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CULTURE OF PRECISION-CUT LIVER SLICES: EFFECT OF SOME PEROXISOME PROLIFERATORS

J. A. BEAMAND, R. J. PRICE, M. E. CUNNINGHAME and B. G. LAKE*
BIBRA Toxicology International, Woodmansterne Road, Carshalton, Surrey SM5 4DS, UK

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Abstract—Precision-cut rat liver slices were prepared with a Krumdieck tissue slicer and cultured in three standard hepatocyte culture media. Rat liver slices cultured in either RPMI 1640 medium or Williams Medium E could be maintained in culture for up to 72 hr. In contrast, Leibovitz's L-15 medium was unsatisfactory in that slice viability, assessed either by morphological examination or by measurement of enzyme activities, could not be maintained for periods greater than 24 hr. As a measure of functional viability liver slices were cultured with some known rodent peroxisome proliferators, namely clofibric acid, nafenopin, ciprofibrate and Wy-14,643. The peroxisome proliferators induced both palmitoyl CoA oxidation and carnitine acetyltransferase activities in 48- and 72-hr slice cultures. Ultrastructural examination of liver slices cultured with either ciprofibrate or Wy-14,643 for 72 hr revealed an increase in the number of peroxisomes. These results demonstrate that rat liver slices may be maintained in culture for up to 72 hr, and that they respond in a similar manner to rat primary hepatocyte cultures to some peroxisome proliferators. Precision-cut liver slices may therefore be a useful alternative *in vitro* system to hepatocyte cultures for screening compounds for effects on enzyme activities and for assessing species differences in response.

INTRODUCTION

Conventional procedures for the evaluation of the toxicity of food and environmental chemicals are lengthy and expensive, and necessitate the use of relatively large numbers of laboratory animals. The development and validation of appropriate *in vitro* systems offers the potential to screen untested chemicals for toxic and other effects more rapidly and economically. Indeed, certain *in vitro* systems permit the comparison of the effects of chemicals in tissue from both experimental animals and humans, and hence may provide valuable data for human hazard assessment (Frazier *et al.*, 1989).

The liver is the major site of xenobiotic metabolism and is often a target organ for xenobiotic-induced toxicity (Zimmerman, 1978). Accordingly, much effort has been devoted to the development of *in vitro* systems for assessing the hepatic effects of xenobiotics. One such system is primary hepatocyte cultures, which have been extensively used for studies of xenobiotic metabolism, toxicity and induction of cytochrome *P*-450 dependent and peroxisomal enzyme activities (Acosta *et al.*, 1985; Chenery, 1988; Gray *et al.*, 1983a; Lake *et al.*, 1984; Paine, 1990; Tyson, 1987).

An alternative to primary cell cultures is the use of precision-cut tissue slices, a technique developed by Krumdieck, Brendel and co-workers (Brendel *et al.*, 1987; Krumdieck *et al.*, 1980; Sipes *et al.*, 1987; Smith *et al.*, 1986 and 1989). With this technique slices of uniform thickness may be readily prepared from the liver and other tissues and maintained in a suitable dynamic organ culture system (Brendel *et al.*, 1987; Smith *et al.*, 1985, 1986 and 1989).

Precision-cut tissue slices have a number of potential advantages over other *in vitro* systems such as primary cell cultures (Azri *et al.*, 1990a; Brendel *et al.*, 1987; Sipes *et al.*, 1987; Smith *et al.*, 1986 and 1989). For example, slices maintain tissue architecture so that all cell types are present and intercellular communication between the various cell types is maintained. This should permit the detection of both selective toxicity (where one or more cell types are affected) and interactive toxicity (where one cell type contributes to the toxicity observed in another cell type). In addition, tissue slicing avoids the damage to cells which may occur during cell isolation procedures employing proteolytic enzymes.

Precision-cut liver slices from laboratory animals have been used to study the *in vitro* metabolism of various xenobiotics including chlorobenzenes (Barr *et al.*, 1991a), diazepam (Dale *et al.*, 1988) and halothane (Ghantous *et al.*, 1990a). In addition, liver slices have been used to study the toxicity of several chemicals including allyl alcohol (Smith *et al.*, 1987), bromobenzene and derivatives (Fisher *et al.*, 1991a; Smith *et al.*, 1987), carbon tetrachloride (Azri *et al.*, 1990b and 1992; Wolfgang *et al.*, 1990), chlorobenzenes (Fisher *et al.*, 1990), cocaine (Connors *et al.*, 1990), halothane (Ghantous *et al.*, 1990b) and valproic acid (Fisher *et al.*, 1991b). Furthermore,

*To whom correspondence should be addressed.
Abbreviations: DMSO = dimethyl sulphoxide; EBSS = Earle's balanced salt solution.

xenobiotic metabolism (Barr *et al.*, 1991b; Gunawardhana *et al.*, 1991) and toxicity (Connors *et al.*, 1990; Fisher *et al.*, 1991c,d) studies have also been conducted with human liver slices.

In the studies with tissue from both laboratory animals and humans described above, the liver slices were exposed to the xenobiotics for various periods up to a maximum of 24 hr. The aim of the present study was to determine whether rat liver slices could be cultured for extended periods (i.e. greater than 24 hr). As a measure of the functional viability of the cultured liver slices, their response to some peroxisome proliferators has also been investigated. Peroxisome proliferators are known to produce organelle proliferation and to induce peroxisomal enzyme activities in the rat liver after *in vivo* administration and *in vitro* in primary hepatocyte cultures (Gray *et al.*, 1982 and 1983a,b; Lake *et al.*, 1984; Lock *et al.*, 1989; Mitchell *et al.*, 1984; Moody *et al.*, 1991).

MATERIALS AND METHODS

Materials. RPMI 1640, Leibovitz's L-15 medium, Williams Medium E, Earle's balanced salt solution (EBSS) and gentamicin were purchased from Gibco BRL (Uxbridge, Middlesex, UK) and foetal calf serum from Sera-Lab Ltd (Crawley Down, Sussex, UK). Insulin, hydrocortisone-21-hemisuccinate, L-methionine and clofibric acid (2-(4-chlorophenoxy)-2-methylpropionic acid) were obtained from Sigma Chemical Co. Ltd (Poole, Dorset, UK) and fungizone from E. R. Squibb and Sons (Wirral, Merseyside, UK). Dimethyl sulphoxide (DMSO) was purchased from FSA Laboratory Supplies (Loughborough, Leics., UK) and Wy-14,643 ([4-chloro-6-(2,3-xyli-dino)-2-pyrimidinylthio]acetic acid) from Chemsyn Science Laboratories (Lenexa, KS, USA). Nafenopin (2-methyl-2-[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]-propionic acid) and ciprofibrate (2-[4-(2,2-dichlorocyclopropyl)phenoxy]-2-methylpropionic acid) were the generous gifts of Ciba-Geigy Ltd (Basle, Switzerland) and Sterling Winthrop Research Centre (Alnwick, Northumberland, UK), respectively. Polystyrene vials were obtained from Luckham Ltd (Burgess Hill, Sussex, UK) and stainless-steel wire mesh from Locker Wireweavers Ltd (Barnet, Middlesex, UK).

Animals. Male Sprague–Dawley rats were obtained from Harlan Olac (Bicester, Oxon, UK) and allowed free access to R and M No. 1 diet (Special Diets Services, Witham, Essex, UK) and water. The animals were housed in accommodation maintained at 22 ± 3°C with a relative humidity of 40–70% and allowed to acclimatize to these conditions for at least 3 wk before use.

Preparation of liver slices. Rats (13–15 wk old) were anaesthetized with sodium pentobarbitone (60 mg/kg ip) and then killed by cervical dislocation. The livers were excised and placed in EBSS containing 25 mM-D-glucose previously gassed with 95% O_2/5% CO_2. Tissue cylinders were prepared using a 10-mm diameter cork borer. From the cylinders tissue slices (200–300 μm) were prepared in oxygenated (95% O_2/5% CO_2) EBSS containing 25 mM-D-glucose at room temperature using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munford, AL, USA).

Culture of liver slices. Liver slices in EBSS containing 25 mM-D-glucose were floated onto inserts made of stainless-steel mesh screens (263-μm pore size). The inserts (each containing two slices) were placed in plastic vials (diameter 14.6 mm and length 40.8 mm) containing 1 ml culture medium and were held fixed by the vial caps. Small holes were made in these plastic caps to facilitate gas exchange. All culture media contained 5% foetal calf serum, 1 μM-insulin, 0.1 mM-hydrocortisone-21-hemisuccinate, 50 μg gentamicin/ml and 2.5 μg fungizone/ml. In addition, the L-methionine content of RPMI 1640 medium was increased to a final concentration of 0.5 mM since this with all the above additions except fungizone is used as a standard rat hepatocyte culture medium in this laboratory. The vials were placed on a roller system housed in a humidified incubator. They were incubated at 37°C in an atmosphere of either 5% CO_2/95% air or air (depending on the culture medium selected) and rotated at approximately 9 rpm. After 2 hr, treatment was commenced by replacing the culture medium with medium containing the required concentration (range 0.02 to 0.5 mM) of the peroxisome proliferators. Subsequently, the medium was replaced and the slices redosed every 24 hr. All the peroxisome proliferators used were dissolved in DMSO and added to the culture medium so that the final DMSO concentration was 0.4% (v/v) in all vials including the control cultures.

Biochemical investigations. At the end of the treatment period slices were washed in 0.154-M-KCl containing 50 mM-Tris–HCl, pH 7.4, and homogenized (two slices in 2 ml) in this medium with an MSE Soniprep 150 Ultrasonic Disintegrator (MSE Scientific Instruments, Crawley, Sussex, UK) and the whole homogenates were stored at −80°C. Cyanide-insensitive palmitoyl-CoA oxidation was assayed as described by Gray *et al.* (1983a) but with the addition of 10 μM-FAD to each cuvette. Carnitine acetyltransferase was determined as described previously (Gray *et al.*, 1982) and protein by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Morphological investigations. Liver slices were fixed in neutral buffered formalin. Paraffin sections of about 5 μm thickness were cut and stained with haematoxylin and eosin. Liver-slice thickness was measured using a VIDS IV Image Analysis System (Synoptics Ltd, Cambridge, UK). For electron microscopy liver slices were placed in 0.1 M-sodium cacodylate buffer (pH 7.39) containing 0.25 M-sucrose for 5 min and fixed in 3% glutaraldehyde in 0.1 M-sodium cacodylate buffer (pH 7.30) for 1 hr. The

slices were rinsed in 0.1 M-sodium cacodylate buffer (pH 7.39) containing 0.25 M-sucrose, post-fixed in 1% osmium tetroxide in Millonig's buffer, rinsed in the cacodylate buffer and dehydrated through graded alcohols, followed by propylene oxide before embedding in Epon Araldite resin. Thin sections (1 μm) of the slices were stained with toluidine blue and examined by light microscopy. Ultrathin sections of selected areas were cut and picked up on uncoated copper grids and stained with uranyl acetate followed by lead citrate (Reynolds, 1963). The sections were examined at a magnification of $\times$ 6600 and 14 photographs of random areas of each liver slice were taken to assess peroxisome numbers.

Statistical analysis. Statistical evaluation of data was performed by one-way analysis of variance. Comparisons between means were made using the least-significant difference test.

RESULTS

Culture of rat liver slices

Morphological examination of freshly cut rat liver slices revealed a normal histological appearance of the cells (Plate 1a). Slice thickness, determined by examination of formalin fixed slices, was 266 ± 17 μm (mean $\pm$ SEM of seven experiments). Rat liver slices were cultured in a dynamic organ culture system (Brendel *et al.*, 1987; Smith *et al.*, 1985, 1986 and 1989) for periods of 6, 24, 48 and 72 hr in one of three standard hepatocyte tissue culture media. Slices cultured in either RPMI 1640 medium or Williams Medium E were maintained in an atmosphere of 5% CO_2/95% air, whereas slices cultured in Leibovitz's L-15 medium were maintained under air. During the culture period, a decrease in slice thickness with time was observed which was associated with a loss of cells from the slices (Plate 1a,b). Slice protein content also decreased with time in culture (Fig. 1). Compared with liver slices maintained in either RPMI 1640 medium or Williams Medium E, the loss of slice protein content was significantly greater in slices cultured in Leibovitz's L-15 medium for 6–48 hr (Fig. 1).

Morphological examination of rat liver slices maintained for 72 hr in RPMI 1640 medium revealed a band of dead/dying cells in the middle of the slice surrounded by two bands of viable cells. The band of viable cells on the edge of the slice attached to the stainless-steel mesh insert was thinner than that on the other edge of the slice. Rat liver slices cultured in Williams Medium E had a similar appearance to those cultured in RPMI 1640, whereas slices cultured in Leibovitz's L-15 medium revealed extensive cell death within the first 24 hr of culture (data not shown). Increasing the medium glucose concentration from the 5.5–11 mM already present in the three standard media to 50 mM did not appear to affect slice viability (data not shown).

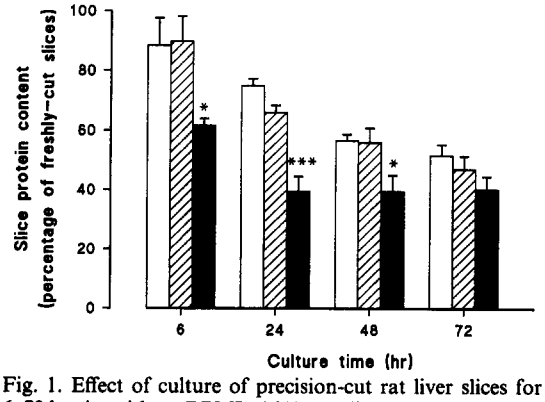

Fig. 1. Effect of culture of precision-cut rat liver slices for 6–72 hr in either RPMI 1640 medium (□), Williams Medium E (▨) or Leibovitz's L-15 medium (■) on slice protein content. Slice protein content is expressed as percentage of freshly cut (0 hr) values, and data are expressed as means $\pm$ SEM of four experiments. The protein content of freshly cut rat liver slices was 3.77 ± 0.21 mg protein/slice (mean $\pm$ SEM of 12 experiments). Asterisks indicate values significantly different from slices cultured in RPMI 1640 medium ($*P < 0.05$; $***P < 0.001$).

Effect of some peroxisome proliferators

Rat liver slices were cultured for up to 72 hr in the presence of some peroxisome proliferators. The effect of the peroxisome proliferators was monitored by measurement of cyanide-insensitive palmitoyl-CoA oxidation and carnitine acetyltransferase activities in rat liver slice whole homogenates. The former activity is a specific marker for the peroxisomal fatty acid β-oxidation cycle (Lazarow and DeDuve, 1976), whereas the latter activity is present in several subcellular fractions including peroxisomes, mitochondria and endoplasmic reticulum (Bieber *et al.* 1981; Ishii *et al.*, 1980).

When rat liver slices were cultured in Leibovitz's L-15 medium, both enzyme activities were only detectable in control (DMSO only) and peroxisome proliferator treated cultures for up to 24 hr and not in either 48- or 72-hr cultures (data not shown). In

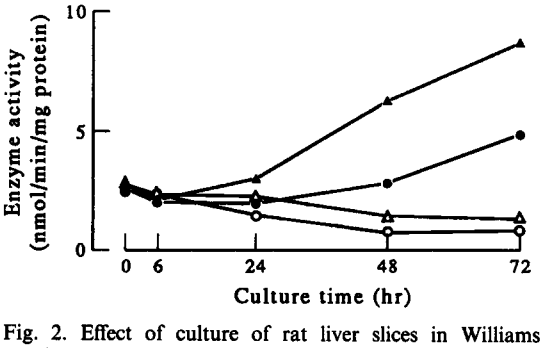

Fig. 2. Effect of culture of rat liver slices in Williams Medium E for up to 72 hr with 0.5 mM-clofibric acid. Compared with rat liver slices cultured in control (DMSO only) medium (open symbols), the addition of clofibric acid (closed symbols) results in a time-dependent induction of cyanide-insensitive palmitoyl-CoA oxidation (○,●) and carnitine acetyltransferase (△,▲) activities. Each point represents the mean of duplicate rat liver slice cultures, and the results of a typical experiment are shown.

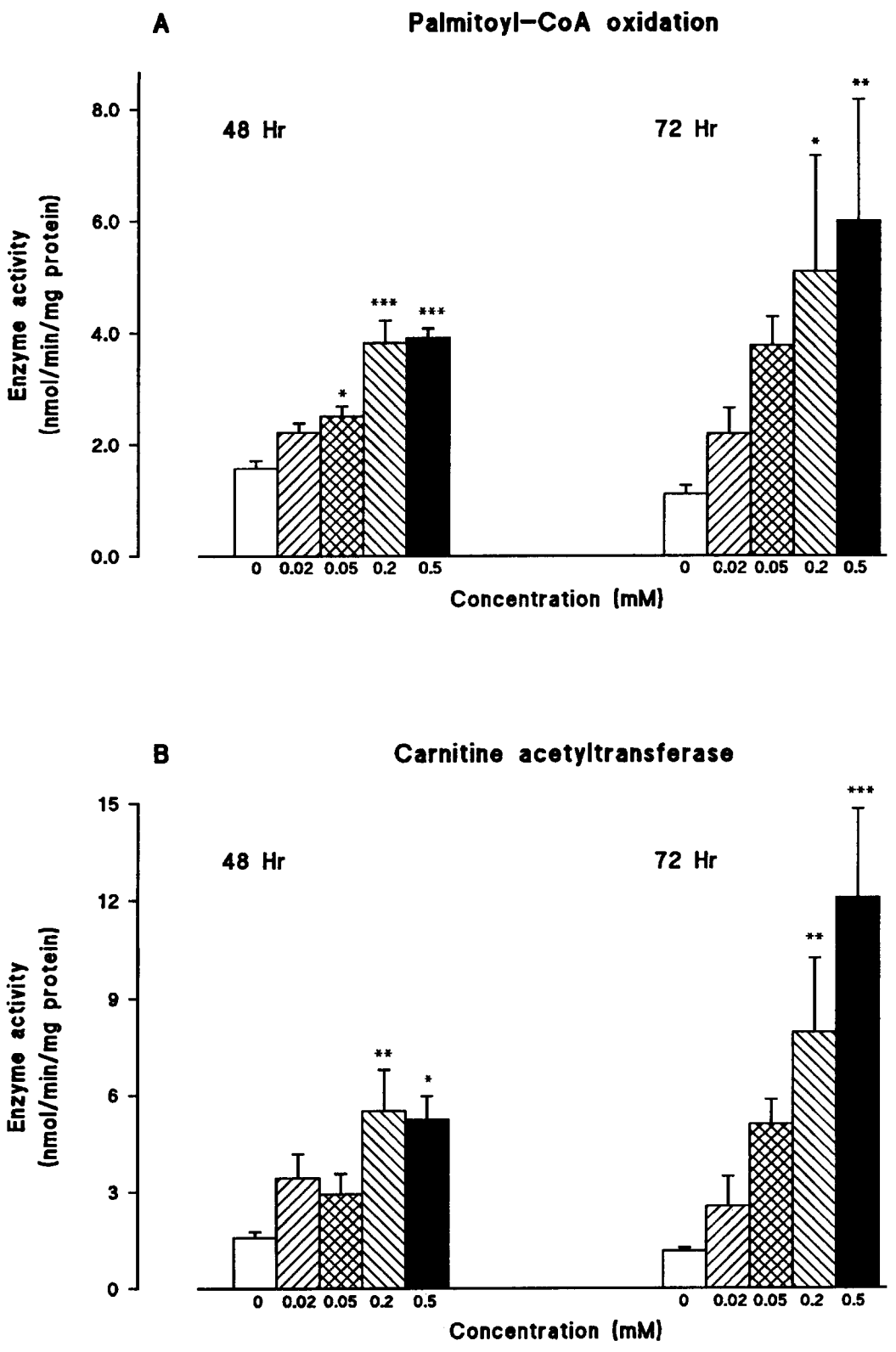

Fig. 3. Effect of culture of precision-cut rat liver slices in RPMI 1640 medium for 48 and 72 hr with 0–0.5 mM-nafenopin on cyanide-insensitive palmitoyl-CoA oxidation (A) and carnitine acetyltransferase (B) activities. Results are expressed as means ± SEM of three experiments. Asterisks indicate values significantly different from corresponding controls (DMSO only) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

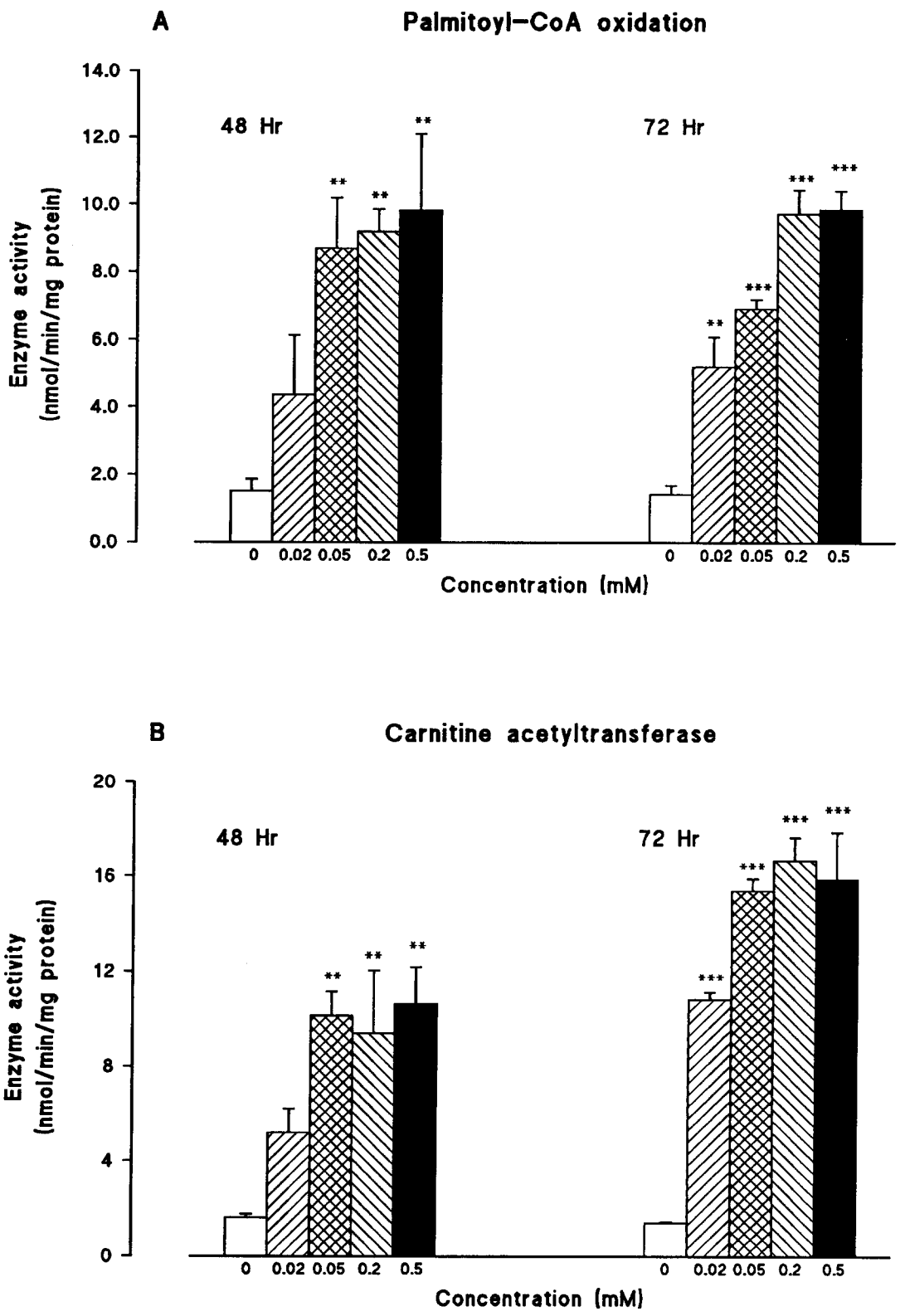

Fig. 4. Effect of culture of precision-cut rat liver slices in RPMI 1640 medium for 48 and 72 hr with 0–0.5 mM-ciprofibrate on cyanide-insensitive palmitoyl-CoA oxidation (A) and carnitine acetyltransferase (B) activities. Results are expressed as means ± SEM of three experiments. Asterisks indicate values significantly different from corresponding controls (DMSO only) (**$P < 0.01$; ***$P < 0.001$).

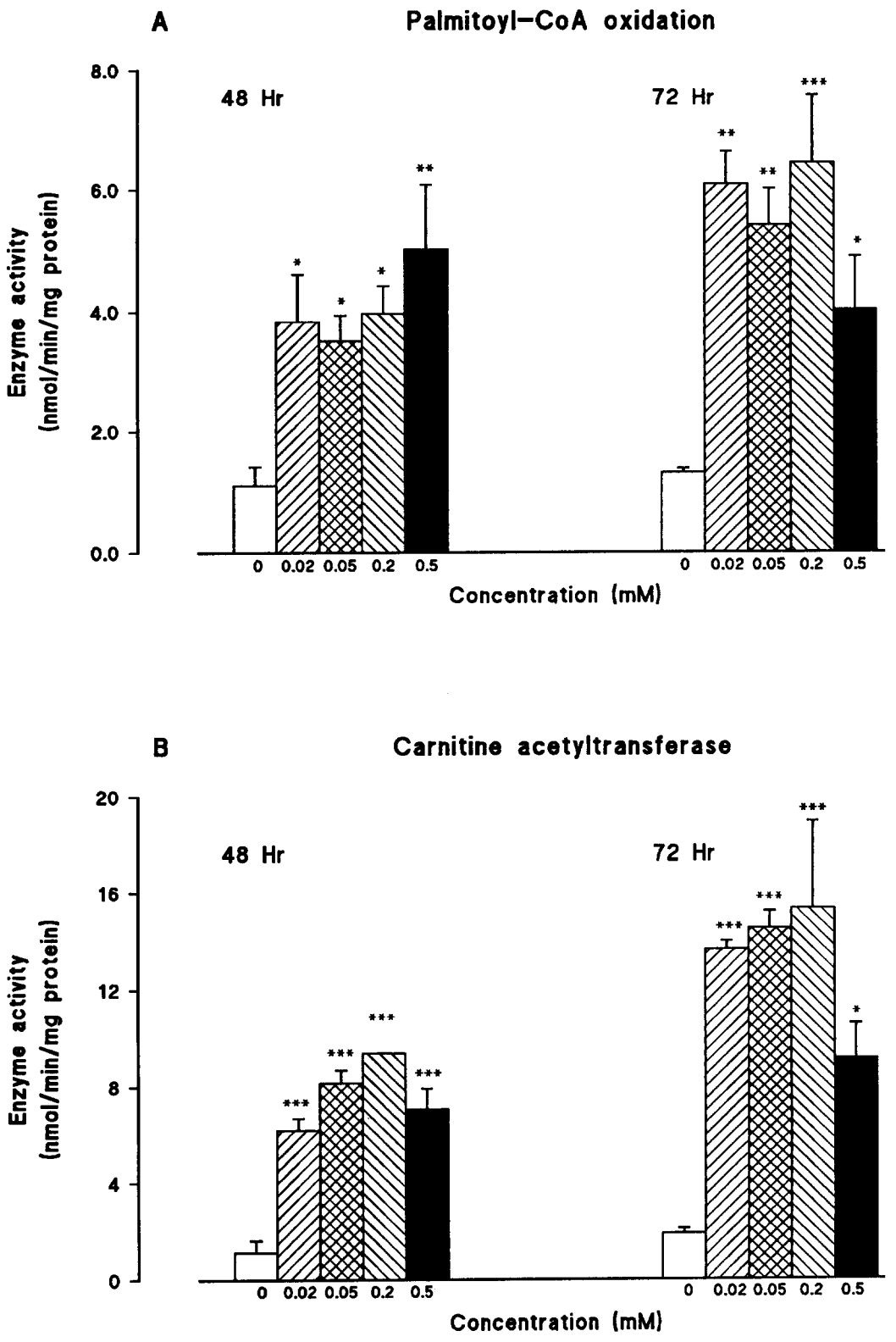

Fig. 5. Effect of culture of precision-cut rat liver slices in RPMI 1640 medium for 48 and 72 hr with 0-0.5 mM-Wy-14,643 on cyanide-insensitive palmitoyl-CoA oxidation (A) and carnitine acetyltransferase (B) activities. Results are expressed as means ± SEM of three experiments. Asterisks indicate values significantly different from corresponding controls (DMSO only) (*$P < 0.05$, **$P < 0.01$; ***$P < 0.001$).

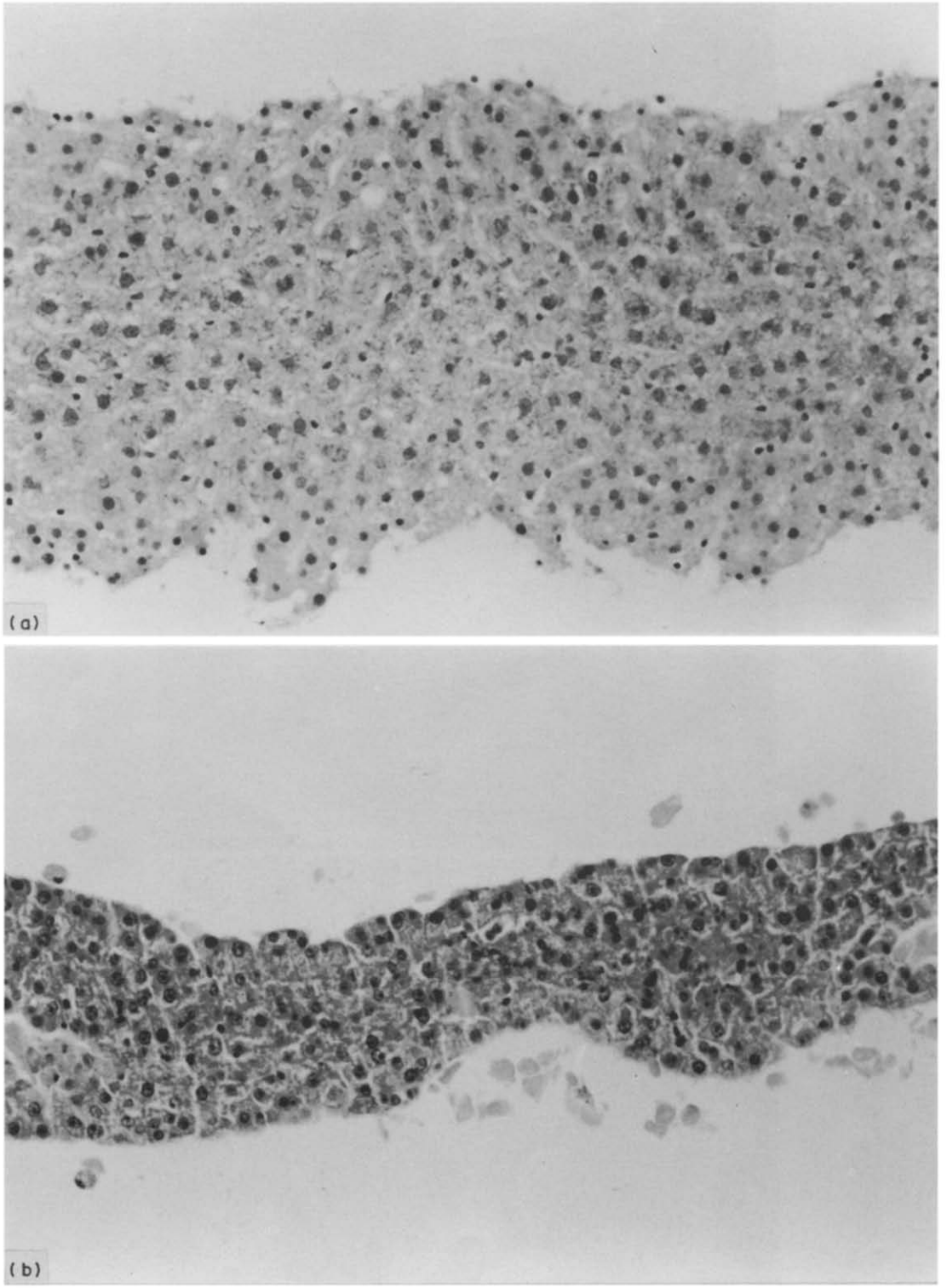

Plate 1. Representative sections of a freshly cut rat liver slice (a) and a rat liver slice cultured for 72 hr in RPMI 1640 medium containing 0.05 mM-nafenopin (b). Haematoxylin and eosin. Magnification ×610.

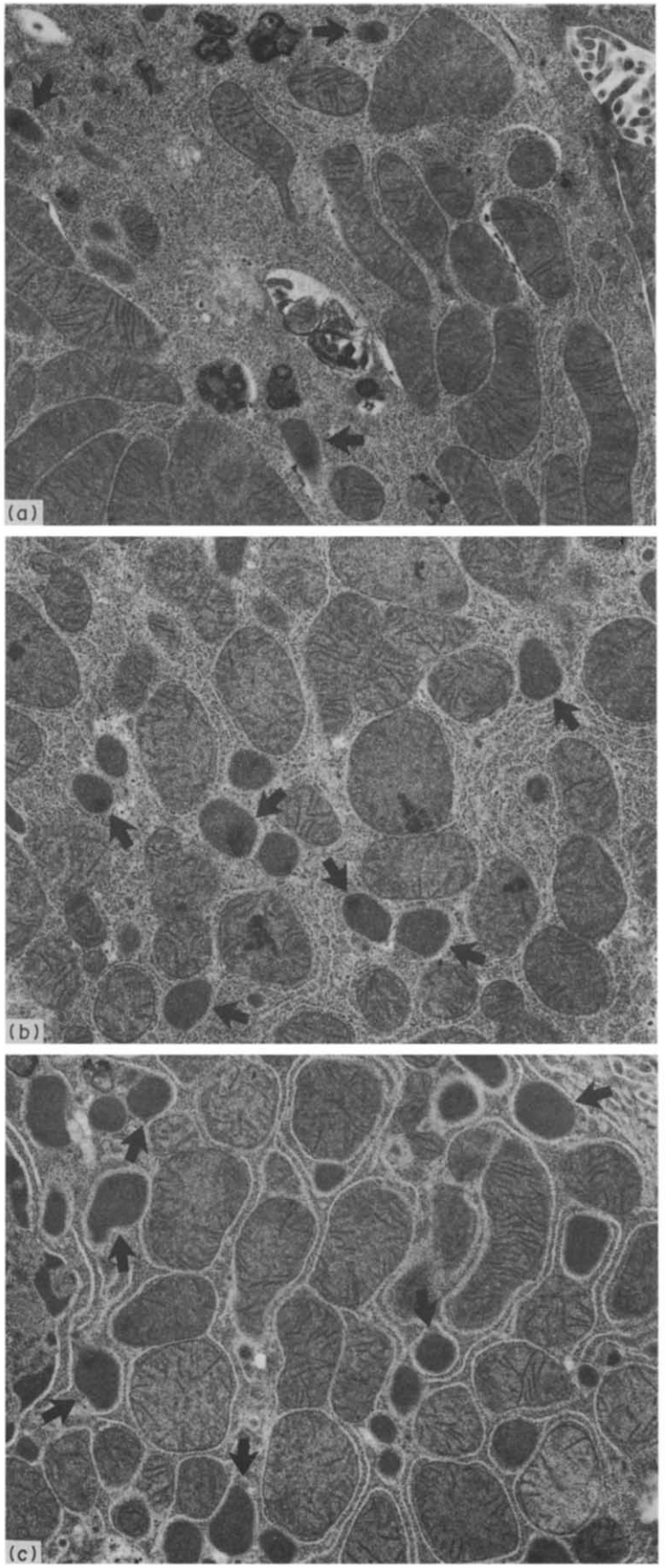

Plate 2. Representative electron micrographs of precision-cut rat liver slices cultured for 72 hr in RPMI 1640 medium. Compared with liver slices cultured in control (DMSO only) medium (a), the culture of liver slices in medium containing either 0.05 mM-ciprofibrate (b) or 0.05 mM-Wy-14,643 (c) results in an increase in the numbers of peroxisomes (arrows) present in hepatocytes. Magnification $\times$ 17,000.

contrast, both enzyme activities were detectable in control and peroxisome proliferator-treated cultures for up to 72 hr in either Williams Medium E (Fig. 2) or RPMI 1640 medium (Figs 3–5).

The activities of both palmitoyl-CoA oxidation and carnitine acetlyltransferase were found to decline in control (DMSO only) cultures. However, both enzyme activities were induced by the addition of 0.5 mM-clofibric acid with a clear stimulation of enzyme activities being observed after either 48 or 72 hr of treatment (Fig. 2). Subsequent studies were performed with rat liver slices cultured for 48 and 72 hr in RPMI 1640 medium containing 0.02–0.5 mM of either nafenopin (Fig. 3), ciprofibrate (Fig. 4) or Wy-14,643 (Fig. 5). None of the three peroxisome proliferators had any marked effect on slice protein content compared with either 48- or 72-hr control (DMSO only) cultures (data not shown). Control levels of palmitoyl-CoA oxidation and carnitine acetyltransferase activities ranged from 1.1 to 1.9 nmol/min/mg protein in 48- and 72-hr cultured slices (Figs 3–5) against 2.54 ± 0.19 (mean $\pm$ SEM of 11 experiments) and 2.35 ± 0.23 nmol/min/mg protein, respectively, in freshly cut slices.

All three peroxisome proliferators at some concentrations examined produced significant increases in both palmitoyl-CoA oxidation and carnitine acetyltransferase activities (Figs 3–5). Generally the induction of enzyme activities was both concentration and time dependent. Moreover, at the higher concentrations of all three peroxisome proliferators, levels of palmitoyl-CoA oxidation and carnitine acetyltransferase activities were greater than those observed in freshly cut slices. Ciprofibrate was found to produce the greatest induction of enzyme activities. After 72 hr of culture, ciprofibrate at concentrations of 0.2 and 0.5 mM maximally induced carnitine acetyltransferase to 1190% of control and palmitoyl-CoA oxidation to 700% of control, respectively (Fig. 4). Supplementing the glucose concentration of RPMI 1640 medium from 11 to 50 mM had no apparent effect on the induction of enzyme activities by either 0.05 mM-ciprofibrate or 0.05 mM-Wy-14,643 (data not shown).

Rat liver slices were cultured in RPMI 1640 medium containing either DMSO only (control), 0.05 mM-ciprofibrate or 0.05 mM-Wy-14,643 for 72 hr and processed for electron microscopy. For each treatment, photographs of 14 random areas of the processed liver slices were taken and numbers of peroxisomes counted. Compared with control (DMSO only) cultured liver slices (Plate 2a), the culture of slices with either 0.05 mM-ciprofibrate (Plate 2b) or 0.05 mM-Wy-14,643 (Plate 2c) was found to approximately double the numbers of peroxisomes in hepatocytes.

DISCUSSION

Previous studies with precision-cut rat liver slices have demonstrated the usefulness of this *in vitro* model system for investigating the metabolism and hepatotoxicity of a wide range of xenobiotics (Azri *et al.*, 1990a; Barr *et al.*, 1991a; Brendel *et al.*, 1987; Sipes *et al.*, 1987; Smith *et al.*, 1989). In the present study we have demonstrated that precision-cut rat liver slices may be maintained in culture for periods of at least 72 hr.

To maintain rat liver slices for up to 72 hr, a dynamic organ-culture system (Brendel *et al.*, 1987; Smith *et al.*, 1985, 1986 and 1989) was used. Previous studies have demonstrated that rat liver slices deteriorate rapidly when cultured in a static system, but remain viable for at least 24 hr when maintained in a dynamic organ-culture system (Brendel *et al.*, 1987; Smith *et al.*, 1985, 1986 and 1989; Trowell, 1959; Wright and Paine, 1992). However, the selection of a dynamic organ-culture system is clearly not the only prerequisite for maintenance of viable slices. In this study three tissue culture media, which had previously been used for studies with primary rat hepatocyte cultures (Gray *et al.*, 1983a; Mitchell *et al.*, 1984; Paine, 1990; Wright and Paine, 1992), were compared. While both RPMI 1640 and Williams Medium E proved suitable for culture of rat liver slices, Leibovitz's L-15 medium was clearly unsatisfactory for maintaining viable slices. In some investigations reported by other workers, media containing either 25 or 50 mM-glucose have been used for studies with rat liver slices (Fisher *et al.*, 1990 and 1991a; Smith *et al.*, 1986). However, in the present study, increasing the glucose concentration of RPMI 1640 medium to 50 mM did not appear to enhance slice viability, as assessed either by morphological examination or by induction of peroxisomal enzyme activities. Further studies are required to establish the optimal tissue culture medium and additions for maintenance of viable rat liver slice cultures.

As a measure of functional viability of the cultured rat liver slices, their response to some known peroxisome proliferators was investigated. In keeping with previous studies with primary rat hepatocyte cultures (Gray *et al.*, 1983b; Lake *et al.*, 1984; Mitchell *et al.*, 1984), the activities of palmitoyl-CoA oxidation and carnitine acetyltransferase declined in rat liver slices cultured in control media. However, a decline in enzyme activities, which has also been observed for cytochrome *P*-450 levels in cultured rat liver slices (Wright and Paine, 1992), does not necessarily preclude their use for enzyme induction studies. Indeed in the present study, in keeping with previous results for rat hepatocyte cultures (Gray *et al.*, 1982 and 1983a,b; Lake *et al.*, 1984; Mitchell *et al.*, 1984), four peroxisome proliferators induced both palmitoyl-CoA oxidation and carnitine acetyltransferase activities in rat liver slices. Furthermore, enzyme induction was not due to an apparent maintenance of enzyme activities, since at the higher concentrations of the test compounds the induced levels of palmitoyl-CoA oxidation and carnitine acetyltransferase activities were greater than those observed in freshly cut slices.

Although a comparison of the response to peroxisome proliferators of rat liver slices and rat hepatocyte cultures was not performed in this study, the present results suggest that somewhat higher concentrations of the test chemicals are required to induce enzyme activities in liver slices. One explanation would be that in hepatocyte monolayer cultures all cells are exposed to the medium and hence to the test chemical, whereas in liver slices only a proportion of the cells are directly exposed to the medium.

The limited ultrastructural studies confirmed the biochemical findings that peroxisome proliferation could be produced *in vitro* in rat liver slices. However, further studies using morphometric analysis would be required to confirm and extend the present findings. Indeed, one possible application of cultured rat liver slices would be to use them for both biochemical and morphological studies of the zonal effects of chemicals in hepatocytes across the liver lobule. For example, a lobular distribution of *in vivo* induction of markers of peroxisome proliferation in rat liver has previously been reported (Bell *et al.*, 1991) and examples of zonal differences in xenobiotic-induced toxicity in liver slices have also been described (Azri *et al.*, 1990a,b and 1992; Azri-Meehan *et al.* 1992; Smith *et al.* 1987).

In summary, precision-cut rat liver slices may be cultured for up to 72 hr in suitable media and appear to retain functional viability in that they respond to some known rodent peroxisome proliferators (Lock *et al.*, 1989; Moody *et al.*, 1991). Thus precision-cut liver slices, like primary hepatocyte cultures, would appear to be a potentially valuable *in vitro* model system for screening chemicals for effects on various enzyme activities and for assessing species differences in response. Such studies are currently being conducted in our laboratory.

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