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EFFECT OF PHENOBARBITAL ADMINISTRATION TO RATS ON THE LEVEL OF THE IN VITRO SYNTHESIS OF CYTOCHROME P-450 DIRECTED BY TOTAL RAT LIVER RNA

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SUMMARY

Total liver RNA has been isolated from male rats at different time points subsequent to a single injection of phenobarbital, and the level of cytochrome P-450 synthesis directed by these RNA preparations in a cell-free translation system has been determined. It is observed that the maximum in vitro synthesis of cytochrome P-450 occurs at 16 hours (3-fold above uninduced level) which is approximately 30 hours prior to the maximum induction of spectrophotometrically detectable cytochrome P-450 measured in liver homogenates. Thus, while cytochrome P-450 mRNA is involved in the induction process, its synthesis does not appear to be rate limiting. In addition, phenobarbital induced cytochrome P-450 is not synthesized in vitro in a form larger than that isolated from endoplasmic reticulum, but rather is also found to have a molecular weight of 50,000.

INTRODUCTION

In rat liver, at least three different forms of the mixed function oxidase, cytochrome P-450, are present whose concentrations can be modified by treatment with different xenobiotics (1). The explanation for this diversity of forms and their responsiveness to different inducing agents will eventually arise from knowledge of the organization of the cytochrome P-450 gene(s). The recent development of specific antibodies to the different forms of rat liver cytochrome P-450 (1,2) makes it possible to initiate studies on the molecular basis of its induction by xenobiotics. In principle, induction may result from an increase in the rate of de novo enzyme synthesis, a decrease in the rate of enzyme degradation, activation of existing enzyme molecules or some combination of these processes. An increase in the rate of enzyme synthesis may be due to an effect of the inducing agent at the transcriptional or translational level. In the present study, we have measured the effect of adminis-

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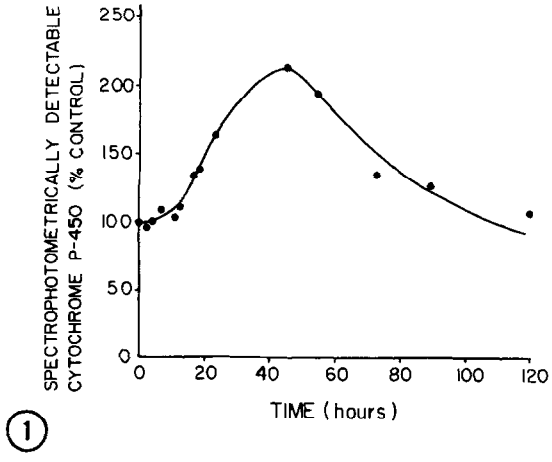
tration of a single dose of phenobarbital to rats on the ability of total liver RNA to direct the synthesis of cytochrome P-450 in a cell-free translation system and have compared these results to the induction of this heme protein as determined spectrophotometrically. Phenobarbital is a well-known inducer of the drug metabolizing enzymes in the liver (3), i.e. the cytochrome P-450 level increases 3 or 4 fold (4). The results presented here suggest that phenobarbital induction is related to an increase in the level of the mRNA able to encode for cytochrome P-450.

METHODS

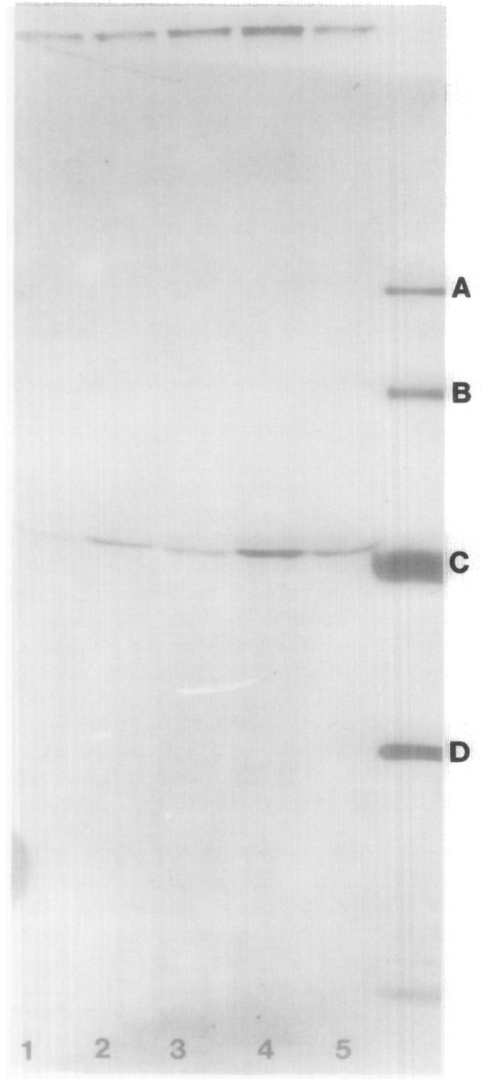
Male Sprague-Dawley rats (180-210 gm) were given a single injection of phenobarbital (80 mg/kg) and sacrificed by exsanguination at the indicated time points. The livers were perfused with 0.15 M NaCl and total RNA isolated by the guanidine hydrochloride extraction procedure (5). The RNA was translated in a rabbit reticulocyte lysate translation system obtained from New England Nuclear using [^{35}S]-methionine. Each RNA sample was titrated in the translation system to ensure that the RNA concentration corresponded to a point on the linear portion of the radioactivity incorporation curve. The concentration of RNA in the translation experiments, measured by absorbance at 260 nm, varied between 2 µg and 6 µg per 25 µl of the translation system. The synthesis of cytochrome P-450 in the lysate, following a 60 minute incubation with rat liver RNA at 37°C, was determined using the antibody described below. Immune IgG (3.7 mg) was added to the lysate following the translation incubation and allowed to react with newly synthesized cytochrome P-450 for 20 minutes at 37°C. Immunoprecipitation of the newly synthesized cytochrome P-450-antibody complex was achieved by addition of partially purified cytochrome P-450 as carrier (0.26 nmoles). In each complete translation experiment the immunoprecipitation of the translation products was normalized as follows. A 2 µl aliquot of each translation system was precipitated on Whatman 3MM filter paper with 6% trichloroacetic acid and the amount of radioactivity determined. Then, in each experiment, the same amount of acid-precipitable radioactivity was immunoprecipitated for each time point. Each immunoprecipitate was thoroughly washed (6), dissolved in SDS-electrophoresis buffer and electrophoresed on a 7.5% to 12.5% polyacrylamide gradient gel following the method of Laemmli (7). The gel was dried onto Whatman 3MM filter paper and exposed to Kodak XR-2 x-ray film. The dried gel was then sliced and the amount of radioactivity in each slice determined following treatment with hydrogen peroxide.

The antibody to purified cytochrome P-450 was raised in New Zealand White rabbits by injection of highly purified phenobarbital induced rat liver cytochrome P-450. This antigen was kindly provided to our laboratory by Dr. Anthony Lu and Susan West of the Merck, Sharp and Dohme Research Laboratory (8). One milligram of the immune IgG used in these studies was shown to precipitate 0.24 nmoles cytochrome P-450 as measured spectrophotometrically, whereas the addition of preimmune IgG resulted in no change in absorbance.

The level of cytochrome P-450 in liver homogenates, prepared at selected times following administration of a single dose of phenobarbital to rats, was determined spectrophotometrically (9). The protein concentration of each liver homogenate was determined by the biuret method (10). The molecular weight of the newly synthesized cytochrome P-450 was determined by measuring the distance of migration on the autoradiogram. Distance of migration of standard



(1)



(2)

Figure 1: Time course of the appearance of spectrophotometrically detectable cytochrome P-450 in liver homogenates following administration of a single dose (80 mg/kg) of phenobarbital to rats. The cytochrome P-450 assay was carried out as a reduced-CO minus CO difference spectrum using an extinction coefficient of 104 mM^{-1} cm^{-1} (9). Each point represents an average of at least two measurements, each carried out on a homogenate prepared by pooling the livers of three rats to minimize individual differences. The uninduced (0 hours) value was found to be 0.27 nmoles cytochrome P-450/mg protein in three separate assays.

Figure 2: Autoradiogram prepared from the SDS-polyacrylamide gel electrophoresis of the immunoprecipitates obtained in experiment 1 (see Figure 3). The different lanes on the gel represent the cytochrome P-450 synthesized from RNA isolated at different time points following phenobarbital administration to rats. Lane 1- 0 hours; Lane 2- 4 hours; Lane 3 - 10.5 hours; Lane 4 - 16 hours; Lane 5 - 24 hours. Radioactive molecular weight markers were electrophoresed in the far right lane of the gel: A - phosphorylase B (92,500); B - bovine serum albumin (69,000); C - ovalbumin (46,000); D - carbonic anhydrase (30,000). The molecular weight of the cytochrome P-450 synthesized *in vitro* was determined to be 50,000 daltons (see Fig. 4).

proteins labeled with [^{14}C] (New England Nuclear) was determined from the same autoradiogram.

RESULTS AND DISCUSSION

Figure 1 shows the time course for the appearance of spectrophotometrically detectable cytochrome P-450 in liver homogenates prepared at various time points following administration of a single dose of phenobarbital. The maximal appearance of the cytochrome P-450 measured in this way occurs in the second half of the second day following phenobarbital administration, a result similar to that reported by others (11,12). To investigate the mechanism by which this induction occurs we isolated total rat liver RNA at various times following administration of phenobarbital, and then measured the level of cytochrome P-450 synthesis in an *in vitro* translation system. The results of such an experiment are shown in Figure 2. When immunoprecipitates from the translation of RNA isolated at different times were thoroughly washed and electrophoresed, a radioactive band was observed on the resultant autoradiogram, which corresponded to a protein having a molecular weight of 50,000 daltons. Control experiments were carried out to ensure that the cytochrome P-450 was quantitatively immunoprecipitated from the translation products.

Figure 3 displays the profile of the *in vitro* synthesis of cytochrome P-450 in two separate experiments. The results are expressed as a per cent of the cytochrome P-450 synthesis directed by RNA isolated from untreated rats. In both experiments, a 3-fold increase in the *in vitro* synthesis is observed using RNA isolated at 16 hours following phenobarbital administration. Using RNA isolated up to 12 hours subsequent to phenobarbital administration or at time periods longer than 16 hours, the *in vitro* cytochrome P-450 synthesis is significantly less than that observed at 16 hours. The radioactivity determined for each time point in Fig. 3 is shown in Table 1.

The results in Figures 2 and 3 show that it is possible to direct the *in vitro* synthesis of cytochrome P-450 using total rat liver RNA. When we compare the induction of spectrophotometrically detectable cytochrome P-450

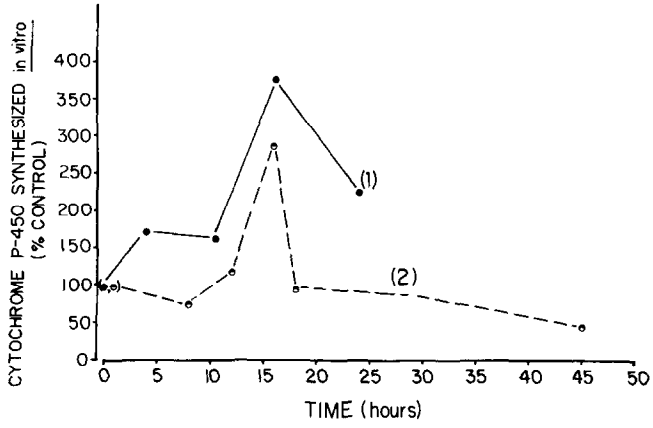


Figure 3: Level of the in vitro synthesis of cytochrome P-450 directed by total rat liver RNA expressed as a per cent of the uninduced (0 hours) level of synthesis. The abscissa is the time at which the total liver RNA was isolated following phenobarbital administration to rats. Radioactivity was extracted with H_2O_2 from gel slices containing the newly synthesized, 50,000 molecular weight cytochrome P-450 precipitated by the immune IgG. In experiment 1 (●) 400,000 cpm were immunoprecipitated at each time point while in experiment 2 (◓) 180,000 cpm were immunoprecipitated at each point. In both experiments, each point represents the level of translation directed by RNA isolated from three pooled rat livers to minimize individual differences.

TABLE I Time Course of the Appearance of Radioactivity in Cytochrome P-450 Synthesized in vitro

Experiment No.	Hours Following Phenobarbital Administration to Rats	CPM Over Background in Gel Slice 17 (50,000 Molecular Weight)
1[a]	0	138
	4	351
	10.5	323
	16	1186
	24	629
2[b]	0	151
	8	87
	12	108
	16	576
	18	130
	45	53

[a] Immunoprecipitation at each time point was from 400,000 TCA precipitable cpm.

[b] Immunoprecipitation at each time point was from 180,000 TCA precipitable cpm.

(Fig. 1) to the induction of the ability of rat liver RNA to direct cytochrome P-450 synthesis in vitro (Fig. 3), we see a difference of approximately 30 hours in the maximum appearance of these two events. Also it is seen that the capacity of RNA to direct cytochrome P-450 synthesis increased 3-fold, while the

level of spectrophotometrically detectable product increased 2-fold. The ability of RNA to direct in vitro cytochrome P-450 synthesis appears much earlier than the spectrophotometrically detectable induction of this heme protein. Therefore, the rate-limiting step in the overall induction process may be an alteration of the newly synthesized cytochrome P-450 apoprotein such as heme binding. It is also possible that the mRNA coding for cytochrome P-450 is not available in a form usable for protein synthesis in vivo until a later time. However, a previous study, the maximal level of immunodetectable radiolabelled cytochrome P-450 synthesized in vivo was observed at 12 hours, followed by a decrease in this level at 18 hours (13).

Figure 3 reveals an apparent lag of approximately 12 hours before an increase in the level of the in vitro synthesis of cytochrome P-450 occurs. In a study of the effect of α-amanitin on the induction of cytochrome P-450 it was observed that administration of this RNA polymerase inhibitor, up to 8 hours following phenobarbital administration, inhibited the induction of cytochrome P-450 and it was concluded that induction required de novo RNA synthesis (14). It is attractive to speculate that the increased ability of total RNA to direct cytochrome P-450 synthesis observed in the present study arises from an increase in the number of cytochrome P-450 specific mRNA sequences, but such a conclusion must await preparation of a probe capable of directly measuring the number of such sequences.

The cytochrome P-450 synthesized in vitro has a molecular weight of 50,000 daltons (Fig. 4), the same as determined for the phenobarbital-induced form isolated from rat liver (1,8,15,16). Therefore, cytochrome P-450 is not synthesized in a form substantially larger than that isolated from endoplasmic reticulum. Proteins have been found to be synthesized in larger precursor forms, which are processed upon insertion into membranes (17-19), as well as in forms containing signal sequences which are not processed (20). It will be interesting to determine the requirements for insertion of cytochrome P-450

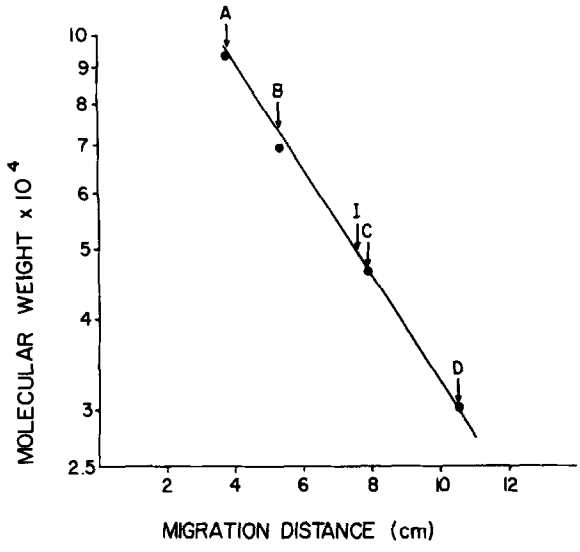


Figure 4: Semi-log plot of protein molecular weight vs migration distance on SDS-polyacrylamide gel electrophoresis. Molecular weight standards are the same as described in Fig. 2. Notation "I" refers to the immunoprecipitated radioactive protein from a 16 hour translation sample from experiment 1 (Lane 4, Fig. 2). The molecular weight of "I" as determined from this graph is 50,000.

into the endoplasmic reticulum, based on the observation that the product synthesized in vitro is indistinguishable in size from that found in vivo.

In summary, we have shown for the first time that following phenobarbital administration to rats, the capacity of isolated liver RNA to direct the in vitro synthesis of cytochrome P-450 increases and that this increase occurs much earlier than the spectrophotometrically detectable increase of cytochrome P-450 measured in liver homogenates. This result indicates that while cytochrome P-450 mRNA is involved in the induction process, its synthesis is not the rate-limiting step. Finally, the cytochrome P-450 synthesized in vitro is indistinguishable in size from that isolated from rat liver endoplasmic reticulum.

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