments with independent cell preparations.

RESULTS

Induction of ECD by known xenobiotic inducers of liver monooxygenase activities

Initial studies using the ECD assay with intact hepatocyte monolayers were designed to characterize the response of this system to known monooxygenase inducers. Figure 1 shows the time course of changes in ECD activity in control monolayers and those continuously exposed to PB, 3-MC or dexamethasone (DEX). In cultures without nicotinamide (Fig. 1A), the activity of control monolayers progressively declined to values after 4 days *ca* 3–5% of that in hepatocytes freshly isolated from untreated rats. This decline was prevented in part by maintaining cells with 30 nM DEX. While 3-MC alone caused clear ECD induction, PB markedly induced activity only in combination with DEX. When monolayers were maintained with 10 mM nicotinamide, the ECD activity of control cultures was preserved at near *in vivo* levels for 2–3 days before declining (Fig. 1B). Furthermore, nicotinamide synergistically enhanced the effectiveness of 3-MC, PB and DEX as inducers. Media for most of the further studies below contained 10 mM nicotinamide.

Figure 2 shows changes in ECD activity when hepatocyte monolayers maintained with nicotinamide were given 24 hr exposures to inducers beginning 4, 28 or 52 hr after plating isolated hepatocytes into culture dishes. Immediately after attachment,

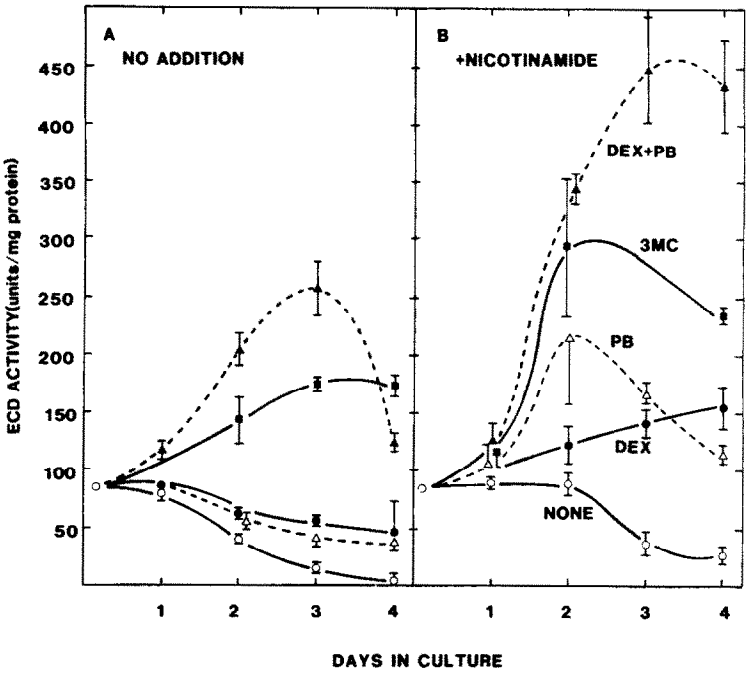

Fig. 1. Effects of xenobiotics and dexamethasone on ECD in hepatocyte cultures maintained with or without nicotinamide. After attachment, hepatocytes were maintained in standard culture medium with or without 10 mM nicotinamide and with additions: ○, None; ●, DEX, 30 nM; △, PB, 2 mM; ■, 3-MC, 2 μM; ▲, DEX, 30 nM plus PB, 2 mM. After various times in culture, sets of dishes were assayed for ECD activity as in Materials and Methods. Values and bars are means ± S.D. from four dishes.

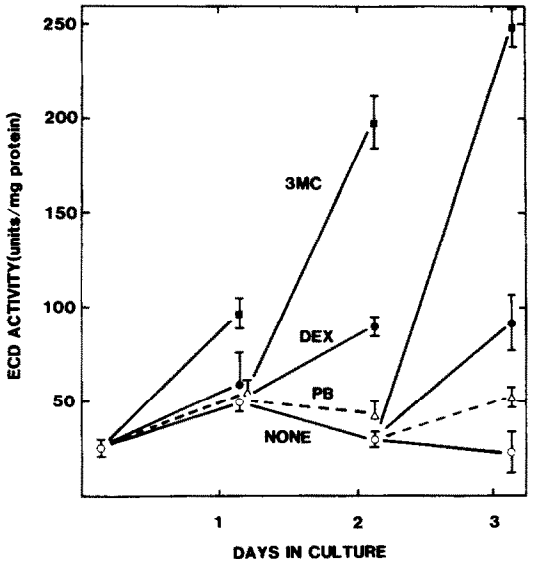

Fig. 2. Response of ECD to xenobiotic inducers and dexamethasone after various times in culture. After attachment, hepatocytes were maintained in standard culture medium plus 10 mM nicotinamide. Inducers (DEX, 3-MC or PB) were added as shown after 4, 28 or 52 hr prior incubation in culture and in each case ECD activity was assayed after a further 24 hr incubation. Points and bars show means ± S.D. from four dishes. Additions were: ○, none; ●, DEX, 30 nM; △, PB, 2 mM; ■, 3-MC, 2 μM.

monolayers were relatively unresponsive to inducers. After a further 24 hr in culture, during which cells spread out on the collagen-coated dishes to form a near-confluent monolayer, the response to inducers was markedly improved. Maximal responses were observed following inducer addition to 2-day cultures, when inducers caused a progressive rise in ECD activities over 1–2 days after an initial lag phase of 5–6 hr (detailed time course not shown).

The concentration dependence of induction by PB, 3-MC and another known xenobiotic inducer of monooxygenase activity, β-naphthoflavone, are shown in Fig. 3. The effects of PB were examined in the presence of DEX since PB was a weak inducer in the absence of glucocorticoid. Significant dose-dependent induction was observed at PB concentrations of 10 μM and above; induction was maximal (3–4-fold) at 1–2 mM but declined very rapidly at concentrations greater than 2 mM, possibly indicating toxicity at these levels. The polycyclic hydrocarbons 3-MC and β-naphthoflavone caused dose-dependent inductions up to 10-fold which were maximal with 2 μM 3-MC and *ca* 50 μM β-naphthoflavone.

That the changes in ECD observed in response to inducers in culture indeed reflected enzyme induction rather than activation was suggested by their time-dependence. Furthermore, elevation of ECD during an 18 hr exposure to inducers was prevented by the addition of either the RNA synthesis inhibitor, actinomycin D (1 μg/ml), or the inhibitor of protein synthesis, cycloheximide (20 μg/ml).

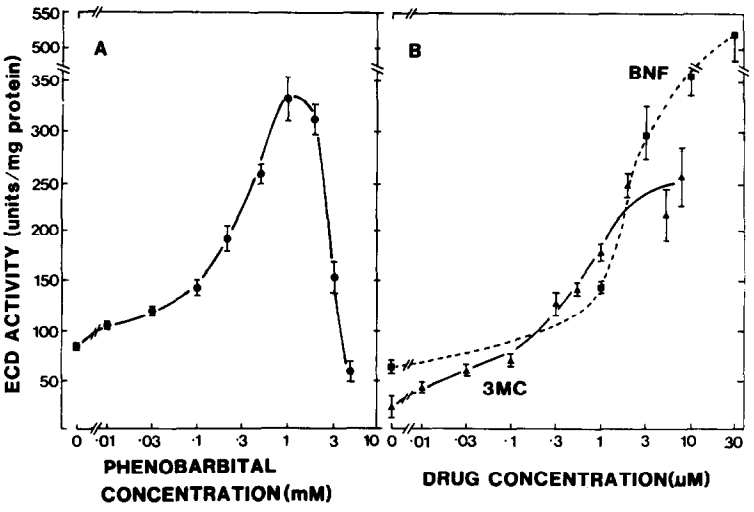

Fig. 3. Concentration dependence of ECD induction by xenobiotics. After attachment, hepatocytes were maintained in standard medium plus 10 mM nicotinamide. In panel A, media also contained 30 nM DEX throughout; varying concentrations of PB were added after 24 hr and ECD assayed after a further 48 hr. In panel B, varying concentrations of β-naphthoflavone (BNF) or 3-MC were added after 44 hr and ECD assayed after a further 24 hr. Points and bars show means ± S.D. from 3–4 dishes.

Comparison of ECD induction in vivo *and in culture*

Table 1 compares the ECD activities of hepatocytes from control cultures or cultures exposed to PB or 3-MC with the activities of hepatocytes isolated after treatments with the same compounds *in vivo*. The protocols for *in vivo* induction were based on those widely used in other laboratories to observe maximal effects of PB or 3-MC. In cultures maintained without glucocorticoids, both control and induced activities were about half the *in vivo* values. However, in the presence of moderate levels of the glucocorticoid, DEX, the levels of ECD induced by either PB or 3-MC were comparable with levels found after *in vivo* induction.

To provide some indication of whether the complement of P-450 isozymes contributing to activity differed *in vivo* and in culture, hepatocytes were assayed in the absence or presence of metyrapone, considered to be a relatively specific inhibitor of certain forms of cytochrome P-450. Compared with hepatocytes freshly isolated from an untreated rat, the ECD activity of hepatocytes after 3 days in culture, with or without glucocorticoid, was less sensitive to metyrapone inhibition. While the low proportion of metyrapone-inhibitable activity after 3-MC exposure was comparable *in vivo* and *in vitro*, the PB-induced activity in culture was clearly qualitatively different from that *in vivo*. No manipulations

Table 1. Comparison of ECD induction in hepatocytes *in vivo* and in monolayer cultures

	ECD activity (units/mg protein)		
Treatment	(A) *In vivo*	(B) In culture, 3 days	
		None	+ DEX
None	74 (63)*	31 (35)	144 (36)
PB	323 (72)	170 (30)	370 (28)
3-MC	496 (10)	237 (6)	499 (−)†

(A) From rats previously untreated, given three daily injections (i.p.) of PB, 80 mg/kg or one injection (i.p.) of 3 MC, 25 mg/kg, cells were isolated after a further 20 hr and the ECD activity of cell suspensions assayed immediately essentially as in Materials and Methods, in the presence or absence of 100 μM metyrapone. Each value is the mean of quadruplicate assays on suspensions from two rats.

(B) Hepatocytes were isolated from untreated rats and after attachment, maintained in culture for 3 days in standard culture medium plus 10 mM nicotinamide with or without additions of 30 nM DEX, 2 mM PB or 2 μM 3-MC. ECD activities were assayed in the presence and absence of 100 μM metyrapone. The values shown for cells from a single rat are typical of a large number of experiments.

* Values in parentheses show % inhibition by 100 μM metyrapone.

† Not determined.

so far tested, including a variety of media or supplements employed in other studies [7, 14, 25, 29, 38], have fully restored an *in vivo*-like proportion of metyrapone-inhibitable ECD activity. Recent experiments have, however, shown that Williams medium E gives higher total ECD activities than those observed with the modified Waymouth medium used in most of this study (see below, Fig. 6).

Effect of nicotinamide and other pyridines on ECD levels

The results in Fig. 1 indicated that nicotinamide favours preservation of ECD activity at near *in vivo* levels in control cultures and also enhances induction by either glucocorticoid or xenobiotics. Pyridine and its derivative metyrapone caused effects similar to nicotinamide (not shown). Metyrapone at the concentration (0.5 mM) shown to be optimal in other studies [10, 15] on cytochrome P-450 was effective alone in maintaining ECD levels, and in combination with DEX (30 nM), increased ECD to activities 4–5 times those in cultures with DEX alone. The concentration dependence of the effects of nicotinamide are shown in Fig. 4. Maximal preservation of ECD activity in control cultures and enhancement of induction by 3-MC or PB were observed with 15 mM nicotinamide. Similar results were obtained with DEX as inducer (not shown). Higher concentrations up to 30 mM gave no additional effect or were suboptimal, possibly due to the inhibition of protein synthesis at higher nicotinamide levels [12]. The 10 mM nicotinamide used in most of the present studies was near optimal in maintaining ECD activities but caused no significant inhibition of general protein synthesis in 24-hr-old hepatocyte monolayers (measured as [^{3}H]leucine incorporation into trichloroacetic acid-precipitable material as in ref. [39]). Furthermore, although nicotinamide is known to bind, like metyrapone, to specific forms of cytochrome P-450 [10, 15] it caused no significant inhibition of ECD activity when present at concentrations up to 20 mM in assays of intact cells.

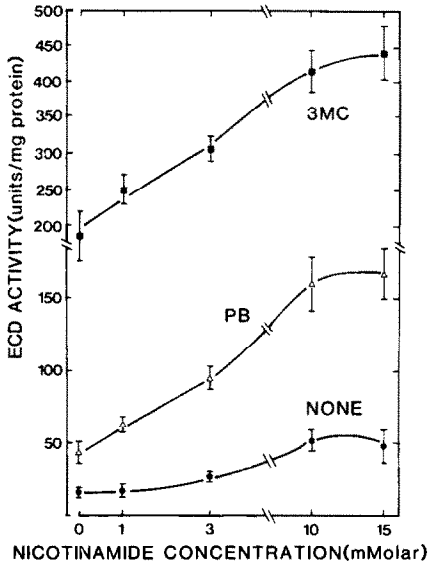

Fig. 4. Effect of nicotinamide concentration on basal and xenobiotic-induced ECD activities in culture. After attachment, monolayers were maintained with standard medium plus the nicotinamide concentrations shown and other additions below. ECD was assayed after 3 days. Points and bars show means ± S.D. from four dishes. Additions were: ○, none; △, PB, 2 mM; ■, 3-MC, 2 μM.

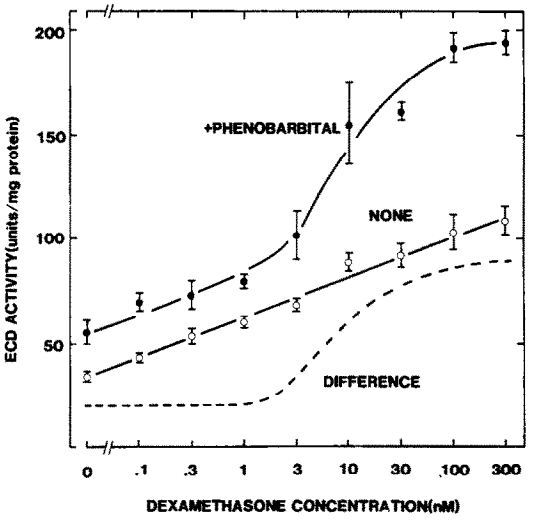

Fig. 5. Concentration dependence of the inducing and potentiating effects of dexamethasone in ECD regulation in culture. After attachment, monolayers were maintained in standard medium plus 10 mM nicotinamide with varying DEX concentrations, without additions (○) or with 2 mM PB (●). ECD activity was assayed after a further 40 hr. Points and bars show means ± S.D. for four dishes.

Potentiating and inducing effects of glucocorticoid in ECD regulation

Figure 1 showed that the addition of DEX alone favoured preservation or induction of ECD and that DEX acted synergistically with PB in ECD induction. For cultures maintained with nicotinamide, the concentration dependence of induction by DEX alone or in combination with an optimal concentration of PB is shown in Fig. 5. DEX caused an increase in ECD activity, measured after 2 days in culture, which was proportional to log (DEX concentration) over the range 0.1–100 nM. At higher levels, DEX did not further increase ECD activity in 2-day cultures although there was an additional effect of concentrations up to 3 μM when ECD was assayed after 4 days (not shown).

In addition to the inducing action of DEX, the steroid strongly potentiated induction by PB which alone caused only a small induction (Fig. 5). This potentiation appeared most marked at later culture times (Fig. 1). Although 3-MC alone was a potent inducer, its action was also potentiated by DEX (Table 2). The concentration dependence of the inducing action of DEX and of its potentiating effect (shown by the difference curve in Fig. 5) were consistent with a mechanism involving the glucocorticoid receptor. In both cases maximal effects were observed at *ca* 0.1 μM steroid. This concentration is also optimal for the glucocorticoid-receptor-dependent

Table 2. Interaction between dexamethasone and induction by 3-methylcholanthrene (3-MC)

Additions (nM)	ECD activity (units/mg protein)	
	None	+ 3-MC
None	57 ± 10	192 ± 25
DEX, 3	81 ± 6	266 ± 21
DEX, 30	122 ± 9	396 ± 43

Monolayers after attachment were incubated for 48 hr in culture medium with 10 mM nicotinamide with or without 3-MC, 2 μM and the concentrations of DEX shown. Values are means ± S.D. from four dishes.

induction of tyrosine aminotransferase by DEX in similar cultures (A. M. Edwards, unpublished results). Some additional evidence that DEX is acting as a glucocorticoid in its effects on ECD is shown in Table 3. The known glucocorticoids DEX, hydrocortisone and corticosterone all clearly induced and (where tested) acted synergistically with PB. The structurally related steroids 20β-hydrocortisol, tetrahydrocortisol and fluoxymesterone, known to be suboptimal-, inactive- and anti-glucorticoids, respectively [40], were less effective or inactive as inducers, and tetrahydrocortisol failed to potentiate PB induction.

Regulatory effects of other steroids

Table 3 also shows that some non-glucocorticoids, namely fluoxymesterone, pregnenolone and PCN, caused small but significant increases in ECD levels. The effect of PCN was approximately additive with that of a maximally effective concentration of DEX suggesting that glucocorticoids and PCN may induce by distinct mechanisms. In a separate experiment similar to that in Table 3, the sex steroids testosterone and 17β-estradiol were found to increase ECD from a control value of 59 ± 14 units/mg protein to 94 ± 20 (with testosterone, 1 μM) and 81 ± 9 (with 17β-estradiol, 1 μM). Either steroid also augmented induction by PB + DEX in an approximately additive fashion.

Effects of glucagon or cyclic AMP derivatives

Contrary to the expectation from previous work [16, 17, 20, 27, 41] that cyclic AMP might increase ECD levels, initial experiments showed that the addition of dibutyryl cyclic AMP markedly decreased ECD in cultures incubated for 2 days with or without inducers. Similar results were obtained when glucagon (0.4 μM) was added to monolayers after attachment (not shown). The results in Table 4 indicate that early exposure of monolayers to dibutyryl cyclic AMP accounted for most of the observed repression of ECD. Addition of dibutyryl cyclic AMP during a 1-day preincubation period had marked effects on ECD activities observed after a further 1-day incubation with or without inducers. When the nucleotide was absent during the preincubation but present during the further incubation ± inducers, its effects were less marked. Additional experiments (not shown) in which nucleotide was added to cultures on days 1, 2 or 3 with exposure to inducers on day 3 suggested that it was the presence of cyclic AMP on day 1 rather than in the phase prior to adding inducers (day 2) which caused the most marked reduction in ECD.

Effects of insulin

In most of the studies reported here insulin was included in standard culture media to promote cell attachment to substrata. Table 5 shows the effects of maintaining cultures with insulin or omitting it after cell attachment. In general, omitting insulin gave higher ECD activities. This was clearly the case for cultures maintained with PB + DEX; in control or PB-treated cultures small (but non-significant)

Table 3. Relative effects of glucocorticoids and steroids of related structure on ECD

Additions	(μM)	ECD activity (units/mg protein)	
		None	+PB
None		41 ± 4	105 ± 13
DEX	(0.1)	115 ± 13**	343 ± 30**
Hydrocortisone	(30)	88 ± 9**	
Corticosterone	(30)	89 ± 5**	263 ± 26**
20β-Hydrocortisol	(30)	62 ± 4**	
Tetrahydrocortisol	(30)	45 ± 5	122 ± 16
Fluoxymesterone	(30)	58 ± 7*	
Progesterone	(30)	43 ± 4	
Pregnenolone	(30)	57 ± 8*	
PCN	(30)	52 ± 4*	
PCN (30) + DEX (0.1)		137 ± 8†	

After cell attachment and maintenance of monolayers in the presence of 10 mM nicotinamide for 24 hr, fresh medium with nicotinamide ± 2 mM PB was added together with the steroids shown. ECD activity was assayed after a further 40 hr. Values are means ± S.D. from 3–4 dishes.

Significance of difference from corresponding controls without steroid: *$P < 0.01$; **$P < 0.001$.

† Significantly greater than DEX alone, $P < 0.01$.

Table 4. Effect of dibutyryl cyclic AMP addition during preincubation of monolayers or during exposure to inducers

Additions (day 2)	ECD activity (units/mg protein)		
	Additions of dbcAMP		
	None	Day 1	Day 2
None	36 ± 9	14 ± 1	35 ± 1
DEX, 30 nM	138 ± 22	34 ± 3	63 ± 10
DEX, 30 nM + PB, 2 mM	208 ± 11	42 ± 5	71 ± 8
3-MC, 2 μM	167 ± 13	37 ± 5	135 ± 24

After attachment in the presence of 10 mM nicotinamide, the medium was changed to standard culture medium with 10 mM nicotinamide. Dibutyryl cyclic AMP (dbcAMP), 50 μM was added to some dishes (day 1) as indicated. After a further 18 hr, media were changed and dbcAMP, 50 μM added to some dishes (day 2) as indicated. Inducers were also added as shown and all dishes incubated for a further 24 hr prior to ECD assay. The values given are means ± S.D. from four dishes.

Table 5. Effects of insulin on ECD activity and extent of inhibition by metyrapone

Inducers	No insulin		+ Insulin	
	ECD†	% Inhib.‡	ECD†	% Inhib.‡
None	53 ± 11	53	34 ± 2*	26
PB, 2 mM	81 ± 3	40	75 ± 9	32
PB, 2 mM + DEX, 30 nM	557 ± 48	42	323 ± 10**	34

After attachment in the presence of 0.3 μM insulin and 10 mM nicotinamide, the medium was changed to standard culture medium plus 10 mM nicotinamide except that for half the dishes insulin (0.3 μM) was omitted. After 24 hr inducers were added as shown. ECD activity was assayed in the presence or absence of 100 μM metyrapone after a further 48 hr.

Significance of differences from corresponding value without insulin: *$P < 0.01$; **$P < 0.001$.

† ECD activity (units/mg protein); mean ± S.D. from 3–4 dishes.

‡ % Inhibition of ECD activity by 100 μM metyrapone.

increases were seen in several experiments. The omission of insulin consistently increased the proportion of ECD activity inhibitable by metyrapone, suggesting that insulin-free cultures contained higher proportions of metyrapone-binding P-450 isozymes, possibly including one recently identified in diabetic rats [42].

Effects of other possible regulatory agents

Previous studies have advocated the inclusion of thyroid hormones to aid preservation or induction of monooxygenase components in hepatocyte cultures [16, 17, 20, 23]. Neither thyroxine nor triiodothyronine at concentrations up to 10^{-5} M had significant effects on ECD activity in the absence or presence of inducers. Similarly, fetal calf serum had only minor effects on activity. Giger and Meyer [27] reported that glucose impaired the induction of cytochrome P-450 by PB in cultured chick embryo liver cells but varying the glucose concentration in rat hepatocyte cultures from 2.8 to 28 mM had little effect on ECD activities in cells incubated with or without inducers.

The role of heme in the regulation of ECD activity

It seems likely that the observed ECD activity in hepatocytes is dependent on levels of a subset of cytochromes P-450, hemoproteins which require the insertion of heme into appropriate apoproteins for activity. Table 6 shows that maintenance of cultures with 10 μM hemin or the heme precursor ALA (at concentrations up to 30 μM) caused a small increase in ECD in the absence of inducers and little effect when inducers were present. When added at higher

Table 6. Effects of cell maintenance with hemin or ALA on ECD levels

Additions	ECD activity (units/mg protein)		
	None	3 MC	DEX + PB
None	52 ± 2	321 ± 34	302 ± 18
Hemin, 10 μM	90 ± 8**	357 ± 19	267 ± 16
ALA, 30 μM	59 ± 3*	374 ± 18*	285 ± 21
ALA, 100 μM	—	183 ± 31**	195 ± 23**

After attachment media were changed to standard culture medium + 10 mM nicotinamide with or without 2 μM 3-MC or 30 nM DEX plus 2 mM PB and with other additions as shown. ECD activity was assayed after a further 2 days. Values are means ± S.D. from four dishes.

Significance of differences from corresponding controls without hemin or ALA: *$P < 0.01$, **$P < 0.001$.

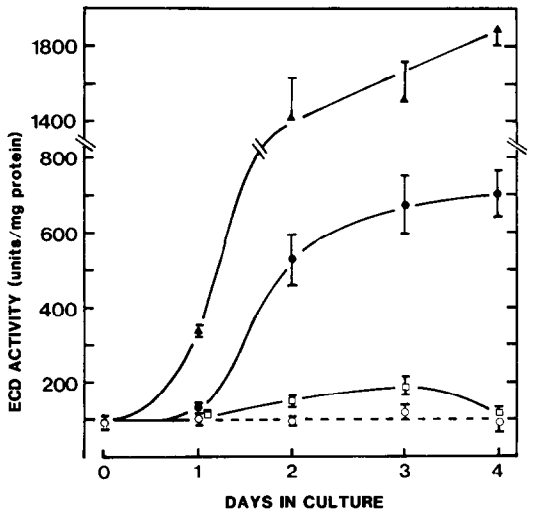

Fig. 6. Basal and xenobiotic-induced ECD activity in hepatocytes maintained in a defined, supplemented culture medium. After attachment, monolayers were maintained in Williams medium E supplemented with penicillin (60 μg/ml), streptomycin (100 μg/ml), nicotinamide 10 mM, DEX 30 nM, testosterone 1 μM and estradiol 1 μM with the following modifications: □, none; ○, testosterone and estradiol omitted; ●, plus PB 2 mM; and ▲, plus 3-MC 2 μM. Points and bars are means ± S.D. from four dishes.

concentrations, however, ALA clearly depressed ECD in cultures with inducers.

These studies on factors influencing levels of ECD in cultured hepatocytes led to the conclusion that high basal levels and inducibility of ECD were favoured in media containing nicotinamide or other substituted pyridines, by the inclusion of glucocorticoid, testosterone and estradiol, by the omission of insulin after cell attachment and also by using Williams medium E as the basic culture medium in place of the modified Waymouth medium employed in most of this study. The use of these conditions in combination with optimal concentrations of PB or 3-MC is illustrated in Fig. 6. Activity was preserved for 4 days at or above the levels of ECD in freshly isolated cells when media contained nicotinamide and steroids. PB caused 7-fold, and 3-MC 18-fold induction under these conditions.

DISCUSSION

The experimental approach adopted in this study was to establish a convenient and sensitive assay of drug-metabolizing enzymes in intact liver cells and use this to survey factors which contribute to the regulation of monooxygenase activity in rat hepatocyte primary cultures. Ethoxycoumarin is a substrate for a number of the cytochrome P-450 isozymes including P-450$_a$, P-450$_b$, P-450$_c$ and P-450$_e$, with P-450$_c$ giving the highest activity [1, 43–46]; P-450$_d$ is inactive [47] and the ECD activity of P-450$_{PCN}$ [48] is unknown. With the simplified and shortened procedure described, assays on 100–200 culture dishes established from a single liver perfusion can be completed in 1–2 days.

The initial phase of this study characterized the changes in hepatocyte ECD activity in response to known xenobiotic inducers of the monooxygenase system. Monolayers were relatively unresponsive to xenobiotic inducers or DEX during the first 24 hr in culture and optimal responses were observed after 48 hr (Fig. 2) in general agreement with related studies [7, 25]. After 24–48 hr, all the known inducers tested increased ECD levels although the effect of PCN, a potent inducer of some monooxygenase components, was small. With a supplemented defined Waymouth medium, the extent of induction by 3-MC or PB was comparable with the induction *in vivo* (Table 1), and with supplemented Williams medium E larger inductions could be produced *in vitro* than *in vivo* (Fig. 6). Findings on the extent to which metyrapone inhibited ECD activity suggested that at least in control and PB-induced cultures the spectrum of P-450 isozymes induced was different from that *in vivo*, in agreement with a number of previous observations [4, 5, 25, 31]. This probably reflects a relative deficiency of P-450$_b$ [25] or possibly of P-450$_e$ or P-450$_{PCN}$. Metyrapone is known to inhibit P-450$_b$ [49] and also to bind (and presumably inhibit) P-450$_e$ and P-450$_{PCN}$ [32]. The qualitative differences between responses of rat hepatocytes *in vivo* and *in vitro* suggests that there are important factors in monooxygenase regulation, presumably in control of individual P-450 isozymes, yet to be identified.

Despite some reservations about qualitative aspects of ECD regulation, hepatocyte cultures respond to a variety of known monooxygenase regulators and provide a convenient means of exploring the effects of various hormones and nutrients with possible regulatory significance. This study has made a relatively comprehensive survey of such factors. In a number of respects, findings for ECD regulation in rat hepatocytes are qualitatively similar to those previously reported for total cytochrome P-450 [5, 11, 12, 14, 15, 20, 22, 25, 38]; PB or polycyclic hydrocarbons induce both parameters with similar concentration-dependence; glucocorticoids, estradiol and testosterone, and nicotinamide and related pyridines all enhance activity.

The present study extends previous reports that glucocorticoids enhance monooxygenase activities in cultured hepatocytes [16–19, 21, 32, 33–35] and clearly shows that these steroids contribute in two ways to monooxygenase regulation. DEX and other glucocorticoids induced ECD in hepatocytes maintained with or without nicotinamide and potentiated induction by PB and 3-MC. The structure/activity relationships (Table 3) and concentration dependence (Fig. 5) of these effects were consistent with the view that both induction and potentiation were examples of glucocorticoid hormone action. One or more further groups of steroids (including testosterone, estradiol, pregnenolone and PCN) apparently contribute to regulation by distinct mechanism(s).

The time-dependent effects of glucocorticoids are probably mediated by enhanced synthesis of monooxygenase components [19] and are distinct from rapid activation by high glucocorticoid concentrations reported for biphenyl-2-hydroxylation in isolated microsomes [50]. Addition of DEX at concentrations up to 10^{-4} M had no short-term effect on

ECD activity in hepatocytes. The relative effects of DEX on ECD activity were similar regardless of the basis for expressing activities. After 3 days the total protein and DNA contents of DEX-treated cultures were both slightly higher (up to 10%) than those of control cultures (cf. ref. [51]). Despite its small effect on total cell protein, DEX is known to exert a wide range of effects on hepatocytes in culture (see [34] for refs.) and it is possible that its role in ECD regulation involves a broad effect on hepatocyte gene expression.

Some agents previously added to rat hepatocytes in hormone/nutrient cocktails with the aim of preserving cytochromes P-450 [16, 17, 20] had small or negligible effects on ECD (thyroid hormones, heme, μM concentrations of ALA) or significantly depressed activity (glucagon or cyclic AMP, insulin, 100 μM ALA). The ability of dibutyryl cyclic AMP or glucagon to depress ECD was most marked if these agents were added to cultures for a period soon after cell attachment. Cyclic AMP may exert an early "imprinting" effect which blocks later ECD induction resembling related, previously reported effects on glucokinase induction [52] and DNA synthesis [53] in culture. The findings with added heme or its precursor ALA suggest that maximal ECD activities can be observed without supplementing the endogenous supply of heme prosthetic groups for those apocytochromes P-450 contributing to ECD activity. Since ALA up to 100 μM caused clear increases in *total* cytochrome P-450 measured spectrophotometrically ([29, 30]; findings in this study not shown) it may be that other isozymes are less readily saturated with heme or are regulated differently by heme levels.

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REFERENCES

1. A. Y. H. Lu and S. B. West, *Pharmac. Rev.* **31**, 277 (1980).
2. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
3. A. Y. H. Lu, R. Kuntzman and A. H. Conney, *Front. Gastrointest. Res.* **2**, 1 (1976).
4. J. W. Grisham, *Int. Rev. exp. Path.* **20**, 124 (1979).
5. A. E. Sirica and H. C. Pitot, *Pharmac. Rev.* **31**, 205 (1980).
6. J. R. Fry and J. W. Bridges, *Naunyn-Schmiedeberg's Archs Pharmac.* **311**, 85 (1980).
7. J. R. Fry, P. Wiebkin and J. W. Bridges, *Biochem. Pharmac.* **29**, 577 (1980).
8. C. J. Maslansky and G. M. Williams, *In Vitro* **18**, 683 (1982).
9. K. F. Nelson and D. Acosta, *Biochem. Pharmac.* **31**, 2211 (1982).
10. U. Giger and U. A. Meyer, *Biochem. Pharmac.* **31**, 1735 (1982).
11. A. J. Paine, L. J. Williams and R. F. Legg, *Life Sci.* **24**, 2185 (1979).
12. P. Villa, L. J. Hockin and A. J. Paine, *Biochem. Pharmac.* **29**, 1773 (1980).
13. F. R. Althaus, S. D. Lawrence, G. L. Sattler and H. C. Pitot, *Biochem. biophys. Res. Commun.* **95**, 1063 (1980).
14. B. G. Lake and A. J. Paine, *Biochem. Pharmac.* **31**, 2141 (1982).
15. A. J. Paine, P. Villa and L. J. Hockin, *Biochem. J.* **188**, 937 (1980).
16. M. Dickens and R. E. Peterson, *Biochem. Pharmac.* **29**, 1231 (1980).
17. K. K. Dougherty, S. D. Spilman, C. E. Green, A. R. Steward and J. L. Byard, *Biochem. Pharmac.* **29**, 2117 (1980).
18. P. Kremers, F. Goujon, J. DeGraeve, J. van Cantfort and J. E. Gielen, *Eur. J. Biochem.* **116**, 67 (1981).
19. F. R. Althaus and U. A. Meyer, *J. biol. Chem.* **256**, 13079 (1981).
20. G. M. Decad, D. P. H. Hsieh and J. L. Byard, *Biochem. biophys. Res. Commun.* **78**, 279 (1977).
21. P. S. Guzelian, R. F. Diegelmann, R. G. Lamb and H. J. Fallon, *Yale J. Biol. Med.* **52**, 5 (1979).
22. G. Michalopoulos, G. L. Sattler and H. C. Pitot, *Life Sci.* **18**, 1139 (1976).
23. J. F. Sinclair, P. R. Sinclair and H. L. Bonkowsky, *Biochem. biophys. Res. Commun.* **86**, 710 (1979).
24. S. Sassa, M. K. Sardana and A. Kappas, in *Biochemistry, Biophysics and Regulation of Cytochrome P-450* (Eds. J. A. Gustafsson, J. Carlstadt-Duke, A. Mode and J. Rafter), p. 187. Elsevier Biomedical Press, Amsterdam (1980).
25. S. Newman and P. S. Guzelian, *Proc. natn. Acad. Sci. U.S.A.* **79**, 2922 (1982).
26. N. A. Elshourbagy, J. L. Barwick and P. S. Guzelian, *J. biol. Chem.* **256**, 6060 (1981).
27. U. Giger and U. A. Meyer, in *Biochemistry, Biophysics and Regulation of Cytochrome P-450* (Eds. J. A. Gustafsson, J. Carlstadt-Duke, A. Mode and J. Rafter), p. 199. Elsevier Biomedical Press, Amsterdam (1980).
28. J. P. Whitlock and H. V. Gelboin, *Pharmac. Ther.* **4**, 587 (1979).
29. A. J. Paine and L. J. Hockin, *Biochem. Pharmac.* **29**, 3215 (1980).
30. P. S. Guzelian and D. M. Bissell, *J. biol. Chem.* **251**, 4421 (1976).
31. W. E. Fahl, G. Michalopoulos, G. L. Sattler, C. R. Jefcoate and H. C. Pitot, *Archs Biochem. Biophys.* **192**, 61 (1979).
32. A. Parkinson, L. W. Robertson and S. Safe, *Biochem. Pharmac.* **31**, 3489 (1982).
33. A. M. Edwards, C. M. Lucas, P. A. Wilson and M. L. Glistak, *Abstr. 12th Int. Congr. Biochem., Perth*, p. 264 (1982).
34. A. M. Edwards, *Cancer Res.* **42**, 1107 (1982).
35. M. J. Whiting and A. M. Edwards, *J. Lipid Res.* **20**, 914 (1979).
36. W. F. Greenlee and A. Poland, *J. Pharmac. exp. Ther.* **205**, 596 (1978).
37. P. Moldeus, T. Hogberg and S. Orrenius, *Meth. Enzym.* **51**, 60 (1978).
38. A. J. Paine, L. J. Hockin and C. M. Allen, *Biochem. Pharmac.* **31**, 1175 (1982).
39. R. J. Mans and G. D. Novelli, *Archs Biochem. Biophys.* **94**, 48 (1961).
40. G. G. Rousseau, *J. Steroid Biochem.* **6**, 75 (1975).
41. C. Manen, M. Costa, I. G. Sipes and D. H. Russell, *Biochem. Pharmac.* **27**, 219 (1978).
42. M. R. Past and D. E. Cook, *Biochem. Pharmac.* **31**, 3329 (1982).
43. D. E. Ryan, P. E. Thomas, D. Korzeniowski and W. Levin, *J. biol. Chem.* **254**, 1365 (1979).
44. P. P. Lau and H. W. Strobel, *J. biol. Chem.* **257**, 5257 (1982).
45. D. J. Waxman and C. Walsh, in *Cytochrome P-450, Biochemistry, Biophysics and Environmental Implications* (Eds. E. Hietanen, M. Laitinen and O. Hanninen), p. 311. Elsevier Biomedical Press, Amsterdam (1982).

46. D. J. Waxman and C. Walsh, *J. biol. Chem.* **257**, 10446 (1982).
47. D. E. Ryan, P. E. Thomas and W. Levin, *J. biol. Chem.* **255**, 7941 (1980).
48. N. A. Elshourbagy and P. S. Guzelian, *J. biol. Chem.* **255**, 1279 (1980).
49. V. Luu-The, J. Cumps and P. Dumont, *Biochem. biophys. Res. Commun.* **93**, 776 (1980).
50. D. J. Benford and J. W. Bridges, *Biochem. Pharmac.* **29**, 2345 (1980).
51. D. M. Bissell and P. S. Guzelian, *Ann. N.Y. Acad. Sci.* **349**, 85 (1980).
52. J. T. Spence, L. Haars, A. Edwards, A. Bosch and H. C. Pitot, *Ann. N.Y. Acad. Sci.* **349**, 99 (1980).
53. D. L. Friedman, T. H. Claus, S. J. Pilkis and G. E. Pine, *Exp. Cell Res.* **135**, 283 (1981).