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INFLUENCE OF MEDIUM COMPOSITION AND CULTURE CONDITIONS ON GLUTATHIONE S-TRANSFERASE ACTIVITY IN ADULT RAT HEPATOCYTES DURING CULTURE

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SUMMARY

Glutathione S-transferase (GST) activity was measured in adult rat hepatocytes during either pure culture or coculture with another rat liver cell type in various media. Addition of nicotinamide, selenium, or dimethylsulfoxide, deprivation of cyst(e)ine and the use of two complex media were tested. Whatever the conditions used, after a constant decrease during the first 24 h, GST remained active over the whole culture period (1–2 wk). However, various patterns were observed: GST activity either remained relatively stable to approximately 50% of the initial value or showed a moderate or strong increase. The highest values were found in pure hepatocyte cultures maintained in the presence of nicotinamide or dimethylsulfoxide. Similar changes were observed using 1-chloro-2,4-dinitrobenzene or 1,2-dichloro-4-nitrobenzene as substrates for GST. Addition of 10^{-4} *M* indomethacin resulted in 37 to 60% inhibition of enzyme activity. Thus, these results demonstrate that GST remained expressed during culture but its levels markedly varied depending on the medium composition and type and age of culture.

Key words: primary culture; rat hepatocytes; glutathione S-transferase; culture medium.

INTRODUCTION

The major enzymatic processes involved in the biotransformation of xenobiotics are classified as phase I oxidation-reduction reactions and phase II conjugation reactions. Phase I reactions have been widely investigated in hepatocyte cultures which have been increasingly used as in vitro models to study biotransformation and cytotoxicity of drugs in the liver. Several studies have shown that in standard culture conditions the level of cytochrome P-450 and the associated mixed function oxidase system of rat hepatocytes exhibit rapid changes, resulting in the loss of up to 50% of the cytochrome P-450 content within the first 24 h (5,10,19,25). However, a variety of culture conditions has allowed to delay that decline for 1 to 3 d or more (3,17,20,24). When cocultured with another liver cell type, rat hepatocytes were found to retain their initial cytochrome P-450 level for at least 10 d (2).

Much less attention has been paid to the maintenance of conjugation reactions in rat hepatocytes during culture that include glucuronidation, sulfation, and glutathione conjugation (12). This latter reaction is catalyzed by glutathione S-transferases (GST) (E.C. 2.5.1.18) which form a group of multifunctional proteins (15). A wide range of hydrophobic electrophiles can be conjugated with glutathione by GST (11). In rat liver, these enzymes are mostly located in the cytosol (29). Some GST activity is also bound to the endoplasmic reticulum; this intrinsic microsomal GST has been purified, characterized, and sequenced (22). These isoenzymes have a different activity toward each known substrate of which 1-chloro-2,4-dinitrobenzene is the most commonly used (1,11).

Many electrophiles that result from the oxidation of drugs by cytochrome P-450 are detoxified by GST, and several agents induce members of both groups of enzymes (16). Therefore, we decided to investigate GST activities in rat hepatocytes cultured in various media which have been reported to favor maintenance of cytochrome P-450 (23,26) and cell survival (13,28). Both pure cultures and cocultures with rat liver epithelial cells were used. When put in coculture adult hepatocytes have been shown to retain their differentiated functions and survive longer (9).

MATERIALS AND METHODS

Chemicals. 1-Chloro-2,4-dinitrobenzene (CDNB), insulin, and albumin were obtained from Sigma Chemical Company, St. Louis, MO, 1,2-dichloro-4-nitrobenzene (DCNB) from Merck-Schuchardt, Darmstadt, Germany. Reduced glutathione and collagenase were purchased from Boehringer Mannheim, Germany. Fetal bovine serum (FBS) was from Eurobio, Paris, and hydrocortisone hemisuccinate

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from Roussel, Paris. Indomethacin was a gift from Certa Nouille/Mehaigne, France. All other compounds were readily available commercial products and were used without further purification.

Cell isolation. Adult hepatocytes were isolated from 2-mo.-old Sprague-Dawley rats (240 to 280 g) by the two-step collagenase perfusion method. In outline, the liver was first washed with N-2 hydroxy-ethylpiperazine-N' 2 ethane sulfonic acid (HEPES) buffer, pH 7.6, then with a 0.025% collagenase solution buffered with HEPES (7).

Rat liver epithelial cells were obtained by trypsinization of 10-d-old Fisher rat livers and used before they underwent spontaneous transformation, i.e. between the 15th and the 30th passage (21).

Cell culture. Hepatocytes were seeded at a density of 2.5×10^6 cells/10-cm² petri dish in 4 ml of medium with 10% FBS. The medium was renewed after 4 h, when cocultures were set up by adding 2.5×10^6 rat liver epithelial cells. All the media were renewed daily thereafter. Most of them were a mixture of 75% minimal essential medium and 25% medium 199 containing 200 μg/ml bovine serum albumin and 10 μg/ml bovine insulin. This FBS-free standard medium (St) was constantly supplemented with 7×10^{-5} *M* hydrocortisone hemisuccinate after 4 h. After this, the following modifications, which have been reported to favor maintenance of cytochrome P-450 and/or cell survival, were tested:

— St with 10% FBS (St + FBS)
— St without cyst(e)ine (St − cyst)
— St with 0.1 μ*M* selenium (St + Se)
— St with 25 m*M* nicotinamide (St + Nic)
— St with 0.1 μ*M* selenium and without cyst(e)ine (St − cyst + Se)
— St with 25 m*M* nicotinamide and without cyst(e)ine (St − cyst + Nic)
— St with 2% dimethylsulfoxide (St + DMSO).

Two additional media were included in this study: a) a mixture of 50% standard medium and 50% Williams' medium added with 2% DMSO (St − W + DMSO) and b) the medium devised by Sells et al. (28) for fetal human hepatocyte cultures (Acs medium). This medium is a serum-free RPMI 1640 medium supplemented with 3.5×10^{-6} *M* hydrocortisone hemisuccinate, 65 ng/ml somatotropin, 5 ng/ml transferrin, 10 μg/ml insulin, and 50 μg/ml epidermal growth factor.

Cell harvesting. After washing with chilled HEPES buffer, pH 7.6, cell monolayers were collected in 4 ml of HEPES by scraping with a rubber policeman and centrifuged at 100 *g*. The pellets were used either

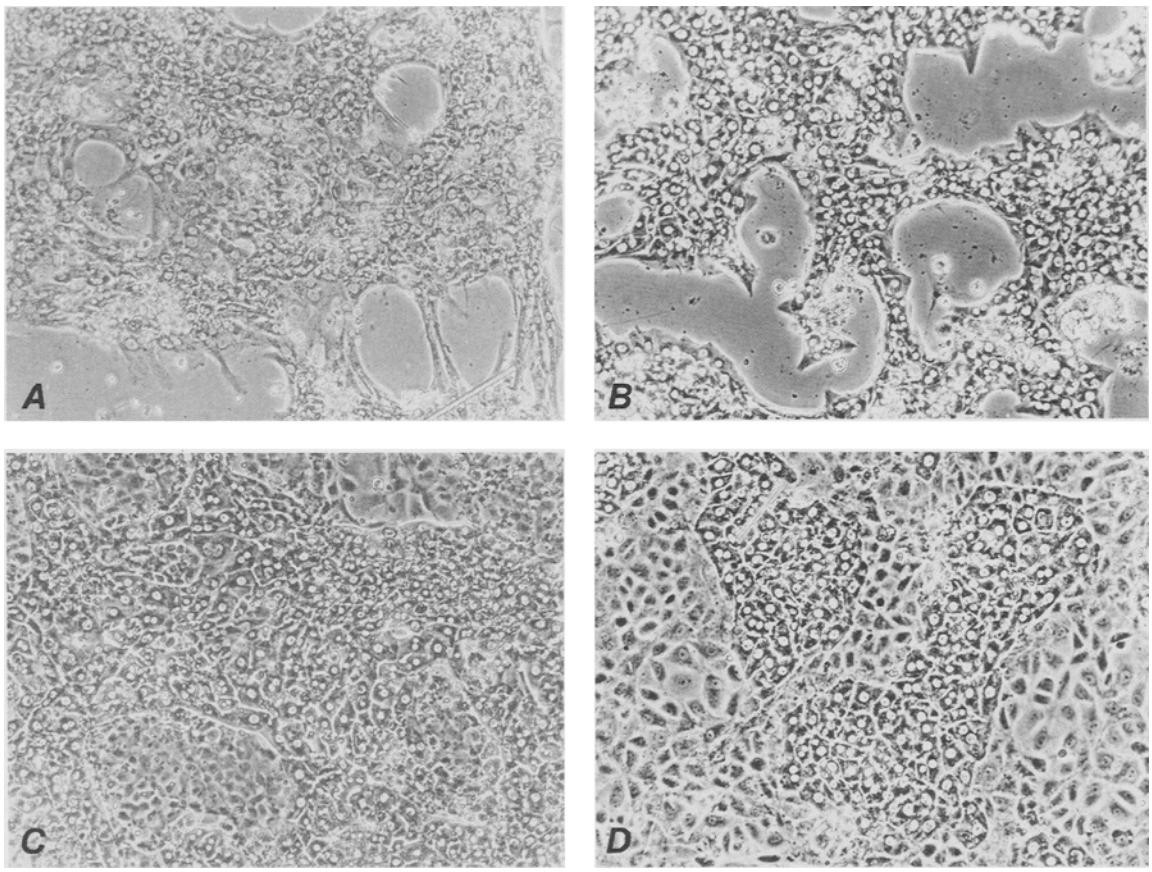

FIG. 1. Primary cultures of adult rat hepatocytes in various media. Pure culture for 4 d in standard medium unmodified (*A*) and for 8 d in a mixture of 50% standard medium and 50% Williams' medium added with 2% DMSO (*B*); cocultures for 12 d in standard medium added with 2% DMSO (*C*) and for 12 d in standard medium added with 0.1 μ*M* Se (*D*). ×172.

immediately or stored at −80° C until analysis. Storage at −80° C over 30 d did not modify GST activity.

Glutathione S-transferase assay. The pellets were resuspended in cold HEPES buffer to reach a concentration of about 10^6 cells/ml. Cell suspensions were then disrupted by sonication. GST activity was determined spectrophotometrically in cell extracts at 340 nm with CDNB as a substrate and at 345 nm with DCNB as a substrate. Spontaneous conjugation was corrected by measuring conjugation toward a blank to which no enzyme was added (11).

The inhibitory effect of indomethacin was measured by adding 10^{-4} to 10^{-7} *M* of this compound in an alcoholic solution to the cell suspension after sonication.

Enzyme activities were calculated as units per milligram of protein ± SE. A unit activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product/min at 30° C (18). Proteins were determined using the Bio-Rad protein assay and bovine serum albumin as the standard.

Statistical analysis. A two-way analysis of variance (ANOVA) test was used for analyzing medium-time interaction. Probability values lower than 0.05 were judged to be significant. A one-way ANOVA was used to control significance as a function of time in one medium. Again probability values lower than 0.05 were judged to be significant. When significance was noticed with the one-way test, a Student's Newman-keuls (SNK) test controlled significance during culture. The SNK test gives significance on a 5% significant level.

RESULTS

Cell Survival and Morphology

Pure culture. As previously reported (9), hepatocytes seeded in a medium supplemented with FBS reaggregate within a few hours, spread, and form typical monolayers of contiguous granular cells after 2 d. Spreading was delayed in a serum-free medium (Fig. 1 *A*). Addition of Se reversed this delay. Marked differences were observed in serum-free medium added with nicotinamide or deprived of cyst(e)ine. The cells attached but did not spread normally. In addition, in the absence of cyst(e)ine the cells detached earlier (around Day 6). Addition of Se or nicotinamide improved cell survival only slightly in cyst(e)ine-free medium. Spreading was also impeded when DMSO was added to the St-W medium (Fig. 1 *B*) and detachment occurred after 10 d. In Acs medium

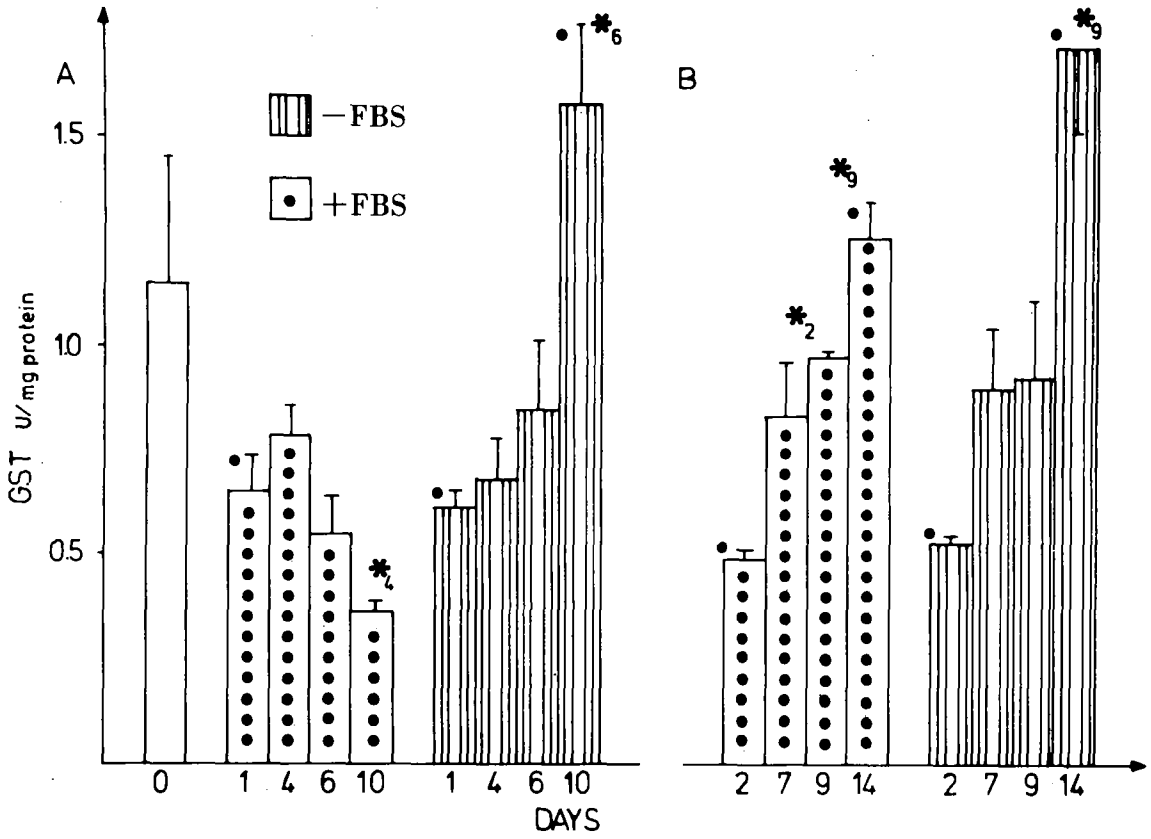

FIG. 2. Glutathione S-transferase activities toward CDNB in pure culture (*A*) and coculture (*B*) of adult rat hepatocytes maintained in standard medium with or without FBS. 0: GST activity of freshly isolated hepatocytes (in the five different cell preparations the extreme values were 0.81 to 1.46 U/mg protein). When GST activity was measured in intact liver and freshly isolated hepatocytes from the same rat the values did not differ more than 5%. Each *bar* represents the mean ± SE of three or five (0) independent experiments (different animals) in duplicate or triplicate. • = Significantly different from freshly isolated cells: 0 day; * = Significantly different from the day indicated under the asterisk.

spreading was also reduced and the cells seemed poorly attached to the substratum.

Coculture. Typical long-term cultures were obtained whether the serum was present or not, as described previously (9). Addition of DMSO (Fig. 1 *C*), Se (Fig. 1 *D*) or nicotinamide or the use of the Acs medium did not affect the morphology and survival of the cells. Interestingly, epithelial cells did not survive for more than 3 d and hepatocytes died a few days later (as in pure culture) in a cyst(e)ine-free medium. When Se was added to this medium, epithelial cells divided and confluent cocultures could be maintained for about 10 d.

Effect of Fetal Bovine Serum on GST Activities Toward CDNB

Pure culture. When CDNB was used as a substrate, hepatocytes cultured in standard medium supplemented with FBS exhibited a 30% decline in GST activity within the first 24 h. From Days 1 to 6, it remained relatively stable, and then decreased again to represent only 30% of the initial value on Day 10 (Fig. 2 *A*). In the absence of serum, the same pattern was observed during the first 4 d, GST activity was significantly lower than in freshly isolated cells ($P < 0.001$). Later, the activity increased, reaching 137% of the initial value after 10 d.

Coculture. In the presence or absence of FBS, the GST activity profile was not significantly different (Fig. 2 *B*). In both media, GST activity remained lower than in freshly isolated cells between Days 2 and 9 ($P < 0.001$). Later, a continuous increase was observed and by Day 14 the levels became significantly higher.

Effect of Various Factors on the GST Activities Toward CDNB

Pure culture. The various conditions tested included addition of Se, nicotinamide, DMSO and depletion of cyst(e)ine. The effects on GST activities are shown in Fig. 3. In all media but one (St + Se) a rapid decrease was observed followed by an increase in which the rates varied from one medium to another. In St + Nic and St − cyst + Nic media the values were higher than those measured in freshly isolated cells, after 6 and 8 d respectively, and further increased to reach values 170 and 125% higher, respectively, than in freshly isolated cells after 10 d of culture. Adding DMSO to the standard medium also showed an increase after the initial decrease. In St − cyst + Se and St − cyst media, the decrease after 4 and 6 d respectively, could be related to cell death. St + Se was the only medium in which the level of GST never reached the initial value, but remained relatively stable at 60 to 80% of the initial content for 8 d.

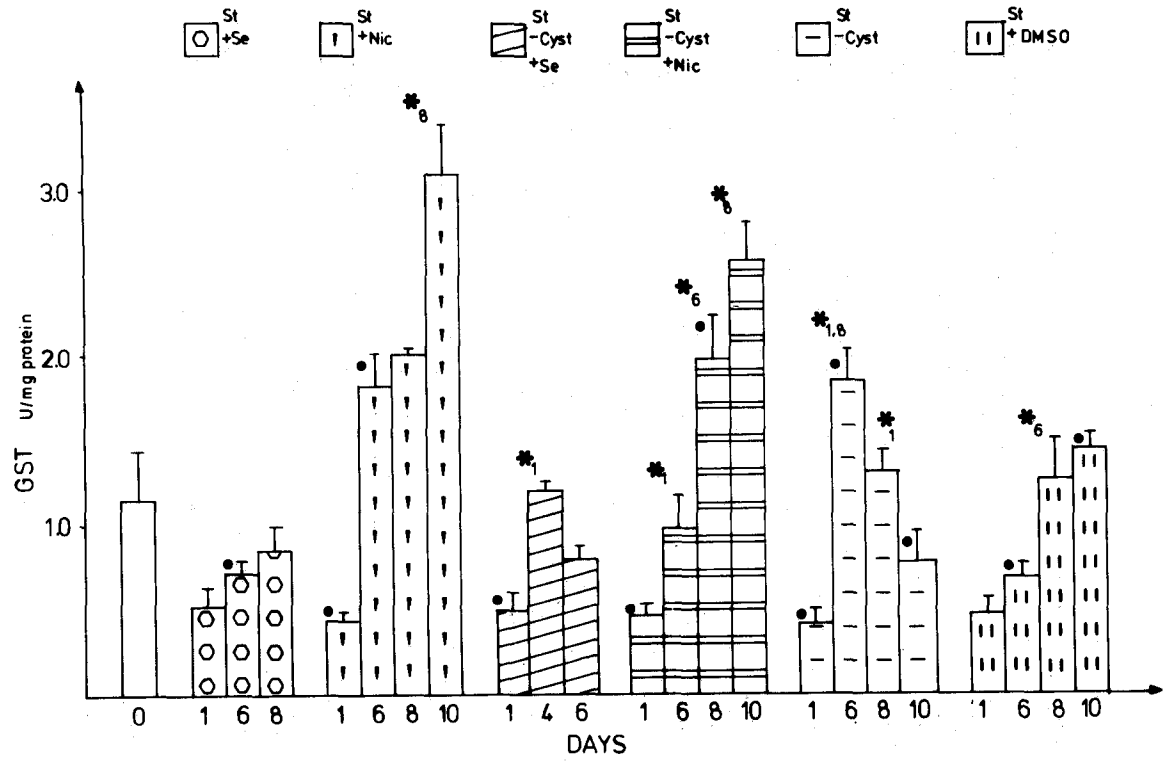

FIG. 3. Influence of various factors on glutathione S-transferase activities toward CDNB in pure cultures of adult rat hepatocytes maintained in the standard medium. The standard medium (St) was either added with Se, Nic, or DMSO or deprived of cyst and supplemented or not with Se or Nic. In all media but St − cyst + Se, GST values measured on Day 4 were lower than those found on Day 6 and were not incorporated in the figure. 0 = GST activity of freshly isolated hepatocytes. Each *bar* represents the mean ± SE of three independent experiments (three different animals) in duplicate or triplicate. • = Significantly different from freshly isolated cells: 0 day; * = Significantly different from the day indicated under the asterisk.

GST activity was also found to decrease in more complex media (50 to 50% mixture of St and Williams' media with DMSO and Acs medium with or without DMSO) (Fig. 4). Later, the activity either increased (St − W + DMSO) or remained relatively stable at a level significantly lower than in freshly isolated cells (Acs medium). Adding DMSO to the St − W medium gave an increase of 200% after 10 d of culture. GST activity could not be measured in St − W medium without DMSO because a great number of cells died during the first 2 d of culture. Adding DMSO to the Acs medium gave an increase of enzyme activity after the initial decrease.

Coculture. The influence of the different media was investigated during 16 d in coculture (Fig. 5). As observed in pure culture, the GST activity decreased significantly ($P < 0.001$) during the first 2 to 4 d whatever the medium used. Later, the activity remained low in Se − supplemented standard medium. In two other media (St + Nic and St − cyst + Se) GST activity increased, becoming either comparable to that found in freshly isolated cells (in Se + Nic medium between Days 6 and 16) or significantly higher (in St − cyst + Se medium by Day 12). Epithelial cells did not survive longer than 4 d in St − cyst and St − cyst + Nic media and did not constitute a coculture with hepatocytes in St + DMSO medium.

Both hepatocytes and epithelial cells died rapidly when cocultured in Acs medium supplemented with DMSO or in St − W medium, but they constituted long-term cocultures in the normal Acs medium and in St − W medium added with DMSO. In the latest conditions, GST activity was also found to decrease during the first 2 d. During the following days, it remained low in Acs medium whereas it increased in St − W medium supplemented with DMSO, reaching values significantly higher than in freshly isolated cells by Day 12 of coculture.

GST activity in rat liver epithelial cells. GST activity was also measured in pure epithelial cell cultures maintained in various media for 4 d at confluency. It varied from 0.17 to 0.28 U/mg protein. The lowest and highest values were found in cells cultured in St and in St − cyst + Se media, respectively.

Glutathione S-transferase Activity Toward DCNB in Cultured Rat Hepatocytes

In freshly isolated parenchymal cells GST activity was much lower toward DCNB than toward CDNB (0.035 ± 0.012 vs. 1.15 ± 0.31 U/mg protein). However, parallel changes in GST activities occurred during both pure culture and coculture whatever the medium used. As examples, the results in pure culture maintained in two different media (St − W + DMSO and St − cyst + Se) are displayed in Table 1.

Inhibition of Glutathione S-transferase Activity by Indomethacin

Indomethacin, which is widely used as an antiinflammatory drug, has been shown to inhibit GST activity in a dose-dependent manner. A concentration of 10^{-4} *M* giving a 50% inhibition of GST activity toward CDNB in freshly isolated hepatocytes was chosen. All the media were tested. The inhibition rate in cultured hepatocytes ranged between 37 and 60%, most often it was close to 50%. The results obtained with St + Se in both pure culture and coculture are given in Table 2.

Discussion

Glutathione S-transferases are thought to play an important role by catalyzing the glutathione conjugation of electrophilic drugs and drug metabolites. There are several cytosolic glutathione S-transferase isozymes, some of them having a typical substrate specificity. Most of them are expressed in the liver. As other phase II enzymes, i.e. UDP glucuronosyltransferases and sulfatases, GSTs have been poorly studied in culture. Recently, Croci and Williams (4) have shown that GST activity toward DCNB fell to 20% of the initial value after 24 h of culture, whereas the activity tested toward CDNB remained relatively stable during the same period. Our results are somewhat different because we found a decrease of about 40% toward both substrates during the same period. The reasons for such a discrepancy are not clear, but are probably related to the

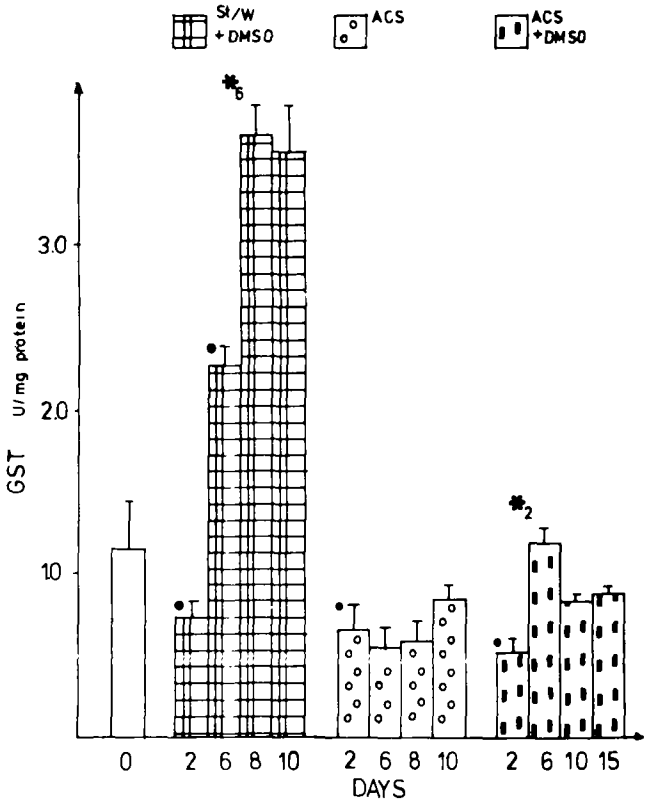

FIG. 4. Glutathione S-transferase activities toward CDNB in pure cultures of adult rat hepatocytes maintained in various complex media. 0 = GST activity of freshly isolated hepatocytes. The composition of the media is given in Materials and Methods. Each *bar* represents the mean ± SE of three independent experiments (three different animals) in duplicate or triplicate. • = Significantly different from freshly isolated cells: 0 day; * = Significantly different from the day indicated under the asterisk.

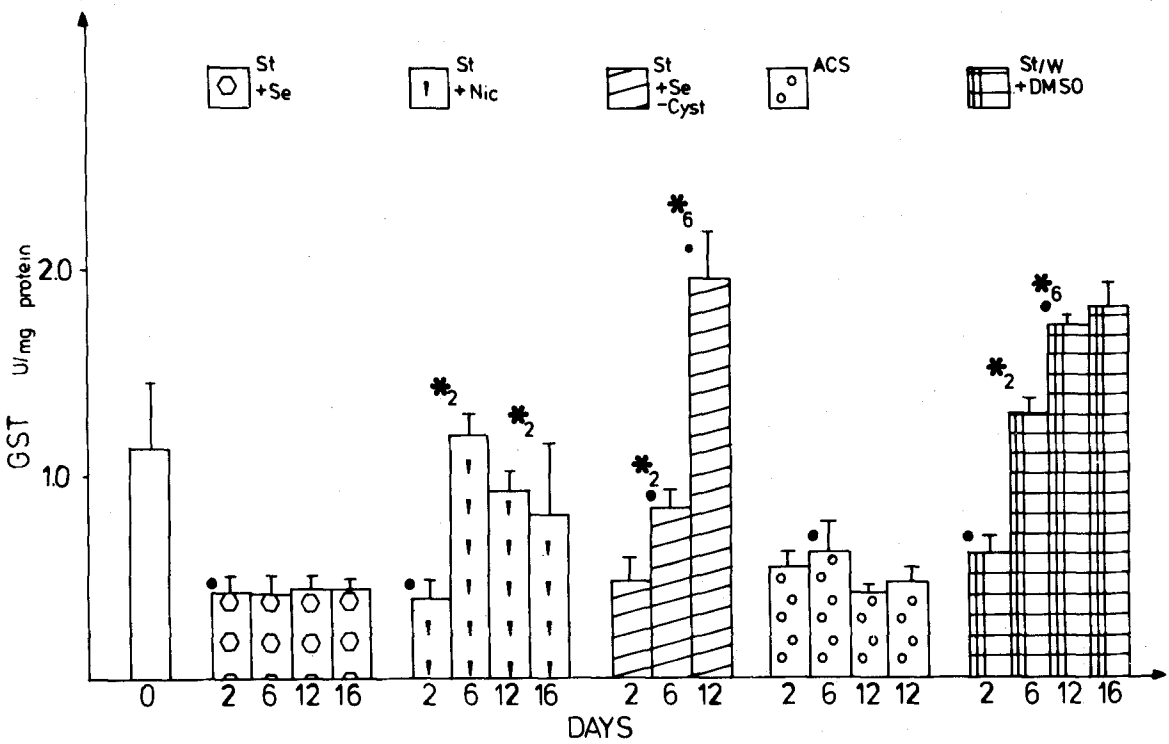

FIG. 5. Influence of various factors on glutathione S-transferase activities toward CDNB in cocultures of adult rat hepatocytes. 0 = GST activity of freshly isolated hepatocytes. Each *bar* represents the mean ± SE of three independent experiments (three different animals) in duplicate or triplicate. • = Significantly different from freshly isolated cells: 0 day; * = Significantly different from the day indicated under the asterisk.

composition of the culture medium, which was different in the two studies, rather than to the strain of rats or another parameter.

After 48 h of culture, different changes in GST activities were observed according to the composition of the medium and condition of culture. FBS affects GST activity differently in pure culture and in coculture. It prevents enzyme increase only in pure culture. These results are consistent with previous observations showing that in pure culture, protein synthesis and transcription of specific genes are better preserved in the absence of serum (14,27) and that in coculture, hepatocytes remain differentiated much longer whether in the absence or presence of serum (6,7). In pure culture, hepatocytes exhibit a rapid shift toward a more fetal state (8), as demonstrated by expression of fetal forms of enzymes

TABLE 1

GLUTATHIONE S-TRANSFERASE ACTIVITIES TOWARD DCNB AS A SUBSTRATE IN PURE CULTURE AND COCULTURE OF ADULT RAT HEPATOCYTES[a]

Days of Culture	Glutathione S-Transferase, U/mg protein			
	Pure Culture		Coculture	
	St − cyst + Se	St − W + DMSO	St + Nic	St − W + DMSO
1	0.00	0.064 ± 0.004		
2			0.00	0.00
4	0.029 ± 0.002	0.11 ± 0.020	0.00	0.00
6	0.05 ± 0.003	0.10 ± 0.018	0.033 ± 0.002	0.044 ± 0.004
8	0.07 ± 0.003	0.16 ± 0.011		
10	0.10 ± 0.008	0.17 ± 0.016	0.021 ± 0.001	0.054 ± 0.004
12			0.016 ± 0.001	0.08 ± 0.006

[a]The cells were cultured in cyst(e)ine-free standard medium added with Se (St − cyst + Se) or in a mixture of St and Williams' media added with DMSO (St − W + DMSO). Each value represents the mean ± SE of two independent experiments in duplicate. Results are expressed in units per milligram protein. GST activities in rat liver and freshly isolated hepatocytes were 0.041 ± 0.004 and 0.031 ± 0.008 U/mg protein, respectively.

TABLE 2

INDOMETHACIN INHIBITION OF GLUTATHIONE S-TRANSFERASE ACTIVITIES IN PURE CULTURE AND COCULTURE OF ADULT RAT HEPATOCYTES MAINTAINED IN St + Se[a]

Days of Culture	Glutathione S-Transferase, U/mg protein			
	Pure Culture		Coculture	
	CDNB	CDNB + Indomethacin	CDNB	CDNB + Indomethacin
1	0.53 ± 0.12	0.20 ± 0.05		
2			0.42 ± 0.07	0.23 ± 0.02
4	0.63 ± 0.04	0.33 ± 0.01	0.43 ± 0.05	0.29 ± 0.02
6	0.72 ± 0.08	0.32 ± 0.02	0.41 ± 0.08	0.20 ± 0.02
8	0.86 ± 0.14	0.39 ± 0.01	0.48 ± 0.03	0.26 ± 0.03
12			0.44 ± 0.05	0.18 ± 0.01
16			0.44 ± 0.09	0.23 ± 0.01

[a]1-Chloro-2,4-dinitrobenzene was used as a substrate and indomethacin was added at the concentration of 10^{-4} *M*. Each value represents the mean ± SE of two independent experiments in duplicate. Results are expressed in units per milligram protein. GST activities in rat liver and freshly isolated hepatocytes were 1.08 and 1.05 U/mg protein, respectively. After addition of indomethacin the values were reduced to 0.38 and 0.45 U/mg protein, respectively.

and secretion of alpha-fetoprotein after a few days. It would be important to determine whether the same GST isozymes were affected in the two culture systems.

A recent study has shown that DMSO improved hepatocyte survival and maintenance of specific functions (13). Our observations agree with such a conclusion. However, it must be emphasized that DMSO effect could be marked depending on the composition of the medium to which this compound was added.

Inasmuch as many reactive metabolites formed by the mixed function oxidase system are conjugated to glutathione it was also interesting to measure GST activity in hepatocytes cultured in media added with various factors which affect cytochrome P-450 maintenance in vitro. None of the conditions tested, i.e. addition of nicotinamide or Se or deprivation of cyst(e)ine, allowed stabilization of GST during the first days of culture. After a constant initial decrease, various patterns, which did not fit with that of cytochrome P-450, were observed; they corresponded to a stabilization throughout the culture period or an increase of GST activity. Differences were observed between pure culture and coculture. Whether these were due to the presence of rat liver epithelial cells in coculture was not directly established. However, several indirect arguments suggested that these cells were not responsible for most of the changes in GST levels found in coculture in relation to the composition of the culture medium. Indeed, their GST content was much lower than that of hepatocytes and the variations found, depending on culture conditions, did not parallel those observed in coculture. From these results we suggest the use of a medium that allows maintenance of relatively stable and elevated levels of GST throughout the culture period: e.g., serum-free standard medium or Acs medium supplemented with DMSO for pure culture and standard medium added with nicotinamide for coculture.

Similar changes in GST activity were obtained with the two substrates. DCNB is a substrate specific for GST forms 3-3 and 3-4 (1). Because GST activity toward this substrate was maintained in culture, indications are that subunit 3 and at least one of the two forms remained expressed in vitro. CDNB is a substrate for most of the isozymes including 1-1, 1-2, 2-2, 3-3, 3-4, and 4-4 (1). The major forms present in rat liver are 1-1, 3-3, and 3-4 (1). Similarly, indomethacin affects several GST forms. At a concentration of 100 μM it inhibits by 50%, 1-2, 2-2, 3-3, 3-4 forms in vivo (1). The same degree of inhibition of GST activity was obtained with this concentration of cultured hepatocytes. To better characterize isozymic changes according to the conditions used, the different forms needed to be analyzed separately. This could be done by high performance chromatography analysis. Work is presently performed in this way. In summary, our study is the first demonstration that GST remained expressed for several days in hepatocyte cultures. It suggests that this system represents a unique tool to investigate the regulation of the different forms of this enzyme under various culture conditions.

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