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CHANGES IN EXPRESSION AND "*DE NOVO*" SYNTHESIS OF GLUTATHIONE *S*-TRANSFERASE SUBUNITS IN CULTURED ADULT RAT HEPATOCYTES

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Abstract—Glutathione *S*-transferase (GST) isoenzymes of conventionally and co-cultured adult rat hepatocytes were purified and the GST subunits were separated by reversed phase HPLC in order to study the development of the GST subunit composition as a function of culture time and culture conditions. Several media conditions were tested, namely medium with and without fetal calf serum and with nicotinamide or dimethyl sulphoxide. Compared to the GST subunit composition of freshly isolated hepatocytes, changes in culture and media conditions result in a modification of the subunit profile. General observations are a decrease of subunits 1 and 2, an increase of subunit 3, a stabilization of subunit 4 and "*de novo*" expression of subunit 7. When [^{35}S]methionine was added to the various culture media, and the thus labelled subunits were purified and separated, it was shown that cultured adult rat hepatocytes are able to synthesize the different GST proteins. Furthermore, the GST subunit composition, measured during various culture conditions, is probably a reflection of the "*de novo*" synthesis *in vitro*.

Cultured adult rat hepatocytes have often been proposed as a suitable *in vitro* model for pharmacotoxicological studies. An *in vitro* model however, has only scientific and practical value if its biotransformation capacity remains as close as possible to the *in vivo* situation. As far as phase I metabolism in cultured hepatocytes is concerned, it is known that important changes may occur as a function of culture time [1–3]. Phase II enzymes and especially glutathione *S*-transferase (EC 2.5.1.18) have been studied in less detail.

Glutathione *S*-transferases (GST‡) comprise a family of multifunctional isoenzymes which catalyse the conjugation of endogenous and exogenous hydrophobic electrophiles with glutathione [4]. Studies in rats and other mammals have shown that GST are dimers of subunits with molecular weights between 23,000 and 30,000. In liver tissue, major subunits are 1, 2, 3 and 4. Subunit 7 has only been found in fetal liver cells [5, 6], in cultured adult rat hepatocytes [5, 7–9] and during hepatocarcinogenesis [8, 10], but not in freshly isolated hepatocytes.

From previous studies, it is known that GST activity [11] and mRNA levels corresponding to the different subunits [5] change considerably depending on the type of culture and media conditions used. These results strongly suggest that changes could also occur in the GST subunit composition during evolution of the hepatocytes as a function of culture time.

In order to obtain more information concerning eventual variations in subunit pattern, isolated rat hepatocytes were seeded in conventional culture and in co-culture with rat liver epithelial cells. Various culture media conditions, known to cause changes in GST activity and mRNA levels encoding GST subunits, were tested, i.e. media with or without fetal calf serum (FCS) and with nicotinamide or dimethylsulphoxide (DMSO). At different time intervals, GST isoenzymes were purified and the constituting subunits separated by reversed phase HPLC. After 4 and 8 days of conventional culture and co-culture respectively, [^{35}S]methionine was added to the different culture media, in which methionine was omitted in order to study the "*de novo*" synthesis of GST isoenzymes *in vitro*.

MATERIALS AND METHODS

Cell isolation and culture. Rat hepatocytes were isolated from 2-month-old Sprague–Dawley rats as described by Guguen *et al.* [12]. They were seeded in culture and co-culture with rat liver epithelial cells, as described previously [13]. Rat liver epithelial cells were obtained by trypsinization of 10-day-old Fischer rat livers and used before they underwent spontaneous transformation, i.e. between the 15th and 30th passage [14]. Hepatocytes were seeded at a density of 10×10^6 cells per 175 cm^2 in 25 mL of medium. The standard medium (St) consisted of 75% minimal essential medium (Gibco, Paisley, U.K.) and 25% medium 199 (Gibco), containing 1.0 mg/mL bovine serum albumin (Sigma Chemical Co., St Louis, MO) and 10 μg/mL bovine insulin (Sigma). Kanamycin monosulfate (39.1 μg/mL

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‡ Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione *S*-transferase; GSH, glutathione; DMSO, dimethylsulphoxide; FCS, fetal calf serum; HPLC, high pressure liquid chromatography.

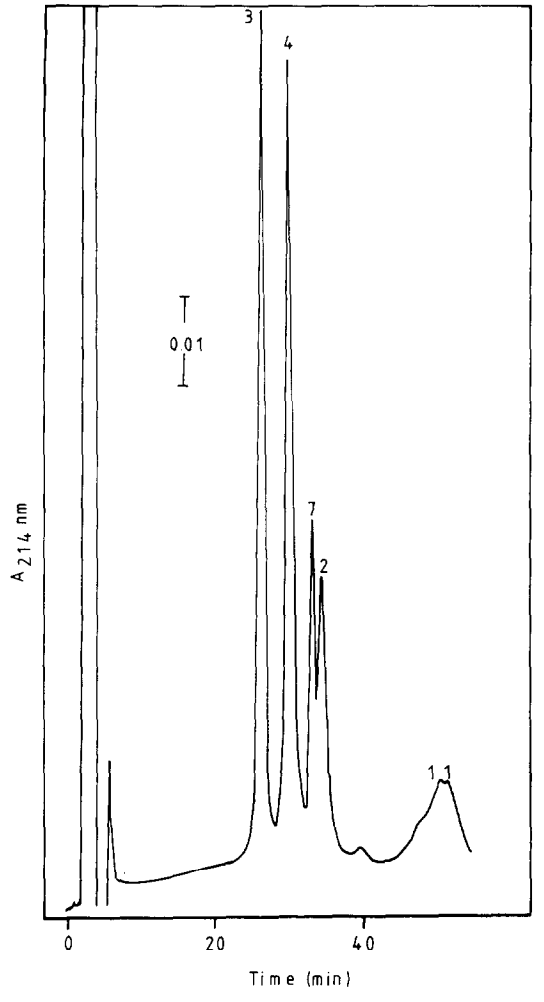

Fig. 1. Separation of GST subunits by reversed phase HPLC. The sample was applied to a μBondapak C18 column and eluted with a gradient of 0.06% trifluoroacetic acid in acetonitrile as described in Materials and Methods. Numbers refer to the GST subunits.

medium, Sigma), benzylpenicillin natrium (7.5 units/mL medium, Continental Pharma, Brussels, Belgium) and streptomycin sulfate (37.5 units/mL medium, Sigma) were used as antibiotics. The medium was renewed 4 hr after cell seeding and every day thereafter. Co-cultures were set up by adding 1.4×10^7 rat liver epithelial cells per flask in order to get confluency within 24 hr. The renewing medium was supplemented with 7×10^{-5} M hydrocortisone hemisuccinate (Roussel, Brussels, Belgium) for conventional cultures or with 7×10^{-6} M for co-cultures. Four different culture media were tested, namely: St medium with 10% FCS (Gibco) (St + FCS); St medium minus FCS (St − FCS); St medium minus FCS containing 25 mM nicotinamide (St − FCS + Nic); 50% St medium and 50% Williams' E medium minus FCS with 2% dimethylsulphoxide (St/W − FCS + DMSO).

Cell harvesting. Cells from three flasks, i.e. approximately 30×10^6 hepatocytes were harvested on days 2, 4 and 6 for conventional culture and on days 4, 8 and 16 for co-culture in order to study the subunit composition. Hepatocytes (1.6×10^6) were harvested on days 4 and 8 for conventional culture and co-culture respectively in order to study "*de novo*" synthesis of GST subunits. After harvesting, cells were stored as previously described [7]. Hepatocytes were not separated from the epithelial cells.

Purification of GST isoenzymes. GST isoenzymes from freshly isolated hepatocytes, cultured hepatocytes and rat liver epithelial cells were purified using a shortened [7] version of the method of Vander Jagt *et al.* [15].

Analysis of GST subunits by reversed phase HPLC. Separation of the GST subunits was carried out by HPLC using a 10×0.8 cm μBondapak C-18 reversed phase column in a Z-module (Millipore Waters, Milford, MA). The solvents were water (A) and acetonitrile (B) (RS and per HPLC, Carlo Erba, Milano, Italy) each containing 0.06% (w/w) trifluoroacetic acid (Merck, Darmstadt, F.R.G.). The samples were injected at 36% B. During a run, a linear gradient was used from 36% to 53% B over 60 min with a flow rate of 1.5 mL/min. Detection was carried out at 214 nm. GST subunits, separated by HPLC, were identified by comparing their retention times with those of purified GST. Quantitative data were obtained by the recoveries of the respective subunits from HPLC and their molar extinction coefficients at 214 nm, as given by Ostlund-Farrants *et al.* [16].

"De novo" *synthesis of GST isoenzymes.* In order to analyse "*de novo*" synthesis of GST proteins in cultured hepatocytes, 150 μCi of [^{35}S]methionine (Amersham, Bucks, U.K.) was added to 1.6×10^6 hepatocytes for 4 hr on day 4 and on day 8 in conventional culture and co-culture, respectively. Carrier methionine was omitted (Gibco). After exposure, media were removed, cells harvested and GST isoenzymes purified as described [7]. After purification, labelled GST subunits were separated by reversed phase HPLC as described above. Twenty second fractions were collected, taken up in 10 mL liquid scintillation cocktail (Insta-gel, Packard, IL) and counted for 10 min by a liquid scintillation system (Beckman LS 7000) using a ^{14}C program.

RESULTS

GST isoenzymes were purified from cytosol using sepharose 6B reacted with glutathione (GSH) as an affinity matrix. Preliminary experiments showed that this matrix retained 75–85% of the 1-chloro-2,4-dinitrobenzene-GSH conjugating activity from the cytosol. The isoenzymes were separated into subunits by reversed phase HPLC and eluted in the order 3, 4, 7, 2 and 1 (Fig. 1). Subunit 1 was resolved into two distinguished peaks, giving further evidence that this subunit is heterogeneous [17, 18]. Identification of GST subunits was based on retention times determined with purified GST, and by migration on sodium dodecyl sulphate polyacrylamide gel electrophoresis.

GST subunit composition of cultured rat hepatocytes

Freshly isolated hepatocytes. Freshly isolated hepatocytes contain average amounts of 35 ± 3 μg,

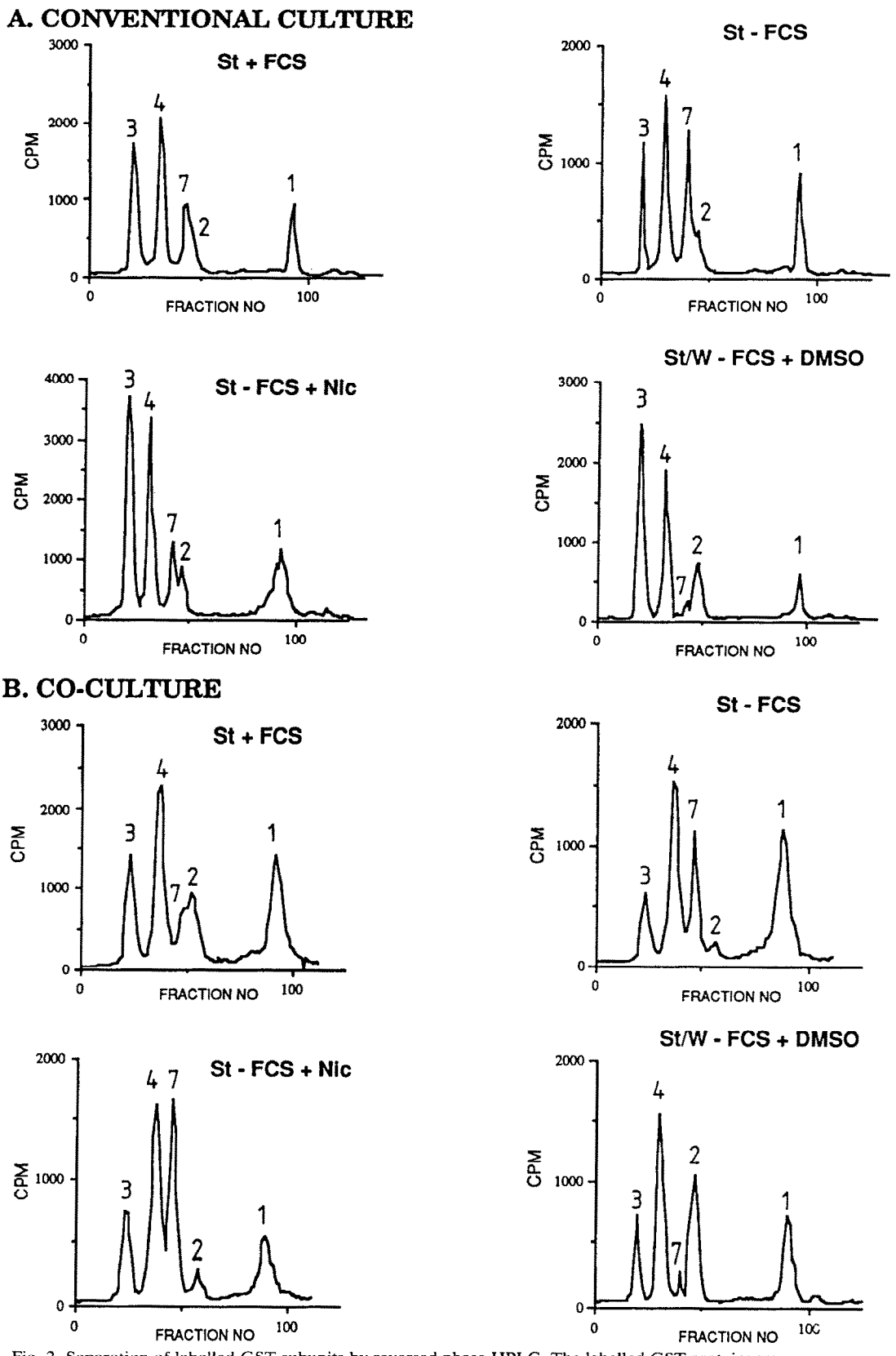

Fig. 2. Separation of labelled GST subunits by reversed phase HPLC. The labelled GST proteins were synthesized over a 4 hr culture period after 4 days of conventional culture (A) or 8 days of co-culture (B). The media conditions used in both culture systems are indicated above each figure. Numbers refer to the GST subunits.

Table 1. Quantitative analysis of GST subunits of conventionally cultured adult rat hepatocytes

Days of culture	GST subunits 1	2	3	4	7
St + FCS					
2	22.1	8.5	8.5	14.9	1.2
4	18.1	11.5	13.9	21.8	2.4
6	5.0	3.4	9.2	13.6	8.6
St − FCS					
2	17.3	9.8	12.2	19.7	3.2
4	18.4	11.6	16.9	38.1	13.9
6	5.9	4.7	18.1	23.1	20.8
St − FCS + Nic					
2	9.4	6.7	11.8	29.5	2.8
4	13.7	12.7	19.4	24.2	4.3
6	13.3	10.2	29.4	55.3	14.7
St/W − FCS + DMSO					
2	13.0	10.8	16.2	29.9	—
4	19.9	12.1	23.3	31.3	—
6	14.7	11.1	25.2	30.2	2.2

Values were obtained after purification of the GST subunits of conventionally cultured rat hepatocytes as described in Materials and Methods. Each point represents a pool of 30×10^6 hepatocytes.

Values are expressed in μg GST per cytosolic protein.

Table 2. Quantitative analysis of GST subunits of co-cultured adult rat hepatocytes

Days of culture	GST subunits 1	2	3	4	7
St + FCS					
4	4.7	4.9	5.9	19.4	7.2
8	7.8	7.7	19.0	29.7	10.2
16	9.1	12.9	31.5	43.7	25.1
St − FCS					
4	9.3	4.2	9.7	23.8	5.2
8	10.2	3.5	15.0	38.1	9.2
16	12.9	3.6	20.5	44.8	15.6
St − FCS + Nic					
4	28.7	7.9	23.2	44.2	7.7
8	12.6	4.9	16.1	32.2	10.7
St/W − FCS + DMSO					
4	7.2	4.6	11.9	26.5	3.7
8	10.6	8.7	22.4	35.4	3.1
16	19.2	9.1	27.0	42.9	3.9

Values were obtained after purification of the GST subunits of co-cultured rat hepatocytes as described in Materials and Methods. Each point represents a pool of 30×10^6 hepatocytes.

Values are expressed in μg GST per mg cytosolic protein. Hepatocytes were not separated from the epithelial cells for purification and separation of the GST subunits.

16 ± 1 μg, 20 ± 6 μg and 34 ± 3 μg of GST subunits 1, 2, 3 and 4 respectively per mg of cytosolic proteins ($N = 5$).

Conventional culture. Seeded in conventional culture (Table 1), whatever the medium conditions involved, a drastic decrease of subunit 1 was noticed in function of culture time. With or without FCS in the culture medium, subunit 2 decreases too in contrast with its behaviour in cells cultured in the presence of nicotinamide or DMSO. In all the media tested, after an initial decrease on day 2, subunits 3 and 4 both increase during the further culture period. Already after 2 days in conventional culture, subunit 7 is expressed, with the exception of the cultures treated with DMSO. In freshly isolated hepatocytes, subunit 7 could never be detected. By the addition of the polar solvent DMSO to the hepatocytes, seeded in conventional culture, the "*de novo*" expression of subunit 7 was started only from day 4 on, and at a lower rate than that observed during the other culture conditions.

Co-culture. In co-culture (Table 2), the GST subunit composition was analysed over a 16-day period except for cells treated with nicotinamide which detach from the petri dishes after 10–14 days. Using nicotinamide supplemented media, subunits 1, 2, 3 and 4 were initially better maintained compared to the cells treated with FCS or without FCS or in the presence of DMSO. For all the treatments involved in co-culture, it was found that subunits 1, 2, 3 and 4 are decreased on day 4 compared to the corresponding amounts in freshly isolated cells. Subunits 1 and 2 are further maintained at the same level over the 16-day period, while the amounts of subunits 3 and 4 increase. Adding rat liver epithelial cells to cultured hepatocytes did not prevent the expression of subunit 7, as was observed by analysing the subunit composition of co-cultured hepatocytes, separated or not from the epithelial cells. Rat liver epithelial cells, used to set up the co-cultures, contain 0.9, 2.1 and 5.4 μg of GST subunits 3, 4 and 7 respectively per mg of cytosolic proteins. The different medium conditions and the effect of co-culture with hepatocytes do not significantly change the subunit composition of rat liver epithelial cells.

"De novo" *synthesis of GST subunits in cultured rat hepatocytes*

By incorporating ^{35}S labelled methionine into the different culture media, the "*de novo*" synthesis of GST subunits could be analysed in both culture systems. Purification and separation of the labelled transferases showed their subunit composition on day 4 and day 8 of conventional culture and co-culture respectively.

Conventional culture (Fig. 2A). The "*de novo*" synthesis reveals also that subunits 3 and 4 are becoming the most important GST in culture. Addition of DMSO or nicotinamide to the cells shows a better production of subunit 2 compared to the medium conditions with or without FCS. The "*de novo*" synthesis of subunit 7 is highest when FCS is absent from the medium and lowest when cells are treated with DMSO.

Co-culture (Fig. 2B). The major production of GST, whatever the medium composition, consists of subunit 4 (34–38% of the total amount). As in conventional culture, synthesis of subunit 2 is best maintained when DMSO is added to the cells. Subunit 7 is lowest in the presence of this organic compound. When the results are expressed in percentage of total production of GST subunits, subunit 1 is

better maintained in co-culture than in conventional culture.

DISCUSSION

GST subunit pattern of cultured hepatocytes

When the observations made here for the GST subunit patterns of conventional cultures and co-cultures under different conditions are interpreted in the light of previous obtained data [5, 11], very consistent results are found which support the hypotheses that variations in GST mRNA levels [5] result in changes in GST protein amounts, reflected in modifying GST activity during the culture period [11].

When parenchymal liver cells are cultured using standard conditions, most remarkable is a drastic loss of subunit 1, an increase of subunit 3 and a "*de novo*" expression of subunit 7. This specific evolution of adult rat hepatocytes as a function of culture time probably reflects dedifferentiation of the cells towards a "fetal-like" state. Indeed, fetal rat hepatocytes are characterized by the presence of a low amount of subunit 1, which is known to increase in function of development, and a high concentration of subunit 3, both as well in mRNA level [6] as in protein amount (unpublished results). Furthermore, subunit 7, which disappears soon after birth [5, 6], is part of the GST subunit composition of fetal rat hepatocytes [5, 6].

Rat hepatocytes, conventionally cultured in the presence of nicotinamide or DMSO, show on day 6 a doubling of the total amounts of subunits 1, 2 and 3 compared with that of cells cultured with or without FCS. This increase should lead to an approximately two-fold elevation of the GST enzyme activity towards 1-chloro-2,4-dinitrobenzene (CDNB), since the isoenzymes derived from these three subunits, i.e. 1–1, 2–2, 3–3, are the most important ones, when their cellular concentration is considered, in comparison with isoenzyme 6–6 [19]. Indeed, this increase was previously observed when the GST activity of conventionally cultured adult rat hepatocytes, treated with nicotinamide or DMSO, was measured using CDNB as a substrate [11].

In co-culture, less variation between the different media conditions could be noticed as far as the GST activity measurements [11] or mRNA level analysis of the different GST subunits [5] were concerned. Further in agreement with previous work, are the results concerning the absolute amounts of the individual GST subunits. They confirm that the influence exerted by the various media conditions is less pronounced in co-culture than in conventional culture, probably due to a stabilization of the hepatocytes by the surrounding rat liver epithelial cells. The presence of these cells however, could not prevent the hepatocytes from expressing "*de novo*" subunit 7. In a recent study [5], the appearance of subunit 7 mRNA in co-culture was already demonstrated by use of a corresponding cDNA sequence.

In both culture systems, as was previously observed by analysis of subunit 7 mRNA level [5], and from this study by quantifying its protein amount, the expression of subunit 7 is inhibited by the addition of DMSO to the culture medium. The results of the *in vitro* "*de novo*" synthesis of this specific subunit also confirm this inhibition. Knowing that subunit 7 is a hepatoma marker [20, 21], the observations made in this paper support the hypothesis that DMSO maintains differentiation in cultured adult rat hepatocytes [22].

The origin of the rat liver epithelial cells, used in co-cultures and isolated by the method of Williams *et al.* [23] has not yet been well identified. They are thought to be bile ductular epithelial cells [24]. Analysis of the GST subunit composition of these cells, showing the presence of subunits 3, 4 and 7, provides further information and supports this hypothesis. Mathis *et al.* [25] suggest, from GST activity measurements using specific substrates and inhibition techniques, that bile ductular epithelial cells contain isoenzymes 3–3 and 3–4. In earlier studies, Redick *et al.* [26] and Rao *et al.* [27] have demonstrated, by the use of immunonohistochemical techniques, that bile duct epithelial cells contain subunits 3 and 7. Northern blot analysis of our rat liver epithelial cell RNA confirmed the presence of subunits 3, 4 and 7 mRNA, while no hybridization to the subunit 1/2 cDNA sequence could be observed (results not shown).

"De novo" *synthesis in cultured hepatocytes*

The experiments with the incorporation of [^{35}S] methione in the culture media, showed for the first time the ability of rat hepatocytes to synthesize *in vitro* GST proteins. When the results of the "*de novo*" synthesis of each subunit are expressed in percentage of the total GST protein production over a 4 hr culture period, on day 4 and on day 8 of conventional- and co-culture respectively, it becomes clear that the GST subunit profile *in vitro* is a reflection of the "*de novo*" synthesis. This probably means that the variations in expression, seen by the addition of different medium factors, are more the result of the *in vitro* "*de novo*" synthesis rather than the result of stabilization of the proteins.

General conclusions of this work are that: due to changes in GST subunit composition, the ability of hepatocytes in culture to conjugate GSH with endogenous or exogenous substrates may differ from hepatocytes *in vivo* or from freshly isolated hepatocytes. Quantitatively, the subunit composition of cultured hepatocytes resembles most that of freshly isolated cells, when 2% DMSO is added to the cultures. For long-term studies, co-cultures should be recommended, since qualitatively, they maintain GST over at least a 16-day culture period.

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