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PHASE I AND PHASE II XENOBIOTIC REACTIONS AND METABOLISM OF THE FOOD-BORNE CARCINOGEN 2-AMINO-3,8-DIMETHYLIMIDAZO[4,5-*f*]QUINOXALINE IN AGGREGATING LIVER CELL CULTURES

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Abstract—Aggregating fetal liver cell cultures were tested for their ability to metabolize xenobiotics using ethoxycoumarin-*O*-deethylase (ECOD), as marker of phase I metabolism, and glutathione *S*-transferase (GST), as marker for phase II reactions. Significant basal activities, stable over 14 days in culture were measured for both ECOD and GST activities. The prototype cytochrome P450 inducers, 3-methylcholanthrene (3-MC) and phenobarbital (PB), increased ECOD and GST activities reaching an optimum 7 days after culturing, followed by a decline in activity. This decline was partially prevented by 1% dimethyl sulfoxide (DMSO) added chronically to the culture medium. DMSO was also found to induce ECOD activity and to a lesser extent GST activity. Furthermore, it potentiated in a dose-dependent manner the induction of ECOD by PB. The food-borne carcinogen 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is metabolically transformed through a number of pathways *in vivo*. It was therefore used to examine the metabolic capacity in fetal and adult liver cell aggregates. Metabolism of MeIQx was mainly through N^2-conjugation, resulting in formation of the N^2-glucuronide and sulfamate conjugates for non-induced fetal liver cells. These metabolites were also found in large amounts in non-induced adult liver cells. Low levels of cytochrome P450-mediated ring-hydroxylated metabolites were detected in both non-induced fetal and adult liver cells. After induction with arochlor (PCB) or 3-MC, the major pathway was ring-hydroxylation (cytochrome P450 dependent), followed by conjugation to β-glucuronic or sulfuric acid. The presence of the glucuronide conjugate of *N*-hydroxy-MeIQx, a mutagenic metabolite, suggested an induction of P450 CYP1A2. The metabolism of MeIQx by liver cell aggregates is very similar to that observed *in vivo* and suggests that aggregating liver cell cultures are a useful model for *in vitro* metabolic studies in toxicology.

Cell culture has become a widely used laboratory tool for scientific, economic and ethical reasons. In toxicology, cell culture models have been developed to investigate xenobiotic metabolism and toxicity. To this end, hepatic cell cultures have been applied using cell lines [1] or primary adult [2, 3] and fetal cells [4]. However, one fundamental technical problem with cell culture is the inability to obtain cells that express a complete set of phase I and phase II metabolic enzymes. For example, primary adult rat hepatocytes have been shown to rapidly lose their drug-metabolizing capability [5]. Therefore, there have been many attempts to maintain the differentiated properties of hepatocytes *in vitro*, notably by medium complementation [2] and supply of extracellular matrix components for cell attachment [6, 7]. Liver cell survival and functionality was also significantly improved in co-culture with other cell types [3], suggesting a key role for cell–cell interactions in the maintenance of liver specific functions.

Aggregating cell cultures which were discovered by Moscona [8], are based on the unique ability of freshly dissociated fetal cells to re-aggregate spontaneously into spheroids. Extensively used with fetal rat brain cells [9, 10], this culture system proved to reproduce very closely the complex cellular organization and maturation of the developing brain *in vivo* [9, 10]. Assuming that the intercellular contacts within such aggregates are crucial for the expression, maturation and maintenance of cell-specific functions, a method has been developed to grow aggregating fetal liver cells in a chemically defined medium [11]. In accordance with the previous work of Moscona [8], these cultures were found to exhibit a histotypic organization [11]. In particular, hepatocytes containing glycogen granules were found to form acini-like structures, and the presence of reticulin fibers suggested the formation of an extracellular matrix [11]. Investigation of the expression of liver-specific genes such as tyrosine aminotransferase, α-fetoprotein and albumin were found to mimic the developmental behavior of perinatal liver *in vivo* [11].

In this study, the capacity of aggregating fetal

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liver cell cultures to metabolize xenobiotics was investigated. As a first approach, markers of phase I and phase II metabolic reactions were examined. Ethoxycoumarin-*O*-deethylase (ECOD*) activity was measured as a parameter for phase I reactions. This enzymatic activity is dependent mainly on the 3-methylcholanthrene (3-MC)-inducible cytochrome CYP1A1, but also on the phenobarbital-inducible cytochromes P450 CYPIIA1, CYPIIB1, CYPIIB2 [12]. For phase II reactions, glutathione *S*-transferase (GST) activity was chosen as a marker, using 1-chloro-2,4-dinitrobenzene (CDNB) which is metabolized by a majority of the GST isoenzymes [13]. The induction of these two enzymes was studied using one prototype of each of the two main classes of inducers: phenobarbital (PB) and 3-MC (a polycyclic hydrocarbon). The metabolic capacity of aggregating liver cell cultures was further examined using the heterocyclic amine 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) as a substrate. This food-borne carcinogen is known to be metabolized in rodent liver through various enzymatic pathways [14, 15], including direct conjugation to the exocyclic amine group by N-acetylation, N-glucuronidation and sulfamate formation (the chemical structures of major metabolites are displayed in Scheme 1). Cytochrome P450-mediated hydroxylation at position 5 of MeIQx followed by conjugation to sulfuric or β-glucuronic acid represent other major routes of detoxification [14, 15]. N-Oxidation catalysed predominantly by CYP1A2 also occurs [14, 15]. The latter reaction appears to be the initial step in the transformation of MeIQx to a carcinogen [16, 17]. The metabolites detected in fetal liver cell cultures were compared with those of adult liver cell aggregates which had shown good maintenance of the adult hepatocyte phenotype [18].

MATERIALS AND METHODS

Cell culture

Aggregate cultures of fetal liver cells. These were prepared from 15–16-day-old rat embryos as described previously in detail [10, 11]. In brief, excised livers were minced, pooled and washed in ice-cold sterile solution D1 (modified Puck's solution) pH 7.4, 340 mOsm, containing 138 mM NaCl, 5.4 mM glucose, 14 μg/mL phenol red, 15 μg/mL gentamicin. The tissue was then dissociated by the sequential digestion technique of Chessebeuf and Padieu [19] using acetylated trypsin (0.2%, Sigma) in Hepes-buffered D1 solution. The resulting supernatants containing the dissociated liver cells were mixed with an equal volume of cold Hepes-buffered D1 solution containing 0.02% of trypsin inhibitor. The combined supernatants were then filtered by gravity flow through a layer of 115 μm nylon mesh and washed twice in solution D1 by centrifugation (10 min, 230 *g*, 4°). The sedimented cells were finally resuspended in serum-free culture medium by gentle trituration. Aliquots (4 mL) of the final cell suspension (2×10^7 cells/mL) were distributed to 25 mL Delong flasks and incubated at 37° in an atmosphere of 10% CO_2 and 90% air under constant gyratory agitation. On day 1, 3.5 mL of medium were removed from each culture and replaced by fresh medium. On day 2, the cultures were transferred to 50 mL Delong flasks by addition of 4 mL of fresh medium. From the onset, the cultures were grown in a serum-free chemically defined medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with transferrin (1 μg/mL), insulin (800 nM), triiodothyronine (30 nM), dexamethasone (10 nM), BME vitamins, vitamin B12, trace amounts of retinol and α-tocopherol [9], L-carnitine (12 μM), 7.6 μM fatty acid (31% palmitic acid, 2.8% palmitoleic acid, 11.6% stearic acid, 13.4% oleic acid, 35.6% linoleic acid and 5.6% linolenic acid) bound to bovine serum albumin (final albumin concentration in the medium 0.2% w/v) and various amounts of trace elements [10, 11]. Finally, L-proline (11.5 μg/mL, from day 1 onwards) and epidermal growth factor (EGF, 10 ng/mL from day 2 onwards) were added to the medium. The rotation speed of the shaker, initially set at 65 rpm was gradually increased to 80 rpm. The media were replenished by replacing 5 mL of medium every 3 days.

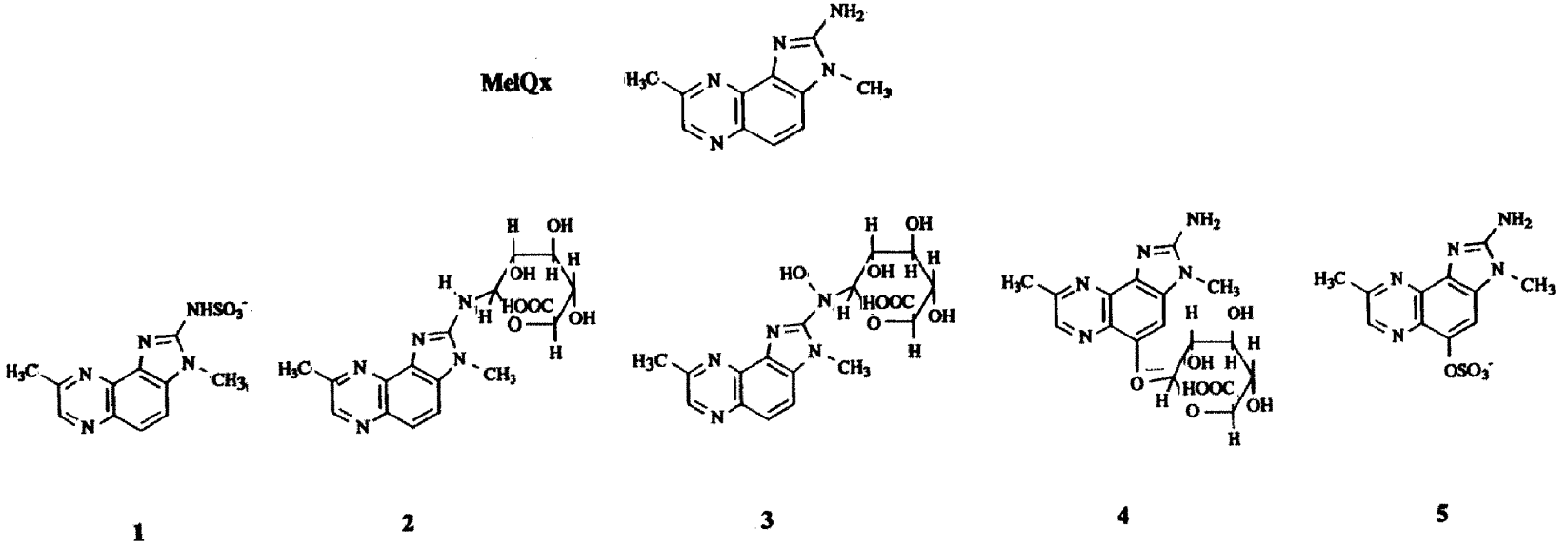
Culture harvest. For protein and GST assays, aggregates were washed twice with phosphate-buffered saline and homogenized in 300 μL of 1 mM phosphate buffer (pH 6.8), using glass–glass homogenizers (Bellco).

Induction procedures. Cultures were treated with either 12.5 μM 3-MC (Sigma), 3.2 mM PB (Sigma) or 20 μg/mL Arochlor-1254 (PCB, Analabs) for 48 hr. The culture medium was replenished daily with fresh medium containing the inducer but no EGF. Stock solutions of PB were prepared in water, those for 3-MC and PCB in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the medium was 0.25% (v/v). Control cultures received the same volume of solvent.

Aggregating adult liver cell cultures. These cultures were prepared as described earlier in detail (Juillerat *et al.*†). Hepatocytes were isolated from adult male Sprague–Dawley rats (IFFA, CREDO, L'Arbresle, France) by a two-step collagenase perfusion method [20]. In parallel, a suspension of non-parenchymal cells was prepared according to Hendriks *et al.* [21]. The different non-parenchymal cell types were purified by elutriation. The purity of all cell fractions

* Abbreviations: 3-MC, 3-methylcholanthrene; CDNB, 1-chloro-2,4-dinitrobenzene; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECOD, ethoxycoumarin-*O*-deethylase; EGF, epidermal growth factor; GST, glutathione *S*-transferase; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PB, phenobarbital; PCB, polychlorinated biphenyl (Arochlor-1254); NSO_3^-, *N*-3,8-dimethylimidazo[4,5-*f*]quinoxaline-2-yl sulfamic acid; N-Gl, N^2-(β-1-glucosiduronyl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; HO-N-Gl, N^2-(β-1-glucosiduronyl)-*N*-hydroxy-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; O-Gl, 2-amino-5-(β-1-glucosiduronyloxy)3,8-dimethylimidazo[4,5-*f*]-quinoxaline; $5\text{-}OSO_3^-$,2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline-5-yl sulfate.

† Juillerat M, Marceau N, Coeytaux S, Sierra F, Kolodziejczyk E and Guigoz Y, "Microliver", or tridimensional culture of liver cells: a model for studying hepatic functions. *Hepatology*, submitted.



Scheme 1. Chemical structure of major metabolites of the food-borne carcinogen MEIQx: (1) NSO_3^-; (2) N-Gl; (3) HO-N-Gl; (4) O-Gl; (5) 5-OSO_3^-.

was determined by light microscopy and conventional methods [21–23]. A final cell suspension was prepared in serum-free culture medium with the different cell types mixed in proportions similar to those found in adult liver *in vivo* [24], i.e. 65% hepatocytes, 23% endothelial cells, 12% Kupffer cells and 4% fat-storing cells. Aggregating cell cultures were obtained after distribution of 4 mL of cell suspension (3.2×10^6 cells) to 25 mL Delong flasks and incubation under gyratory agitation in the same culture conditions as described for fetal liver cell aggregate cultures.

Assays

ECOD. ECOD activity was determined by a modification of the method described by Moldeus *et al.* [25] measuring the O-deethylation of 7-ethoxycoumarin. The assay was performed with intact aggregates, in the presence of salicylamide, a competitive inhibitor of 7-hydroxycoumarin conjugation. Briefly, the aggregates (the content of one or two culture flasks per 0.5 mL of medium) were maintained under gentle agitation in culture medium (without EGF) at 37°, under a carbogen atmosphere (95% O_2/5% CO_2). The reaction was initiated by adding 5 μL of methanol containing 10 mM 7-ethoxycoumarin and 20 mM salicylamide. At the start of the reaction and after 2 and 4 min of incubation, 100 μL aliquots of aggregate suspension were taken and mixed immediately with 100 μL of 0.01 N HCl in methanol. After vortexing and centrifugation the supernatants were quickly frozen in dry ice and stored at −80°. For the 7-hydroxycoumarin analysis, the samples were diluted 10-fold in 0.01 N NaOH and measured fluorimetrically at 366 nm excitation and 466 nm emission wavelengths, using a Perkin–Elmer LS-30 fluorimeter. The linearity of the assay was controlled for up to 8 min of incubation.

GST. GST was assayed in cell homogenates according to the method of Habig and Jakoby [13]. The activity was determined spectrofluorimetrically at 340 nm using CDNB as a substrate. The portion of non-enzymatic conjugation was determined by omission of the enzyme in the assay mixture and subtracted from the total activity.

The metabolism of MeIQx. This was studied in 7-day-old liver cell cultures prepared either from fetal or adult tissue. 2-^{14}C-Labeled MeIQx (37 mCi/mmol, Toronto Research Chemicals) was given to the culture media (devoid of EGF) at a concentration of 57 μM. After 15 hr of incubation the reaction was terminated by lysing the cells with 2 volumes of cold ethanol and subsequent centrifugation (20,000 *g*, 15 min, 4°). The pellet was resuspended in 1 volume of deionized water, precipitated a second time by addition of ethanol and centrifugation (20,000 *g*, 15 min, 4°). The pooled supernatants were stored at −80°. Prior to HPLC analysis, aliquots were rotary evaporated and resuspended in deionized water. Aliquots of a bile extract from PCB-treated adult rats containing unlabeled MeIQx metabolites were added as internal standards. The HPLC analysis was performed using the method and instrumentation described earlier [14, 15].

Protein was determined by the Folin phenol method of Lowry *et al.* [26] using bovine serum albumin as a standard.

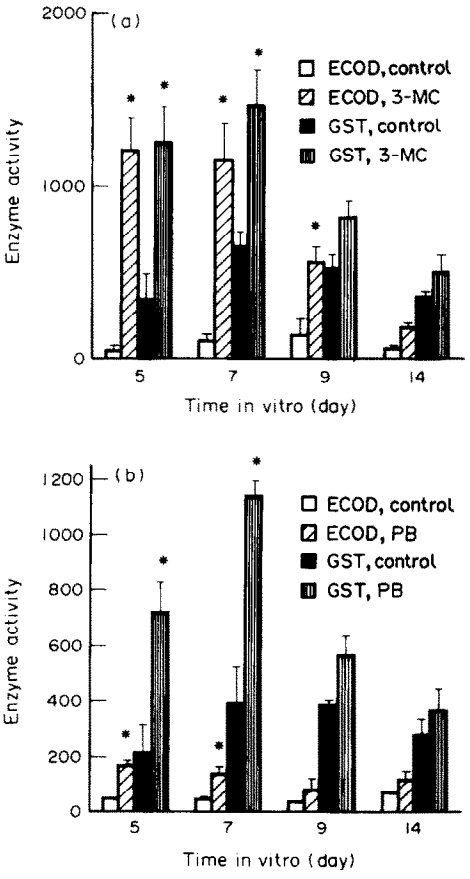


Fig. 1. Inducibility of ECOD and GST as a function of the culture time. The cultures were exposed to 12.5 μM 3-MC (a) or 3.2 mM PB (b) during a period of 48 hr before the ECOD and GST assays performed on day 5, 7, 9, 14. The ECOD activity is given in pmol/min/mg of protein and GST in nmol/min/mg of protein. For the 3-MC-induction, the results are expressed as mean ± SD of triplicate cultures. For PB-induction, the measures were performed on the content of two cultures pooled just before the assays. In this case, the results are expressed as mean ± SD of duplicate pooled cultures. *Significant differences from corresponding non-induced controls (determined by Student's *t*-test, $P < 0.05$).

RESULTS

Induction of ECOD and GST activities

The induction of ECOD and GST by 3-MC and PB was studied in aggregates maintained in culture for different time periods. The cultures were exposed from day 3, 5, 7 or 12 for 48 hr to either 3-MC (12.5 μM) or to PB (3.2 mM) The results (Fig. 1a and b) show no significant age-dependent changes in ECOD and GST activities in control cultures, whereas the induction of these enzymes varied considerably. In 3-MC-induced cultures the highest activities of ECOD and GST were found on days 5 and 7. Thereafter, induction by 3-MC of either

Table 1. Effects of 3-MC plus PB on the ECOD and GST activities

	EOCD	GST
Control	86 ± 37	443 ± 126
Control DMSO	340 ± 25*	551 ± 19
3-MC	888 ± 148*	1140 ± 118*
PB	352 ± 25*	973 ± 296*
3-MC + PB	1932 ± 105*	2476 ± 142*

Cultures were exposed on day 5 to both, 3-MC (12.5 μM) and PB (3.2 mM), or to each inducer alone and were assayed for ECOD and GST activities on day 7. The ECOD activity is given in pmol/min/mg of protein and GST in nmol/min/mg of protein. The measures were performed on the content of two cultures pooled just before the assays and the results are expressed as mean ± SD of duplicate pooled cultures.

* Significant differences from corresponding non-induced controls (determined by Student's t-test, $P < 0.05$).

enzyme was significantly lower ($P < 0.05$), showing a progressive decline as a function of time in culture (Fig. 1a). The induction of ECOD and GST by PB showed a similar age-dependency (Fig. 1b).

The combined effects of 3-MC and PB were examined at day 7. Compared to the individual stimulatory effects of each inducer, 3-MC and PB acting simultaneously produced an additive increase of GST activity as well as a synergistic increase (i.e. a 50% increase above the arithmetic sum of the individual effects) in ECOD activity (Table 1).

Effects of DMSO

The effects of DMSO on the induction of ECOD and GST were investigated. Aggregating fetal liver cell cultures were treated on day 5 with either 3-MC or PB, in the presence of DMSO at various concentrations (0–1%), and assayed for ECOD and GST activities on day 7. The results (Fig. 2a and b) show that in the absence of any inducer, DMSO significantly increased ($P < 0.05$) ECOD activity in a dose-dependent manner. At the highest concentration used (1% DMSO) ECOD activity was increased 5-fold above control values. However, the induction of ECOD by 3-MC was not altered by DMSO (Fig. 2a). In contrast, the induction of ECOD by PB was strongly increased in the presence of DMSO (Fig. 2b). This effect was dose-dependent, reaching at 1% DMSO a level of ECOD activity 14-fold higher than in controls (Fig. 2b). The same pattern of response to DMSO was observed for GST (Fig. 2a and b).

As shown in Fig. 1a and b the induction of ECOD and GST declined with time in culture. To examine the effects of DMSO on this age-dependent loss of liver functions, the induction of ECOD and GST activities by 3-MC was determined in cultures maintained from day 2 onwards in a medium supplemented with 1% DMSO. For the induction 48 hr prior to the enzymatic analysis, the culture medium was replaced by a medium containing 12.5 μM 3-MC and 0.25% DMSO. Control cultures received 0.25% DMSO during the same period. The

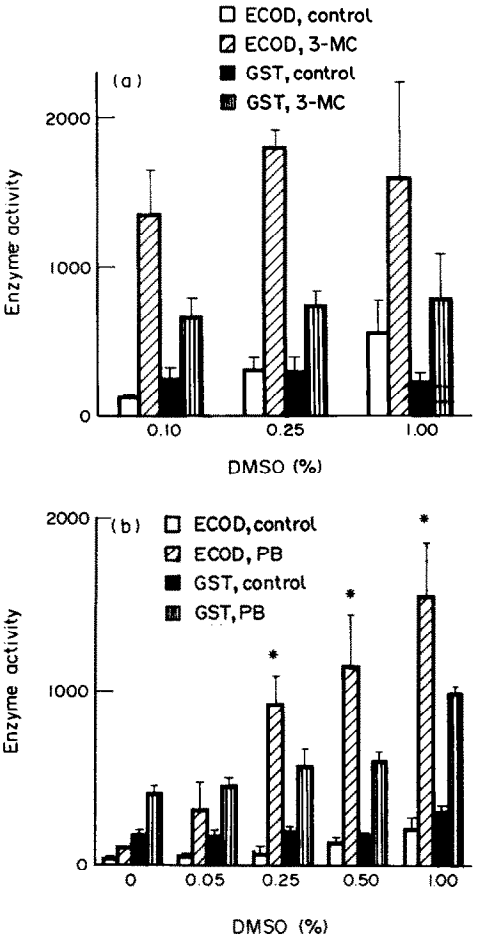


Fig. 2. Effects of DMSO on the 3-MC- and PB-induction of ECOD and GST activities. Cultures were exposed on day 5 to 12.5 μM of 3-MC (a) or 3.2 mM (b) in the presence of various concentrations of DMSO and assayed on day 7 for ECOD and GST activities. The ECOD activity is given in pmol/min/mg of protein and GST in nmol/min/mg of protein. The results are expressed as mean ± SD of triplicate cultures. *Significant differences from corresponding values measured in the absence of DMSO for PB-induction or in the presence of 0.1% DMSO for the MC-induction (determined by Student's t-test, $P < 0.05$).

results (Fig. 3) show in accord with previous observations on day 7 (Fig. 2a and b) that exposure to DMSO resulted in a significantly higher ECOD activity. Furthermore, the enzyme induction increased by 75% in DMSO-treated cultures. In contrast, GST activity and its induction by 3-MC were not significantly affected by DMSO (Fig. 3). Confirming the data of Fig. 1a, a strong reduction of ECOD and GST response was observed in 14-day cultures as compared to those of day 7 (Fig. 3). This decline was partially prevented by the presence of DMSO (1%) in the culture medium. At day 14, DMSO-treated cultures showed a significant ($P < 0.05$) induction of ECOD (5-fold) and GST (2.5-fold). For GST, the specific activities at day 14

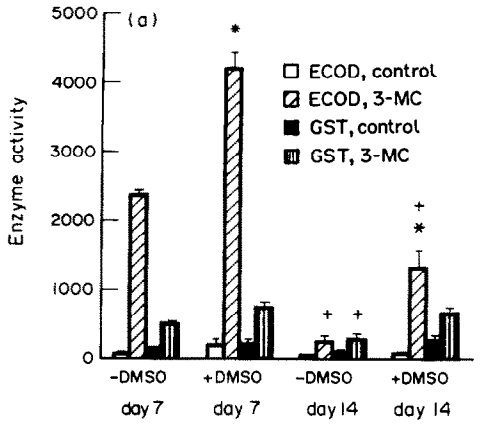


Fig. 3. Effects of DMSO on the age-dependent loss of 3-MC-induction of ECOD and GST activities. The induction of ECOD and GST activities were measured after 48 hr of treatment with 12.5 μM of 3-MC, in cells maintained from day 2 in a medium supplemented with 1% DMSO or in cells maintained in normal conditions. The assays were performed on day 7 and on day 14. The ECOD activity is given in pmol/min/mg of protein and GST in nmol/min/mg of protein. The results are expressed as mean ± SD of triplicate cultures. *Significant differences from the corresponding values measured in absence of DMSO. +Significant differences of the values obtained on day 14 from corresponding values measured on day 7 (determined by Student's t test, $P < 0.05$).

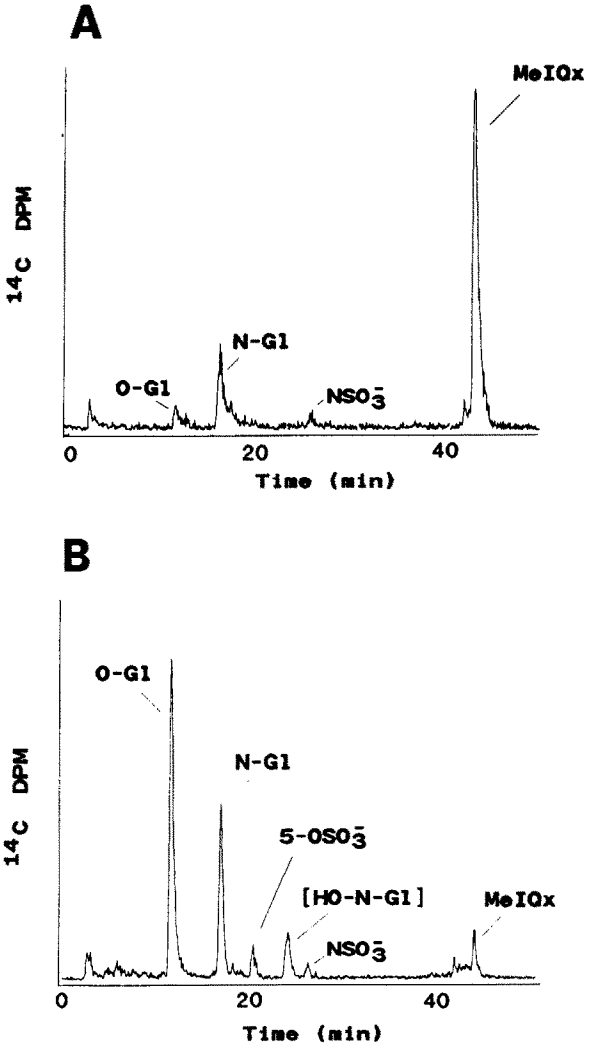


Fig. 4. Representative HPLC profile of MeIQx metabolites isolated from control (A), PCB-treated (B) re-aggregating fetal liver cell cultures. MeIQx was given to 7-day-old cultures and the metabolite pattern was analysed after 15 hr of incubation.

corresponded to those measured at day 7, whereas for ECOD they were clearly lower than at day 7 both in controls (−25%) and in DMSO-treated cultures (−58%). However, if expressed as total enzyme activities per flask, ECOD basal activities and its induction by 3-MC remain unchanged, while GST activities show an increase both in the non-induced and in the 3-MC-induced cultures, as compared to 7-day cultures (data not shown).

MeIQx metabolism

The capability of aggregating fetal liver cell cultures to perform complex metabolic reactions was examined by studying the biotransformation of MeIQx, a carcinogen which is activated/detoxified through various phase I and phase II enzymatic routes in the rat liver [14, 15]. MeIQx was given to 7-day cultures, and the resulting pattern of metabolites was analysed after 15 hr of incubation. Representative HPLC profiles of metabolites detected in controls and PCB-treated cultures are shown in Fig. 4A and B. The quantitative results for the major metabolites, expressed as percentages of the initial dose of MeIQx are presented in Table 2. In non-induced control cultures, about 35% of the initial dose of MeIQx was metabolized, mainly via exocyclic N^2-conjugation. The major metabolite produced by these cultures was the N^2-glucuronide (N-Gl), followed by the sulfamate (NSO_3^-) in lower amounts. Low levels of the cytochrome P450 activity were detected through MeIQx-*O*-glucuronide (O-Gl) formation. Pretreatment with PCB or 3-MC greatly enhanced the metabolism of MeIQx, resulting in a large increase in the formation of the ring-hydroxylated conjugates. In the induced cultures, O-Gl was the most abundant metabolite and the sulfate ($5\text{-}OSO_3^-$), which was not detectable in control cultures, also was found at high levels. Furthermore, metabolic activation of MeIQx through N-hydroxylation was demonstrated by the relatively large amounts of the *N*-glucuronide conjugate (HO-N-Gl) found exclusively in the induced cultures. PCB and 3-MC induction had little influence on the N^2-conjugation; N-Gl formation was unchanged, and NSO_3^- formation was somewhat decreased as compared to control cultures.

In order to compare the metabolic pattern of fetal liver cell aggregates with that of differentiated liver cells, MeIQx metabolism was also investigated in

Table 2. Distribution of major metabolites of MeIQx produced by fetal or adult liver cell aggregates

Metabolite	Fetal			Adult		
	Non-induced	PCB	3-MC	Non-induced	PCB	3-MC
Polar	4.8 ± 0.3	9.0 ± 0.1	10.6 ± 0.1	4.2	4.6	4.3
MeIQx-O-Gl	3.9 ± 0.5	39.5 ± 1.5	47.1 ± 1.7	0.8	9.3	19.0
MeIQx-N-Gl	17.3 ± 0.6	19.6 ± 0.4	19.8 ± 0.6	14.6	30.5	28.4
MeIQx-5-OSO_3^-	—	7.7 ± 0.2	3.8 ± 0.8	2.7	15.8	12.1
MeIQx-[HO-N-Gl]	—	6.0 ± 0.4	8.1 ± 0.3	—	1.7	6.6
MeIQx-NSO_3^-	2.3 ± 0.2	1.1 ± 0.7	1.7 ± 0.1	27.4	6.8	1.4
MeIQx	64.7 ± 1.0	8.6 ± 1.3	—	35.5	16.4	5.5

Data are presented as per cent of the dose of MeIQx given to cell cultures. N = 3 ± SD of pooled fetal cell cultures, while single values are reported for adult cultures.

Analysis of MeIQx metabolites was done by HPLC after a 15 hr incubation in 7-day-old cultures.

aggregate cultures of adult rat liver cells. The results are shown in Table 2. As observed in fetal cell cultures, adult hepatocyte cultures (non-induced controls) produced mainly N^2-conjugates. The sulfamate was the most abundant metabolite, while the N-Gl metabolite was found in lower amounts. The glucuronide (O-Gl) and sulfate (5-OSO_3^-) conjugates were found in relatively low quantities. Pretreatment of these cultures with PCB or 3-MC greatly induced MeIQx metabolism, enhancing, as in fetal cultures, the production of ring-hydroxylated conjugates such as O-Gl and 5-OSO_3^- as well as HO-N-Gl, a marker of increased N-hydroxylation activity. The formation of N^2-conjugation products also differed; N-Gl was significantly increased, and NSO_3^- was considerably reduced.

DISCUSSION

The present results show that aggregating fetal liver cell cultures contain significant activities of the two marker enzymes for drug metabolism, ECOD (phase I, activation) and GST (phase II, conjugation). The specific activities of ECOD measured on day 5 were in good agreement with those reported in liver cell monolayer cultures [27] and in newborn rat liver [28]. As known from studies *in vivo* and in other cell culture systems, both ECOD and GST activities were markedly enhanced after exposure to the two prototype inducers, 3-MC and PB.

It was found that the induction of ECOD and GST activities by either 3-MC or PB declined markedly after culture day 7, while non-induced aggregate cultures exhibited relatively stable ECOD and GST activities up to day 14 (Fig. 1a and b). A similar decrease in response has been reported for aryl hydrocarbon hydrolase and epoxide hydrolase in monolayer cultures of fetal liver cells [29], suggesting that with time in culture there is a loss in the induction of drug-metabolizing enzymes in fetal liver cells. The reason for this functional loss is not understood, but part of the problem may be inadequate culture conditions. While homologous cultures prepared from fetal brain attain a very high level of cellular differentiation and can be maintained in serum-free medium for many months [9, 10], liver cell aggregates already deteriorate after a few weeks *in vitro*. This deterioration may be due to nutritional deficits and/or diffusional barriers. Aggregates of fetal liver cells are enveloped by a relatively tight layer of epithelial cells [11] which could prevent the access of nutrients and/or growth factors to cells within the aggregates.

DMSO significantly enhanced ECOD activity in a dose-dependent manner (Fig. 2a and b). As demonstrated in adult rat hepatocyte cultures [30], the present data indicate that DMSO is an inducer of mono-oxygenase activities. Furthermore, in aggregate cultures, DMSO caused a strong potentiation of the induction of ECOD by PB. This effect could explain the synergistic stimulation of ECOD activity found in cultures exposed simultaneously to 3-MC and PB (Table 1). This synergism is probably a consequence of the additive effect of 3-MC and PB, and the potentiation by DMSO, the solvent used for 3-MC. GST was less responsive to DMSO. Only chronic treatment with 1% DMSO increased significantly and reproducibly GST activity (Fig. 3), and with a weak potentiation by DMSO on GST induction by PB (Fig. 2b), suggesting a stronger modulation of DMSO for phase I than phase II enzyme activities. Chronic treatment of aggregate cultures with 1% DMSO maintained the induction of ECOD and GST activities at least 2 weeks. The concomitant increase in total protein content would suggest that DMSO exerts a general stimulatory effect on hepatocyte cultures. This view is supported by observations in adult rat hepatocyte cultures, showing that DMSO treatment (i) retards the loss of cytochrome P450 [30–32], (ii) increases cell survival [31], and (iii) maintains the morphological [31, 32] and biochemical [31–33] characteristics of differentiated liver cells. Furthermore, DMSO has been shown to induce a differentiated phenotype in certain cell lines [34, 35], to stimulate heme synthesis [34], and to induce various specific proteins [30, 36]. This generalized action of DMSO might be brought about by a modifying effect on the plasma membrane [37] and/or by its function as a hydroxyl radical scavenger. The latter has been shown to play a central role in the maintenance of cytochrome P450 content and ECOD activity in adult rat hepatocytes [30].

The study of MeIQx metabolism revealed that

aggregating fetal liver cell cultures express a variety of phase I and phase II enzymes. A similar metabolic capacity was reported in fetal liver cell monolayer cultures, capable of transforming benzo[*a*]pyrene into sulfate and glucuronide conjugates [38]. The major route of MeIQx biotransformation in non-induced fetal liver cell aggregates was through exocyclic N^2-conjugation, producing mainly N-Gl and low amounts of the NSO_3^- conjugate. The P450-dependent ring hydroxylation was demonstrated in these cultures by the presence of O-Gl, although this activity was very low, probably because of low P450 levels in non-induced cell cultures. Similar to the fetal cell aggregates, non-induced adult liver cell aggregates metabolized MeIQx mainly through N^2-conjugation, although the relative proportions of the metabolites were different. In adult cultures the major metabolite found was the NSO_3^- conjugate, but there were also relatively high amounts of N-Gl. Furthermore, both the O-Gl and the 5-OSO_3^- ring-hydroxylated conjugates were detected. The pattern of metabolites observed in adult cultures closely resembled that reported *in vivo* [15]; however, it is interesting to note that in freshly isolated adult rat hepatocytes the N^2-glucuronide of MeIQx is a minor metabolite [14]. In non-induced cultures the main difference between adult and fetal cells in MeIQx metabolism was the relatively lower production of sulfamate and sulfate conjugates in fetal cells, suggesting that immature liver cells express high levels of glucuronyltransferase but a low amount of sulfotransferase.

After induction with PCB or 3-MC, the major metabolic pathway in both fetal and adult liver cell aggregates was the cytochrome P450-mediated hydroxylation of MeIQx at position 5, followed by conjugation to sulfuric or β-glucuronic acid. Also, N^2-glucuronidation remained a major route of MeIQx biotransformation in both types of aggregate cultures. In adult liver cell aggregates, the latter reaction was considerably greater than in fetal cell cultures. Induced adult liver cell aggregates also produced large amounts of O-Gl and 5-OSO_3^- at the expense of sulfamate formation. These results are in good agreement with observations in rat liver *in vivo* [15] and in freshly isolated hepatocytes [14]. However, in contrast to these *in vivo* and *ex vivo* findings, aggregate cultures, in particular those of fetal cells, showed a preference for glucuronidation as compared to the sulfamate formation pathway.

Metabolic activation of MeIQx through N-hydroxylation, forming the mutagenic metabolite *N*-hydroxy-MeIQx was demonstrated in rat hepatic microsomes [16, 17] and in freshly isolated hepatocytes [14]. This unstable metabolite was not found in biological fluids of rodents [15]. However, *N*-hydroxy-MeIQx is a substrate of UDP-glucuronyltransferase forming a metastable *N*-glucuronide conjugate (HO-N-Gl) which was detected in the urine and bile of control and PCB-induced rats [15]. In non-induced aggregate cultures of fetal or adult liver cells HO-N-Gl, the marker of N-hydroxylation was undetectable, probably due to the low cytochrome P450 levels in these cultures. Following treatment with PCB or 3-MC, fetal as well as adult cell aggregates produced substantial amounts of HO-N-Gl, indicating a highly inducible N-hydroxylation activity. This reaction is catalysed mainly by CYP1A2, and to a lesser extent by CYP1A1 [17, 39]. It has been shown that CYP1A2 is not expressed in a hepatic cell line [1]. Furthermore, in the mouse CYP1A2 is developmentally regulated and inducible around the time of birth [40]. Thus, the high amount of HO-N-Gl produced by the fetal cell aggregates could be interpreted as a CYP1A2 induction, which would suggest that these cultures followed the developmental schedule of perinatal liver *in vivo*. The presence of dexamethasone in the culture medium could contribute to this developmental process. It has been shown that dexamethasone is required for the expression of the adult forms of P450 in monolayers of fetal liver cells [4, 27]. Furthermore, Silver *et al.* [41] demonstrated that the positive regulation of CYP1A2 by 3-MC in adult hepatocyte cultures is dependent on dexamethasone. However, the involvement of a specific fetal P450 capable of N-hydroxylation cannot be excluded. Kitada *et al.* [42] reported that the mutagenic activation of 2-amino-3-methylimidazo[4,5-*f*]-quinoline, another structurally related arylamine, was catalysed by two forms of P450 in human fetal liver, one immunologically related to CYP1A2 and another termed P450 HFLa.

It was previously shown that fetal liver cells are able to rearrange themselves into histotypic structures and to display a developmental pattern of gene expression comparable to that of perinatal rat liver *in vivo* [11]. This report demonstrates in these cultures the presence and inducibility of various enzymes involved in phase I and phase II xenobiotic biotransformation. Ethoxycoumarin used to measure ECOD, and CDNB used for GST are metabolized by several isoforms of cytochrome P450 [12] and GST [13], respectively. Thus, the activities for ECOD and GST reported here represent global activities and do not show possible developmental changes of specific isoenzymes. However, since aggregating cell cultures can be grown in a chemically defined medium and can be made in large quantities and are reproducible, this system appears very promising for studying the developmental regulation of cytochrome P450 and GST isoenzymes. Furthermore, the metabolism of the MeIQx in aggregating liver cell cultures demonstrate that aggregating cell cultures offer a suitable model to study the developmental toxicity as well as the biotransformation of chemicals. Thus, aggregating cell cultures are complementary to cytochrome P450s cDNA expression systems used in studies of metabolism and risk assessment of contaminants [43].

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