Biochemical Pharmacology, Vol. 29, pp. 2117–2124.

0006-2952/80/0801-2117 $02.00/0

PRIMARY CULTURES OF ADULT MOUSE AND RAT HEPATOCYTES FOR STUDYING THE METABOLISM OF FOREIGN CHEMICALS*

KATHLEEN K. DOUGHERTY, STANLEY D. SPILMAN, CAROL E. GREEN, A. RUTH STEWARD and JAMES L. BYARD

Department of Environmental Toxicology, University of California, Davis, CA 95616, U.S.A.

(*Received* 10 *March* 1979; *accepted* 21 *January* 1980)

Abstract—A primary hepatocyte culture was developed as a model system to investigate the metabolism of foreign chemicals. Hepatocytes were prepared from adult male Charles River CD-1 mice and adult male Sprague–Dawley rats by *in situ* pre-perfusion of the liver with ethyleneglycol-bis-(β-amino-ethyl ether) *N*, *N'*-tetra-acetic acid (EGTA) followed by perfusion with calcium and collagenase. The digested liver was dispersed, and hepatocytes were isolated by filtration and differential centrifugation yielding 10^8 hepatocytes per mouse liver and 5×10^8 hepatocytes per rat liver. More than 90 per cent of the hepatocytes excluded trypan blue. Hepatocytes were prepared aseptically, plated on tissue culture dishes coated with rodent tail collagen (2.5×10^6 cells/60 mm dish), and cultured in serum-free modified Waymouth's medium. Within 4 hr the hepatocytes attached to the collagen, and by 24 hr they had flattened and formed a monolayer. A non-metabolizable alanine analog, α-aminoisobutyric acid, accumulated in mouse hepatocytes with peak incorporation occurring at 24 hr. Cultured mouse and rat hepatocytes were able to *N*-demethylate para-chloro-*N*-methylaniline (PCMA). An NADPH-generating system stimulated *N*-demethylation 2.75-fold in freshly isolated mouse hepatocytes, but did not stimulate metabolism in cultured mouse hepatocytes. SKF 525-A inhibited PCMA *N*-demethylation in cultured mouse hepatocytes with an I_{50} of 3.75×10^{-5} M. Hormonal supplementation of the culture medium stimulated PCMA metabolism measured in 24- and 48-hr cultures. These studies demonstrate the utility of rodent hepatocyte cultures as models of hepatic metabolism of foreign chemicals.

The metabolism of foreign chemicals has been studied *in vivo* with perfusion systems, organ slices, cells, homogenates, subcellular fractions and isolated enzymes. The study of metabolism in primary cell cultures is advantageous for two reasons: the culture medium can be completely defined, and the cultures more closely approximate *in vivo* conditions, compared to homogenates or subcellular fractions, while still allowing replicate experiments from one animal. Since the liver is the major site of metabolism of most foreign chemicals, liver cell cultures offer a good model system for the study of the hepatic metabolism of foreign chemicals.

The collagenase perfusion method has been widely used to isolate adult rat [1–5] and mouse [6] hepatocytes. The primary culture of rat hepatocytes has been reported from several laboratories [7–12]. Both freshly isolated and cultured hepatocytes have been used to study various hepatic functions including reactions catalyzed by mixed function oxidases (MFO)†, probably the most important enzyme activity for the metabolism of foreign chemicals. Several reactions catalyzed by MFO have been studied with suspensions of freshly isolated adult rat hepatocytes. Hayes and Brendel [13] observed the hydroxylation of quinine and the *N*-demethylation of antipyrine and dansylamide. Holtzman *et al.* [14] reported the oxidation of ethylmorphine, aniline and 3,4-benzo[*a*]pyrene. Burke *et al.* [15] reported the metabolism of benzo[*a*]pyrene to organic soluble and aqueous soluble metabolites and the effect of inhibitors on the formation of benzo[*a*]pyrene metabolites that bind to DNA. Bock *et al.* [16] found that naphthylene was converted to naphthalene dihydrodiol glucuronide. Moldéus *et al.* [17] observed the metabolism of alprenolol to polar metabolites, and the metabolism of *p*-nitroanisole and *p*-nitrophenol [18]. Corona *et al.* [19] used freshly isolated rabbit hepatocytes to study the metabolism of amitryptiline to its basic metabolites. Hepatocytes isolated from adult rhesus monkeys, and cultured in medium containing 10% calf serum, were shown by Poole and Urwin [20] to convert nicotine to cotinine. Leffert *et al.* [21] showed that 2-acetylaminofluorene was *N*-hydroxylated by adult rat liver cells cultured with fetal bovine serum. Decad *et al.* [22], using rat hepatocytes cultured in a defined medium, demonstrated the conversion of aflatoxin B_1 to aflatoxin

* This work was supported in part by the National Institute of Environmental Health Sciences Training Grant (ES00125). It was also supported in part by Hatch and State Critical Research Funds (CA-D-ETX-3395-H/CA-D-ETX-3650-H). A preliminary report of these findings was presented at the Annual Meeting of the Society of Toxicology, Toronto, Canada, March 1977, and San Francisco, CA, March 1978.

† Abbreviations used are as follows: MFO, mixed function oxidases; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulfonic acid; BSA, bovine serum albumin; EGTA, ethyleneglycol-bis-(β-amino ethyl ether) *N*,*N'*-tetra-acetic acid; HBSS, Hanks balanced salt solution; PBS, phosphate-buffered saline; AIB, α-aminoisobutyric acid; 3-MG, 3-*O*-methyl-D-glucose; PCMA, para-chloro-*N*-methylaniline; PDAB, paradimethylaminobenzaldehyde; PCA, para-chloroaniline; and SDS, sodium dodecylsulfate.

M_1, polar metabolites, and metabolites covalently bound to macromolecules.

To investigate MFO catalyzed reactions and their induction at the cellular level, a technique was developed for the isolation and primary culture of mouse and rat hepatocytes. The *N*-demethylation of PCMA was used to assess the utility of hepatocyte cultures as a model for hepatic metabolism of foreign chemicals and as a model for the induction of this metabolism.

MATERIALS AND METHODS

Animals. Adult male CD-1 mice, 4 weeks post weaning, were obtained from Charles River Breeding Laboratories (Charles River, MA). Adult male Sprague–Dawley rats, 180–210 g, were obtained from Simonsen Laboratories (Gilroy, CA). The animals were housed in temperature- and light-controlled rooms prior to use. The light–dark cycle was maintained at 12 hr light and 12 hr dark. Food (Purina Lab Chow, Ralston Purina Co., Saint Louis, MO) and water were allowed *ad lib.*

Chemicals. PDAB (B grade), PCMA (A grade), PCA (A grade), and HEPES were obtained from CalBiochem (San Diego, CA). Pentex BSA, fraction V, fatty acid poor, was obtained from Miles Laboratories (Kankakee, IL). Waymouth's medium 752/1 and Swim's medium S-77 were obtained as powdered formulations from the Grand Island Biological Co. (Grand Island, NY). Gentamicin sulfate was obtained from the Schering Corp. (Kenilworth, NJ). Insulin U-40 was obtained from Eli Lilly & Co. (Indianapolis, IN). [1-^{14}C]-A1B (2–10 mCi/mmole) and 3-*O*-[^{14}C]MG (20–55 mCi/mmole) were obtained from the New England Nuclear Corp. (Boston, MA). All other biochemicals were obtained from the Sigma Chemical Co. (Saint Louis, MO).

Perfusates and culture medium. EGTA perfusate consisted of 100 ml HBSS [23] (10× without NaCl), 5.945 g NaCl, 11.9 g HEPES, 350 mg $NaHCO_3$, 100 mg streptomycin sulfate, 2 ml gentamicin sulfate (50 mg/ml), 0.125 ml insulin (40 units/ml), 190 mg sodium EGTA, and H_2O (distilled, deionized) to 1 liter. Collagenase perfusate consisted of 100 ml HBSS (10× without NaCl), 5.945 g NaCl, 11.9 g HEPES, 350 mg $NaHCO_3$, 100 mg streptomycin sulphate, 2 ml gentamicin sulfate (50 mg/ml), 0.125 ml insulin (40 units/ml), 735 mg $CaCl_2 \cdot 2H_2O$, and H_2O to 1 liter. The working collagenase solution was prepared by dissolving 75 mg collagenase in 150 ml of collagenase perfusate immediately before use. The culture medium contained Waymouth's medium 752/1 supplemented with 2 g BSA, 5 mg oleic acid, 5 mg linoleic acid, 11.2 mg alanine, 24 mg asparagine, 12.8 mg serine, 8 mg insulin, 100 mg streptomycin sulfate, 100 mg gentamicin sulfate, 2.24 g $NaHCO_3$ and H_2O to 1 liter. Perfusates and culture medium were equilibrated with air: CO_2 (95% : 5%) at 37° before adjusting the pH to 7.4 with NaOH. Perfusates and culture medium were sterilized by filtration through a 0.2 μm membrane filter and were stored at 4° until ready for use.

Collagen. Collagen was extracted from mouse and rat tail tendons according to Elsdale and Bard [24] and Wood and Keech [25] as modified by Pariza *et al.* [11, 12]. The collagen was sterilized by centrifugation at 17,000 *g* for 21 hr. Protein was determined by the method of Lowry *et al.* [26] using calf skin collagen as a standard. Collagen coated dishes were prepared according to the method of Pariza* by pipetting 2.0 ml of Swim's medium S-77, pH 7.4, into a Falcon tissue culture dish (60 mm), and then adding 100 μg collagen previously diluted to 0.5 mg/ml with 1 : 10 Swim's medium S-77, pH 4.2. The dishes were swirled to mix the solutions, allowed to stand for 4 hr at room temperature, and incubated at 37° overnight before use.

Preparation of parenchymal cells. Cells were isolated by a two-step perfusion based on methods developed by Seglen [3–5] and Bonney *et al.* [8, 10]. Animals were anesthetized with ether. The abdomen was shaved, washed with pHisoHex (Winthrop Laboratories, New York, NY), rinsed with water, swabbed with Betadine (The Purdue Frederick Co., Norwalk, CT), and rinsed with 95% ethanol. The animal was moved to a sterile hood. All procedures from this point on were carried out under sterile conditions. The abdomen was opened with a midline and two lateral incisions. The portal vein was exposed and cannulated with an 18-gauge needle for the rat and a 19-gauge needle for the mouse. The abdominal inferior vena cava was severed and EGTA perfusate at 37° was pumped through the liver. A flow rate of 8 ml/min for the mouse and 50 ml/min for the rat was maintained by means of a peristaltic pump. As soon as the liver began to clear of blood, the chest cavity was opened and the thoracic inferior vena cava was severed. After a 7-min pre-perfusion, EGTA perfusate was replaced by collagenase perfusate and the perfusion continued until the liver was digested (about 20 min for rat and mouse). In the mouse, this procedure was also carried out by perfusing the liver through the abdominal inferior vena cava, although the yield of hepatocytes was less by this method. In the rat, it was necessary to recirculate the collagenase perfusate to conserve collagenase; the liver was removed from the body after pre-perfusion with EGTA perfusate and suspended on a wire screen in a funnel. The collagenase perfusate was pumped through the liver and collected in a flask by way of the funnel. The perfusate in the flask was constantly gassed with CO_2 : O_2 (5% : 95%) and recirculated to the liver. Following digestion, the liver was transferred to a plastic centrifuge tube containing approximately 20 ml of ice-cold EGTA perfusate and was drawn slowly into and out of a wide bore Pasteur pipette until the cells were dispersed. The suspension was filtered through 263 μm mesh, and then through 64 μm mesh nylon bolting cloth. The resulting filtrate was centrifuged twice for 2 min at 50 *g*. After each centrifugation, the supernatant fraction was aspirated with a Pasteur pipette and the pellet was resuspended in ice-cold EGTA perfusate by gentle swirling. Following the second centrifugation, an aliquot of the hepatocytes was counted using a hemocytometer, and the viability was determined by trypan blue exclusion (0.4% solution, pH 7.4, 10-min incubation). The remaining

* Michael W. Pariza, Food Research Institute, University of Wisconsin, Madison, WI, personal communication.

hepatocyte suspension was centrifuged at 50 *g* for 2 min and the resulting pellet was suspended in culture medium. The hepatocytes were plated on tissue culture dishes precoated with rodent tail collagen at a density of 2.5×10^6 cells/60 mm dish or 20×10^6 cells/150 mm dish. Cells were incubated at 37° in an atmosphere of air: CO_2 (95% : 5%). The medium, with or without hormones, was changed 4 hr after plating, 24 hr after plating, and every 24 hr thereafter.

AIB uptake. The method of Kletzien *et al.* [27] was used to measure the accumulation of the non-metabolizable alanine analogue, AIB, in mouse hepatocytes. The culture medium was removed from 60 mm dishes of cells after 10.5, 22.5, 34.5 and 46.5 hr in culture. HBSS (2 ml) containing 8 mM glucose, 0.02 M HEPES, pH 7.4 (HBSS–HEPES-glucose) and 0.5 mM [^{14}C]AIB (0.1 to 0.2 μmCi/ml) was added to the cells. The cells were incubated for 3 hr at 37° in an atmosphere of air : CO_2 (95% : 5%) without shaking. After removing the medium and rinsing with ice-cold PBS, 1 ml of alkaline SDS (5% SDS plus 0.2 N NaOH) was added to each dish. Cells were then removed from the dish by scraping with a tygon policeman. Aliquots were taken for protein determination and for scintillation counting. Protein was determined by the method of Lowry *et al.* [26], using BSA as a standard. Aliquots were added to Patterson–Greene scintillation fluid [28] and assayed for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer. To determine the intracellular concentration of AIB, the amount of intracellular water was determined by measuring the uptake of non-metabolized 3-MG by the method of Kletzien *et al.* [29]. The distribution ratio was then calculated as the ratio of intracellular to extracellular AIB concentration. To determine AIB uptake by freshly isolated mouse hepatocytes, the above procedure was modified as follows: freshly prepared cells (6–8 × 10^6) were rinsed with ice-cold HBSS–HEPES–glucose, and centrifuged at 50 *g* for 2 min; then the wash medium was removed by aspiration. Six milliliters of HBSS–HEPES–glucose containing 0.5 mM [^{14}C]AIB (0.2 μCi/ml) were added. Two-ml portions of this suspension of cells were added to 60 mm tissue culture dishes (without collagen) and incubated at 37° for 30 min. The cells (which did not attach to the dishes) were aspirated into test tubes and kept on ice. Aliquots were taken for protein determination and for scintillation counting. The cells to be assayed for radioactivity were sedimented by centrifugation (50 *g* for 2 min). The medium was aspirated, and the cells were transferred to a Millipore filter (1.2 μm pores), and immediately rinsed six times with 2 ml of cold PBS. The filter and cells were added to a scintillation vial along with 1 ml of water and 10 ml of Patterson–Greene scintillation fluid. The experiment was completed within 3 hr following isolation of the cells.

Determination of N-*demethylase activity.* The procedure for measuring *N*-demethylation of PCMA, described previously by Kupfer and Bruggerman [30], was modified for use in hepatocyte cultures as follows: The medium was aspirated, and the hepatocytes were washed with 2 ml HBSS. The cells were then incubated with 3 μmoles PCMA in 2.0 ml HBSS in air : CO_2 (95% : 5%) at 37°. At the end of the incubation period the incubate was aspirated to a test tube. The cells were washed twice with 1 ml HBSS, and the combined washes were added to the original incubate. The washed cells were resuspended (using a rubber policeman) in 0.5 ml HBSS for DNA determination. In some experiments, 60 mM HEPES was added to HBSS (HBSS–HEPES) to increase buffering capacity. PDAB, in sulfuric acid, was added to the combined incubate and washes to convert the metabolite, PCA, to a yellow complex. The solution was centrifuged at 8000 *g* for 10 min, the supernatant fraction was removed, and its absorbance was measured at 445 nm. The micromoles of PCA formed were determined using a standard curve.

Inhibition of PCMA N-*demethylation by SKF-525A.* Mouse hepatocytes cultured for 24 hr were washed with 2 ml HBSS and incubated for 1 hr with 2 ml HBSS or 2 ml HBSS containing 10^{-3}, 10^{-4} or 10^{-5} M SKF-525A. After a 1-hr incubation, the HBSS solutions were aspirated and the cultures were washed with 2 ml HBSS. Determination of *N*-demethylase activity was carried out as described above.

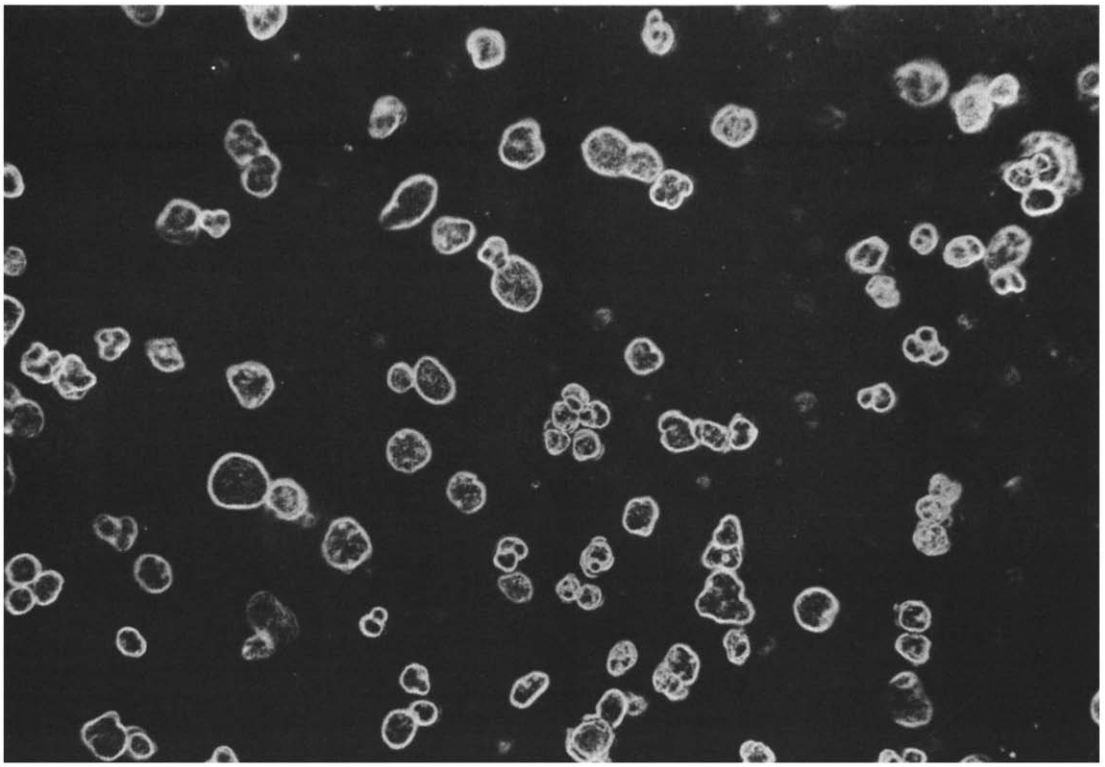
DNA determination. DNA was determined colorimetrically by the diphenylamine method [31].

RESULTS

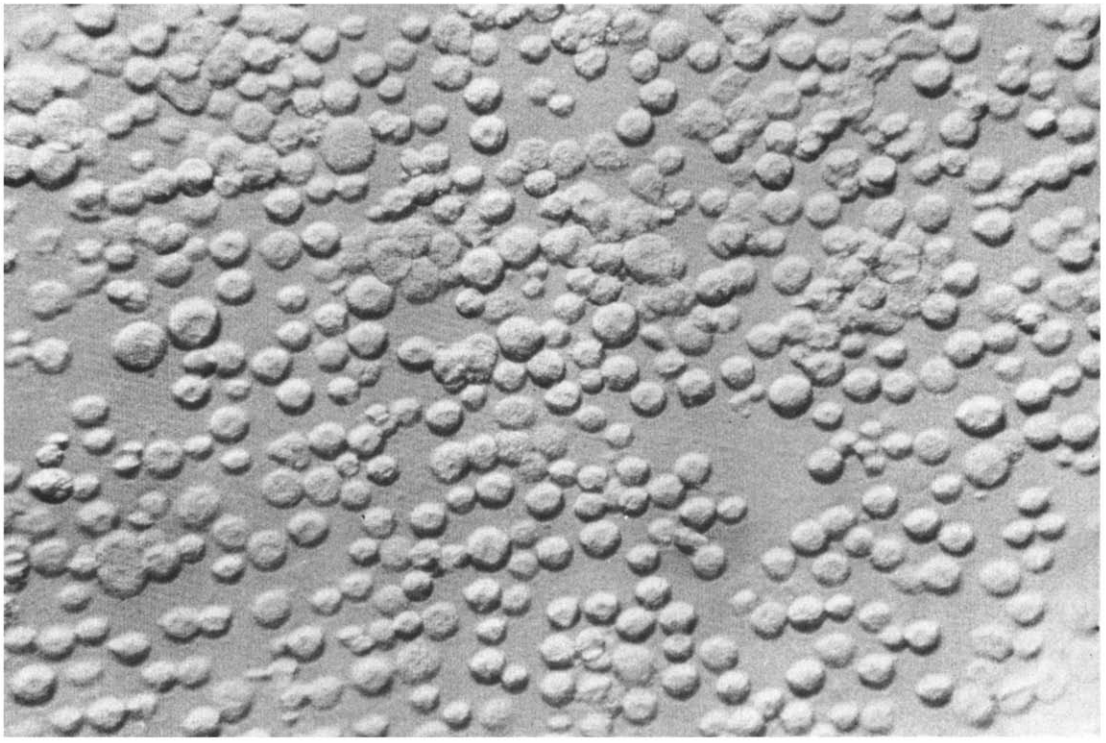
Isolation and culture of hepatocytes. Light microscopic observation revealed that freshly isolated cells were more than 99 per cent hepatocytes (Fig. 1A). The yield was approximately 10^8 hepatocytes per mouse liver and 5×10^8 hepatocytes per rat liver. The viability of freshly isolated hepatocytes was 90 per cent or greater. The hepatocytes attached firmly to rodent tail collagen within 4 hr (Fig. 1B) and flattened out until contact was made with neighboring cells, resulting in a monolayer (Fig. 1, panels C and D).

AIB uptake. The distribution ratio of AIB after a 30-min incubation with mouse hepatocytes cultured for 21 hr was 3.34 ± 0.07 (S.E.). The distribution ratio of AIB for freshly isolated hepatocytes from the same preparation was only 0.11 ± 0.02, suggesting that these cells had sustained membrane damage, so that any attempt to wash radioactivity from their surface resulted in the removal of AIB from the cytosol. AIB uptake for cells at various times in culture is shown in Fig. 2 (3-hr incubations). The maximum uptake occurred in hepatocytes cultured for 24 hr, producung a distribution ratio of 9.5.

PCMA metabolism. Two considerations guided the choice of a time in culture at which to carry out metabolic studies. First, freshly isolated cells were found to have leaky membranes. Whilst most freshly isolated hepatocytes excluded a large molecule such as trypan blue (mol. wt 961), they were leaky to a smaller molecule such as AIB (mol. wt 103). The AIB data indicate that by 24 hr in culture the membrane damage had been repaired (Fig. 2). The second consideration was the general deterioration of cul-

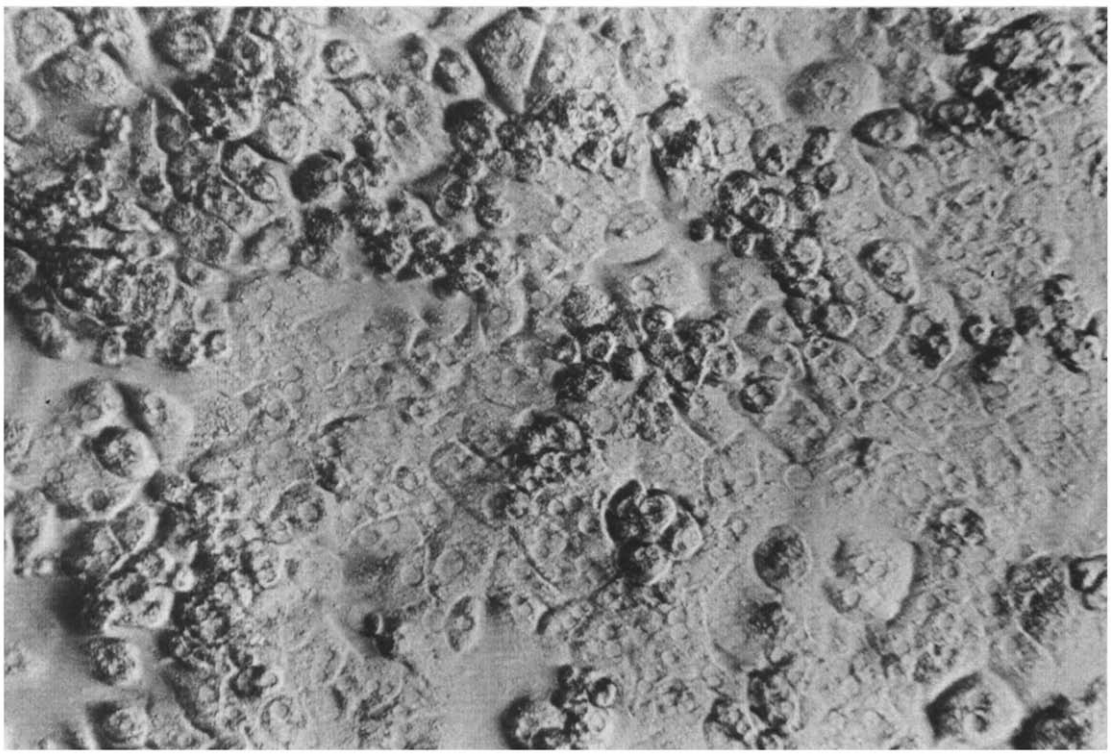


A



B

C



D

Fig. 1. Light microscopic photographs of hepatocytes. (A) Freshly isolated mouse hepatocytes (dark field illumination, 200 ×). (B) Mouse hepatocytes cultured for 4 hr (200 ×). (C) Mouse hepatocytes cultured for 24 hr (200 ×). (D) Rat hepatocytes cultured for 24 hr (200 ×).

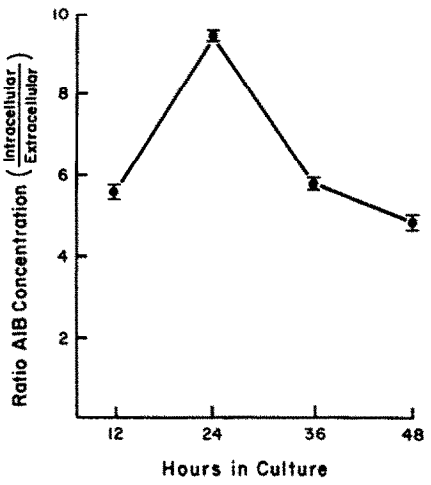


Fig. 2. Time course of α-aminoisobutyric acid uptake in cultured mouse hepatocytes. Each point (●) represents the mean ± S.E. of three dishes from one animal. The incubation time was 3 hr and the AIB concentration was 0.5 mM in Hanks–HEPES–glucose.

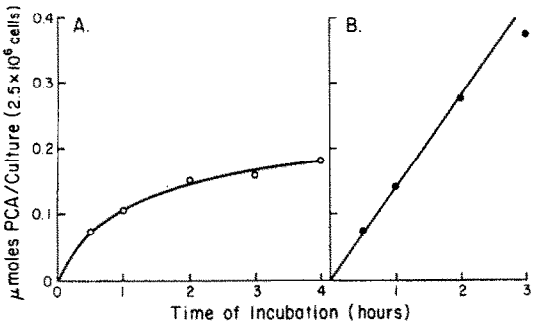


Fig 3. Time course of para-chloro-*N*-methylaniline demethylation in cultured mouse hepatocytes. (A) Incubation in HBSS. Each point (○) represents the mean value from two dishes from one animal. Values from duplicate dishes had a range between 5 and 20 per cent of the mean value. The PCMA concentration was 1.5 mM. (B) Incubation in HBSS–HEPES. Each point (●) represents the mean value of four dishes from one animal. The PCMA concentration was 1.5 mM.

tures with time as would be expected from cells which have been removed from the humoral factors which maintain the differentiated state *in vivo*. This deterioration was indicated by microscopic observation of the cells after 24 hr in culture (at 48 hr in culture the cells began to appear granular and show blebs on the surface of the plasma membranes), and also by the declining ability of the cells to concentrate AIB (Fig. 2). Using these data as a guideline, 24 hr was chosen as a compromise between intact membranes and the declining state of the cell.

Both rat and mouse hepatocyte cultures were able to metabolize PCMA to PCA in the absence of added cofactors (Table 1). The addition of an NADPH-generating system stimulated PCMA metabolism in freshly isolated mouse hepatocytes but did not stimulate PCMA metabolism in cultured mouse hepatocytes. This result, in agreement with AIB uptake data in freshly isolated versus cultured hepatocytes, indicates that freshly isolated cells are leaky while cultured hepatocytes are relatively more intact. All subsequent studies were done with cultured mouse hepatocytes.

Following the initial observations that PCMA *N*-demethylation could be measured colorimetrically in cultured hepatocytes, experiments were conducted to maximize the assay conditions. When HBSS was used as the incubation medium, the rate of metabolism of PCMA to PCA increased with time up to 4 hr (Fig. 3A); the rate, however, decreased with time of incubation. When HBSS-HEPES was used as the incubation medium, the rate of *N*-demethylation increased and was linear for up to 2 hr (Fig. 3B), presumably because the additional buffering capacity prevented acidification of the incubation medium. Using a 1-hr incubation with 2 ml HBSS, 3.0 μmoles of PCMA was found to be the optimum amount of substrate (Fig. 4).

To determine that PCMA was being metabolized by the hepatocytes and not by some unknown component of collagen or the incubation medium, a control experiment (results not shown) was conducted using collagen-coated culture dishes contain-

Table 1. *N*-Demethylation of PCMA in cultured rat and mouse hepatocytes

Species	Hepatocytes*	Cofactors†	(pmoles PCA‡/μg DNA·min)
Mouse	Freshly isolated	−	105 ± 22 (5)
		+	289 ± 18 (5)
	Cultured 24 hr	−	18 ± 5 (4)
		+	16 ± 2 (3)
Rat		−	32 ± 1 (3)

* Incubations with suspensions of freshly isolated hepatocytes (10^6 cells/dish) were carried out in 60 mm diameter plastic tissue culture dishes without collagen. Hepatocytes (2.5×10^6/dish) were cultured for 24 hr on 60 mm diameter plastic tissue culture dishes coated with rodent tail collagen.

† Cofactors were present during the 2-hr incubation with PCMA and included: NADP, 0.8 mM; glucose-6-phosphate, 8 mM; $MgCl_2$, 15 mM; and glucose-6-phosphate dehydrogenase, 1 unit per dish.

‡ Each incubation was for 2 hr at 37° in air : CO_2 (95% : 5%). PCMA concentration was 3 μmoles in 2 ml HBSS–HEPES. Average values are shown with the standard deviation and number of dishes assayed. DNA determinations were by the diphenylamine method [31].

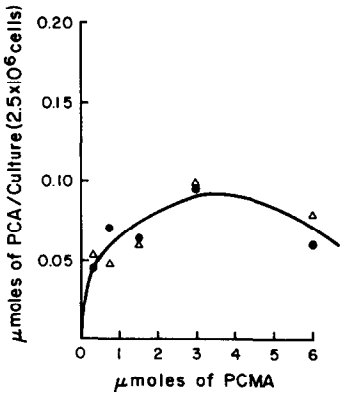


Fig. 4. Effect of substrate concentration on para-chloro-*N*-methylaniline demethylation in cultured mouse hepatocytes. The symbols (△) and (●) represent the mean values from two separate experiments (two dishes/point). Within each experiment, values from duplicate dishes had a range between 2 and 33 per cent of the mean value.

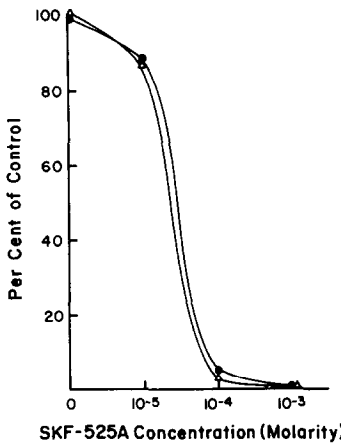


Fig. 5. Inhibition of para-chloro-*N*-methylaniline demethylation in cultured mouse hepatocytes pretreated with SKF-525A. The symbols (△) and (●) represent the mean values from two separate experiments (three dishes/point). Hepatocytes cultured for 24 hr were incubated for 1 hr with SKF-525A (10^{-3}, 10^{-4}, 10^{-5} M). The SKF-525A was washed from the dishes and 1.5 mM PCMA was incubated with each culture for 2 hr.

ing HBSS but no hepatocytes. Metabolism of PCMA did not occur in the absence of hepatocytes.

Experiments were conducted to determine if PCA was degraded or further metabolized under the conditions of the assay. After a 2-hr incubation of PCA with hepatocytes, approximately 92 per cent was recovered in the incubation medium while 4–5 per cent of the PCA was found unchanged in the cells. Further metabolism of PCA by conjugation was investigated by heating the aspirate with 1 N HCl for 5 min at 100°. No additional absorbance at 445 nm was produced after adding PDAB, indicating that no PCA was released from acid- and/or heat-hydrolyzable conjugates.

To determine if PCMA metabolism in cultured hepatocytes is mediated by MFO, experiments were conducted to examine the effect of SKF-525A, a known inhibitor of MFO catalyzed reactions, on the metabolism of PCMA to PCA. A dose-dependent inhibition of PCMA *N*-demethylation was observed in each of two experiments with mouse hepatocytes (Fig. 5), indicating that the *N*-demethylation of PCMA was mediated by MFO. The I_{50} for this inhibition was found to be 3.8×10^{-5} M.

Hormonal supplementation of the culture media stimulated the rate of *N*-demethylation of PCMA (Table 2). The difference in metabolism between hepatocytes cultured with and without hormones increased with the age of the culture (Table 2).

DISCUSSION

The isolation and culture of mouse and rat hepatocytes reported here are based on methods published by Seglen [3–5], Bonney *et al.* [8–10] and Pariza *et al.* [11, 12]. Perfusion through the portal vein with EGTA followed by collagenase was modified from the work of Seglen [3–5]. Our experience supports Seglen's findings that pre-perfusion with EGTA leads to a more complete digestion of the liver, permitting the use of less mechanical force to disperse the cells. Cell dispersion, filtration and centrifugation were adapted from the procedure developed by Bonney *et al.* [8–10]. These procedures resulted in a cell pellet which consisted of single cells or small groups of cells with high viability. The use of rodent tail collagen coated tissue culture dishes for culturing hepatocytes was adopted from methods modified by Pariza *et al.* [11, 12], and greatly facilitated cell attachment and formation of a monolayer in the absence of serum. The advantage of using serum-free medium in metabolism studies is that all the components of the culture are known, providing a more chemically defined cellular model for liver metabolism. The usefulness of freshly isolated,

Table 2. Effect of hormones on the *N*-demethylation of PCMA in primary cultures of mouse hepatocytes

Expt.	Age of culture (hr)	Hormones*	PCA† (pmoles/culture/min)
1	24	−	800 ± 200 (6)
		+	1700 ± 300‡(6)
2	24	−	1800 ± 400 (6)
		+	2300 ± 400§(5)
3	48	−	190 ± 70 (5)
		+	1000 ± 60‡(3)
4	48	−	310 ± 40 (3)
		+	1030 ± 370‡(3)

* The following hormones were added to the culture media: D-thyroxine, 10^{-5} M; glucagon, 5×10^{-8} M; testosterone, 10^{-6} M; estradiol, 10^{-6} M; corticosterone, 10^{-5} M; and progesterone, 10^{-5} M. δ-Amino levulinic acid, 10^{-6} M, and vitamin E, 5 mg/ml, were also added.

† Each incubation was carried out for 2 hr at 37° in air : CO_2 (95% : 5%). PCMA concentration was 3 μmoles in 2 ml HBSS–HEPES. Each culture represents 2.5×10^6 plated cells. Average values are shown with the standard deviation and the number of dishes assayed.

‡ $P < 0.01$, Student's *t*-test.

§ $0.05 < P < 0.1$, Student's *t*-test.

compared to cultured, hepatocytes as cell models for liver metabolism rests with an understanding of the advantages and disadvantages of each method. Probably the major advantage of the use of freshly isolated hepatocytes is that they contain *in vivo* levels of MFO. Additional advantages are that the cell preparation is easier and faster, and bacterial contamination is not a consideration. One of the disadvantages of using freshly isolated hepatocytes is that they have been shown to be leaky to small molecules, such as AIB [27]; depleted of ATP [7]; and do not exhibit normal liver cell morphology, i.e. there are no cords of cells with tight junctions between adjacent cells and no bile cannuliculi. An advantage of studying metabolism in cultured, compared to freshly isolated, hepatocytes is that cultured hepatocytes morphologically more closely resemble hepatocytes *in vivo*. The cell membrane is intact, as indicated by the AIB uptake and PCMA metabolism data, and the cells have re-established many features of normal liver cell morphology, i.e. cells are attached to collagen substratum, flattened, and have formed bile cannuliculi and tight junctions between adjacent cells [11, 32, 33]. Added cofactors are not needed for optimal activity, and metabolic studies can be carried out non-destructively; solutions of chemicals can be added to the culture and removed without disrupting the cells. Cultured cells are firmly attached and hence the medium is more easily manipulated than with freshly isolated cells. For metabolic studies, a serious disadvantage of the culture method is that the cytochrome P-450 concentration decreases when cells are cultured in the absence of *in vivo* humoral control.

In rat hepatocyte cultures, cytochrome P-450 concentration has been reported to decrease to 10–20 per cent of control after 24 hr in culture [34], greatly limiting the usefulness of these cultures as a model for *in vivo* hepatic metabolism of foreign chemicals. In a previous report from this laboratory [22], *in vivo* levels of MFO had been maintained in rat hepatocyte cultures for 24 hr by hormonal supplementation of the culture medium. A similar decline in MFO was seen in mouse hepatocyte cultures. The addition of hormones (those known to affect MFO levels *in vivo*) maintained MFO activity at levels higher than those obtained in the absence of hormones. These results suggest that there is also an appropriate hormonal supplementation of the medium which will produce mouse hepatocyte cultures whose MFO activity reflects that of the liver *in vivo*.

REFERENCES

1. R. B. Howard, A. K. Christensen, F. A. Gibbs and L. A. Pesch, *J. Cell Biol.* **35**, 675 (1967).
2. M. N. Berry and D. S. Friend, *J. Cell Biol.* **43**, 506 (1969).
3. P. O. Seglen, *Expl. Cell Res.* **74**, 450 (1972).
4. P. O. Seglen, *Expl. Cell Res.* **76**, 25 (1973).
5. P. O. Seglen, *Expl. Cell Res.* **82**, 291 (1973).
6. D. M. Crisp and C. I. Pogson, *Biochem. J.* **126**, 1009 (1972).
7. D. M. Bissell, L. E. Hammaker and U. A. Meyer, *J. Cell Biol.* **59**, 722 (1973).
8. R. J. Bonney, P. R. Walker and V. R. Potter, *Biochem. J.* **136**, 947 (1973).
9. R. J. Bonney, J. E. Becker, P. R. Walker and V. R. Potter, *In Vitro* **9**, 399 (1974).
10. R. J. Bonney, *In Vitro* **10**, 130 (1974).
11. M. W. Pariza, J. E. Becker, J. D. Yager, Jr., R. J. Bonney and V. R. Potter, in *Differentiation and Control of Malignancy of Tumor Cells* (Eds. W. Nakahara, T. Ono, T. Sugimura and H. Sugene), p. 267. University of Tokyo Press, Tokyo (1974).
12. M. W. Pariza, J. D. Yager, Jr., S. Goldfarb, J. A. Gurr, S. Yanagi, S. H. Grossman, J. E. Becker, T. A. Barber and V. R. Potter, in *Gene Expression and Carcinogenesis in Cultured Lines* (Eds. L. E. Gerschenson and E. B. Thompson), p. 137. Academic Press, New York (1975).
13. J. S. Hayes and K. Brendel, *Biochem. Pharmac.* **25**, 1495 (1976).
14. J. L. Holtzman, V. Rothman and S. Margolis, *Biochem. Pharmac.* **21**, 581 (1972).
15. M. D. Burke, H. Vadi, B. Jernström and S. Orrenius, *J. biol. Chem.* **252**, 6424 (1977).
16. K. W. Bock, G. Van Ackeren, F. Lorch and F. W. Birke, *Biochem. Pharmac.* **25**, 2351 (1976).
17. P. Moldéus, R. Grundin, H. Vadi and S. Orrenius, *Eur. J. Biochem.* **46**, 351 (1974).
18. P. Moldéus, H. Vadi and M. Berggren, *Acta pharmac. tox.* **39**, 17 (1976).
19. G. L. Corona, G. Stanagostino, R. M. Facino and D. Pirillo, *Biochem. Pharmac.* **22**, 849 (1973).
20. A. Poole and C. Urwin, *Biochem. Pharmac.* **25**, 281 (1976).
21. H. L. Leffert, T. Moran, R. Boorstein and K. S. Koch, *Nature, Lond.* **267**, 58 (1977).
22. G. M. Decad, D. P. H. Hsieh and J. L. Byard, *Biochem. biophys. Res. Commun.* **78**, 279 (1977).
23. J. H. Hanks and R. E. Wallace, *Proc. Soc. exp. Biol. Med.* **71**, 196 (1949).
24. T. Elsdale and J. Bard, *J. Cell Biol.* **54**, 626 (1972).
25. G. C. Wood and M. K. Keech, *Biochem. J.* **75**, 588 (1960).
26. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
27. R. F. Kletzien, M. W. Pariza, J. E. Becker, V. R. Potter and F. R. Butcher, *J. biol. Chem.* **251**, 3014 (1976).
28. M. S. Patterson and R. C. Greene, *Analyt. Chem.* **37**, 854 (1965).
29. R. F. Kletzien, M. W. Pariza, J. E. Becker and V. R. Potter, *Analyt. Biochem.* **68**, 537 (1975).
30. D. Kupfer and L. L. Bruggeman, *Analyt. Biochem.* **17**, 502 (1966).
31. G. M. Richards, *Analyt. Biochem.* **57**, 369 (1974).
32. G. S. Chapman, A. L. Jones, U. A. Meyer and D. M. Bissell, *J. Cell Biol.* **59**, 735 (1973).
33. J. C. Wanson, P. Drochmans, R. Mosselmans, and M. F. Ronveaux, *J. Cell Biol.* **74**, 858, 1977.
34. D. M. Bissell, in *Progress in Liver Disease* (Eds. H. Popper and F. Schoffner), p. 69. Grune & Stratton, New York (1976).