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SYNTHESIS OF LIVER CYTOCHROME P-450b IN A CELL-FREE PROTEIN SYNTHESIZING SYSTEM

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SUMMARY. Liver polysomes isolated from rats that had been treated with phenobarbital (PB) are able to incorporate [^{3}H]leucine into total protein *in vitro* at a rate almost five times that of polysomes prepared from control animals. Specific immunoprecipitation of translational products has shown that polysomes from induced animals synthesize cytochrome P-450b at a rate almost seven times greater than polysomes from control animals. The increased protein and cytochrome P-450b synthesis can be detected as early as 6 h following phenobarbital administration and reaches a maximum at 12-18 h. The results suggest that PB administration effects an increase in mRNA for cytochrome P-450b.

It has been known for quite some time that a wide variety of drugs and polycyclic aromatic hydrocarbons are able to markedly increase the activity of the mixed function oxidase enzymes such as aryl hydrocarbon hydroxylase (1-3). In particular, phenobarbital administration is known to increase the activity of certain species of the cytochrome P-450 dependent enzymes (4-6). The major PB inducible enzyme in rat liver, referred to as cytochrome P-450b, has been isolated and purified to apparent homogeneity (7-9). Although the mechanism by which phenobarbital is able to elevate the level of this cytochrome is not fully understood, previous work has earmarked transcription and translation as the sites of action. Thus, after phenobarbital administration, an increased incorporation of precursors into ribosomes and ribosomal RNA was noted (10-12). Microsomal enzyme induction has also been reported to be blocked by the prior

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administration of amino acid analogs (13), and by inhibitors of translation (14-16) and transcription (14, 16-18).

In the present study, we have investigated the effects of phenobarbital administration on cytochrome P-450b synthesis using a liver polysomal-wheat germ lysate. Cytochrome P-450b synthesis is markedly enhanced, suggesting an elevated mRNA content of the polysomes. Maximum polysome activity was demonstrable at 12-18 h post treatment of the rats with the barbiturate.

MATERIALS AND METHODS

Polysome preparation. Male Sprague-Dawley rats (90-100g) fed and watered ad libitum were used for all studies. Phenobarbital (PB) was administered i.p. at 75 mg/kg body weight at various times preceding sacrifice. Control animals received the solvent alone, i.e., 0.9% saline. Polysomes were prepared by centrifugation through a 1.5 M sucrose cushion as described by McNelis and Cutroneo (19). The RNA concentration of the polysomes was determined assuming 1 mg/ml as equivalent to an absorbancy at 260 nm of 20.

Cell-free protein synthesis. Incorporation of [^{3}H]leucine (Amersham-Searle, 50 Ci/mmole, 0.2 μCi/assay) into protein was performed using a wheat germ in vitro translational system obtained from Bethesda Research Laboratories. Liver polyribosomes (10 μg polysomal RNA) were added to the wheat germ extract containing 155 mM K^{+2}, 2.6 mM Mg^{+2} and 80 μM spermidine in a total volume of 30 μl. This combined wheat germ-liver polysome system is a modification of that which was published by McNelis and Cutroneo (19). All incubations were for 1 h at 25°C. The reactions were terminated by the addition of 1.5 M Tris, pH 10.0 and a further incubation was conducted at 37°C for 20 min. Protein was precipitated with 10% trichloroacetic acid (TCA), and collected on 0.45 μ Millipore filters, followed by several washings with 5% TCA. The radioactivity was determined in a toluene-based scintillation fluid. The incorporation of [^{3}H]leucine into protein was completely dependent upon the addition of polysome fractions.

Immunoprecipitation. Cytochrome P-450b was purified to apparent homogeneity from microsomes of PB-treated rats (8). Antibodies to cytochrome P-450b were prepared in rabbits (20) and were made monospecific by employing affinity chromatography (7). One nmole of cytochrome P-450b was completely neutralized by 15 mg of antibody. Following translation of the polysomes in the wheat germ system, the reaction mixtures were diluted to 1 ml with solution A (50 mM Tris, pH 7.5-200 mM NaCl-0.1% sodium dodecyl sulfate (SDS)-1% Triton X-100 0.5% sodium deoxycholate-50 mM leucine). Carrier cytochrome P-450b (3.5 μg) and anticytochrome P-450b were added. The reaction mixture was incubated at 25°C for 45 min and then overnight at 4°C. On the second day, 75 μl of Pansorbin A (Calbiochem-Behring Corp., La Jolla, CA) was added and incubation was conducted at room temperature for 1 h. The Pansorbin antigen-antibody complex was spun through a discontinuous gradient of 0.5 M sucrose over 1.0 M sucrose in solution B (50 mM Tris, pH 7.5 - 200 mM NaCl-20 mM EDTA 2% Triton X-100-2% sodium deoxycholate), according to the method of Taylor and Schimke (21). The pelleted material was resuspended in solution B and the centrifugation was repeated. The final pellet was suspended in 0.4% Emulgen 911 and an aliquot was counted for radioactivity.

SDS Polyacrylamide gel electrophoresis. Aliquots of the immunoprecipitates or other standard protein samples were dissolved in a minimal volume of 62 mM

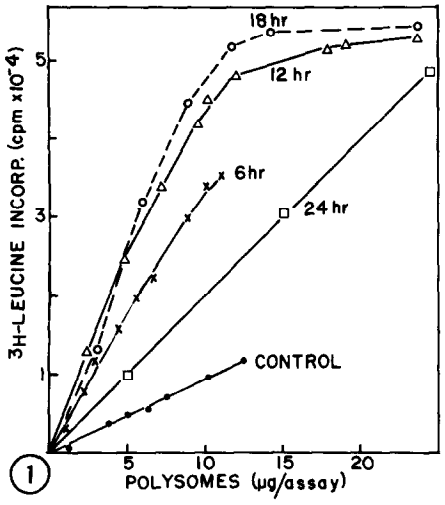
Tris, pH 6.8 - 1% SDS - 0.01% bromphenol blue - 10% glycerol - 5% β mercaptoethanol. The samples were boiled for 5 min and electrophoresed on 7.5% polyacrylamide gels as described by Laemmli (22). After visualization of the protein by Coomassie blue R-250 staining, 1 mm gel slices were cut and treated with 30% H_2O_2- 1% NH_4OH in scintillation vials. After neutralization of the contents of each vial, the samples were counted in a Triton X-100-toluene based scintillation counting fluid. In all cases, the position of authentic cytochrome P-450b superimposed upon the major peak of radioactivity (excluding gel front).

RESULTS AND DISCUSSION

Polysomes isolated at various times following phenobarbital administration showed a differential ability to incorporate [^{3}H]leucine into protein in an *in vitro* translation system. Fig. 1 shows that increased protein synthesis was observed as early as 6 h post-treatment of the rats with phenobarbital, reached a maximum at 12-18 h, and decreased significantly by 24 h. At its maximum, [^{3}H]leucine incorporation was stimulated 5-fold over control values.

When the polysome-wheat germ reaction mixtures were incubated with monospecific antibody to cytochrome P-450b in the presence of carrier antigen, newly-synthesized apoprotein and other incomplete cytochrome P-450b polypeptides were immunoprecipitated. The incorporation of [^{3}H]leucine into total protein and cytochrome P-450b is reported in Table 1 as a function of time after administration of phenobarbital. Maximal incorporation into the apoprotein followed a similar time course as total protein synthesis. Cytochrome P-450b synthesis was 4.2 times greater than control at 6 h post-injection; at 18 h, a 6.7 fold elevation was apparent. Synthesis of cytochrome P-450b at 25 h was only 2.4 fold greater than control values. Immunoprecipitated translational products from the polysomes of control rats did not show a peak of radioactivity in the region of cytochrome P-450b following gel electrophoresis. This is consistent with the low level of cytochrome P-450b (7% of total cytochrome P-450) in control rat liver (7).

When immunoprecipitates from the *in vitro* translational experiments were electrophoresed on SDS-polyacrylamide gels, a single peak of radioactivity was observed in gel slices as seen in Figure 2 (excluding the radioactivity in the small molecular weight material at the gel front). When unlabeled authentic cytochrome P-450b was mixed with the immunoprecipitate, the protein staining



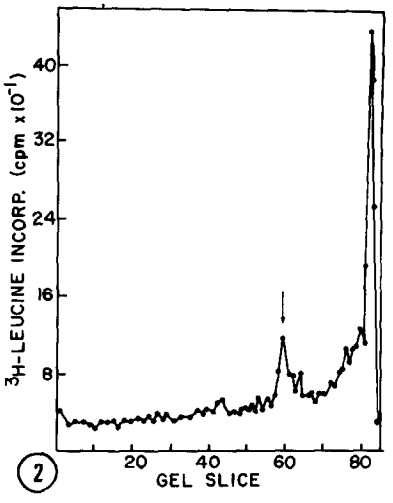


Figure 1: Polysomes isolated 6-24 hours after PB administration, or from control animals were incubated for 60 min at 25°C in the wheat germ translational system. Incorporation of [^{3}H]leucine into trichloracetic acid precipitable protein was measured as a function of polysomal RNA concentration. Optimal concentration was chosen as 10 µl assay volume.

Figure 2: Polysomes isolated 18 hours post PB administration were translated as described in Figure 1 at a concentration of 50 µg per 150 µl reaction volume. Cytochrome P-450b labelled *in vitro* with [^{3}H]-leucine was immunoprecipitated and electrophoresed on a 7.5% SDS-polyacrylamide gel. Aside from the gel front; the major peak of radioactivity was coincident with the Coomassie blue staining region of purified cytochrome P-450b run in an adjacent well.

band was coincident with the peak of radioactivity (position of arrow in Figure 2). Thus, the *in vitro* synthesized product was indistinguishable from cytochrome P-450b by SDS-polyacrylamide gel electrophoresis.

TABLE 1

Incorporation of [^{3}H]Leucine into Total Protein and Cytochrome P-450b.

	[^{3}H]Leucine Incorporation			
Time After Phenobarbital Administration (hr)	Total Protein CPM	Total Protein Experimental / Control	Cytochrome P-450b CPM	Cytochrome P-450b Experimental / Control
Control	9,600	1	292	1
6	33,000	3.4	1,220	4.2
12	43,800	4.6	1,301	4.5
18	47,300	4.9	1,941	6.7
24	20,300	2.1	691	2.4

Legend: Liver polysomes were prepared from rats that had been pretreated with either saline or phenobarbital and were assayed for translational activity in the wheat germ system as described in the text. In all cases, CPM's reported are per 10 µg of polysomal RNA in the incorporation experiments.

The data presented in this report indicate that the polysomes isolated from phenobarbital-treated rats are considerably more active in the translation of cytochrome P-450b. They also appear more active in the incorporation of amino acids into total protein. Previously Craft et al (23) had demonstrated the in vitro incorporation of amino acids into microsomal preparations and the immunoprecipitation by antibodies to a mixture of cytochrome P-450's. However, their data were derived completely from the analysis of radioactivity in the immune complex. Furthermore, the antibody preparation employed by Craft et al (23) was not monospecific for a single form of cytochrome P-450, c.f. ref (20). Our SDS gel electrophoresis results would lead us to suspect that antibody reacts with a considerable quantity of partially completed or degraded protein; the latter appear at the front with the tracking dye. Electrophoresis would seem a technical requirement in these types of studies. Bhat and Padmanaban (24) have also recently reported the synthesis of cytochrome P-450 using a post-mitochondrial supernatant fraction from the livers of phenobarbital-treated rats. Their in vitro data which are also based upon quantitation of the immune complex indicate, as do ours, an early enhancement in messenger activity after phenobarbital administration; they noted maximal activity by 12 h.

We are currently attempting the immunoprecipitation of cytochrome P-450b bearing polysomes with the aim of ultimately purifying this messenger activity. Our results so far would indicate the feasibility of this approach.

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