SPECTRAL STUDIES ON DRUG-CYTOCHROME P-450 INTERACTION IN ISOLATED RAT LIVER CELLS

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Summary

Various drugs including hexobarbital, lidocaine and nortriptyline were added to suspensions of liver cells isolated from untreated and phenobarbital-treated male rats. Upon drug addition, there was a fast binding to cytochrome P-450, as revealed by the appearance of a rapidly growing type I spectral change in the difference spectrum. When this had reached optimal magnitude, an absorption peak at 437 nm could often be seen to appear and quickly disappear, followed by yet another increase in absorption at about 446 nm; the latter and the type I spectral change then rapidly disappeared. These spectral changes were most pronounced with liver cells from phenobarbital-treated rats which contained markedly increased levels of cytochrome P-450. Also the rate of hexobarbital binding to cytochrome P-450 seemed to be increased after phenobarbital pretreatment. Finally, evidence was obtained that the major part of cytochrome P-450 in the isolated liver cells is present in an oxidized, non-substrate-bound form.

Introduction

Drug oxidation by the cytochrome P-450-linked monooxygenase system of liver microsomes is thought to involve the following reaction sequence (cf. ref. 1 for review): (a) binding of the drug to oxidized cytochrome P-450, (b) reduction of the cytochrome-substrate complex by way of cytochrome P-450 reductase, (c) interaction of the reduced cytochrome-substrate complex with molecular oxygen, (d) acceptance of a second electron with the formation of a yet unidentified active oxygen-cytochrome-substrate complex and (e) transfer within this complex of one oxygen atom to the substrate and uptake of two protons, whereafter the complex dissociates into oxidized cytochrome, H_2O and product.

Although the above reaction sequence is still partly only hypothetical, at least two of the various states of cytochrome P-450 have been associated with characteristic optical absorption changes in the microsomal difference spectrum. Thus, the binding of the drug substrate to oxidized cytochrome P-450 has been

shown to produce the type I spectral change (λ_{max} at about 385 nm and λ_{min} at about 420 nm) (2), whereas an increase in absorption at about 440 nm, observed with liver microsomes during the aerobic steady state in the presence of certain drugs, has been associated with the formation of an oxygenated cytochrome P-450 (3), in analogy with previous findings with the cytochrome $P\text{-}450_{cam}$ system from Pseudomonas putida (4)

The present paper describes spectral studies on cytochrome P-450 in isolated liver cells from control and phenobarbital-pretreated rats and reports changes in optical absorption associated with drug uptake and steady state monooxygenation.

Methods

Male Sprague-Dawley rats (200 g) were used. Sodium phenobarbital was given i.p. in a daily dose of 80 mg per kg body-weight for three days.

The rats were starved over-night and anaesthetized with ether. Liver cells were isolated by perfusion via the portal vein in the presence of collagenase and hyaluronidase according to the technique described by Quistorff et al. (5) with the following modifications: the perfusion rate was 35-40 ml per min; all media contained 2% bovine serum albumin and the isolated cells were kept at 4°C in a Krebs-Henseleit buffer (6), pH 7.5, also containing 2% albumin.

Cell suspensions were always examined microscopically for trypan blue exclusion. A high percentage (~90%) of unstained cells was routinely observed. Stimulation of oxygen uptake by the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone was a further criterion of cell viability. A yield of 2-3 ml of tightly packed cells per liver (~7 g) was obtained. Cell counting was performed in a Bürker chamber.

Difference spectra were recorded in an Aminco DW-2 UV-VIS Spectrophotometer.

Results

As shown in Fig. 1A, introduction of CO into the sample cuvette - both cuvettes containing liver cells isolated from control rats in a buffered medium - produced a rapid increase in light absorption with a maximum at about 453 nm. Subsequent addition of hexobarbital caused a further increase in absorption

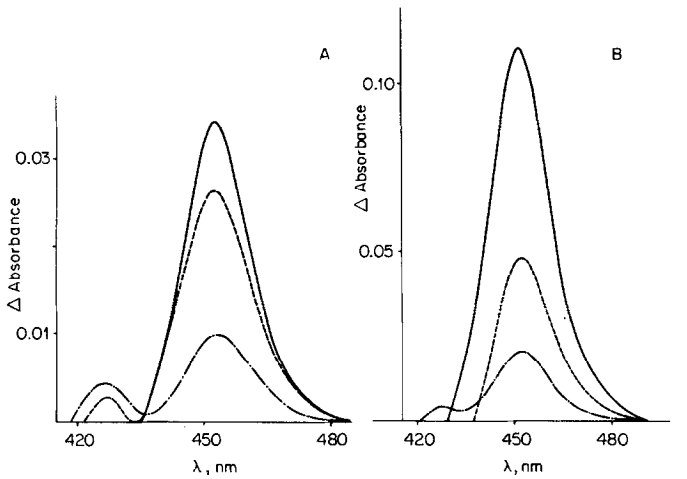

Fig. 1. Carbon monoxide difference spectra of isolated rat liver cells. Each cuvette contained ~2×10^6 cells per ml of buffered medium (see Methods). CO was bubbled for 20 sec into the sample cuvette and the difference spectrum recorded (—·—). Hexobarbital was then added to the same cuvette to a final concentration of 2 mM (---). Finally, a few grains of $Na_2S_2O_4$ were added to the sample cuvette (—). A represents cells from control rat and B cells from phenobarbital-treated rat. Temperature was 37°C.

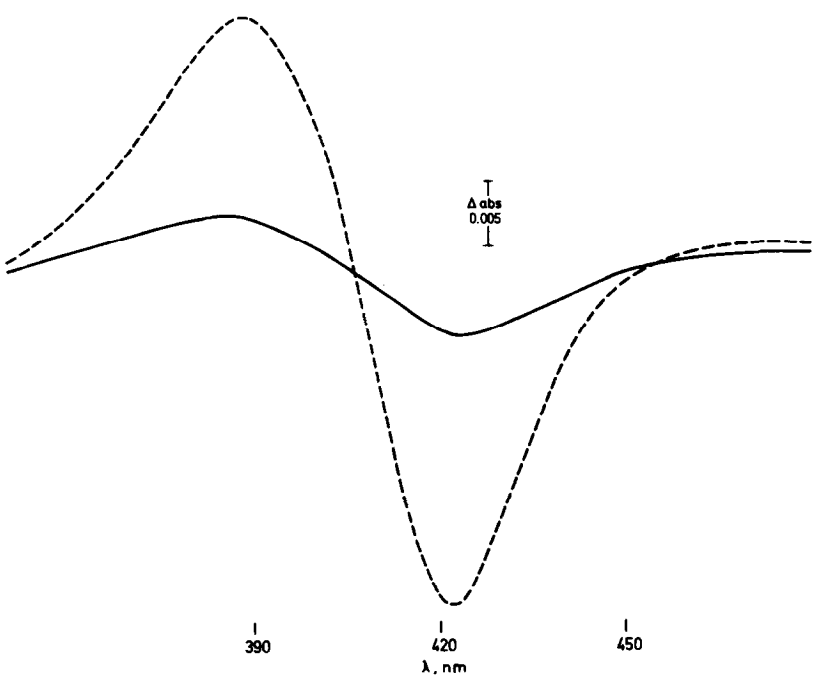

Fig. 2. Hexobarbital-induced type I spectral change in liver cells from contro (—) and phenobarbital-treated rats (---). Each cuvette contained ~2×10^6 cells per ml. Hexobarbital was added to the sample cuvette to a final concentration of 2 mM.

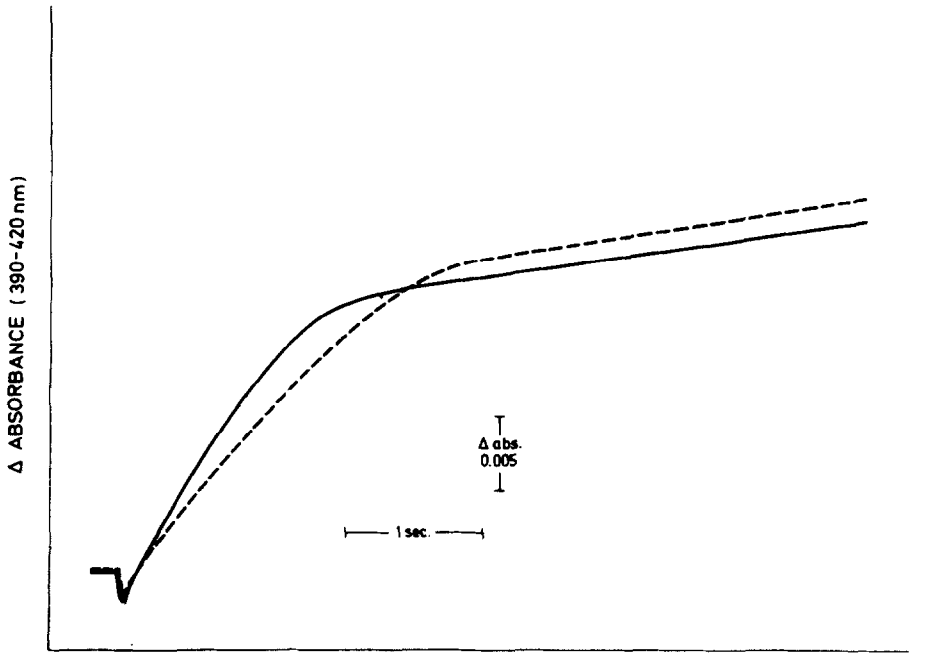

Fig. 3. Rate of formation of hexobarbital-induced type I spectral change in liver cells from control (---) and phenobarbital-treated rats (—). Cell concentrations were adjusted to give the same cytochrome P-450 levels in both instances. The reaction was started by plunging 50 µl of 0.1 M hexobarbital into the 3 ml suspension. Binding was complete in 7-8 sec. Temperature was 20°C.

with a slight shift in peak position towards 450 nm. Sodium dithionite addition, finally, produced maximal light absorption in this region. When the same experiment was performed with liver cells isolated from phenobarbital-treated rats, similar findings were obtained (Fig. 1B). The apsorption changes were, however, of a much greater amplitude.

When a drug such as hexobarbital was added to the isolated liver cells, a type I spectral change was recorded (Fig. 2). Its magnitude was enhanced several fold by phenobarbital pretreatment of the rats. The uptake of hexobarbital - estimated by the rate of formation of the type I spectral change - was rapid and usually occurred within a few seconds after addition. This rate seemed to be further enhanced by phenobarbital pretreatment of the animals (Fig. 3).

Thus, as already stated above, the addition of various drugs to suspensions of isolated liver cells gave rise to the rapid development of a type I spectral

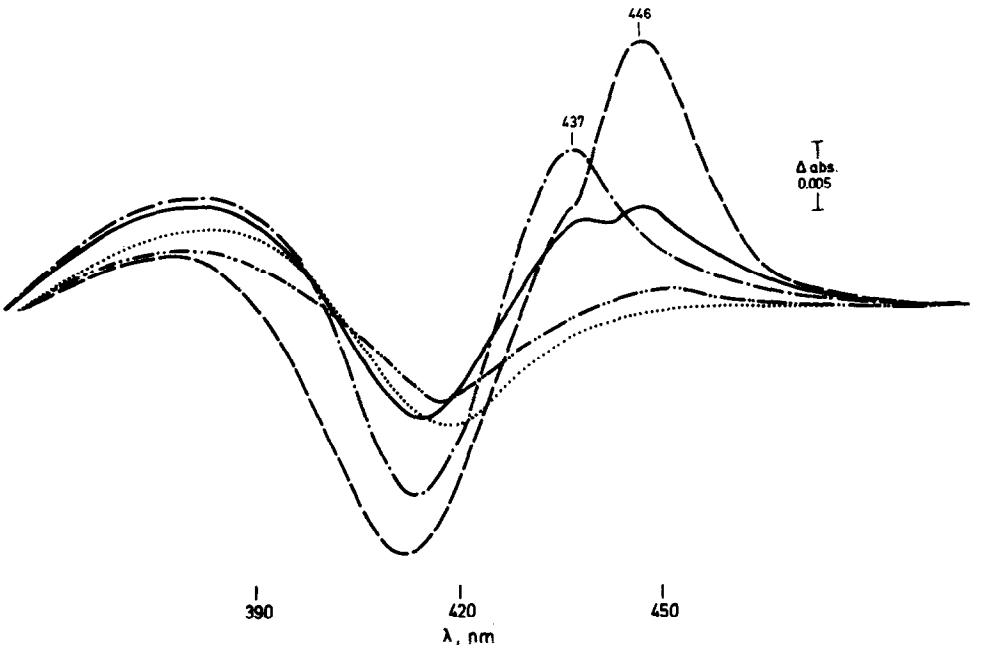

Fig. 4. Spectral changes produced by nortriptyline addition to control rat liver cells. Each cuvette contained ~3×10^6 cells per ml. Nortriptyline was added to the medium to a final concentration of 20 μM and repetitive scanning was performed. (····) 15 sec, (—·—) 90 sec, (—) 120 sec, (--) 180 sec and (—··—) 240 sec after nortriptyline addition. Temperature was 37°C.

change. When this had reached optimal magnitude, an absorption peak at about 437 nm could often be seen to appear and quickly disappear, followed by yet another increase in absorption in the 446 nm region of the difference spectrum; the latter and the type I spectral change then rapidly disappeared. This sequence is shown in Fig. 4 for nortriptyline with liver cells from control rats and in Fig. 5 for lidocaine with liver cells from phenobarbital-treated rats. It should be added that the peaks at 437 nm and 446 nm varied in amplitude between different experiments but were always seen under aerobic conditions.

When aniline was added to the isolated rat liver cells to a final concentration of 10 mM, a more complex difference spectrum, probably including a type I as well as a type II component, was seen. Ethanol addition, finally, produced no observable rapid changes in the difference spectrum.

Discussion

This study has shown that certain optical absorption characteristics of liver cytochrome P-450 in some of its various states can also be observed in

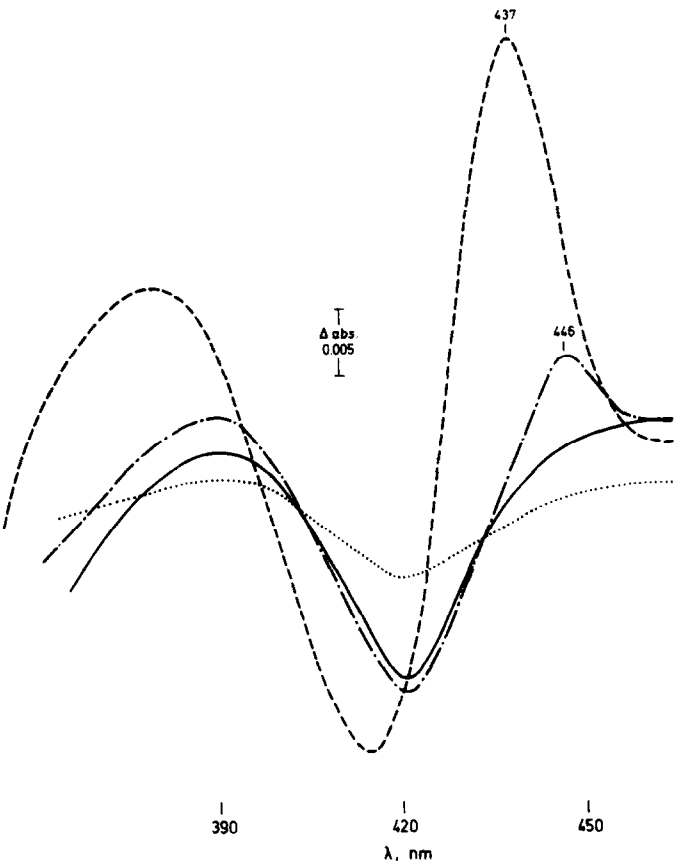

Fig. 5. Spectral changes produced by lidocaine addition to liver cells from phenobarbital-treated rat. Each cuvette contained ~1x10^6 cells per ml. Lidocaine was added to the medium to a final concentration of 20 μM and repetitive scanning was performed. (—) 15 sec, (--) 90 sec, (—·—) 120 sec and (···) 180 sec after lidocaine addition. Temperature was 37°C.

isolated liver cells. As expected, the cellular level of cytochrome P-450, as well as the absorption changes associated with drug binding and monooxygenation, were markedly enhanced by phenobarbital pretreatment of the animals. It was of interest to note, that only a minor portion of the cytochrome P-450 present was endogenously reducible in the absence of added substrate. This fraction, which was larger in liver cells from phenobarbital-treated rats, was considerably increased upon the addition of hexobarbital to the medium. It is tempting to speculate that only the substrate-bound fraction of cytochrome P-450 can accept endogenous reducing equivalents and thus interact with CO. This would in turn imply that the major fraction of the cytochrome P-450 in the isolated liver cells is present in the oxidized, non-substrate-bound state.

This hypothesis is further supported by the finding that the addition of certain drugs to suspensions of liver cells produced a type I spectral change. Although drug uptake into the isolated cells - as measured by the formation of the type I spectral change - was rapid and took place within seconds, it was considerably slower than with liver homogenates or isolated microsomes. Whether drug uptake into the isolated liver cells is actually an active process is, however, too early to decide.

Further experiments are also needed to elucidate the nature of the absorption peaks at about 437 nm and 446 nm observed during steady state drug monooxygenation. The fact that they appeared early after drug addition to the medium under aerobic conditions, had relatively short halflives and were increased in magnitude in liver cells isolated from phenobarbital-treated rats suggest that they may reflect different cytochrome P-450 states during the monooxygenation process. In this case, the 437 nm peak could possibly correspond to an increase in absorption observed in the same region of the difference spectrum of liver microsomes during steady state monooxygenation of drug substrates, which has been ascribed to an oxygenated form of cytochrome P-450 (3). It is still too early to speculate about the nature of the 446 nm peak.

In conclusion, the fact that it is possible to study the interaction of drug substrates with cytochrome P-450 in isolated liver cells makes this system a powerfull tool in the study of drug metabolism and its cellular regulation.

Acknowledgements

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References

1. V. Ullrich, Angew. Chem. Internat. Ed. Engl., 11, 701(1972).
2. J.B. Schenkman, H. Remmer and R.W. Estabrook, Mol. Pharmacol., 3, 113(1967).
3. R.W. Estabrook, A.G. Hildebrandt, J. Baron, K.J. Netter and K. Leibman, Biochem. Biophys. Res. Commun., 42, 132(1971).

4. C.A. Tyson, R. Tsai and I.C. Gunsalus, J. Amer. Oil. Chem. Soc., 47, 7(1970).

5. B. Quistorff, S. Bondesen and N. Grunnet, Biochim.Biophys.Acta, 320, 503(1973).

6. H.A. Krebs and K. Henseleit, Hoppe-Seylers Z. Physiol. Chem., 210, 33(1932).