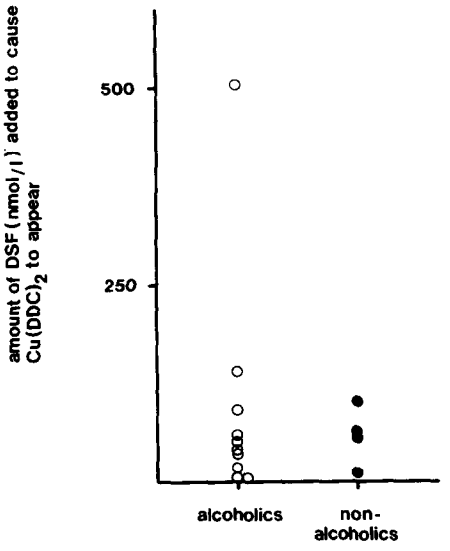

Fig. 3. The appearance of $Cu(DDC)_2$ in fresh heparin plasma drawn from alcoholics ($n = 11$) and a non-alcoholic reference group ($N = 4$) after addition of DSF at concentrations ranging from 100 to 800 nmoles/l.

The plasma concentrations of $Cu(DDC)_2$ in two samples from the *in vivo* studies were found to be just above the detection limit of the HPLC assay. However, the concentration of $Cu(DDC)_2$ found in the eluent fractions collected for measurements by MS was too low to give a definite identification of the compound. The very low plasma concentrations obtained from patients treated with a single dose of DSF could possibly be due to the individual capacity to form $Cu(DDC)_2$ from copper protein bound DDC [11] or to the extremely lipophilic character of the metal complex resulting in its distribution through cell membranes or its deposition into fat tissue. This is in agreement with the recently reported redistribution of endogenous copper into brain tissue when rats and mice were treated with tetramethylthiuram disulfide and DDC, respectively [12, 13]. The $Cu(DDC)_2$ partition between 1-octanol and water was found to be infinite into the organic phase. This finding supports our observation that the compound is highly hydrophobic. Moreover, preliminary results from five patients treated with repeated doses of DSF (400 mg) for 2 weeks show detectable plasma levels of $Cu(DDC)_2$ in all five samples. In the present study no evidence was found that administration of DSF could cause a decrease in the glutathione content of the erythrocytes, nor in the total sulfhydryl activity in blood or plasma. If erythrocyte glutathione was involved in the reduction of DSF, this would probably bring about a decline of the glutathione level in erythrocytes *in vivo* [2].

In summary, the *in vitro* and *in vivo* formation of a bis-(diethyldithiocarbamato) copper complex in humans has been observed. The *in vitro* formation has been clearly verified by MS. However, the plasma levels found *in vivo* are too low to be definitely identified by any available technique and this is probably due to the lipophilic character of the compound.

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Maintenance of cytochrome P-450 in cultured adult human hepatocytes*

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The liver is the primary organ involved in metabolism of xenobiotics. One major metabolic pathway is represented by cytochrome-P-450-dependent mono-oxygenases. Because *in vivo* liver functions are influenced by various endogenous and exogenous factors, a number of investigators have turned to simpler experimental models. Hepatocyte cultures theoretically represent the only *in vitro* model to perform long-term studies in a well-defined environment. However, cultivation of pure populations of adult rodent hepatocytes under various conditions is associated with a selective loss of biotransformation capacity and consequently, to limitation of potential use of this system for pharmaco-toxicological studies [1–3]. Moreover extrapolation of the results obtained with rodent hepatocyte cultures to the human situation has evident shortcomings since qualitative and quantitative interspecies differences commonly exist in biotransformation of xenobiotics by the cytochrome P-450 system [4]. In this study we have measured the cytochrome P-450 content and two

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major isozymes in pure and mixed primary cultures of adult human hepatocytes.

Material and methods

Cell isolation and culture. Adult human hepatocytes were obtained by collagenase perfusion of the left hepatic lobe of four kidney donors aged between 19 and 25 years [5]. Three donors (A–C) had a normal liver and received low medication, if any; the fourth one (D) died from barbiturate intoxication (Table 1). When necessary, cell dissociation was achieved by gentle stirring in the enzymatic solution at 37°. Freshly isolated parenchymal cells were seeded at the density of 15×10^6 viable cells/175 cm^2 plastic flask in 25 ml of Ham F_{12} medium containing 10 μg/ml porcine insulin, 0.2% bovine albumin and 10% fetal calf serum. After 3–4 hr, the medium was renewed. A part of the cultures was set up as pure cultures, the other was set up as co-cultures by adding 20×10^6 rat liver epithelial cells [6, 7]. These latter cells obtained by trypsinization of 10-day-old rat livers, were subcultivated and used before they underwent spontaneous transformation [8]. Cell confluency was reached in co-culture within 24–36 hr. Then the medium of both pure and mixed cultures was renewed daily and supplemented with 3×10^{-6} M hydrocortisone hemisuccinate. Hepatocyte monolayers survived for 2–3 weeks in conventional culture and for up to 2 months in co-culture [6].

Total cytochrome P-450. After two washes with Hepes buffer pH 7.4, hepatocyte cultures were scraped with a rubber policeman and pelleted. The cells were homogenized with a Potter Elvejhem device in 100 mM phosphate buffer containing glycerol (20% v/v), 1 mM EDTA and 0.2% emulgen. Cell homogenate was centrifuged at 12,000 *g* and the amount of cytochrome P-450 was quantified in the supernatant according to Omura and Sato [9].

For the preparation of microsomes, cell pellets were homogenized with a Potter Elvejhem device in 100 mM Tris–HCl buffer containing 250 mM sucrose and 1 mM EDTA. Cell homogenate was centrifuged at 800 *g* for 10 min, at 13,500 *g* for 20 min and at 105,000 *g* for 60 min.

Cytochrome P-450 *isozymes.* Cytochromes P-450-5 and 8 and the corresponding antibodies were those described by Wang *et al.* [10]. Cytochromes P-450-5 and P-450-8 correspond to two major isozymes present in adult human liver. They did not cross-react immunologically.

Purified P-450-5 and P-450-8 and microsomal proteins were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli [11] with slight modifications: the concentration of acrylamide was 9 instead of 7.5%. Proteins were then electrotransferred to a nitrocellulose sheet as described [12, 13] Nitrocellulose sheets were saturated with phosphate buffered saline (PBS) containing 3% bovine serum albumin, 10% fetal calf serum and 0.5% Tween 20 for 30 min at 37° before incubation with an appropriate dilution of antisera in the buffer used for saturation. The sheets were washed six times with PBS containing 0.5% Tween 20 at room temperature and then incubated with peroxidase-labelled anti-rabbit immunoglobulins. After washing peroxidase was revealed with 50 mM Tris–HCl buffer pH 7.6 containing 20% methanol, 0.02% and 2.8 mM 4-chloro-1-naphthol. Coloration was stopped by thorough washing of the nitrocellulose sheet which was then dried between filter papers. Quantification of the spots was performed by densitometry (PHI 6E, Vernon, France) as previously described [13]. A standard curve was obtained with increasing amounts of purified antigens.

Albumin secretion. The amount of albumin secreted into the medium was determined by laser immunonephelometry [14] using specific antibodies prepared for immunonephelometry (ATAB, Scarborough, ME). Appropriate dilutions of standard human albumin solutions and culture media were mixed with human anti-albumin antibodies in 0.1 M phosphate buffer containing 2% polyethylene glycol and incubated at room temperature for 1 hr. Controls included the absence of cross-reactivity with other proteins present in the medium and the absence of detectable albumin in the medium of pure cultures of rat-liver epithelial cells.

Expression of the results. Cytochrome P-450 content was expressed as picomoles per milligram of hepatocyte protein in the 12,000 *g* supernatant and albumin secreted rate as micrograms per 10^6 hepatocytes per day. Proteins were determined by the Folin-phenol colorimetric method [15]. In co-culture the number of hepatocytes and the amount of proteins were calculated from 1-day, sister pure cultures taking into account that there was no obvious proliferation or detachment of parenchymal cells during the first weeks. This was supported by the very few percentage of labelled co-cultured hepatocytes after a 24-hr incubation in the presence of [methyl-^{3}H]thymidine and the absence of floating cells or increased release of lactate dehydrogenase in the culture medium. Similar time-dependent variations were obtained when the values were expressed per flask. The same observations were made with co-cultures of rat hepatocytes [16]. All the determinations of cytochrome P-450 were done in duplicate or triplicate.

Results and discussion

Cytochrome P-450 *content.* Cytochrome P-450 was determined during the first 8 days. The data from the four cases are displayed separately in Fig. 1. Similar results were obtained with pure or mixed cultures from the three normal patients (A–C). The initial values of cytochrome P-450 in human hepatocytes were slightly lower than those normally found in freshly isolated normal rat hepatocytes [1, 2]. The same observations were made with tissue extracts and microsomes from human and rat liver [17]. In freshly isolated barbiturate-treated hepatocytes (case D) cytochrome P-450 content was much higher, suggesting that it was induced prior to cell isolation. Rat-liver epithelial cells did not contain detectable amounts of cytochrome P-450.

In pure culture, cytochrome P-450 diminished during the first days in the three normal cases but this decrease was much lower than in cultured rat hepatocytes. After 6–8 days human hepatocytes still contained around 50% of the initial value while in rat cells cultured in similar conditions the decline reached 90% on day 4 [1]. A 3-day daily addition of 3.2 mM sodium phenobarbital to cultures from case B resulted in a 1.3-fold increase of cytochrome P-450 on day

Table 1. Summary of kidney donors from whom hepatocytes were obtained

Case	Sex	Age	Cause of death	Duration of coma	Medication
A	M	24	Suicide	40 hr	Isoprenalin,* dopamine*
B	M	22	Traffic accident	48 hr	Dopamine
C	M	19	Traffic accident	72 hr	
D	F	25	Suicide (barbiturates)	5 days	Isoprenalin,* dopamine

* Isoprenalin (Winthrop, Dijon, France) and dopamine were given only a few hours before death, and therefore could not be considered as inducers.

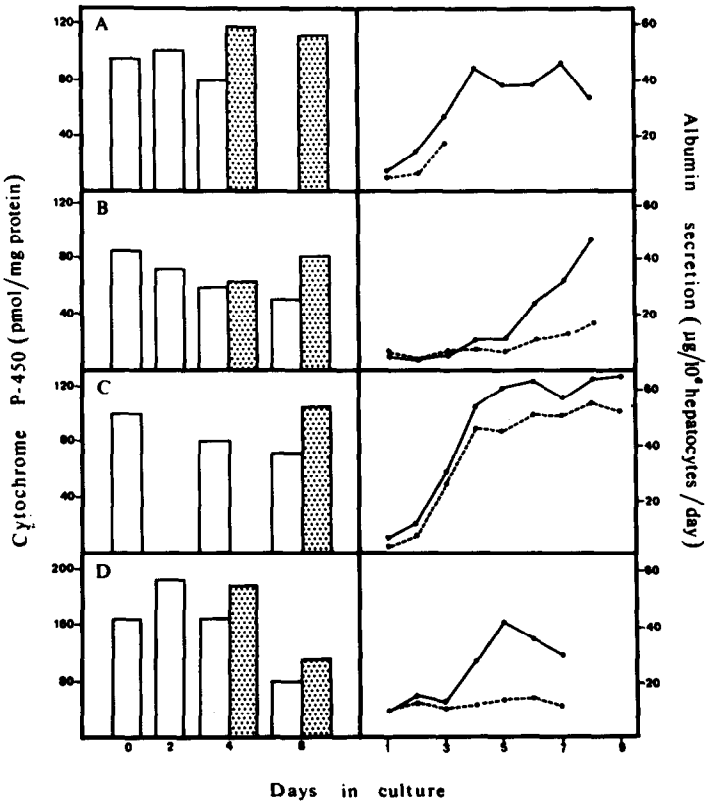

Fig. 1. Cytochrome P-450 concentration (left) and albumin secretion (right) in human-hepatocyte cultures from 4 donors (see Table 1). Cytochrome P-450 levels in pure (□) and mixed (▩) cultures are expressed as pmoles per milligram of hepatocyte protein in the 12,000 *g* supernatant. All determinations were done in duplicate or triplicate. The amount of albumin secreted in the medium of pure (●···●) and mixed (●——●) cultures were quantified by laser immunonephelometry and expressed as micrograms per 10^6 hepatocytes per day. Values are means of 2–10 flasks.

8 (64.5 pmol vs 51 pmol/mg protein in control cells). This finding is in agreement with that of Newman and Guzelian [18] who demonstrated *de novo* synthesis of cytochrome P-450 in rat hepatocyte cultures after exposure to phenobarbital. *In vivo* barbiturate-treated hepatocytes showed a higher decrease of cytochrome P-450, presumably due to the progressive loss of phenobarbital-induced isozyme(s). During the same period, the secretion rate of albumin, a liver-specific protein, augmented as previously observed [6]. These observations support the conception of standard culture conditions as an inappropriate environment for maintaining *in vitro* the normal hepatocyte phenotype [19]. However, as suggested by Strom *et al.* [20], our results definitively indicate that adult human hepatocytes are much less sensitive than rat hepatocytes to an inadequate culture environment. In the latter, in addition to the rapid loss of cytochrome P-450, albumin secretion rate decreased after a few days [21].

When mixed with rat-liver epithelial cells human hepatocytes retained a relatively stable concentration of total cytochrome P-450 during the first 8 days. Addition of 3.2 mM phenobarbital (3 times daily) was followed by a net increase (1.7- to 2.1-fold) of the cytochrome P-450 level on day 8 in co-cultures from cases A and B. The levels were 143 and 139 pmol vs 70 and 82 pmol/mg protein in untreated cells, respectively, from cases A and B. In all co-cultures, albumin secretion rate also increased, reaching higher levels and remaining more elevated than in pure cultures. The delayed increase in cultures from case B could be related to a temporary decline in the cytochrome P-450 level (Fig. 1). This could be explained by a delay in the establishment of interactions between the two cells types and/or by functional alterations of hepatocytes during trauma. It has been shown that the nutritional status of the donors might influence energetic metabolism of parenchymal cells greatly [22]. The maintenance of initial level and inducibility of cytochrome P-450 concomitantly with that of other liver functions has never been demonstrated in traditional hepatocyte cultures. Only temporary stabilization of cytochrome P-450 by ligands [23] or by modifying the culture medium composition [24] has been reported in rat hepatocyte cultures and it remains unclear whether this compound is normally inducible and whether other liver functions are expressed in these conditions.

In order to verify whether the different forms of cytochrome P-450 normally present *in vivo*, remained expressed in culture, we studied the maintenance of two major isozymes (P-450-5 and P-450-8) by immunostaining after protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transfer to a nitrocellulose sheet. The two isozymes were found in both pure and mixed cultures over a 10-day period. Quantification of both forms gave similar values in pure and mixed cultures whatever the time after cell seeding, except for P-450-8 which reincreased to a value close to that found in freshly isolated cells on day 10 of co-culture (Fig. 2). These findings suggest that no dramatic changes occur in cytochrome P-450 isozymic pattern of human hepatocytes during the first days of culture. We have recently confirmed the *in vitro* maintenance of the two isozymes by using the immu-

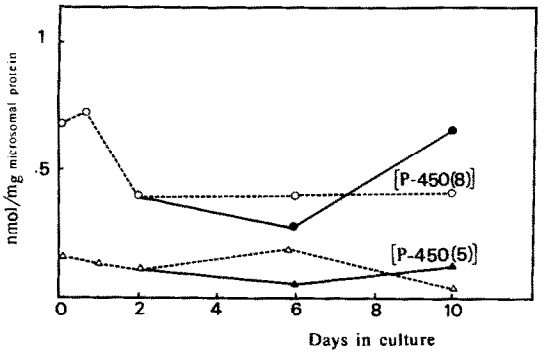

Fig. 2. Immunoquantification of cytochromes P-450-5 and -8 in human-hepatocyte cultures (case C). Cytochromes P-450-5 and -8 were electrophoresed, transferred to nitrocellulose and immunochemically stained. The spots were quantified by densitometry and compared with a standard curve obtained with increasing amounts of purified antigens. The values found in pure (---) and mixed (——) cultures are expressed as nmoles per milligram of microsomal proteins. All determinations were performed in duplicate.

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noperoxidase technique (Ratanasavanh *et al.*, unpublished observations). Both P-450-5 and P-450-8 were not increased after addition of phenobarbital to pure and mixed cultures and were not detected in rat-liver epithelial cells. This is interesting since the most abundant isozyme (P-450-8) is absent in fetal liver (T. Cresteil, personal communication) which indicates that human hepatocytes do not exhibit an evident shift towards a more fetal-like state. It may be assumed that when specific cell-cell interactions are recreated *in vitro* between hepatocytes and rat-liver epithelial cells, proper signals are induced resulting in stimulation or maintenance of liver functions [19, 21].

Additional work is required in order to determine whether human hepatocytes, particularly when placed in co-culture, retain their various drug-metabolizing enzyme activities as well as interindividual differences allowing the distinction between low and high metabolizers. However, the results reported here, together with those showing that various drug metabolic pathways (including glucuronidation) were maintained for several days [25] suggest that cultured human hepatocytes represent an unique system to study cytotoxicity and metabolic pathways of drugs in man as well as to determine mutagenic or carcinogenic effects of various compounds. The use of such as *in vitro* approach might provide a suitable alternative for experimental animals in various studies.

In summary, our observations show that: (1) total cytochrome P-450 decreases progressively to about 50% of its initial value for the first 8 days in pure adult human-hepatocyte cultures while it remains relatively stable during the same period when parenchymal cells are co-cultured with rat-liver epithelial cells; (2) cytochrome P-450 is increased in the presence of phenobarbital; and (3) two major isozymes (cytochromes P-450-5 and -8) do not show important change or decrease in both cultures. This suggests that in cultured human hepatocytes the cytochrome P-450 is less sensitive to an inappropriate environment than in rat hepatocytes and that there is no dramatic changes in the isozymic pattern for several days.

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Effects of D,L-4-fluoroglutamic acid on glutamylation of methotrexate by hepatic cells *in vitro*

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Folypolyglutamate synthetase catalyzes the γ-glutamylation of folates as described in reaction 1.

$$H_4PteGlu_n^* + ATP + L\text{-}Glu \rightarrow H_4PteGlu_{n+1} + ADP + P_i \quad (1)$$

The enzyme is somewhat nonselective with regard to the structure of the folate: numerous folates and antifolates can serve as substrates [1], provided no alterations are made in the glutamate portion of the molecule. The glutamate being added cannot be changed greatly, although some modifications in this structure are possible [1]. An example is replacing the L-Glu by *threo*-FGlu, in which case a single *threo*-FGlu molecule can be added to the folate, resulting in the formation of H_4PteGlu *threo*-FGlu [2]. Further glutamylation of this molecule is prevented by the presence of the fluorine atom adjacent to the γ-carboxyl. Thus, the *threo*-FGlu acts as a terminator of glutamate chain elongation.

The inhibitory properties of FGlu with isolated folylpolyglutamate synthetase necessitated an examination of the effects of this analog on glutamylation in intact cells. For this study we chose to investigate the glutamylation of the antifolate MTX in hepatic parenchymal and H35 hepatoma cells *in vitro*. The advantages of methotrexate are the limited number of glutamylation products and their exact resolution and identification, based upon the earlier characterization of the glutamylation reaction [3–5]. A preliminary account of the results of the current study has been presented [6].

Materials and methods

Male Lewis rats were obtained from Charles River Breeding Laboratories. They were maintained on a 12-hr inverted light–dark schedule and fed Wayne Lab Blox F6 and water *ad lib*. Swims medium S77, folic acid-free Swims medium, Liebowitz L-15 medium, fetal calf serum and horse serum were obtained from Gibco Lab. MTX and [3′,5′,7-^{3}H]MTX were obtained from Lederle Laboratories and Moravek Biochemicals respectively. Both were purified with DEAE cellulose, and the concentrations were determined by ultraviolet spectroscopy [7]. The final specific activity of the radioactive methotrexate was between 5×10^4 and 50×10^4 dpm/nmole. D,L-*erythro,threo*-FGlu and each of the pure *erythro* and *threo* diastereomers were obtained from Calbiochem, and 4-NH_2-10-CH_3PteGlu-γ-FGlu was obtained by chemical synthesis [2].

Hepatocytes were isolated, cultured as monolayers, and incubated with [^{3}H]MTX as previously described [8, 9]. H-11-EC3 cells (referred to as H35 cells) from the Reuber rat hepatoma cells were also cultured as monolayers as described [7]. After 72 hr in culture the cells were changed to folate-free Swims medium S77, for 30 hr, then to fresh medium containing 0.1 μM insulin for 18 hr, at which point a 4-hr incubation with [^{3}H]methotrexate was begun. Incorporation of methotrexate into polyglutamates was measured the same way in hepatocytes and hepatoma cells, and the reaction products containing from two to five glutamate residues were resolved by high-performance liquid chromatography [3, 4]. Standard enzymatic techniques were used for the measurement of ATP and glutamate [10, 11].

The *erythro,threo* mixture of FGlu had no effect on glutamylation in hepatocytes at concentrations up to 5 mM. Glutamylation was reduced in H35 cells with an I_{50} of 0.7 mM when 10 μM MTX and 4 mM glutamine were present in the medium. The *threo* derivative (I_{50} = 0.26 mM) was more effective than the *erythro* derivative (I_{50} = 0.74 mM) of FGlu in inhibiting this reaction. The amounts of all of the individual methotrexate polyglutamate derivatives were reduced by 5 mM *threo* FGlu but the most dramatic decreases were noted with longer-chain-length derivatives (Glu_3, Glu_4 and Glu_5). The presence of 20 mM glutamic acid restored the methotrexate polyglutamate profile of *threo*-FGlu-treated cells to that of the control cells.

We sought to determine if the end product of the reaction of methotrexate and *threo*-FGlu could be detected as has been observed with rat liver folylpolyglutamate synthetase [2]. MTX-FGlu elutes between 4-NH_2-10-CH_3PteGlu$_2$ and Glu_3 on DEAE cellulose [3] and HPLC [4]. None of this material was observed in extracts of FGlu-inhibited cells nor in the medium following incubation. These results suggest that the 4-FGlu was inhibiting polyglutamylation by a mechanism different than that observed with folylpolyglutamate synthetase [3].

* Abbreviations: H_4PteGlu, 5,6,7,8-tetrahydrofolic acid, 5,6,7,8-tetrahydropteroylglutamic acid; methotrexate, MTX, 4-NH_2-10-CH_3PteGlu, 4-amino-10-methylpteroylglutamic acid; L-Glu, L-glutamic acid; FGlu, D,L-4-fluoroglutamic acid (an equimolar mixture of the *erythro* and *threo* diastereomers); methotrexate-γ-FGlu, 4-$NH_2$10-CH_3PteGlu-γ-FGlu, 4-amino-10-methylpteroylglutamyl-γ-D,L-4-fluoroglutamic acid.