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EFFECTS OF A HORMONE-SUPPLEMENTED MEDIUM ON CYTOCHROME P-450 CONTENT AND MONO-OXYGENASE ACTIVITIES OF RAT HEPATOCYTES IN PRIMARY CULTURE*

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Abstract—Cytochrome P-450 content and associated mono-oxygenation activities (7-ethoxycoumarin-*O*-deethylase, biphenyl-4-hydroxylase and 7-ethoxyresorufin-*O*-deethylase) of rat hepatocytes were found to decrease during the first 48 hr in primary culture in control (WOBA-M_2) medium. However, by culturing the hepatocytes in a hormone-supplemented medium (AB medium), all of these enzymes were maintained at higher levels after 12, 24 and 48 hr in culture. In particular, 7-ethoxyresorufin-*O*-deethylase activity was markedly enhanced after 12 and 24 hr in culture in AB medium to levels greater than that in isolated hepatocytes. Metabolic capacities of the cytochromes P-450 present in hepatocytes cultured in WOBA-M_2 medium vs AB medium were also quantitatively different at 12, 24 and 48 hr when specific activities/pmole of hemoprotein were compared. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) experiments further suggested that a cytochrome P-450-related protein was maintained to a greater extent in AB medium than in WOBA-M_2 medium. It is proposed that AB medium may maintain a higher cytochrome P-450 concentration in cultured primary rat hepatocytes by increasing both the rate of heme synthesis and the synthesis of a cytochrome P-450-related protein.

In recent years, a number of workers have successfully isolated rat liver parenchymal cells and developed the technique of primary hepatocyte culture [1–4]. Such cultures offer a number of advantages over isolated hepatocytes in that they can be maintained for several days under closely defined conditions, readily respond to a variety of hormonal stimuli [5,6], and reassociate with each other in monolayers, thus resembling their former *in situ* state [7, 8]. A major disadvatage of this culture system is that levels of cytochrome P-450, the terminal oxidase responsible for metabolism of many drugs, carcinogens and endogenous substrates, have been shown to fall dramatically after 24 hr in culture [9, 10]. A corresponding decrease in microsomal mono-oxygenation has also been observed [10, 11]. A number of workers, however, have shown that cytochrome P-450 can be maintained in primary hepatocyte culture by the inclusion of certain hormones in the culture medium [9, 12, 13].

Previous studies using sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) have demonstrated that a number of liver microsomal proteins of molecular weight 50,000–60,000 are derived from multiple forms of rat hepatic cytochrome P-450 [14, 15]. Recently, Fahl *et al.* [16] showed the presence of multiple forms of cytochrome P-450 in microsomes from hepatocytes in primary culture. These investigators found that a cytochrome P-450-related protein, that typically increased *in vivo* following treatment with 3-methylcholanthrene, was induced after the carcinogen was added to the medium of cultured rat hepatocytes [16]. An increase in the phenobarbital inducible protein was not detected in this system, despite an increase in the cytochrome P-450 content of the hepatocyte microsomes. This suggests that control of cytochrome P-450 synthesis in cultured hepatocytes may be different from that found *in vivo*.

Recently, Decad *et al.* [12] reported the maintenance of cytochrome P-450 in hepatocytes cultured for 24 hr in a hormone-supplemented medium and showed that the metabolic profile of a cytochrome P-450 substrate, aflatoxin B_1, is similar to that found *in vivo*. These results suggest that the cytochrome P-450 species present after 24 hr in culture in this medium may be similar to those found in rat liver *in vivo*. Other results [9], however, suggest that hydrocortisone, present in the supplemented medium of Decad *et al.* [12], preferentially maintains a form of cytochrome P-450 with a spectral absorbance maximum at 448 nm when reduced and complexed with carbon monoxide.

The objectives of the present study were to investigate the effects of the hormone-supplemented medium on cytochrome P-450 and the metabolic capacity of the hemoprotein(s) present after various times in culture, and to compare these values with those found using a conventional medium. The gel electrophoretic profiles of microsomal proteins isolated from cells cultured for different time periods in the two media were also investigated.

METHODS

Materials. Waymouths MB 752/1 medium was purchased from KC Biological Inc., Lenexa, KS; genta

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micin sulfate from the Schering Corp., Kenilworth, NJ; glucagon from Eli Lilly, Indianapolis, IN; and sodium pentobarbital from Abbot Laboratories, Chicago, IL. 7-Ethoxyresorufin was synthesized by Dr. S. R. Challand of the Wellcome Research Laboratories, Beckenham, U.K. All other chemicals or biochemicals were obtained from the Aldrich Chemical Co., Milwaukee, WI, or the Sigma Chemical Co., St. Louis, MO.

Animals. Male Holtzman rats (180–230 g) from the Holtzman Co., Madison, WI, were allowed food (Purina chow) and water *ad lib.* and were maintained on a 12-hr light/dark cycle (7:00 a.m.–7:00 p.m. light) at 22–24°. Rats were fasted overnight before use.

Preparation of isolated hepatocytes. The rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and strict aseptic technique was maintained throughout the following procedure. The abdomen was opened by a midline incision and the intestines were displaced to the left. The hepatic portal vein was cannulated with an 18 gauge catheter placement unit (Jelco Lab., Raritan, NJ), and perfusion of the liver *in situ* was initiated at a flow rate of 15 ml/min with a Masterflex pump (Cole Parmer, Chicago, IL). To allow escape of the blood and perfusate from the liver the inferior vena cava was cut. After about 100–150 ml of buffer had been perfused through the liver, the thoracic cavity was opened, the superior vena cava cannulated with a 14 gauge catheter placement unit, and the inferior vena cava ligated. This diverted the outflow of liver perfusate to the superior vena cava cannula which was returned to the perfusion bottle for recirculation through the liver of the animal. Throughout the entire procedure the perfusion buffer used was Ca^{2+}-free Hanks' balanced salt solution (pH 7.5) supplemented with insulin (10^{-7} M) and gentamicin sulfate (50 μg/ml). It was maintained at 37° and gassed continuously with 95% O_2 and 5% CO_2. After recirculation of the perfusion buffer was established, collagenase (Sigma type I, 60 mg dissolved in 5 ml of perfusion buffer) was added via a 0.45 μm filter (Millipore) to the bottle of recirculating perfusion buffer (about 100 ml), and perfusion was continued for 15–20 min by which time the liver had swollen. The liver was removed to a beaker containing warmed (37°) perfusion buffer (50 ml) to which collagenase had not been added, and the capsule was ruptured with sterilized scissors. Hepatocytes were released by gentle swirling of the liver and pipetting with a large bore pipette. The cell suspension was filtered through a 250 μm nylon mesh into a beaker placed on ice. The filtrate was transferred to ice-cold, sterile 50 ml centrifuge tubes (Falcon), and the hepatocytes were sedimented by centrifugation at 50 *g* for 4 min. The pellets were washed two to three times and the final sediment was resuspended in perfusion buffer (about 20 ml) and stored on ice until further use. The viability of the final cell suspension was assessed by mixing 100 μl of the suspension with 900 μl of 0.4% (w/v) Trypan Blue in 0.95% (w/v) NaCl and allowing the mixture to stand for 5 min on ice. A sample was transferred to a Neubauer counting chamber for determination of the viable and nonviable cells. The total number of parenchymal cells released from the liver was estimated in this manner.

Culture conditions. The cell suspension was diluted to 1.2×10^6 cells/ml in the appropriate medium (WOBA-M_2 or AB) and 3 ml were pipetted into 60 × 15 mm plastic Petri dishes (Falcon) precoated with 100 μg of rat tail collagen [8]. The WOBA-M_2 medium consisted of serum-free Waymouth's MB 752/1 medium [17] as modified by Pariza *et al.* [8]. AB medium consisted of the WOBA-M_2 medium plus the hormones and other supplements described by Decad *et al.* [12]. The compositions of the two media are compared in Table 1. After hepatocytes were inoculated into collagen-coated culture dishes, they were incubated at 37° in a humidified 5% CO_2/95% air incubator (Forma Scientific Co., Marietta, OH) for 4, 12, 24 or 48 hr, and the medium was changed after 4 and 24 hr.

Preparation of whole cell homogenates. All experiments were carried out using whole cell homogenates. After hepatocytes had been in primary culture

Table 1. Composition of WOBA-M_2 and AB media*

Constituent	Final concentration WOBA-M_2	AB
Waymouth's MB 752/1 powdered medium	14.05 mg/ml	14.05 mg/ml
Insulin	1.0×10^{-7} M	1.0×10^{-7} M
Albumin, bovine serum (fraction V)	2.0 mg/ml	2.0 mg/ml
Bicarbonate, sodium	2.67×10^{-2} M	2.67×10^{-2} M
Oleic acid, sodium	2.0×10^{-5} M	2.0×10^{-5} M
Gentamicin sulfate	50.0 μg/ml	50.0 μg/ml
L-Serine	5.32×10^{-4} M	1.22×10^{-4} M
L-Alanine	4.09×10^{-4} M	1.3×10^{-4} M
L-Asparagine		1.6×10^{-4} M
Linoleic acid, sodium		1.8×10^{-5} M
δ-Aminolevulinic acid, hydrochloride		1.0×10^{-6} M†
D,L-α-Tocopherol		5.0 μg/ml†
Hydrocortisone-21-acetate		1.0×10^{-5} M†
D-Thyroxine		1.0×10^{-5} M†
17-β-Estradiol		1.0×10^{-6} M†
Testosterone		1.0×10^{-6} M†

* Medium pH was adjusted to 7.4 before use.

† Added as a suspension in propylene glycol (4 ml/l AB medium) immediately before use.

for 4, 12, 24 or 48 hr, the medium (WOBA-M_2 or AB) was removed by aspiration and the attached hepatocytes were rinsed with 3 ml of ice-cold 66 mM Tris–HCl buffer (pH 7.4). After rinsing the cells, 1 ml of fresh Tris buffer was added to each culture dish, and the attached hepatocytes were scraped off with a rubber policeman. The cells and the buffer from four scraped plates (maintained under identical culture conditions) were transferred to the same centrifuge tube and the scrape–transfer process was repeated a second time. The scraped cells from four plates in 8 ml of buffer were pelleted by centrifugation at 1000 *g* for 10 min and buffer was removed. The pelleted cells were resuspended in 6 ml of fresh Tris buffer and pelleted a second time by centrifugation. The buffer was removed and the pellet was resuspended in 2 ml of 0.25 M sucrose and homogenized using a Potter–Elvehjem homogenizer (ten complete strokes). The homogenate was stored at −70° until use. Enzyme and cytochrome P-450 assays were carried out within 24 hr after whole cell homogenates were prepared.

Analytical methods. 7-Ethoxycoumarin-*O*-deethylase [18], 7-ethoxyresorufin-*O*-deethylase [19] and biphenyl-4-hydroxylase [20] were determined by published methods. For all assays, product formation was linear with respect to time and protein concentration. Cytochrome P-450 content was measured by the method of Omura and Sato [21]; concentrations below 60 pmoles/mg protein were determined using the 460–490 nm absorbance change and $\varepsilon = 48\ mM^{-1}\ cm^{-1}$ [22]. Protein was determined by the method of Lowry *et al.* [23] and DNA was quantitated as described by Richards [24].

Preparation of microsomes. Microsomes were prepared from rat liver, isolated hepatocytes and cultured hepatocytes (using the cells scraped from twenty plates for each microsomal preparation) by centrifugation of homogenates in 66 mM Tris–HCl (pH 7.4) at 15,000 *g* for 15 min. The supernatant fractions were centrifuged at 140,000 *g* for 60 min to obtain the microsomal pellets. Microsomes obtained from rat liver were resuspended in 66 mM Tris–HCl (pH 7.4) and recentrifuged at 140,000 *g* for 60 min. The final pellets were resuspended in 66 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose and 5.4 mM EDTA and stored at −70°.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE was carried out according to the methods of Laemmli and Favre [25]. The 3 mm thick separating gel contained 7.5% (w/v) acrylamide and the stacking gel 3%. The final SDS concentration was 0.1% (w/v), and 50–75 μg of microsomal protein were applied to each well. Electrophoresis was initiated at 10 mA current until the tracking dye had entered the separating gel and was maintained at 30 mA thereafter. When electrophoresis was complete the gels were stained with Coomassie Brilliant Blue R-250, destained, and scanned at 550 nm using an ISCO gel scanner accessory in combination with an ISCO type 6 optical unit and type UA-5 absorbance monitor.

RESULTS

Properties of isolated hepatocytes and primary hepatocyte cultures. The yields of isolated hepatocytes from rat livers perfused with collagenase (Table 2) were similar to those obtained by other workers [2, 12, 13, 26, 27], and viability of the cells was 83 per cent, as assessed by Trypan Blue exclusion. The mean DNA and protein content per cell were 19.7 pg and 1.13 ng respectively. Attachment efficiency, based on the DNA content per plate after 4 hr in culture, was routinely 50–60 per cent. Figure 1 shows that the amounts of protein and DNA on each plate after various times in culture were similar in both WOBA-M_2 and AB media. The levels of DNA remained constant throughout the 48-hr culture period, whereas the protein content showed a small decrease after 24 and 48 hr in both media.

Table 2. Yield, viability, DNA and protein content of isolated rat hepatocytes*

	Mean ± S.E.M.	Range
Yield of cells ($\times 10^6$)	528 ± 50	442–766
Viability of cells (%)	83 ± 5	78–92
DNA content (pg/cell)	19.7 ± 0.8	17.0–23.0
Protein content (ng/cell)	1.1 ± 0.1	0.8–1.4

* Results are for isolated hepatocyte preparations from six to eight rats. Hepatocytes from these same preparations were subsequently cultured in WOBA-M_2 and AB media.

Phase contrast microscopy of hepatocytes cultured for 24 hr (Fig. 2) revealed large areas of flattened cells which excluded Trypan Blue (> 80 per cent of the adherent cells after 24 hr). This was typical of hepatocytes cultured in both WOBA-M_2 and AB media. About 30 per cent of adult rat hepatocytes are binucleate [28] and a number of binucleated cells were readily visible in primary hepatocyte culture after 24 hr (Fig. 2). A number of cells (approximately 10 per cent of those attached) remained rounded and were attached to the surfaces of the flattened cells. Such cells were removed largely by changing

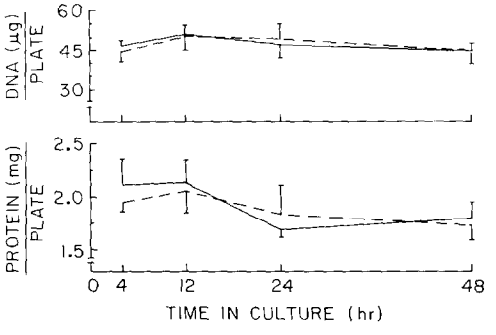

Fig. 1. Total DNA and protein contents of hepatocytes on a per plate basis after various times in culture. Each plate received 3.6×10^6 hepatocytes/3 ml of either WOBA-M_2 or AB medium and was assayed at 4, 12, 24 and 48 hr. All values are means ± S.E.M. of cultures from five rats. Key:(———) designates hepatocytes cultured in WOBA-M_2 medium, and (– – –) hepatocytes cultured in AB medium.

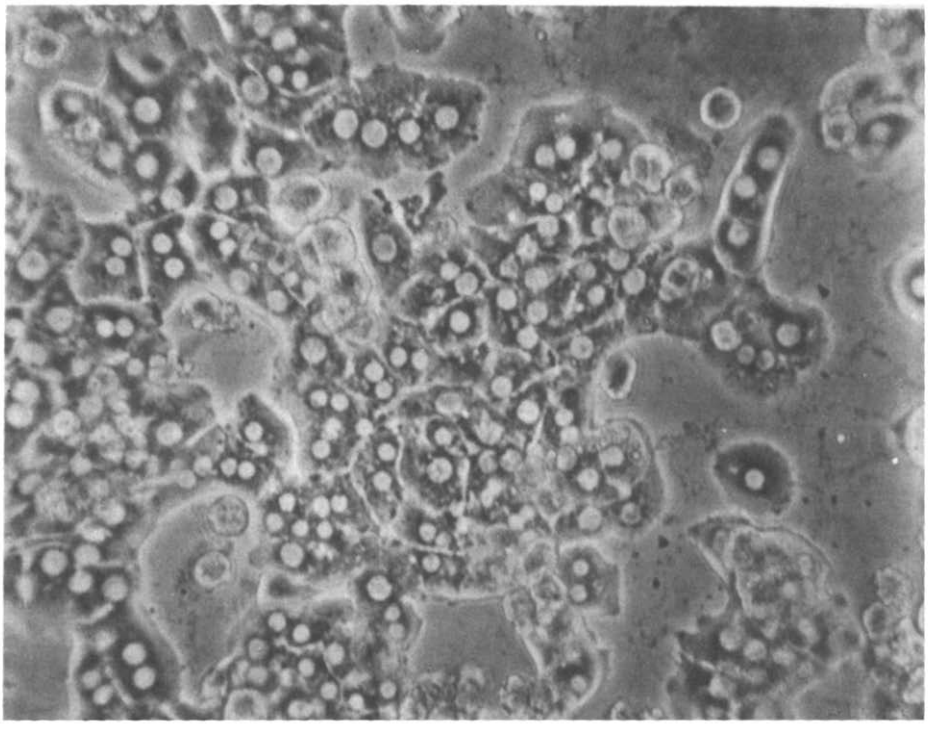

Fig. 2. Photomicrograph of hepatocytes cultured for 24 hr in WOBA-M_2 medium. The cells were cultured on coverslips on the bottom of 60 mm Petri dishes coated with rat tail collagen. The coverslips were removed after 24 hr, rinsed in buffer, and placed in fixative (2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). The photomicrograph was taken with a Zeiss research microscope with camera attachment (× 240).

the culture medium and were presumed to be damaged hepatocytes since they stained readily with Trypan Blue.

Cytochrome P-450 content and associated mono-oxygenase activities of hepatocytes cultured in WOBA-M_2 and AB media. Initial (0 hr) levels for cytochrome P-450 content and mono-oxygenase activities were determined in isolated hepatocytes prior to their being inoculated into culture dishes (Table 3). This was done by suspending the cells in WOBA-M_2 or AB medium for 10–20 min at 25° and then washing and homogenizing an aliquot of the cells. Cytochrome P-450 content and mono-oxygenase activities of hepatocytes isolated from the same rat and suspended in different media were essentially identical, so the results were combined. Table 3 shows that there was a large variation in cytochrome P-450 content and mono-oxygenase activities for isolated hepatocytes prepared from different rats. This was apparently caused by animal-to-animal variation rather than by wide variation in the quality of the isolated hepatocyte preparations. This was suggested by Trypan Blue exclusion and attachment efficiency being similar in all our preparations, while enzyme activities were vastly different. To control for this variable it was decided to express cytochrome P-450 content and mono-oxygenase activities for each isolated hepatocyte preparation at 0 hr as 100 per cent. Enzyme activities (measured on a per mg protein basis) for cultured hepatocytes derived from the

Table 3. Cytochrome P-450 content and activities of mono-oxygenase enzymes in whole cell homogenates of isolated rat hepatocytes*

Enzyme	Units	Mean ± S.E.M.	Range
Cytochrome P-450	pmoles/mg protein	170.5 ± 20.8	78.1–218.7
7-Ethoxycoumarin-*O*-deethylase	pmoles product/min/mg protein	410.0 ± 45.7	158.8–537.4
7-Ethoxyresorufin-*O*-deethylase	pmoles product/min/mg protein	203.9 ± 34.2	96.4–317.9
Biphenyl-4-hydroxylase	pmoles product/min/mg protein	590.2 ± 99.3	202.7–879.1
Cytochrome P-450	pmoles/μg DNA	9.1 ± 1.5	3.0–11.7
7-Ethoxycoumarin-*O*-deethylase	pmoles product/min/μg DNA	24.0 ± 4.8	6.7–39.3
7-Ethoxyresorufin-*O*-deethylase	pmoles product/min/μg DNA	10.5 ± 2.4	5.4–18.7
Biphenyl-4-hydroxylase	pmoles product/min/μg DNA	34.1 ± 10.9	8.6–69.6

* Results are for isolated hepatocyte preparations from five to seven rats. Hepatocytes from these same preparations were subsequently cultured in WOBA-M_2 and AB media.

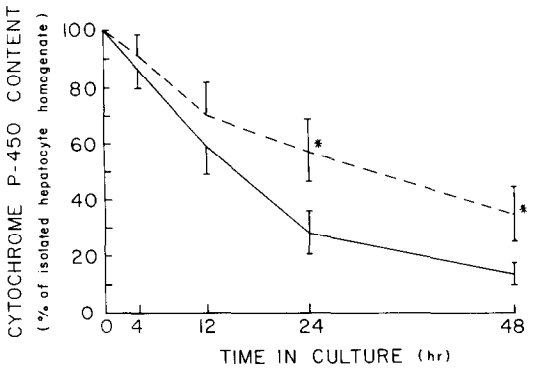

Fig. 3. Percentage of initial cytochrome P-450 content of hepatocyte homogenates after various times in culture. All values are means ± S.E.M. of cultures from four rats. Key: (———) designates hepatocytes cultured in WOBA-M_2 medium, and (– – –) hepatocytes cultured in AB medium; (*) denotes $P < 0.05$, as determined by a paired t-test.

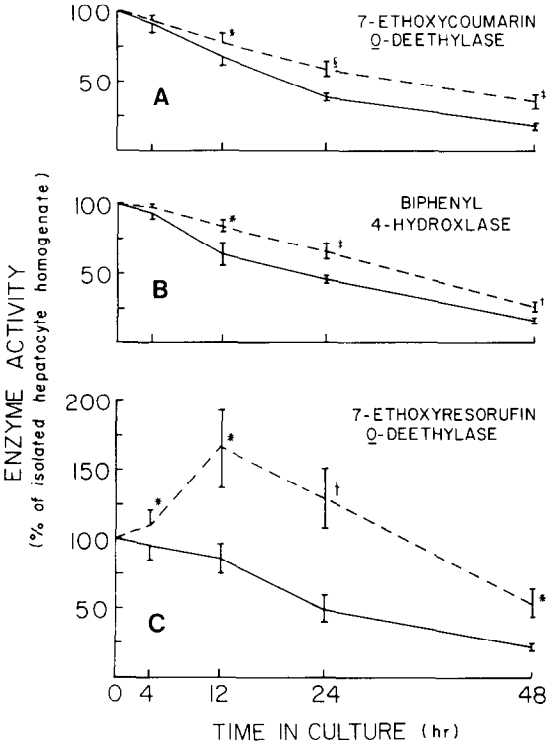

Fig. 4. Percentage of initial 7-ethoxycoumarin-O-deethylase, biphenyl-4-hydroxylase and 7-ethoxyresorufin-O-deethylase activities of hepatocyte homogenates after various times in culture. Biphenyl-2-hydroxylase activity was not detectable by the assay procedure used in isolated hepatocytes or in cultured hepatocytes maintained in either WOBA-M_2 or AB medium. All values are means ± S.E.M. of cultures from five to seven rats. Key: (———) designates hepatocytes cultured in WOBA-M_2 medium, and (– – –) hepatocytes cultured in AB medium; (*) denotes $P < 0.05$, (†) $P < 0.02$, (‡) $P < 0.01$, and (§) $P < 0.001$, as determined by a paired t-test.

same preparation are expressed as a percentage of the initial (0 hr) activity in Figs. 3 and 4.

Figure 3 illustrates the decrease in per cent cytochrome P-450 content of cultured hepatocytes with time in WOBA-M_2 and AB media. In agreement with other workers, cytochrome P-450 content decreased after 24 hr in WOBA-M_2 medium to 28 per cent of the level found initially. However, in AB medium, cytochrome P-450 content was 57 per cent of the initial hemoprotein content after 24 hr in culture. After 48 hr, cytochrome P-450 content was 12 per cent of the initial value for cells cultured in WOBA-M_2 medium and 35 per cent in AB medium.

The time courses of three cytochrome P-450-dependent enzyme activities, 7-ethoxycoumarin-O-deethylase, 7-ethoxyresorufin-O-deethylase and biphenyl-4-hydroxylase, were also measured after 4, 12, 24 and 48 hr in culture (Fig. 4). The decreases, in per cent of initial activity, of all three enzymes in cells cultured in WOBA-M_2 medium were similar and approximately paralleled the decline of cytochrome P-450 shown previously in Fig. 3. Hepatocytes cultured in AB medium showed significant increases in 7-ethoxycoumarin-O-deethylation and biphenyl-4-hydroxylation after 12, 24 and 48 hr when compared with cells cultured in WOBA-M_2 medium. These greater enzyme activities in the AB medium also closely paralleled the greater cytochrome P-450 content of cells cultured in AB medium. In contrast, 7-ethoxyresorufin metabolism in hepatocytes cultured in AB medium was enhanced to levels greater than those of cytochrome P-450 and the other two cytochrome P-450-dependent enzymes. The activity of 7-ethoxyresorufin-O-deethylase after 4, 12, 24 and 48 hr in AB culture medium was, respectively, 110, 165, 130 and 55 per cent of the initial (0 hr) rate and was approximately 2-fold greater than the activities of 7-ethoxycoumarin-O-deethylase and biphenyl-4-hydroxylase and the cytochrome P-450 content. The activity of 7-ethoxyresorufin-O-deethylase in primary hepatocytes in AB medium was also significantly greater than in WOBA-M_2 medium at all times in culture.

Figure 5 shows the specific activities of the enzymes expressed as pmoles of product produced per pmole of cytochrome P-450. 7-Ethoxycoumarin-O-deethylase and biphenyl-4-hydroxylase in hepatocytes cultured in AB medium had specific activities that were similar (within 20 per cent) to that of isolated hepatocytes at 0 hr. However, the specific activities of both enzymes were enhanced after 24 and 48 hr in WOBA-M_2 medium. The specific activity of 7-ethoxyresorufin-O-deethylase was increased after 12, 24 and 48 hr in WOBA-M_2 medium and (in contrast to the other two enzymes) was further enhanced after 12 and 24 hr in AB medium.

SDS–PAGE of rat hepatic microsomes from isolated hepatocytes and hepatocytes cultured in WOBA-M_2 and AB media. The gel electrophoresis profiles of microsomal proteins isolated from normal rat livers or from isolated hepatocytes were very similar in the 50,000–60,000 molecular weight region (Fig. 6). Microsomal proteins from cultured hepatocytes in this molecular weight region have been shown to have an electrophoretic mobility similar to that of the proteins in rat hepatic microsomes that specifi-

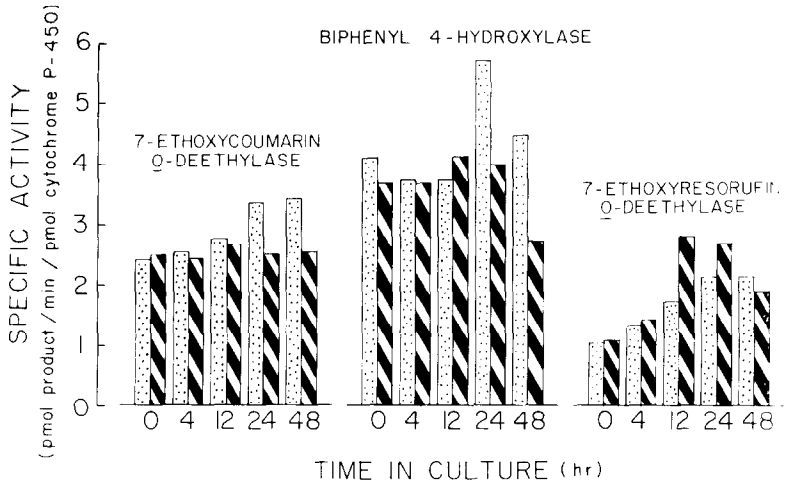

Fig. 5. Specific activities (pmoles product/min/pmole cytochrome P-450) of 7-ethoxycoumarin-*O*-deethylase, biphenyl-4-hydroxylase and 7-ethoxyresorufin-*O*-deethylase in cultured hepatocytes. All values are the means of cultures from five rats. Dotted bars designate hepatocytes cultured in WOBA-M_2 medium and slashed bars hepatocytes cultured in AB medium. All 0 hr values were obtained on isolated hepatocytes and 4, 12, 24 and 48 hr values on cultured hepatocytes.

cally increase following treatment *in vivo* with either phenobarbital or 3-methylcholanthrene [16]. In our studies, the proteins labeled Forms 1 and 2 correspond to the protein inducible by phenobarbital and to the major protein increased by 3-methylcholanthrene treatment respectively [16].

After 12 hr in culture in WOBA-M_2 medium, the major protein in the 50,000–60,000 molecular weight region was Form 2. This pattern was also seen after 24 hr in culture in WOBA-M_2 medium (Fig. 6). The electrophoretic pattern of microsomes from cells cultured in AB medium for 4 hr (not shown) was similar to that for hepatocytes cultured in WOBA-M_2 medium for 4 hr (Fig. 6); their cytochrome P-450 contents were also similar (Fig. 3). However, after 24 hr in culture the amount of Form 1 was greater in microsomes from hepatocytes cultured in AB medium than in WOBA-M_2 medium (Fig. 6). This difference was observed with three hepatocyte preparations and was associated with a small increase in the microsomal protein content of hepatocytes cultured for 24 hr in AB medium as compared to WOBA-M_2 medium (0.94 ± 0.12 vs 0.77 ± 0.11 mg protein, means ± S.E.M.).

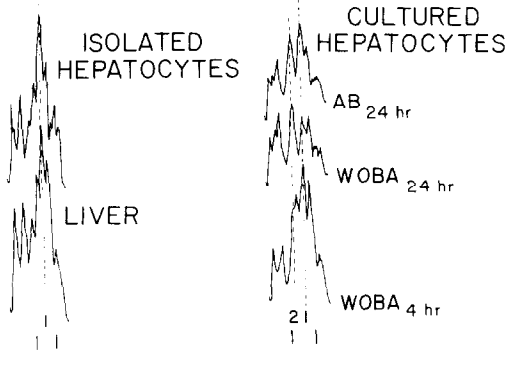

Fig. 6. Densitometric scans of SDS–PAGE experiments on microsomes isolated from control rat liver, isolated rat hepatocytes and cultured rat hepatocytes. In all experiments 50–75 μg of microsomal protein was applied to each sample well. The molecular weight region 50,000–60,000 is marked at the bottom of the scans by (‖).

DISCUSSION

The AB medium containing a number of steroid hormones and the heme precursor, δ-aminolevulinic acid (ALA), has been shown to maintain the cytochrome P-450 content of hepatocytes in primary culture for 24 hr at levels close to those found *in vivo* [12, 13]. This is in contrast to results obtained using more conventional media in which hepatocyte cytochrome P-450 levels decreased markedly after 24 hr in culture [9, 10, 12, 13]. The decrease with time of hepatocyte cytochrome P-450 in culture in a medium such as WOBA-M_2 has been associated with an observed increase in heme oxygenase activity [29]. However, more recent work [13] has shown that cytochrome P-450 may be maintained using the AB medium despite the presence of similar high levels of heme oxygenase. This may be due to the formation or alteration of existing species of cytochrome P-450 to forms which are less susceptible to degradation by heme oxygenase, or to an increased rate of heme synthesis stimulated by the presence of ALA in the medium. ALA has been shown to partially maintain cytochrome P-450 [30], although these effects are pronounced only at relatively high concentration of 10^{-4} M compared with 10^{-6} M in AB medium. Lipid peroxidation has also been associated with the degradation of cytochrome P-450 *in vitro*. Estradiol (present in AB medium at a concentration of 10^{-6} M) has been shown to block lipid peroxidation [31] and this effect may also be partially responsible for the maintenance of cytochrome P-450 in culture. Omission of estradiol from AB medium has been shown to reduce markedly the enhancement of cytochrome P-450 levels by AB medium after 24 hr in culture [12].

The extent to which AB medium maintained the

hemoprotein content of the hepatocytes in the present study (about 60 per cent of the initial value after 24 hr) was lower than values of 100 per cent [12] and 80 per cent [13] reported by others under similar culture conditions. The lower stimulation obtained in the present study did not appear to be due to the cell preparations being of poor quality. Assessment of cultured cells at various times during the culture period by phase contrast microscopy and Trypan Blue exclusion indicated that the cells readily adapted to the culture conditions of both media and were intact. Paine and Legg [13] showed that inclusion of the hormonal supplements to the perfusion buffer increased the cytochrome P-450 content and this, together with a possible strain difference in the animals used, may offer a partial explanation for the smaller stimulation of cytochrome P-450 reported here.

The main finding of the present study was that cytochrome P-450 and associated mono-oxygenase activities, when hepatocytes were cultured, were maintained at higher levels in AB medium than in WOBA-M_2 medium. Although cytochrome P-450 content and 7-ethoxycoumarin-*O*-deethylase and biphenyl-4-hydroxylase activities were enhanced to a similar extent after various times in culture in AB medium, 7-ethoxyresorufin-*O*-deethylase was increased after 12 and 24 hr in culture to a level of activity greater than that of isolated hepatocytes at 0 hr. Furthermore, when expressed in terms of pmoles of product per min per pmole of cytochrome P-450, only 7-ethoxyresorufin-*O*-deethylation was markedly enhanced in hepatocytes cultured in AB medium. In contrast, the specific activities of all three mono-oxygenases were enhanced in hepatocytes cultured in WOBA-M_2 medium after 24 and 48 hr. Similar increases in specific activity of cultured hepatocyte mono-oxygenases have been observed by other workers. After 24 hr the cytochrome P-450 content of primary rat hepatocytes was 20 per cent of the initial amount and the rates of benzo[*a*]pyrene and 4-nitroanisole metabolism were 40 and 85 per cent of the initial rate respectively [10]. Similarly, after 10 days in culture, Fahl *et al.* [16] found that the cytochrome P-450 content of primary rat hepatocytes was 10 per cent of the amount in isolated hepatocytes and benzo[*a*]pyrene hydroxylation was 70 per cent of the initial activity.

Taken together, the results of this study and those of other investigators demonstrate that the metabolic capacity of the cytochromes P-450 in cultured hepatocytes changes even after short culture periods. The present results further suggest that qualitative differences may exist in the species of cytochrome P-450 present in hepatocytes cultured in WOBA-M_2 and AB media. The specific increase of 7-ethoxyresorufin-*O*-deethylase in AB medium suggests that a species of cytochrome P-450, which catalyzes the metabolism of this substrate, may be enhanced or preferentially maintained, i.e. a form resembling cytochrome P-448.* In support of this argument, hydrocortisone present in AB medium at a concentration of 10^{-5} M has been shown [9] to elicit a change characteristic of cytochrome P-448, i.e. a spectral shift of the absorbance maximum of the reduced carbonyl complex from 450 to 448 nm.

Evidence of the synthesis of cytochrome P-450 apoprotein in hepatocytes cultured in AB medium was provided by the results obtained on microsomes that were isolated from cultured cells. The SDS–PAGE experiments suggest that the major form of cytochrome P-450-related protein is reduced after various times in culture. However, the apparent increase in a protein (Form 1) in the 50,000–60,000 molecular weight region, together with an increase in total microsomal protein and cytochrome P-450 in the hepatocytes cultured in AB medium for 24 hr (compared with those in WOBA-M_2 medium), suggests that *de novo* synthesis of cytochrome P-450 may have occurred.

In summary, our results suggest that AB medium maintains levels of cytochrome P-450 and associated mono-oxygenation of hepatocytes *in vitro* to a greater extent than WOBA-M_2 medium which does not contain ALA and a number of endogenous hormones. ALA has been shown to maintain the cytochrome P-450 of cultured hepatocytes at about 30 per cent of initial levels (approximately twice the level in control cultures) after 24 hr in culture by increasing both heme and cytochrome P-450 synthesis [30]. The gel electrophoretic evidence presented here suggests that a cytochrome P-450-related protein may also be enhanced in AB medium. Thus, the additional endogenous compounds present in AB medium may have a dual role in the maintenance of cytochrome P-450 in culture by increasing both heme synthesis and the synthesis of cytochrome P-450 apoprotein. However, the enhancement of 7-ethoxyresorufin metabolism in hepatocytes cultured in AB medium may indicate the formation of a hemoprotein with catalytic properties more similar to cytochrome P-448 than 'normal' cytochrome P-450.

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REFERENCES

1. D. M. Bissell, L. E. Hammaker and U. A. Meyer, *J. Cell Biol.* **59**, 722 (1973).
2. R. J. Bonney, *In Vitro* **10**, 130 (1974).
3. G. Michalopoulos and H. C. Pitot, *Expl Cell Res.* **94**, 70 (1975).
4. G. M. Williams, E. Bermudez and D. Scaramuzzino, *In Vitro* **13**, 809 (1977).
5. R. F. Kletzien, M. W. Pariza, J. E. Becker and V. R. Potter, *J. cell. Physiol.* **89**, 641 (1976).
6. R. F. Kletzien, M. W. Pariza, J. E. Becker, V. R. Potter and F. R. Butcher, *J. biol. Chem.* **251**, 3014 (1976).

* Cytochrome P-448 is a form of hepatic microsomal cytochrome P-450 which is inducible by polycyclic aromatic hydrocarbons and is readily distinguished from 'normal' cytochrome P-450 by its spectral, catalytic, electrophoretic and immunological properties.

7. M. W. Pariza, J. E. Becker, J. D. Yager, R. J. Bonney and V. R. Potter, in *Differentiation and Control of Malignancy of Tumour Cells* (Eds. W. Nakahara, T. Ono, T. Sugimura and H. Sugano), pp. 267–284. University Park Press, Baltimore (1974).
8. M. W. Pariza, J. D. Yager, 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 Liver* (Eds. L. E. Gerschenson and E. B. Thompson), pp. 137–167. Academic Press, New York (1975).
9. G. Michalopoulos, G. L. Sattler and H. C. Pitot, *Life Sci.* **18**, 1139 (1976).
10. P. S. Guzelian, D. M. Bissell and U. A. Meyer, *Gastroenterology* **72**, 1232 (1977).
11. K. W. Renton, L. B. DeLoria and G. J. Mannering, *Molec. Pharmac.* **14**, 672 (1978).
12. G. M. Decad, D. P. H. Hsieh and J. L. Byard, *Biochem. biophys. Res. Commun.* **78**, 279 (1977).
13. A. J. Paine and R. F. Legg, *Biochem. biophys. Res. Commun.* **81**, 672 (1978).
14. A. P. Alvares and P. Siekevitz, *Biochem. biophys. Res. Commun.* **54**, 923 (1973).
15. A. F. Welton and S. D. Aust, *Biochem. biophys. Res. Commun.* **56**, 898 (1974).
16. W. E. Fahl, G. Michalopoulos, G. L. Sattler, C. R. Jefcoate and H. C. Pitot, *Archs Biochem. Biophys.* **192**, 61 (1979).
17. C. Waymouth, *J. natn. Cancer Inst.* **22**, 1003 (1959).
18. W. F. Greenlee and A. Poland, *J. Pharmac. exp. Ther.* **205**, 596 (1978).
19. M. D. Burke and R. T. Mayer, *Drug Metab. Dispos.* **3**, 245 (1974).
20. P. Wiebkin, J. R. Fry, C. A. Jones, R. K. Lowing and J. W. Bridges, *Xenobiotica* **6**, 725 (1976).
21. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
22. J. Kowal, E. R. Simpson and R. W. Estabrook, *J. biol. Chem.* **245**, 2438 (1970).
23. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
24. G. M. Richards, *Analyt. Biochem.* **57**, 369 (1974).
25. U. K. Laemmli and M. Favre, *J. molec. Biol.* **80**, 575 (1973).
26. W. R. Ingebretson and S. R. Wagle, *Biochem. biophys. Res. Commun.* **47**, 403 (1972).
27. J. D. Yager and J. A. Miller, *Cancer Res.* **38**, 4385 (1978).
28. D. N. Wheatley, *Expl Cell Res.* **74**, 455 (1972).
29. D. M. Bissell and P. S. Guzelian, in *Gene Expression and Carcinogenesis in Cultured Liver* (Eds. L. E. Gerschenson and E. B. Thompson), pp. 119–136. Academic Press, New York (1975).
30. P. S. Guzelian and D. M. Bissell, *J. biol. Chem.* **251**, 4421 (1976).
31. M. Jacobson, W. Levin, A. Y. H. Lu, A. H. Conney and R. Kuntzman, *Drug Metab. Dispos.* **1**, 766 (1973).