enables one to make more meaningful assumptions concerning the intracellular fraction of the label. The stimulation of influx due to purified C5a and to NFP gives supportive evidence that the influx due to AS is related to its chemotactic potential.

Gallin and Rosenthal (*18*) demonstrated a stimulation of calcium efflux by activated serum. We also observed an increase in the washout of label from cells incubated for 1 hour in a medium containing ^{45}Ca and exposed to AS. Lanthanum chloride in concentrations up to 1.0 m*M* did not inhibit this effect. The stimulation of calcium efflux by AS may be due to release of a bound intracellular pool of calcium, which is subsequently pumped from the cell. Wilkinson (*19*) has postulated that intracellular release of calcium has a role in neutrophil chemotaxis. However, the data presented here suggest that release of an intracellular pool is insufficient to promote chemotaxis or that such a pool is dependent on calcium influx.

It appears that calcium influx is correlated with chemotaxis, and the amount of calcium that moves into the cell is sufficient to activate a contractile process. The observed calcium influx is unlikely to be an energy-requiring event since the gradient of Ca^{2+} across the plasma membrane would favor calcium movement into the cell as in other well-studied cellular systems. An interaction between La^{3+} and the chemotactic factor is not supported by the data, since concentrations of La^{3+} below 1.0 m*M* selectively blocked calcium influx due to AS. Furthermore, La^{3+} inhibited cellular motility in the absence of a chemoattractant. The role of Ca^{2+} efflux may be secondary since concentrations of La^{3+} that blocked chemotaxis inhibited only calcium influx. Although there was no gross chemical gradient of the chemotactic agents during our calcium flux experiments, these agents may exert their cellular effects in the absence of a gradient. To induce chemotaxis, the gradient would provide direction through a sensing mechanism. The directional polarization may be separate but related to the contractile action initiated by the absolute concentration of the attractant.

MARK M. BOUCEK
Department of Pharmacology, University of Miami School of Medicine, Miami, Florida 33152
RALPH SNYDERMAN
Division of Rheumatic and Genetic Diseases, Department of Medicine, Duke University School of Medicine, Durham, North Carolina 27710

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Cytochrome P-450 Induction by Phenobarbital and 3-Methylcholanthrene in Primary Cultures of Hepatocytes

Abstract. *The characteristic hepatocellular changes resulting from phenobarbital administration in vivo, namely an increase in the levels of cytochrome P-450 and proliferation of membranes of the smooth endoplasmic reticulum, have been demonstrated in primary cultures of nonreplicating hepatocytes on floating collagen membranes. Addition of methylcholanthrene to the medium resulted in an increase in cytochrome P-448 within 48 hours, whereas the phenobarbital induction of P-450 required 5 days. These results demonstrate that responses induced in adult liver cells in vivo by phenobarbital can be reproduced in cultured hepatocytes, contrary to previous reports.*

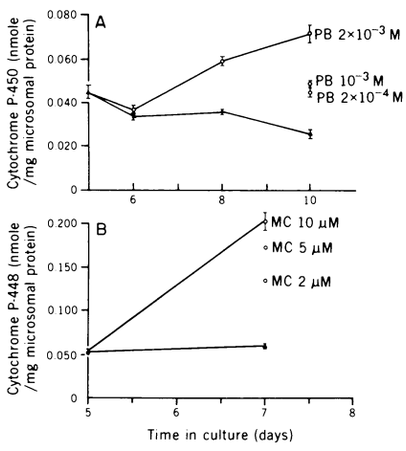
Cytochrome P-450 plays a crucial catalytic role in the activities of several liver microsomal enzymes which, known collectively as mixed function oxidases, are involved in the activation of carcinogens and the detoxification of drugs by liver (*1*). The concentration of cytochrome P-450 in liver is increased by the administration in vivo of a number of chemical compounds, including phenobarbital and 3-methylcholanthrene (MC) (*2*). The molecular forms of cytochrome P-450 induced by these two compounds exhibit difference spectra with absorption maxima at different wavelengths (*3*); they also give different signals when tested by electron paramagnetic resonance (*4*). We use the terms cytochrome P-450 and cytochrome P-448 for the cytochromes induced by treatment with phenobarbital and MC, respectively (*5*).

In addition to its effect on cytochrome P-450, phenobarbital administered to animals causes a characteristic proliferation of membranes of the smooth endoplasmic reticulum (SER) in the cytoplasm of hepatocytes and a simultaneous increase in the levels of several microsomal mixed function oxidases (*2*, *6*).

In the systems of primary cultures of hepatocytes reported so far (*7*, *8*), the administration of such compounds as phenobarbital and MC has not resulted in quantitative changes in cytochrome P-450 levels, nor has it elicited the characteristic, phenobarbital-associated proliferation of SER membranes. The inability to induce cytochrome P-450 and to elicit the characteristic responses to phenobarbital administration in primary cultures of hepatocytes has restricted the use of these systems in studies of drug and carcinogen metabolism in vitro. It has also raised the question of whether it is possible to induce in any culture system the phenobarbital-characteristic form of cytochrome P-450 and the associated proliferation of SER (*8*).

Here we report the induction of cytochrome P-450 by phenobarbital, and cytochrome P-448 by MC, in primary cultures of adult rat hepatocytes on floating collagen membranes. We also report that the proliferation of vesicles of smooth endoplasmic reticulum occurs in the cytoplasm of the cultured hepatocytes after the administration of phenobarbital.

In a system (*9*) for the primary culture of parenchymal hepatocytes on floating collagen gels, the cultured hepatocytes



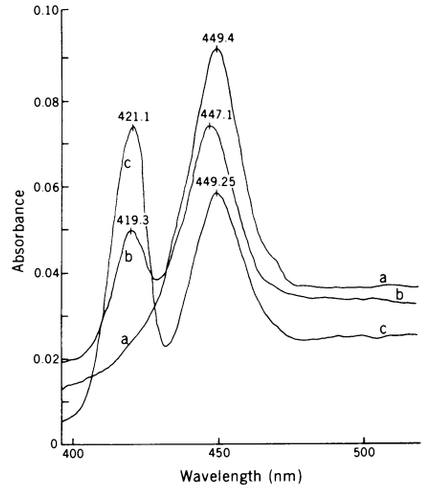


Fig. 1 (left). Effects of (A) phenobarbital (*PB*) on the concentration of cytochrome P-450 and (B) 3-methylcholanthrene (*MC*) on the concentration of cytochrome P-448 in primary cultures of rat hepatocytes. Points represent the mean of five experiments ± standard error. Bottom curves in each graph show data for control experiments. (A) Absolute values are expressed as nanomoles of cytochrome P-450 per milligram of microsomal protein. Cells at 5 days: 0.045 ± 0.003; control cells at 10 days: 0.026 ± 0.002; treated cells at 10 days: 0.072 ± 0.004. (B) Absolute values are expressed as nanomoles of cytochrome P-450 per milligram of microsomal protein. Cells at 5 days: 0.054 ± 0.003; control cells at 7 days: 0.065 ± 0.001; treated cells at 7 days: 0.204 ± 0.017. (MC 5 μM and MC 2 μM are values from experiments performed once.) Fig. 2 (right). Comparison of the carbon monoxide difference spectra of dithionite-reduced microsomes of (a) normal rat liver, (b) MC-treated cells, and (c) phenobarbital-treated cells. [Concentrations of microsomal protein: (a) 1.05 mg/ml, (b) 1.29 mg/ml, and (c) 1.70 mg/ml.]

maintain epithelial morphology and respond to hydrocortisone, glucagon, and cyclic adenosine monophosphate with an increase in the activity of tyrosine aminotransferase (E.C. 2.6.1.5) for periods of at least 20 days. Cytochromes P-450 and b_5 decrease in concentration within the first 5 days of culture but remain at measurable levels for at least 10 days, and cytochrome P-448 can be stimulated to higher levels if hydrocortisone is administered during the culture period (*10*).

For the study reported herein, primary cultures of hepatocytes from adult rat liver were prepared as described (*9*). Fetal calf serum was added to a final concentration of 5 percent by volume to the nutrient medium. Phenobarbital sodium (Baker) was dissolved in the nutrient medium, and the solutions were titrated to *p*H 7.4 with hydrochloric acid. 3-Methylcholanthrene (Calbiochem) was dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide in the MC-treated and control samples was 0.5 percent by volume. For the analysis of the cytochromes the floating collagen gels carrying the hepatocytes (60-mm plates, 30 plates per sample) were homogenized with a glass homogenizer in ice-cold 0.5*M* tris-HCl buffer [tris-(hydroxymethyl)aminomethane] at *p*H 7.5. The homogenates were spun at 12,000*g* for 10 minutes to remove the nuclei, mitochondria, and insoluble collagen gel fragments. The supernatants were frozen at −70°C. Microsomes were prepared on the day of the assay by centrifugation of the thawed supernatant at 108,000*g* for 90 minutes. The sedimented microsomal pellets were resuspended by sonication in 0.5*M* tris-HCl buffer, *p*H 7.5, to a final volume of 2.2 ml. Two portions (0.1 ml) of the microsomal suspension were taken for the protein assays. Spectral assays of cytochromes P-450 and P-448 were performed in a Cary spectrophotometer (model 15) calibrated with a crystal of holmium oxide. The method of Omura and Sato (*11*) was used to measure cytochrome P-450 and cytochrome P-448. Protein was measured by the method of Lowry *et al.* (*12*). Student's *t*-test was used for the statistical evaluation of the results.

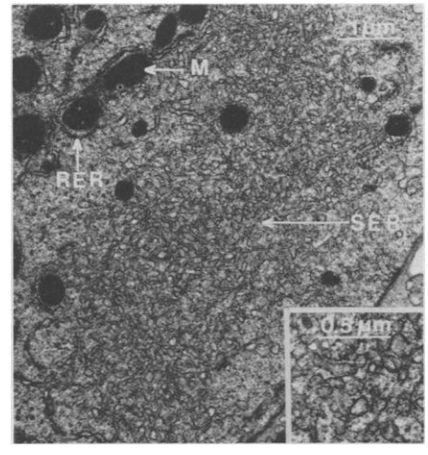


Fig. 3. Electron micrograph of hepatocytes cultured on floating collagen gel for 10 days. Phenobarbital was added to the medium on day 5. The area containing abundant smooth endoplasmic reticulum (*SER*) lacks rough endoplasmic reticulum (*RER*), mitochondria (*M*), and other typical cytoplasmic organelles (× 17,500). Inset shows a higher magnification of SER (× 43,000).

The effects of phenobarbital on cytochrome P-450 are shown in Fig. 1A. Phenobarbital ($2 \times 10^{-3} M$) added to the culture on day 5 significantly increases the amount of cytochrome P-450 compared to that in untreated cultures both at day 5 ($P < .01$) and day 10 ($P < .001$). The time course of these effects in vitro follows closely the rate of induction of cytochrome P-450 by phenobarbital in rat liver in vivo, where the concentration of P-450 usually reaches a maximum after 4 to 5 days of continuous phenobarbital administration (*2, 6*). The level of induction, however (160 percent and 275 percent over the control cells at days 5 and 10, respectively), is less than that occurring in the liver of the intact animal where phenobarbital usually causes a four- to fivefold increase in cytochrome P-450.

The effects of MC on microsomal cytochromes are shown in Fig. 1B. There is a significant ($P < .001$) increase in cytochrome P-448. Again the rapid time course of the action of MC and the amounts of induction are comparable to the results obtained in vivo (*2*). The total concentration of cytochrome P-450, as well as the percentages of induction (377 percent and 313 percent over the control cells at days 5 and 7, respectively) obtained with MC, are higher than the corresponding levels obtained with phenobarbital. This is in contrast to the results in vivo, where the opposite usually occurs. The reason for this difference is unknown.

The difference spectra of the cytochromes induced by phenobarbital and MC in the hepatocyte cultures are compared in Fig. 2. The peak of absorption of the phenobarbital-induced cytochrome P-450, located at 449.25 nm, is almost identical to that obtained with cytochrome P-450 from normal rat liver (located at 449.4 nm). With MC-treated cells, the absorption peak shifts toward the blue part of the spectrum by about 2 nm to give a maximum at 447.1 nm, which characterizes this form of cytochrome as P-448. Thus, the cytochromes induced in these cultured hepatocytes by phenobarbital and MC ex-

hibit the same spectral characteristics as those induced in vivo (*5*).

Cytochrome P-420, considered to be a degradation product of cytochrome P-450 (*13*), occurred in large amounts in the microsomes isolated from cultured cells but not in the microsomes isolated from rat liver under identical conditions. We cannot ascertain whether the cytochrome P-420 in vitro is intracellular, as appears to be the case with Morris hepatomas (*14*), or whether a factor or factors (such as lysosomal proteases) liberated from ruptured cells during homogenization produce cytochrome P-420 during the isolation procedure.

Hepatocytes cultured in the presence of phenobarbital ($2 \times 10^{-3}M$) from day 5 to day 10 were examined by electron microscopy and compared with cells cultured in the absence of phenobarbital. The hepatocytes on the floating collagen gels were fixed first with 3 percent glutaraldehyde at room temperature and then with 2 percent osmium oxide on ice. The cells were stained with a 2 percent aqueous uranyl acetate; the sections were stained with lead citrate. Subcellular organelles and lamellar rough endoplasmic reticulum were adequately preserved. Clusters of tightly aggregated vesicles of SER were seen in abundance (Fig. 3) in the cytoplasm of the phenobarbital-treated hepatocytes. The proliferation of SER was not restricted to any particular region of the cytoplasm and occurred in most of the phenobarbital-treated hepatocytes. Comparable proliferation of vesicles of SER did not occur in cells cultured in the absence of phenobarbital.

In previous studies of primary hepatic cultures and of cell lines derived from normal and neoplastic liver there were dissimilarities in the structure and biochemical function of these cells compared with liver parenchymal cells in vivo [see Bissel *et al.* (*15*)]. In this laboratory we have demonstrated (*16*) the similarity between the ultrastructure of hepatic cells maintained on floating collagen membranes and the ultrastructure of these cells in vivo. We have also shown that the enzyme ornithine oxoacid aminotransferase (E.C. 2.6.1.13) can be induced by glucagon in vitro as well as in vivo (*17*). The data reported herein may be useful in studies of drug metabolism, carcinogenesis in vitro, and the rapid assay of environmental carcinogens.

GEORGE MICHALOPOULOS
CAROL A. SATTLER
GERALD L. SATTLER
HENRY C. PITOT

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison 53706

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Ischemic Areas in Perfused Rat Hearts: Measurement by NADH Fluorescence Photography

Abstract. *Fluorescence emission of reduced nicotinamide adenine dinucleotide (NADH) from the surface of perfused rat hearts was photographed to provide a two-dimensional recording of NADH levels. Sodium Amytal inhibition of NADH oxidation resulted in a homogeneous increase in NADH fluorescence, while lowering the perfusion pressure from 55 to 10 torr caused a heterogeneous increase in NADH fluorescence, reflecting the heterogeneous oxygen delivery at this low pressure. Local ischemia resulted in a well-defined region of high NADH fluorescence that corresponded to the region of ischemic insult. The sharp transition between the ischemic and normoxic areas demonstrated that the hypoxic interface separating the two areas must be quite small.*

Coronary artery occlusion, the common cause of heart attacks, results in regional derangements of normal myocardial function and metabolism that may result in irreversible tissue damage. Efforts now under way to minimize the size of the ischemic area caused by a coronary occlusion (*1*) require techniques suitable to determine the exact size of the ischemic area (*2*).

We report the direct measurement of the area of the epicardium after an ischemic insult in a perfused rat heart. The basis of the technique is the fluorescence of the reduced nicotinamide adenine dinucleotide (NADH) component of the NADH-NAD couple. This fluorescent property has been particularly useful in monitoring metabolic processes in tissues, especially processes occurring during mitochondrial metabolism (*3*). Mitochondrial NADH fluorescence is an efficient indicator of intracellular oxygen concentrations (*4*) and changes in metabolic states (*5*).

Two-dimensional scanning (*6*) and photographic recording (*7*) of NADH fluorescence in the cerebral cortices of rats have been reported; however, the methods used had insufficient time resolution to "freeze" mechanical and metabolic fluctuations in a perfused heart beating three to five times a second. Therefore, an approach was developed whereby the ultraviolet component of a xenon flash was used as an excitation source. This technique allows photographic recording of NADH fluorescence emission from the heart with good time and spatial resolution.

Fluorescence photographs were taken with a Fairchild oscilloscope camera (model 453-1) with a 40-mm focal length lens added to the front of the existing lenses to provide an image on the film twice the actual size of the heart. A filter pack containing a Corning 9788 and a Wratten 45 filter was placed over the camera lens to allow transmission of NADH fluorescence in the 430- to 510-nm region. Photographs were recorded on Kodak Royal Pan film.

Fluorescence excitation was provided by two xenon flashtubes (EG&G FX-100), one mounted on either side of the camera lens. Flashtubes were covered by Corning 5840 filters to provide 330- to 380-nm excitation. Triggering of the flash was synchronized to occur during the diastolic phase of the cardiac cycle.

Hearts were rapidly excised from male Sprague-Dawley rats (weighing 200 to 220 g and anesthetized with sodium pentobarbital) and immediately perfused at 30°C with Krebs-Ringer bicarbonate buffer containing 5 m*M* glucose and 2.5 m*M*