



Role of ATP and glycogen reserves in both paracetamol sulfation and glucuronidation by cultured precision-cut rat liver slices

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Abstract

Precision-cut rat liver slices (PCLS) were used to investigate the formation of paracetamol conjugates. The time course of biochemical markers such as ATP and GSH content, glycogen levels and protein synthesis rates was recorded over a period of time of 26 h and taken as index of slices viability. Low values of ATP (3.6 nmol/mg prot), GSH (7.1 nmol/mg prot) and protein synthesis rates (94.1 pmol leu/mg prot \times min⁻¹) were initially observed. Thereafter, they gradually recovered up to 6 h but decreased values were seen after 20 h. Glycogen, however, dropped rapidly during the first 6 h, being no longer detected after 20 h of incubation. The reincubation of PCLS in a fresh medium for 6 h allowed a strong recovery of GSH, ATP and protein synthesis rates, but no gluconeogenesis was observed. Meanwhile, paracetamol sulfate formation was fairly constant (about 3 μ g/mg protein) while glucuronide gradually disappeared. The amount of both UGT1A1 and ST1A1 did not correlate with their respective enzymatic activities. We suggest that loss of glycogen impair glucuronide conjugation by decreasing the availability of UDPGA, and that low values of ATP are largely enough to support sulfotransferase activity. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Paracetamol metabolism; Rat liver slices; Sulfation; Glucuronidation

1. Introduction

Isolated hepatocytes are widely used to study drug metabolism and drug-induced hepatotoxicity as well as in the screening of metabolic profiles of potentially new drugs (McMahon, 1980; Berry et al., 1992). The use of isolated cells, however, has some limitations and drawbacks such as damaged cellular membranes and disruption of cell-to-cell contacts by the use of proteolytic enzymes; loss of normal polarity of hepatocytes as well as of the architecture of the whole liver, etc. To overcome many of these problems, the in vitro model of precision-cut liver slices (PCLS) was introduced some years ago (Krumdieck et al., 1980; Smith et al., 1985; Brendel et al., 1987; Dogterom, 1993). The main advantages of PCLS are: (a) preservation of a higher

level of the tissue organization which are normally found in the intact organ and thus better reflection of the in vivo situation; (b) preservation of a differentiated state due to the maintenance of cell–cell and cell–matrix interactions; (c) preservation of different cell types, a critical issue since non-parenchymal cells such as Kupffer cells may significantly influence the viability and function of hepatocytes (Neyrinck et al., 1999); and (d) its facilitated histological and histochemical evaluation as an alternative, or as a complement, to biochemical tests.

In order to evaluate the suitability of PCLS as in vitro model for pharmaco-toxicological studies, their capacity to metabolize paracetamol over a period of 26 h was investigated. Paracetamol (acetaminophen), a widely used analgesic, is mainly metabolized by phase II biotransformation pathways, such as sulfate, glucuronide, and glutathione conjugations (Jollow et al., 1974). As UDP-glucuronyltransferase 1A1 (UGT1A1) and sulfotransferase 1A1 (ST1A1) are important isoforms involved in the metabolism of paracetamol in rats (Bock et al., 1993; Duffel et al., 1998), the production of paracetamol conjugates were compared to the amount of

Abbreviations: FCS, fetal calf serum; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; PCLS, precision-cut rat liver slices; UDPGA, UDP-glucuronic acid; WME, Williams' medium E.

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protein UGT1A1 and ST1A1 in liver slices. For such studies it is of the utmost importance to assess not only the viability but also the functionality of the liver slices. The viability of the liver slices during incubation was determined by ATP content and protein synthesis rates. Moreover, important liver functions were assessed: glycogen level and glutathione concentration. In our study, histological examination was also performed and correlated to the measurement of biochemical parameters.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 250–300 g were purchased from Iffa-Credo (Les Oncins, France) and housed in individual cages in a temperature- and light-controlled room (12 h dark/light cycle). They received a standard diet AO3 (U.A.R., Villemoisson-sur Orge, France) and water ad lib.

2.2. Chemicals

Williams' medium E (WME) and fetal calf serum (FCS) were purchased from Gibco BRL (Middlesex, UK). Gentamicin sulfate, leupeptine, PMSF, antipain, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA) and Triton X-100 were obtained from Sigma Chemicals (St Louis, MO, USA). Paracetamol (Janssen Pharmaceutica, Beerse, Belgium), 2-acetamidophenol (Aldrich Chemicals Co., Dorset, UK) and insulin (Actrapid HM, Novo Nordisk, Bagsvaerd, Denmark) were commercially available. Polyclonal sheep antibodies raised against purified recombinant rat ST1A1 were generously provided by Dr. M. Coughtrie (University of Dundee, Scotland, UK). Polyclonal rabbit antibodies raised against rat UGT1A1 were a gift of Dr Shin-ichi Ikushiro (Himeji Institute of Technology, Hyogo, Japan). Anti-rabbit IgG-POD was purchased from Boehringer-Mannheim (Germany) and anti-sheep Ig POD was from Chemicon International (Temecula, CA, USA). Western immunoblotting kit employing enhanced chemiluminescence detection and nitrocellulose membranes was obtained from Amersham (UK). All other chemicals used were of the purest grade available.

2.3. Preparation and incubation of precision-cut liver slices

Rat surgical procedures were carried out under pentobarbital (60 mg/kg) anaesthesia. PCLS (250–300 μ m thickness) were prepared by using the Krumdieck tissue slicer according to procedures previously described (Goethals et al., 1992). They were stored 30 min at 4 °C in WME containing FCS (10%), glutamine (2 mM) and

insulin (100 nM). After preincubation, PCLS were transferred to vials (25 ml Erlenmeyer) containing WME (2 slices/4 ml) supplemented with glutamine (2 mM), insulin (100 nM) and 50 μ g/ml gentamicin sulfate. PCLS were incubated in a shaking water-bath (100 cycles/min) at 37 °C under a continuous flow of O₂/CO₂ (95%:5%) for 4, 6 and 20 h. After 20 h of incubation, PCLS were rinsed with saline and further incubated for 6 h in a fresh WME as reported before.

2.4. ATP content

Liver slices were taken, washed twice in saline and sonicated immediately in 1 ml of 2% perchloric acid. The intracellular ATP content was measured on neutralized perchloric acid extracts using the ATP Bioluminescence Assay Kit CLS II from Boehringer-Mannheim (Germany). The results are expressed as nmol ATP/mg protein.

2.5. GSH content

GSH content was assayed using a modified method of Hissin and Hilf (1976). Liver slices were taken, washed twice in saline and sonicated in 1 ml 6% trichloroacetic acid and samples were then centrifuged for 30 min at 3600 g at 4 °C. Aliquots of the standards and precipitated slices were added to tubes containing phosphate/EDTA buffer (pH 8) and 1 mg/ml *o*-phthalaldehyde (200 μ l), then stored in the dark (15 min). Fluorescence was measured (excitation 350 nm, emission 420 nm) using a spectrofluorimeter, Perkin-Elmer LC-B. The results are expressed as nmol GSH/mg protein.

2.6. Protein synthesis

Protein synthesis was estimated by measuring the incorporation of [¹⁴C]leucine (sp. act. 94 μ Ci/mmol, 0.8 mM unlabelled leucine) into the protein pellet obtained by perchloric acid precipitation as described by Seglen (1976). At the indicated times PCLS were rinsed in saline, further incubated for 2 h in a fresh medium containing unlabelled leucine in the presence of [¹⁴C]leucine. The results are expressed as pmol leucine incorporated/mg protein. The amount of protein was determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin as standard.

2.7. Glycogen content

Liver slices were taken, washed twice in saline and sonicated in 1 ml 1 M KOH. They were heated at 100 °C for 10 min. After neutralization with acetic acid and centrifugation, the supernatant was incubated in the presence of α -amylglucosidase in acetate buffer (pH 5).

The glucose produced was quantified by an enzymatic reaction as previously described (Krack et al., 1980). The results are expressed as μg glycogen/mg protein.

2.8. Paracetamol metabolism

Paracetamol glucuronide, paracetamol sulfate and paracetamol glutathione conjugates were quantified by using reverse-phase HPLC according to the procedure of Lau and Critchley (1994). PCLS were incubated for 2 h in medium supplemented with 5 mM paracetamol. Throughout the 2-h incubation time, the metabolism of paracetamol to its glucuronide, sulfate and glutathione conjugates was linear (data not shown). At the end of the incubation, aliquots of incubation medium were stored at $-20\text{ }^{\circ}\text{C}$. After centrifugation, 25 μl of samples together with 50 μl of the internal standard 2-acetamidophenol (400 mg/mg) were injected on a Nova-Pak C18 column. The mobile phase (0.1 M KH_2PO_4 /0.1% acetic acid/0.75% propane-2-ol) was delivered to the column at a flow rate of 1.50 ml/min. The results are expressed as μg metabolites/mg protein.

2.9. Western immunoblotting

Liver slices (two) were sonicated in Tris-HCl buffer (pH 7.4) containing PMSF (0.1 mM), leupeptine (2 $\mu\text{g}/\text{ml}$), antipain (2 $\mu\text{g}/\text{ml}$), DTT (2 mM), EDTA (0.1 mM) and Triton (0.4%). After centrifugation of liver slices homogenates, the supernatant was stored at $-20\text{ }^{\circ}\text{C}$. The proteins were subjected to SDS-PAGE (10% separating gel) followed by electroblot to nitrocellulose membranes. The membrane was blocked 1 h at $25\text{ }^{\circ}\text{C}$ in Tris-buffered saline (TBS) (pH 7.4) containing 5% (w/v) powdered milk protein followed by an incubation with diluted antibodies at $25\text{ }^{\circ}\text{C}$ in a fresh solution of powdered milk protein (1%, w/v) in TBS buffer. The membrane was washed and incubated with a dilution of secondary antibody at $25\text{ }^{\circ}\text{C}$. The primary and secondary antibodies were diluted respectively 1/4000 and 1/10

000 for UGT1A1, and 1/10 000 and 1/15 000 for ST1A1. Immunodetection was performed using the ECL detection kit (Amersham). The film was scanned and the density of the bands was calculated using the program Image Master from Pharmacia Biotech Bencelux (Roosendaal, The Netherlands).

2.10. Histological and histochemical methods

Specimens were fixed in Carnoy's fixative and embedded in paraffin. Brachet's methylgreen-pyronin staining was used for nucleic acids detection and periodic acid-Schiff (PAS) method for glycogen staining.

2.11. Statistical analysis

Each experiment was performed in triplicate and the results are mean values \pm standard error of the mean (S.E.M.) of at least three different rats. Data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni test for significant differences between means. The level of significance was set at $P < 0.05$.

3. Results

3.1. Time course of biochemical markers in PCLS

Table 1 shows that the slices were viable for at least 26 h as seen by the values of ATP and GSH and protein synthesis rates. Immediately after slicing the ATP content in the PCLS was low (3.6 nmol ATP/mg protein), after 2–4 h it increased thus reaching maximum levels (17.2 nmol ATP/mg protein) at 6 h, but after 20 h of incubation the ATP concentration decreased to 7.2 nmol ATP/mg protein. When PCLS were rinsed in saline and further incubated in a fresh WME medium for 6 h, a slight increase was observed thus reaching 9.8 nmol/mg protein at 26 h of incubation. It represented

Table 1
Viability of PCLS over a period of 26 h incubation

Time (h)	ATP (nmol/mg protein)	GSH (nmol/mg protein)	Glycogen content ($\mu\text{g}/\text{mg}$ protein)	Protein synthesis rates (pmol leu/mg prot \times min $^{-1}$)
0	3.6 \pm 0.6	7.1 \pm 0.1	470.5 \pm 157.9	94.1 \pm 6.9
4	13.4 \pm 1.6 ^a	12.8 \pm 1.6 ^a	136.3 \pm 54.1	123.8 \pm 12.5 ^{a,b}
6	17.2 \pm 1.5 ^a	16.4 \pm 1.9 ^a	94.1 \pm 30.7	92.9 \pm 11.6
20	7.2 \pm 0.2 ^{a,b}	12.9 \pm 1.8 ^a	Nd	70.2 \pm 9.8 ^a
24	7.8 \pm 0.6 ^{a,b}	12.9 \pm 1.7 ^a	Nd	98.2 \pm 5.2
26	9.8 \pm 0.3 ^{a,b}	16.6 \pm 1.9 ^a	Nd	112.8 \pm 7.9 ^a

PCLS were incubated for 26 h at $37\text{ }^{\circ}\text{C}$. After 20 h, PCLS were rinsed in saline and further incubated for 6 h in fresh WME. At the indicated times the ATP content and GSH level, protein synthesis rates and glycogen were measured as described under Materials and Methods. Values represent the mean \pm S.E.M. from at least three rats. Nd, non-detectable.

^a $P < 0.05$ as compared to values at zero time.

^b $P < 0.05$ as compared to values at 6 h.

about 80% of the standard ATP values, which ranging between 10 and 15 nmol/mg protein.

The level of GSH was also low immediately after slicing (7.1 nmol GSH/mg protein). Nevertheless, its time course was less affected by the incubation time. After 20 h a slight decrease was observed, but as soon as the liver slices were placed in a fresh WME, the GSH levels increased reaching about 95% of the standard GSH values (15–20 nmol/mg protein).

Protein synthesis rates by liver slices was measured by the incorporation of radiolabeled leucine into acid-precipitable proteins. As previously observed for ATP and GSH values, protein synthesis rates were initially low, then it increased to 123.8 pmol/mg prot \times min⁻¹, and again it decreased after 20 h of incubation. Reincubation of PCLS with fresh WME resulted in a marked recovery, at the end reaching 112.8 pmol leucine incorporated/mg protein \times min⁻¹.

Finally, Table 1 also shows that the glycogen level in PCLS decreased to 94 μ g/mg protein, which represented about 20% of the value measured at time zero during the first 6 h of incubation. After 20 h, the glycogen content in the liver slices was no longer detectable.

3.2. Histological analysis of PCLS

Plate 1(A) shows that immediately after preparation liver slices retained the trabecular structure of the liver and the intensity of nucleic acids staining by Brachet's method. This normal pattern was homogeneously distributed in all cells across the whole thickness of the slice. Plate 1(B) shows that after 6 h of incubation the trabecular structure of the liver and intensity of both nucleic acids was well maintained. The cytoplasmic RNA staining was more diffuse than at state zero. However, in rare, dispersed hepatocytes (arrows) a decrease or lack of both nucleic acids staining could be seen, indicating their serious deterioration or cell death. Plate 1(C) shows that after 20 h of incubation the volume of the hepatocytes decreased, most probably due to the complete disappearance of glycogen or partial dehydration, thus inducing in certain zones the increase of the space between the trabeculi and a loss of intercellular contact. The intensity of cytoplasmic RNA staining was considerably decreased and DNA was well preserved in nuclei. Some of these nuclei had a pycnotic aspect. In addition, some areas appeared with seriously

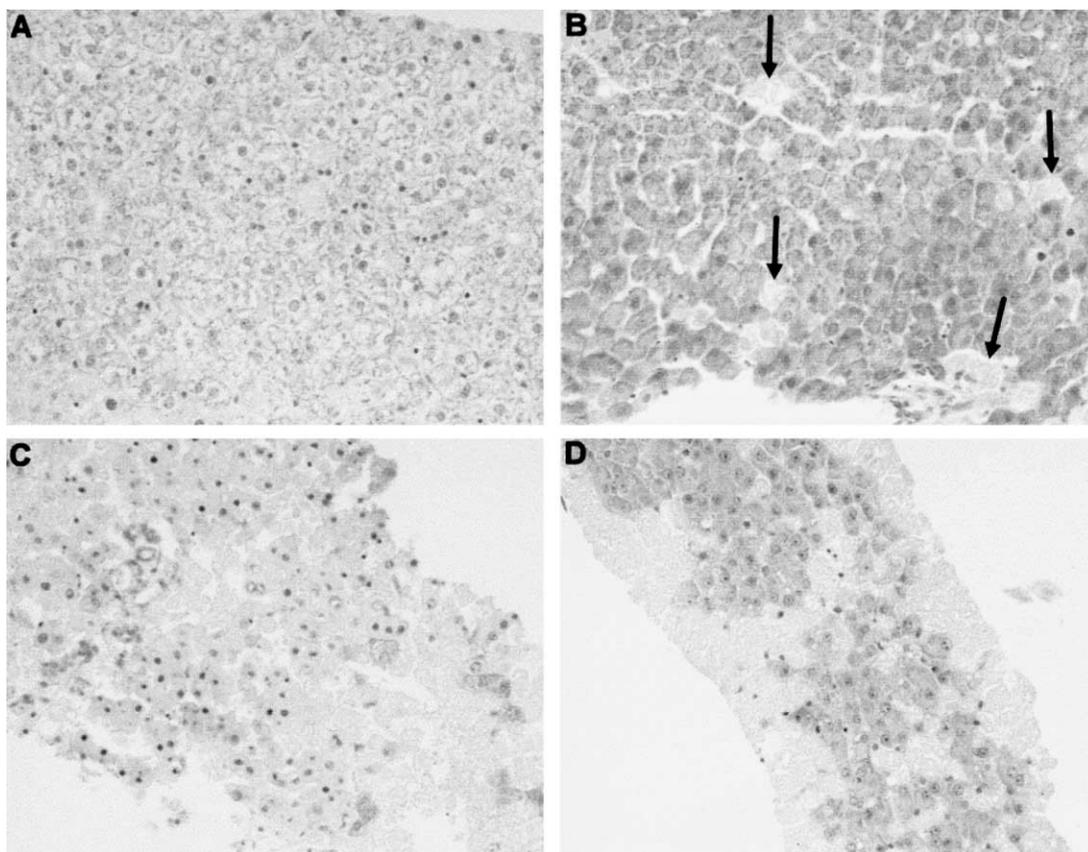


Plate 1. Nucleic acids staining of PCLS after (A) zero time, (B) 6 h, (C) 20 h and (D) 24 h of incubation. Both nucleic acids staining is homogeneously distributed in all hepatocytes (A). Trabecular liver structure is well preserved and cytoplasmic RNA is more diffuse in (B), with rare, dispersed hepatocytes without staining of both nucleic acids (arrows). A progressive decrease of nucleic acids staining with increase of areas completely lacking both nucleic acids staining can be observed in (C) and (D). More important areas of complete lack of both nucleic acids staining are mainly distributed on the surface of slice in (D). Brachet's methylgreen-pyronin staining \times 250.

altered hepatocytes without any staining of both nucleic acids. Plate 1(D) shows that after 24 h of incubation, the layers of deteriorated hepatocytes were larger and localized mainly on the surface of the slice. The staining of nucleic acids was in these cells very weak or absent. In the median zone of liver slice, the trabecular structure of liver parenchyma and nucleic acids staining were, however, preserved.

Plate 2(A) shows that at time zero of incubation glycogen (detected by PAS method) was present in all hepatocytes in the form of fine, red cytoplasmic granules. These granules were often polarized mainly in the surface zone. Plate 2(B) shows that after 6 h of incubation a distinct decrease or complete disappearance of glycogen staining existed in the cytoplasm of all hepatocytes. Basically, only blue contraststaining of nuclei was visible. It was further found, that after 20 h of incubation, PAS staining did not reveal any glycogen in the cytoplasm of hepatocytes (data not shown).

3.3. Drug biotransformation by PCLS

The capacity of PCLS to metabolize paracetamol (5 mM) was measured over a period of 26 h incubation (Fig. 1). As compared to the initial metabolic activity, the formation of paracetamol glucuronide was decreased by 42 and 71%, after 4 and 20 h of incubation, respectively. After 26 h of incubation, no paracetamol glucuronide was detectable in the incubation medium. On the other hand, the formation of paracetamol sulfate also decreased, but to a lower extent, and 38% of the initial metabolic activity was retained after 20 h of incubation and remained more or less constant the next 6 h. Glutathione conjugates of paracetamol were detectable only during the first 6 h, representing 6.7% of the total metabolites produced at zero time.

The amounts of UGT1A1 and ST1A1 proteins detected by Western immunoblotting are presented in Fig. 2(a) (blots) and (b) (optical densities \times mm²). Conversely to glucuronides formation, the amount of the protein UGT1A1 increased during incubation reaching 88% after 20 h of incubation of liver slices. Afterwards, a decrease in the amount of protein to more or less the same level at zero time was observed. As compared to the initial levels of ST1A1, the amount of this protein was decreased by about 20 and 50% after 20 and 26 h of incubation, respectively.

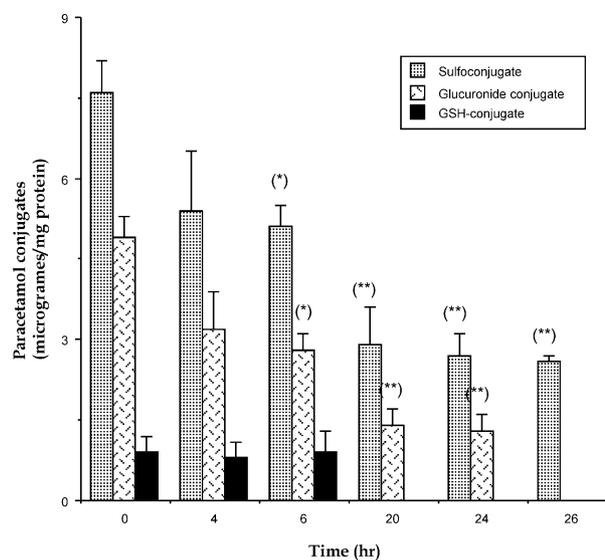


Fig. 1. Effect of time on the formation of paracetamol metabolites by liver slices. Formation of paracetamol metabolites released in the culture medium by PCLS after 2 h of incubation in the presence of paracetamol (5 mM) at various incubation times. Results represent the mean \pm S.E.M. ($n=5$). * $P < 0.05$ as compared to values at zero time. ** $P < 0.05$ as compared to values at 6 h.

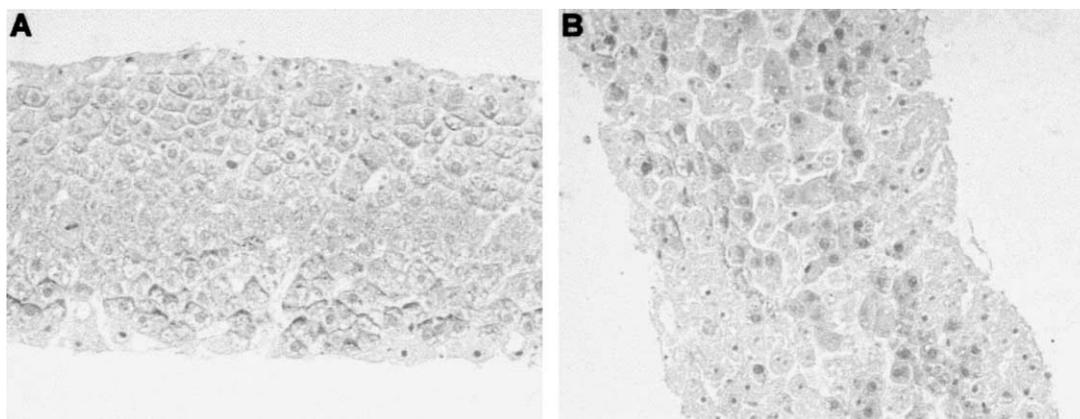


Plate 2. At time zero of incubation, glycogen (detected by PAS method) is visible in all hepatocytes in the form of fine, red cytoplasmic granules, often polarized (A). After 6 h of incubation, glycogen is no longer detectable. At that time, only the contraststained blue nuclei are visible (B). PAS staining $\times 250$.

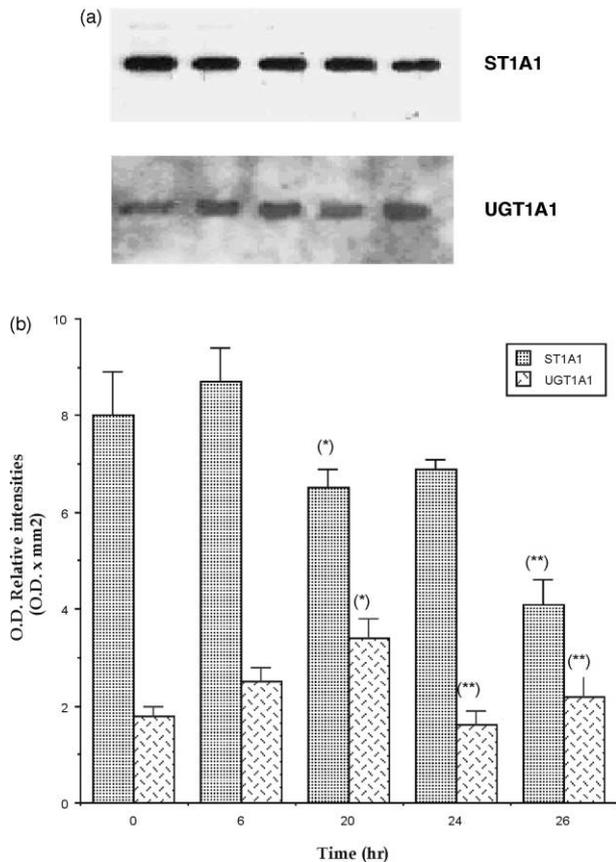


Fig. 2. Immunoblotting of UGT1A1 and ST1A1. Precision-cut rat liver slices were homogenized and immunoblots were performed as described under Materials and Methods. The supernatant of slices (10 μ g protein per lane for UGT1A1 and 4 μ g protein per lane for ST1A1) were prepared from freshly cut slices (lane 1) and from liver slices exposed 6 h (lane 2), 20 h (lane 3), 24 h (lane 4) and 26 h (lane 5) of aerobic incubation at 37 °C (A). Photographs of the developed films were densitometrically quantified and data were expressed as OD \times mm² for the various incubation time (B). * P < 0.05 as compared to values at zero time. ** P < 0.05 as compared to values at 20 h.

4. Discussion

The measurement of a number of functional parameters demonstrated that the biochemical integrity of the liver slices was retained up to 26 h. The level of ATP and GSH were initially low and then recovered after 4–6 h in culture reaching values already reported in fresh tissues. This phenomenon may be explained as a temperature-dependent re-equilibration that occurs when slices are warmed when transferred from ice-cold buffer to the warmer conditions of the incubation. In agreement with the report of Miller et al. (1993), the ATP concentration decreased after 20 h of incubation of liver slices, but a marked recovery was observed when they were reincubated in a fresh medium. Despite the decrease in the ATP content, the GSH levels were less affected and the recovery after 20 h of incubation was almost complete.

In our study, we have tried to correlate the biochemical measurement of parameters to the histological

examination of liver slices. In agreement with the report of Hashemi et al. (1999a) and Olinga et al. (1997), the histomorphology showed that the PCLS maintained an excellent viability for incubation times up to 10 h but began to show signs of damage by 20 h. Interestingly, the analysis of the histological section, after 20 h of incubation, revealed a complete loss of glycogen. The disappearance of glycogen content which was measured biochemically is in full accordance with the results of histological examination. Nevertheless, the morphological observations of PCLS during a 24-h incubation showed that despite the appearance of layers of deteriorated hepatocytes showing weak nucleic acid staining, they were capable of maintaining normal lobular architecture and cellular morphology. In addition, PCLS are able to synthesize proteins at normal rates, and as shown by Lake et al. (1996) and Lupp et al. (2001), PCLS retain their ability to respond to P450-inducing agents. Further studies are required, however, to explain how protein synthesis remains still active with such a decrease of nucleic acids staining.

Protein synthesis as assessed by the incorporation of radiolabeled leucine into acid-precipitable proteins, was utilized as an additional index of biochemical competence because it is one of the major functions of the hepatocytes. The maintenance of this process requires amino acids uptake and their incorporation into proteins. After 20 h of incubation, protein synthesis, while still active, follows the same profile of the time course of both ATP and GSH content. Once PCLS are reincubated in fresh medium, rates return to normal values as previously observed during short-term incubations of freshly isolated rat hepatocytes, namely 150 pmol/mg protein \times min⁻¹ (Tinton et al., 1997).

The maintenance of glycogen reserves is also a specific function of the liver cell. In the present study, the capacity of slices to maintain such a glycogen level over a prolonged period of incubation was examined. The hepatocellular glycogen content results from a balance between glycogen synthesis and degradation. In agreement with the report of Smith et al. (1986), a decrease of glycogen content was observed both biochemically and histochemically, being undetectable after 20 h of incubation. Two major reasons may explain the decline in the glycogen levels: (1) through ionic disturbances, especially loss of calcium homeostasis, it may follow the activation of glycogen phosphorylase *a* as reported elsewhere (Burcham and Herman, 1989); or (2) to a low glucose concentration in the culture medium (about 11 μ M). Actually, regarding the influence of glucose concentration, Smith et al. (1986) reported that in liver slices cultured in the presence of 25 mM glucose, the hepatic glycogen level was restored and maintained for up to 12 h. In agreement with that, we have recently showed that in slices incubated for 24 h in a medium supplemented with 25 mM glucose, the level of glycogen

in PCLS represented about 66% of the value measured at zero time (Vanhulle et al., 2001).

The primary routes of paracetamol metabolism are by conjugation via glucuronosyltransferase and sulfotransferase to form non-toxic metabolites. In addition to the amount and integrity of their respective enzyme proteins, the rate of glucuronidation and sulfation depends on the availability of their cofactors, UDP-glucuronic acid (UDPGA) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS), respectively. The synthesis of UDPGA and PAPS is dependent on several enzymes: UDP-glucose pyrophosphorylase and UDP-glucose dehydrogenase for UDPGA and ATP-sulfurylase and APS-kinase for PAPS. A decrease in the activity and/or content of these enzymes could also explain a decrease in the metabolic activity of PCLS. Nevertheless, the ATP content over the 26 h of incubation is large enough to support metabolic activities.

Previous *in vivo* studies indicated that sulfation was the major route of elimination of paracetamol: 57.7% of sulfate conjugates and 32.3% of glucuronide conjugates (Price and Jollow, 1982). As the dose of paracetamol increased, the proportion of the glucuronide conjugate increased, because sulfation of paracetamol in rats has been characterized as a high-affinity and low-capacity conjugation reaction (Moldeus, 1978). Our results agree with the observations made by Miller et al. (1993) and Oddy et al. (1997) indicating that the sulfation is the major and the predominant route of paracetamol biotransformation. Actually, Miller et al. (1993) reported that glucuronides, sulfates and glutathione conjugates represent 40.2, 57.0 and 2.8%, respectively. Our results are close to that, but higher amounts of glutathione conjugates (6.7%) were found.

The amount of paracetamol glucuronide and sulfate in the medium of the liver slices began to decrease after 4 h of incubation and fell to 29 and 38%, respectively after 20 h of incubation. Hashemi et al. (1999b) also described such a decrease in UDP-glucuronyltransferase and sulfotransferase activity after 24 h of incubation. Our results show that the amount of UGT1A1 or ST1A1 protein, measured by Western blots, did not correlate with their respective enzyme activity. Indeed, while sulfation is decreased by 62% after 20 h of incubation, ST1A1 is only decreased by 19%. Moreover, glucuronidation is decreased by 71% after 20 h of incubation, whereas UGT1A1 is increased by 88%.

As decreased enzymatic activities are unlikely to be explained by changes in the enzyme protein, and protein synthesis ability is not impaired, another regulating factor for glucuronidation may be the carbohydrate metabolism (Thurman and Kauffman, 1979). UDPGA is derived from glucose-1-phosphate and UTP and involves NAD⁺. It has been shown that UDP-glucuronic acid for glucuronidation is provided by glycogenolysis (Banhegyi et al., 1988). In fact, when the rate

of glycogenesis in isolated rat hepatocytes was stimulated by high amounts of glucose, fructose or insulin, both glycogenolysis and the glucuronidation were inhibited (Banhegyi et al., 1991). It has been reported that the decreased energy state involved reduction of glucuronidation and sulfation of xenobiotics (Aw and Jones, 1982). Sulfation may be much more sensitive to changes in energy state than glucuronidation (Thurman and Kauffman, 1979). The dependence of PAPS synthesis on ATP level is supported by many *in vitro* studies in which several substances leading to a decrease of ATP level strongly decreased PAPS levels and/or sulfation (Klaassen and Boles, 1997). The total depletion of glycogen in PCLS after 20 h of incubation and the decrease of ATP after 20 h may therefore explain the decrease of glucuronidation and sulfation capacity of liver slices observed after 20 h of incubation. Despite the decrease of ATP in slices after 20 h, our results showed that PCLS are still capable to maintain their sulfotransferase activity after 20 h of incubation. It appears that when the slices are reincubated in fresh medium they recover their capacity to synthesize ATP, but in any case such a "refreshment" is sufficient to reactivate the gluconeogenesis. These results suggest that such ATP values while reduced, are large enough to support sulfotransferase activity.

Regarding conjugates with glutathione, our results show that during the 120 min of incubation of slices with paracetamol, a small amount of NABQI has been formed during the first 6 h of incubation. Nevertheless, after 20 h of incubation, no NABQI seems to be formed. One possible explanation is that glutathione *S*-transferase (GST) activity was decreased over the 26 h of incubation, thus explaining the maintenance of GSH levels. Hashemi et al. (1999b) reported that GST regarding two different substrates (CDNB and DCNB) was decreased by about 40 and 70%, respectively, after 24 h of incubation. As this conjugation requires first the activation of paracetamol by P450 enzymes to form the reactive intermediate (NABQI), another possibility is that the P450 involved are less active to induce the formation of such reactive intermediate. Indeed, two major isoforms involved in this process, namely CYP2E1 and CYP3A2 were decreased by more than 60 and 95%, respectively, after 20 h of incubation (data not shown).

In conclusion, rat liver slices are a suitable model system for studying the phase II conjugation reactions of xenobiotics such as paracetamol. The data obtained confirm that in liver slices, sulfation is the major route of paracetamol metabolism.

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