Nossal, N. G., & Peterlin, B. M. (1979) *J. Biol. Chem. 254*, 6032-7.
Reynolds, R. J. (1978) *Mutat. Res. 50*, 43-56.
Scherzinger, E., Lanka, E., Morelli, G., Seiffert, D., & Yuki, A. (1977) *Eur. J. Biochem. 72*, 543-58.
Setlow, P., Brutlag, D., & Kornberg, A. (1972) *J. Biol. Chem. 247*, 224-31.
Studier, F. W. (1965) *J. Mol. Biol. 11*, 373-90.
Thomas, K. R., & Olivera, B. M. (1978) *J. Biol. Chem. 253*, 424-9.
Wang, T. S.-F., Sedwick, W. D., & Korn, D. (1974) *J. Biol. Chem. 249*, 841-50.
Wang, T. S.-F., Eichler, D. C., & Korn, D. (1977) *Biochemistry 16*, 4927-34.
Waser, J., Hubscher, U., Kuenzle, C. C., & Spadari, S. (1979) *Eur. J. Biochem. 97*, 361-8.
Weiss, B., Live, T. R., & Richardson, C. C. (1968) *J. Biol. Chem. 243*, 4530-42.
Weissbach, A. (1977) *Annu. Rev. Biochem. 46*, 25-47.
Wickner, S. H. (1978) *Annu. Rev. Biochem. 47*, 1163-91.

Phenobarbital Induction of NADPH-Cytochrome *c* (P-450) Oxidoreductase Messenger Ribonucleic Acid†

Frank J. Gonzalez and Charles B. Kasper*

ABSTRACT: Total RNA isolated from polysomes tightly bound to the rat liver endoplasmic reticulum directed the synthesis of NADPH-cytochrome *c* oxidoreductase (EC 1.6.2.4) by means of a rabbit reticulocyte protein synthesizing system. The oxidoreductase was immunoprecipitated by a monospecific antibody from total translation products and shown to be identical with the enzyme synthesized in vivo in terms of molecular weight and peptide fingerprint patterns, indicating that the oxidoreductase is not synthesized in a form larger than the native enzyme. RNA isolated from free polysomes and polysomes loosely bound to the endoplasmic reticulum did not direct the synthesis of the enzyme in the cell-free system. Phenobarbital administration resulted in a threefold increase in translatable levels of oxidoreductase mRNA; this maximum level was reached 4 h after a single dose of the drug. Cordycepin decreased this response by 50% when administered 20 min before phenobarbital. After reaching its maximum level, induced oxidoreductase mRNA declined with an estimated half-life of 9 h. Phenobarbital administration also resulted in a threefold increase in the in vivo rate of incorporation of [^{3}H]leucine into oxidoreductase 3 h after the maximal increase in oxidoreductase mRNA was obtained. The rate of incorporation of isotope into total microsomal protein, however, did not increase. Cytochrome b_5, an intrinsic microsomal membrane protein not inducible under these conditions, was synthesized from free and loosely membrane-bound polysomal RNA, and translatable levels of its mRNA did not increase upon phenobarbital administration. Serum albumin, which was synthesized from tightly membrane-bound polysomal RNA, also was not increased by phenobarbital administration. These data demonstrate that two intrinsic microsomal membrane proteins, both of which are anchored to the membrane via hydrophobic segments of their polypeptide chain, are synthesized on separate populations of polysomes. Induction of enzymes by phenobarbital does not result solely from a stabilization of protein or mRNA or from an increase in the rate of translation of certain mRNAs. Accumulation of mRNA results from either an increased rate of transcription or posttranscriptional processing and nucleocytoplasmic transport of specific mRNAs.

Chronic phenobarbital administration results in an increased rate of oxidative drug metabolism which is attributed to an increase in activity of the endoplasmic reticulum mixed function oxidase system (Conney et al., 1960; Remmer & Merker, 1963; Orrenius et al., 1965). This change is correlated with a proliferation of smooth endoplasmic reticulum and an increase in the level of certain enzymes involved in drug metabolism (Ernster & Orrenius, 1965; Orrenius & Ericksson, 1966; Conney, 1967; Lu et al., 1969). The mechanism of phenobarbital induction is poorly understood, however. Maximum increase in an inducible enzyme is obtained only after daily administration of the drug and peaks between 4 and 5 days after the first inoculation (Jick & Shuster, 1966; Kuriyama et al., 1969). This is in contrast to another class of drug metabolism inducing agents, which includes the polycyclic aromatic hydrocarbons, in that a maximal increase in specific activity of inducible enzymes occurs 1 to 2 days after a single dose of the inducer with no increase in smooth endoplasmic reticulum (Conney et al., 1957; Conney, 1967; Parke, 1976). The mechanism of these inducing agents appears to be similar to that of steroid hormones, where a receptor-ligand complex influences a battery of genes by promoting transcription (Poland & Glover, 1976). No direct evidence for a phenobarbital receptor, however, has been demonstrated. Another important biochemical distinction between the two types of inducing agents is that phenobarbital preferentially induces certain enzymes in the endoplasmic reticulum but does not induce their nuclear envelope counterparts; yet, polycyclic aromatic hydrocarbons induce certain enzymes in both membrane systems (Kasper, 1971; Khandwala & Kasper, 1973; Fahl et al., 1978).

The enzyme NADPH-cytochrome *c* oxidoreductase has been extensively studied with respect to its induction by phenobarbital. Early reports demonstrated that an increase in the rate of synthesis of the enzyme occurred early after a single dose of the drug; however, the rate of synthesis of another membrane-bound protein, cytochrome b_5, did not increase (Jick & Shuster, 1966; Kuriyama et al., 1969). In

† From the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706. *Received September 17, 1979.* This work was supported by Grants CA-23076 and CA-17300 from the National Cancer Institute, Department of Health, Education, and Welfare.

0006-2960/80/0419-1790$01.00/0

addition, the rate of degradation of both enzymes decreased following phenobarbital administration (Jick & Shuster, 1966; Kuriyama et al., 1969). These observations suggest that phenobarbital may both increase the synthesis and decrease the degradation of certain enzymes. In order to probe the mechanism by which phenobarbital causes a specific increase in synthesis of certain microsomal membrane proteins, we have investigated the levels of mRNA coding for the inducible NADPH–cytochrome *c* oxidoreductase and noninducible cytochrome b_5 at early times after administration of the drug. Serum albumin mRNA levels were also measured as an added control. Intracellular sites of synthesis of the two intrinsic microsomal membrane proteins were also examined.

Materials and Methods

Materials. Radioactive L-[^{35}S]methionine (500–1000 Ci/mmol), L-[4,5-$^{3}H_2$]leucine (40–60 Ci/mmol), and protosol were obtained from New England Nuclear. Cordycepin, creatine phosphate, creatine phosphokinase type I, equine hemin, dithiothreitol, heparin, yeast RNA, Triton X-100, 2-mercaptoethanol, rat serum albumin, and all unlabeled amino acids were products of Sigma Chemical Co. Micrococcal nuclease was purchased from Boehringer Mannheim, and special, enzyme grade sucrose was obtained from the Schwarz/Mann Div. of Becton, Dickinson and Co. Electrophoresis grade acrylamide, *N,N'*-methylenebis(acrylamide), and sodium dodecyl sulfate were Bio-Rad products. Oligo(dT)–cellulose type 3 was purchased from Collaborative Research, Inc. All other chemicals were reagent grade. The salt and buffer solutions were autoclaved prior to use, and the sucrose was autoclaved dry before being dissolved in the appropriate buffer. All glassware that came in contact with RNA was heated at 160 °C for several hours.

Animals. Male Sprague-Dawley rats weighing 180–200 g were obtained from Holtzman Co., Middleton, WI. The rats were maintained ad libitum on a standard laboratory chow diet for 1 week and fasted for 15 h prior to sacrifice. For induction studies, animals were injected in a caudal tail vein with a 10% solution of sodium phenobarbital in 0.15 M NaCl at a dose of 100 mg/kg. Cordycepin (3 mg/100 g) was injected interperitoneally. Control animals received an intravenous injection of 0.15 M NaCl adjusted to pH 10.0 (the same pH as a 10% sodium phenobarbital solution) of the same volume given the experimental animals. In vivo labeling studies were performed by giving intraperitoneal injections of 60 μCi of L-[^{3}H]leucine in 0.15 M NaCl 40 min before the animals were killed.

Isolation of Polysomes. Rat liver free and tightly and loosely membrane-bound polysomes were isolated as described by Ikehara & Pitot (1973), with the following modifications. Heparin was added to the postmitochondrial supernatant to give a final concentration of 0.5 mg/mL and was included at this concentration in all buffer solutions and membrane dilutions throughout the isolation procedure. Loosely membrane-bound polysomes were isolated from rough endoplasmic reticulum by use of the high salt wash of Kruppa & Sabatini (1977) and sedimentation of the polysomes through a 2.0 M sucrose cushion as described (Ikehara & Pitot, 1973). High salt washed rough endoplasmic reticulum was detergent treated in the presence of heparin, 0.6 M KCl, and 50% high-speed supernatant (Kruppa & Sabatini, 1977) as described previously (Ikehara & Pitot, 1973) to liberate the tightly membrane-bound polysomes.

Isolation of Total Polysomal RNA. Extraction of polysomal RNA was carried out by a modification of the phenol and chloroform procedure of Perry et al. (1972), as described by Moore et al. (1977). After extraction and precipitation, the RNA was washed 3 times with 3 M sodium acetate (pH 6.0) and dialyzed exhaustively against doubly distilled water at 4 °C. The RNA was lyophilized to dryness and resuspended in an appropriate volume of doubly distilled water for translation.

Transfer RNA from rat liver was purified by extraction of the high-speed supernatant, as described above, and deacylated according to the procedure of Roberts et al. (1973). RNA concentrations were determined spectrophotometrically; an absorbance of 1.0 at 260 nm was considered equivalent to 45 μg/mL RNA.

Lysate Preparation and Translation Conditions. The rabbit reticulocyte lysate was prepared according to the scheme of Villa-Komaroff et al. (1974) and stored in small aliquots at −80 °C. The translation assay used was essentially that of Pelham & Jackson (1976). The final reaction mix contained 0.75 mM $Mg(OAc)_2$, 100 mM KCl, and 500 μCi/mL L-[^{35}S]methionine. Deacylated tRNA from rat liver was included at a concentration of 120 μg/mL, and polysomal RNA translated at a concentration of 0.75 mg/mL. Addition of this amount of total membrane-bound polysomal RNA stimulated translation 10-fold over background.

The translation reactions were incubated at 30 °C for 50 min and terminated by cooling the sample in an ice bath and diluting with 2 volumes of 50 mM Tris-HCl, pH 7.4, containing 75 mM KCl and 5 mM $MgCl_2$. A stock solution of 10% each of Triton X-100 and sodium deoxycholate was added to a 1% final concentration, and polysomes, ribosomes, and ribosomal subunits were sedimented by centrifugation at 90000*g* for 3 h in a Beckman 25 rotor. The volumes of the supernatants were measured, and 2 μL was spotted onto a Whatman No. 3 MM filter. Filters were washed by being boiled in 10% trichloroacetic acid for 10 min and rinsed twice each with 5% trichloroacetic acid, methanol, and anhydrous ether, and then they were treated with hydrogen peroxide and the radioactivity was measured. The remaining supernatant was used for immunoprecipitation.

Purification of NADPH–Cytochrome c Oxidoreductase and Cytochrome b_5. Oxidoreductase was purified as described by Zimmerman & Kasper (1978). Cytochrome b_5 was purified according to the scheme of Omura & Takesue (1970).

Preparation of Antibody. Antisera to rat serum albumin, oxidoreductase, and cytochrome b_5 were prepared with male albino rabbits and the injection and bleeding scheme of Zimmerman & Kasper (1978). γ-Globulin was isolated from the serum as described by Masters et al. (1971).

Immunoprecipitation and Quantitation of Translation Products. Immunoprecipitation was carried out in a final volume of 2 mL containing 1% each of Triton X-100 and sodium deoxycholate, 10 mL L-methionine, 0.15 M NaCl, 10 mM borate buffer (pH 7.4), 10 μg of carrier, and 1.5 times the equivalent amount of rabbit γ-globulin. The mixture was incubated at 37 °C for 30 min and then further incubated at 4 °C for 12–15 h before sedimentation of the immunoprecipitate through a discontinuous sucrose gradient containing 0.5 and 1.0 M sucrose in the above detergents and buffer as described by Taylor & Schimke (1973). The precipitates were washed 3 times in the detergents and buffer, solubilized in $NaDodSO_4$[1] sample buffer (Laemmli, 1970), and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis in 8.75 or 15% stacking gels as described by Laemmli (1970). The gels were stained, dried, and exposed to Kodak X-Omat X-ray film

[1] Abbreviation used: $NaDodSO_4$, sodium dodecyl sulfate.

as described by Lam & Kasper (1979a). Radioactive bands were localized in the gel and excised, the gel slice was treated with protosol, and the radioactivity was determined with RIA II liquid scintillation solution. Three control regions above and below the labeled band were excised and counted to determine the background level of radioactivity in the gel.

Isolation of Microsomal Membrane and Quantitation of NADPH–Cytochrome c Oxidoreductase. Microsomal membrane was prepared according to the method described by Blackburn et al. (1976). Quantitation of oxidoreductase protein for determination of in vivo specific activity was performed with the radial immunodiffusion assay of Mancini et al. (1965), as described in detail by Zimmerman & Kasper (1978). The rate of synthesis of oxidoreductase after phenobarbital administration was determined by multiplying the fractional increase in oxidoreductase protein relative to control at each time point by the specific activity of oxidoreductase at that time point. This correction accounts for the increased percentage of oxidoreductase protein at each time point and therefore yields the true rate of synthesis (Kuriyama et al., 1969).

Partial Enzymatic Cleavage on Sodium Dodecyl Sulfate Gel. Partial enzymatic cleavage on sodium dodecyl sulfate gel was carried out according to Cleveland et al. (1977), with modifications similar to those recently described by Bordier & Crettol-Jarvinen (1979) and Lam & Kasper (1979b). A cylindrical stacking gel was run as described (Laemmli, 1970). After electrophoresis, the cylindrical gel was shaken in an equilibrating buffer composed of 80 mM Tris-HCl, pH 6.8, 0.1% $NaDodSO_4$ (w/v), 1 mM ethylenediaminetetraacetic acid, and 20% glycerol (v/v) for 30 min. For partial enzymatic cleavage, the preequilibrated cylindrical gel, followed by the reservoir buffer, was loaded on top of a slab stacking gel (14 × 9 × 0.3 cm). Then 2 mL of equilibrating buffer containing 0.8 mg of α-chymotrypsin was carefully added on top of the loaded cylindrical gel, and the gels were then run at 10 mA/slab.

Results

Translation of NADPH–Cytochrome c Oxidoreductase, Cytochrome b_5, and Serum Albumin. The postmitochondrial supernatant was fractionated into free and endoplasmic reticulum bound polysomes to elucidate the intracellular site of synthesis of NADPH–cytochrome *c* oxidoreductase, cytochrome b_5, and serum albumin and to enrich their respective mRNAs relative to other mRNAs. The endoplasmic reticulum bound polysomes were further fractionated into loosely and tightly membrane-bound polysomes. Loosely membrane-bound polysomes were first described as those which are susceptible to removal from the membrane by using a high salt wash (Robash & Penman, 1971a,b). RNA was extracted from each class of polysomes and used to direct translation in the mRNA-dependent reticulocyte lysate system. Figure 1 shows typical autoradiographs of $NaDodSO_4$–polyacrylamide gel electrophoretograms for the antigen–antibody complexes of the oxidoreductase (gel 1), cytochrome b_5 (gel 2), and serum albumin (gel 3). With the exception of serum albumin, each immunoprecipitate yielded a single radioactively labeled polypeptide, confirming the absence of prematurely terminated peptide chains. The minor component directly below serum albumin has also been detected by others (Taylor & Schimke, 1973) with a reticulocyte in vitro translation system. Furthermore, in a separate experiment, NADPH–cytochrome *c* oxidoreductase (M_r 78 000), cytochrome b_5 (18 000), and serum albumin (68 000) synthesized in vitro were shown to comigrate with their respective counterparts synthesized in vivo. Oxidoreductase and serum albumin were coded for by mRNA in the tightly membrane-bound polysomal fraction and represented 0.023 and 4.8%, respectively, of the protein synthesized. Cytochrome b_5 was synthesized from free polysomal RNA and represented 0.022% of the protein synthesized. Cytochrome b_5 was also detected in loosely membrane-bound polysomal RNA translation products at levels similar to that translated from free polysomal RNA. Prolonged exposure of gels containing oxidoreductase immunoprecipitates from free or loosely membrane-bound polysomal RNA translation products did not reveal the presence of NADPH–cytochrome *c* oxidoreductase. A band corresponding to cytochrome b_5 was also not detected in gels of immunoprecipitates derived from tightly membrane-bound polysomal RNA translation products upon prolonged exposure. Serum albumin was present in translation products from free and loosely membrane-bound polysomal RNA; however, the levels were less than 1% of the level in tightly membrane-bound polysomal RNA translation products.

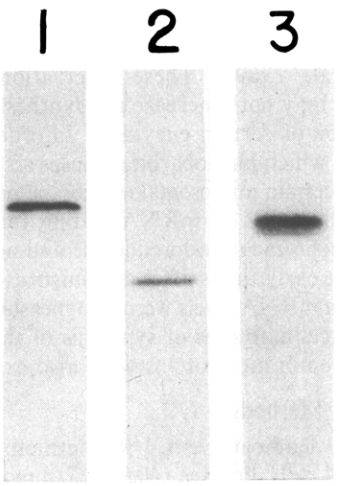

FIGURE 1: Synthesis of NADPH–cytochrome *c* oxidoreductase, cytochrome b_5, and serum albumin in reticulocyte lysates. Immunoprecipitates of each enzyme translated from 0.5 mg/mL free and loosely or tightly membrane-bound polysomal RNA in the reticulocyte lysate were run on $NaDodSO_4$–polyacrylamide gels. The immunoprecipitate of NADPH–cytochrome *c* oxidoreductase from 4.0×10^6 dpm of tightly bound polysomal RNA translation products is displayed on an 8.75% $NaDodSO_4$–polyacrylamide gel (lane 1). The immunoprecipitate of cytochrome b_5 from 4.0×10^6 dpm of free polysomal RNA translation products is displayed on a 15% $NaDodSO_4$–polyacrylamide gel (lane 2). The immunoprecipitate of serum albumin from 5.0×10^5 dpm of tightly bound polysomal RNA translation products is displayed on an 8.75% $NaDodSO_4$–polyacrylamide gel (lane 3). Gels in lanes 1 and 2 were exposed to photographic film for 48 h, and the gel in lane 3 was exposed for 4 h. Migration was from top to bottom.

Peptide Mapping of the in Vitro and in Vivo Synthesized NADPH–Cytochrome c Oxidoreductase. Comparison with the enzyme synthesized in vivo was performed by using a peptide mapping procedure in order to establish unequivocally that the protein synthesized in vitro was the oxidoreductase. An immunoprecipitate containing 100 μg of unlabeled oxidoreductase from microsomes was mixed with an immunoprecipitate of ^{35}S-labeled oxidoreductase from tightly membrane-bound polysomal RNA translation products. The combined immunoprecipitates were electrophoresed on a cylindrical $NaDodSO_4$–polyacrylamide gel and digested in the gel with α-chymotrypsin, and the resulting peptides were separated on $NaDodSO_4$–polyacrylamide gels as described under Materials and Methods. For the purpose of clarity, only the stained region of the gel containing oxidoreductase is displayed in Figure 2, with the heavy and light chain frag-

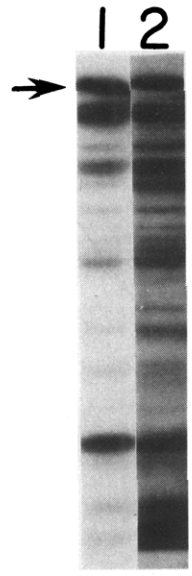

FIGURE 2: Peptide mapping of in vivo and in vitro synthesized NADPH–cytochrome *c* oxidoreductase. An immunoprecipitate of NADPH–cytochrome *c* oxidoreductase synthesized from tightly bound polysomal RNA translation products was mixed with an immunoprecipitate of the enzyme derived from detergent-solubilized microsomes. The combined immunoprecipitates were run on an 8.75% $NaDodSO_4$–polyacrylamide gel and then treated in the gel with α-chymotrypsin as described under Materials and Methods. Partial proteolytic products were electrophoretically separated on a 12% $NaDodSO_4$–polyacrylamide gel. The fluorograph of in vitro synthesized oxidoreductase proteolytic fragments is presented in lane 1, and the Coomassie blue stained gel of the in vivo oxidoreductase proteolytic fragments appears in lane 2. The arrow indicates the position of migration of undigested oxidoreductase.

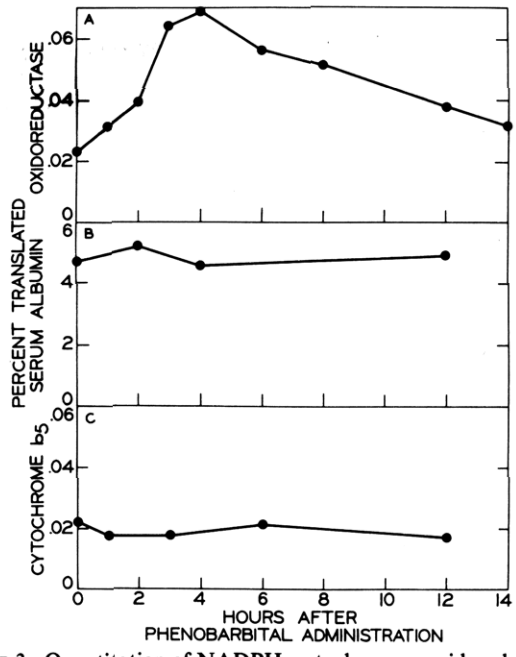

FIGURE 3: Quantitation of NADPH–cytochrome *c* oxidoreductase, cytochrome b_5, and serum albumin mRNA after phenobarbital administration. Free and tightly membrane-bound polysomes were isolated from the livers of animals sacrificed at various times after a single dose of phenobarbital, and the RNA was purified as described under Materials and Methods. NADPH–cytochrome *c* oxidoreductase and serum albumin were immunoprecipitated from tightly membrane-bound polysomal RNA translation products, and cytochrome b_5 was immunoprecipitated from free polysomal RNA translation products. The specific radioactivity of each protein translated was quantitated from $NaDodSO_4$–polyacrylamide gels as described under Materials and Methods and expressed as a percentage of the polysome-released protein in the translation system. Released chains were typically 75% of total protein translated. The dpm values of released protein which had been translated from RNA preparation from each time point were 4.0×10^6, 4.0×10^6, and 5.0×10^5 for oxidoreductase, cytochrome b_5, and serum albumin, respectively. NADPH–cytochrome *c* oxidoreductase (A); serum albumin (B); cytochrome b_5 (C).

mentation products of the immunoglobulin not included. All ^{35}S-labeled proteolytic fragments from the oxidoreductase synthesized in vitro (Figure 2, lane 1) comigrated with Coomassie blue stained chymotryptic peptides derived from the enzyme synthesized in vivo (Figure 2, lane 2). Certain Coomassie blue stained peptides, however, did not have corresponding radioactively labeled counterparts. These presumably represent peptides lacking methionine.

Quantitation of NADPH–Cytochrome c Oxidoreductase, Cytochrome b_5, and Serum Albumin mRNAs after Phenobarbital Administration. Tightly membrane-bound and free polysomes were isolated from the livers of animals at 2-h intervals from 1 to 14 h after a single dose of phenobarbital. The percentage of oxidoreductase and serum albumin in translation products coded for by tightly membrane-bound polysomal RNA was quantitated. The level of oxidoreductase mRNA increased as early as 1 h after phenobarbital administration and peaked at 4 h, at a level threefold higher than that of the control (Figure 3A). Oxidoreductase mRNA levels then declined with an approximate half-life of 9 h. This was determined by replotting the data in Figure 3A on a semilog plot. Serum albumin mRNA levels were not affected by phenobarbital (Figure 3B), and levels of cytochrome b_5 translated by free polysomal RNA from induced and control animals were similar (Figure 3C). Loosely membrane-bound polysomal RNA from phenobarbital-induced animals was not analyzed for cytochrome b_5 mRNA.

Examination of the radioactivity incorporated into total protein in the above experiments revealed that more protein was synthesized per unit of RNA from phenobarbital-induced tightly membrane-bound polysomal RNA than from the control (F. J. Gonzalez and C. B. Kasper, unpublished experiments). This increase was apparent as early as 2 h after phenobarbital administration. The following experiment was performed to explore this phenomenon further and to determine whether the percentage of oxidoreductase synthesized varied with the RNA concentration translated. The percentage of oxidoreductase in total translation products was determined for different concentrations of 4-h induced and noninduced, tightly membrane-bound polysomal RNA in the translation reaction. The percentage of total translation products represented by oxidoreductase was independent of the RNA concentration translated (Figure 4B), and the translation efficiency of 4-h induced RNA was significantly higher than that of noninduced RNA (Figure 4A). This increase in translation efficiency was reproducible by using different preparations of RNA and was specific for tightly membrane-bound polysomal RNA since free polysomal RNA from 4-h induced animals translated at the same efficiency as control free polysomal RNA (F. J. Gonzalez and C. B. Kasper, unpublished experiments). The possibility that preferential degradation of control tightly membrane-bound polysomal RNA was responsible for this difference was excluded for the following reasons. Poly(A) mRNA which was isolated from control and 4-h induced tightly membrane-bound polysomal RNA by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972) translated at the same efficiency. The average sedimentation rate of control poly(A) mRNA was 16 S, indicative of high molecular weight mRNA (F. J. Gonzalez and C. B. Kasper, unpublished experiments). In addition, autoradiograms of oxidoreductase and serum albumin immunopreci-

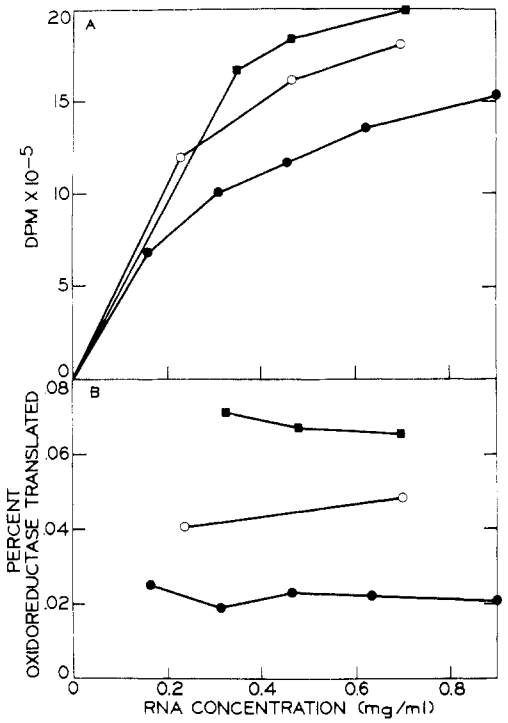

FIGURE 4: Synthesis of total protein and NADPH–cytochrome *c* oxidoreductase as a function of RNA concentration translated in reticulocyte lysates. Various concentrations of tightly membrane-bound polysomal RNA isolated from control (●), 4-h phenobarbital-induced (■), and 4-h phenobarbital-induced plus cordycepin-treated (○) animals were translated. Total counts incorporated into released chains (A) and percent oxidoreductase translated (computed as in Figure 3 legend) (B) were determined.

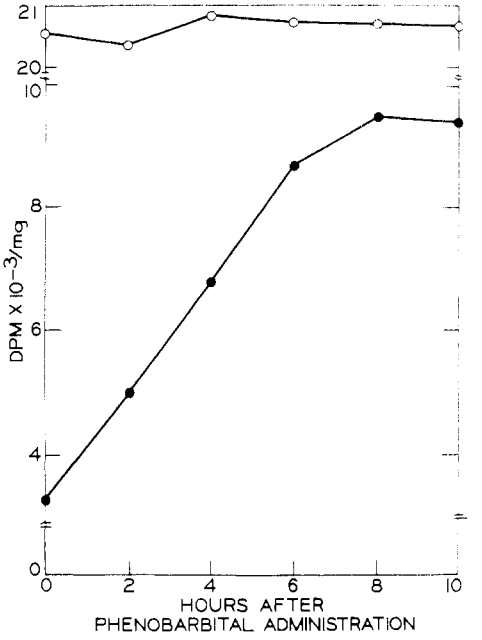

FIGURE 5: Rate of synthesis of NADPH–cytochrome *c* oxidoreductase in vivo. Groups of three control and phenobarbital-induced animals were injected with 60 μCi of L-[^{3}H]leucine 40 min before sacrifice. Microsomes were isolated, and the specific radioactivity of total protein (○) and the corrected specific radioactivity of oxidoreductase (●) were determined as described under Materials and Methods.

pitates from induced RNA translation products were identical with those of the controls with no indication of translation products from degraded mRNA (Figure 1). The possibility exists, however, that noninduced RNA preparations contain an inhibitor of translation such as double-stranded RNA (Levin & London, 1978).

Cordycepin (3′-deoxyadenosine) was tested for its ability to block induction in order to establish whether the induction of oxidoreductase was mediated through a specific increase in newly synthesized mRNA. This RNA synthesis chain terminator was found to inhibit cytoplasmic accumulation of mRNA by blocking transcription and/or polyadenylation of precursor mRNA (Darnell et al., 1971; Beach & Ross, 1978). Administration of cordycepin 20 min before phenobarbital resulted in a 50% decrease in the induced level of translatable oxidoreductase mRNA at 4 h and a slight decrease in the translation efficiency (parts A and B of Figure 4). The level of translatable cytochrome b_5 mRNA did not change under these conditions (F. J. Gonzalez and C. B. Kasper, unpublished experiments). Conditions in which total inhibition of induction is achieved were not sought experimentally, since the combined evidence of mRNA accumulation soon after phenobarbital administration and the blocking effect of cordycepin and actinomycin D is strongly supportive of transcriptional or posttranscriptional nuclear effects (see Discussion).

Effect of Phenobarbital Administration on the in Vivo Rates of Synthesis of NADPH–Cytochrome c Oxidoreductase. In vivo labeling studies were performed to investigate whether the rate of synthesis of oxidoreductase correlated with the increase in oxidoreductase mRNA measured in vitro. The rate of synthesis of oxidoreductase after phenobarbital administration was examined with a 40-min pulse of L-[^{3}H]leucine before sacrifice. This pulse time was chosen on the basis of date from Kuriyama et al. (1969), where incorporation of L-[^{3}H]leucine into total protein and oxidoreductase was approximately constant from 20 to 180 min after injection of the label. Since the percentage of microsomal protein represented by oxidoreductase increased after phenobarbital administration (Kuriyama et al., 1969), the specific radioactivity obtained at each time point was not indicative of the true rate of synthesis, which was computed as described under Materials and Methods. The rate of oxidoreductase synthesis increased almost threefold at 6 h, peaked at 8 h, and remained elevated at 10 h after phenobarbital administration (Figure 5). However, the specific activity of total microsomal protein did not increase above control levels (Figure 5).

Discussion

The use of a well characterized in vitro translation system and immunoprecipitation as primary investigative tools allows both the qualitative and quantitative analyses of mRNA coding for specific enzymes. These techniques have been used to study the early events occurring after the administration of phenobarbital and in the determination of the site of synthesis of two intrinsic membrane proteins, NADPH–cytochrome *c* oxidoreductase and cytochrome b_5. The rationale for the selection of these two proteins is that oxidoreductase is induced by phenobarbital whereas cytochrome b_5 is not (Orrenius et al., 1965; Kasper, 1971). In addition, both proteins are attached to the membrane via hydrophobic segments of their polypeptide chains, with their catalytic portions exposed to the cytoplasm (Omura & Takesue, 1970). This similar orientation and the differential effects of phenobarbital offer the opportunity to analyze the interrelationships between sites of membrane protein synthesis, the mechanism of phenobarbital induction, and the biogenesis of the endoplasmic reticulum.

Administration of phenobarbital resulted in a threefold increase in translatable oxidoreductase mRNA. This maximal increase occurred 4 h after administration of the drug and was followed by an enhanced in vivo rate of synthesis of the enzyme. Interestingly, the time required to achieve a maximum

oxidoreductase mRNA level and the magnitude of this increase after phenobarbital administration are strikingly similar to the results for tyrosine aminotransferase and tryptophan oxygenase mRNA upon glucocorticoid induction (Roewekamp et al., 1976). Translatable levels of mRNA coding for cytochrome b_5 did not increase soon after phenobarbital administration, suggesting that only levels of mRNA coding for induced endoplasmic reticulum bound enzymes are increased. The specificity of the mRNA response is further illustrated by the finding that phenobarbital did not affect serum albumin mRNA levels. This indicates that the induction effect is a phenomenon that may not be generalized to all mRNAs associated with tightly membrane-bound polysomes but may be generalized only to those mRNAs coding for induced enzymes.

Our data suggest that phenobarbital induces enzymes by increasing levels of their mRNA. Furthermore, it has been postulated that phenobarbital increases specific mRNA levels by causing a decrease in mRNA degradation (Cohen & Rudden, 1971; Venkatesan & Steele, 1975; Lindell et al., 1977). Considering the extreme case in which oxidoreductase mRNA degradation would be completely blocked, a threefold increase in the level of the drug 4 h after administration could occur only if oxidoreductase mRNA had a half-life of 40 min, a value much shorter than our estimated half-life of 9 h for the induced oxidoreductase mRNA. This suggests that phenobarbital may not increase mRNA levels by decreasing the rate of degradation. However, an effect on mRNA degradation can only be completely ruled out if the half-life of oxidoreductase mRNA is measured under conditions in which RNA synthesis is inhibited. Phenobarbital may act primarily at the level of the nucleus where it increases the rate of transcription or posttranscriptional processing and nucleocytoplasmic transport of specific mRNAs. This is further supported by our finding that cordycepin, an RNA synthesis chain terminator, partially inhibits the oxidoreductase mRNA response to phenobarbital and by other studies that indicate a blocking action of actinomycin D on the induction process (Conney & Gilman, 1963; Orrenius et al., 1965; Nebert & Gielen, 1971). These results appear to favor a mode of action for phenobarbital that is similar to the model proposed for steroid hormones where hormone–receptor complexes interact with chromatin and cause an increase in the rate of transcription of specific mRNAs (Mueller et al., 1971; Roewekamp et al., 1976; O'Malley et al., 1977). Interestingly, however, RNA polymerases I and II do not increase in activity early after phenobarbital administration (Lindell et al., 1977) in contrast to steroid hormone stimulation (Barry & Gorski, 1971; Borthwick & Smellie, 1975). This finding, in addition to the ability of the drug to stabilize certain enzymes (Jick & Shuster, 1966; Kuriyama et al., 1969) and induce ligandin without increasing ligandin mRNA (Daniel et al., 1977), makes the phenobarbital induction process unique and intriguing.

It is interesting to note that oxidoreductase and cytochrome b_5 mRNAs differ not only with respect to their inducibility by phenobarbital but also with respect to their location within the cell. The present study supports the possibility that microsomal proteins inducible by phenobarbital may be synthesized exclusively on tightly membrane-bound polysomes. This point is illustrated by the fact that oxidoreductase mRNA is associated only with polysomes tightly bound to the endoplasmic reticulum whereas cytochrome b_5 is associated with free polysomes and endoplasmic reticulum loosely membrane-bound polysomes. The location of cytochrome b_5 mRNA in loosely membrane-bound polysomal RNA is not surprising, since loosely membrane-bound polysomes may be partially or exclusively composed of free polysomes adsorbed to the membranes during the isolation procedure as proposed by Ramsey & Steele (1976). This is supported by the observation that two-dimensional peptide analysis of abundant translation products from free and loosely membrane-bound polysomal RNA shows many similarities (F. J. Gonzalez and C. B. Kasper, unpublished experiments).

Our studies clarify the divergent results of previous investigations on the site of synthesis of oxidoreductase. Oxidoreductase has been found associated with polysomes that were membrane bound and free (Ragnatti et al., 1969), free (Lowe & Hallinan, 1973), and loosely membrane bound (Harano & Omura, 1978). All of these studies are similar in one respect: they analyze nascent chains on polysomes for either enzymatic activity (Ragnatti et al., 1969) or specific radioactivity (Lowe & Hallinan, 1973; Harano & Omura, 1978), and in no case was either activity unequivocally shown to be associated with oxidoreductase. The oxidoreductase quantitated in this report by immunochemical means was compared with its in vivo counterpart and shown to be identical with respect to molecular weight and peptide fingerprint pattern.

Since two intrinsic membrane proteins, sharing similar orientations within the endoplasmic reticulum, are synthesized at different sites within the cell, the question arises as to how intracellular membranes are assembled. Experimental results strongly indicate that synthesis and insertion of intrinsic membrane proteins proceed via more than one pathway. Hence, the signal hypothesis (Blobel & Dobberstein, 1975) could explain how the oxidoreductase is inserted into the microsomal membrane, even though a cleavable signal sequence was not detected in our experiments. On the other hand, the site of synthesis of cytochrome b_5 suggests that it may be inserted via a mechanism similar to that described by the membrane trigger hypothesis of Wickner (1979).

References

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Barry, J., & Gorski, J. (1971) *Biochemistry 10*, 2384–2390.

Beach, L. R., & Ross, J. (1978) *J. Biol. Chem. 253*, 2628–2632.

Blackburn, G. R., Bornens, M., & Kasper, C. B. (1976) *Biochim. Biophys. Acta 436*, 387–398.

Blobel, G., & Dobberstein, B. (1975) *J. Cell Biol. 67*, 852–862.

Bordier, C., & Crettol-Jarvinen, A. (1979) *J. Biol. Chem. 254*, 2565–2567.

Borthwick, N. M., & Smellie, R. M. S. (1975) *Biochem. J. 147*, 91–101.

Cleveland, D. W., Fisher, S. G., Kirschner, M. W., & Laemmli, V. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Cohen, A. M., & Rudden, R. W. (1971) *Mol. Pharmacol. 7*, 484–489.

Conney, A. H. (1967) *Pharmacol. Rev. 19*, 317–366.

Conney, A. H., & Gilman, A. G. (1963) *J. Biol. Chem. 238*, 3682–3685.

Conney, A. H., Miller, E. C., & Miller, J. A. (1957) *J. Biol. Chem. 228*, 753–766.

Conney, A. H., Davison, C., Gustel, R., & Burns, J. J. (1960) *J. Pharmacol. Exp. Ther. 130*, 1–8.

Daniel, V., Smith, G. J., & Litwack, B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1899–1902.

Darnell, J. E., Philipson, L., Wall, R., & Adesnik, M. (1971) *Science 174*, 507–510.

Ernster, L., & Orrenius, S. (1965) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 24*, 1190–1199.

Fahl, W. E., Jefcoate, C. R., & Kasper, C. B. (1978) *J. Biol. Chem. 253*, 3106–3113.
Harano, T., & Omura, T. (1978) *J. Biochem. (Tokyo) 84*, 213–223.
Ikehara, Y., & Pitot, H. C. (1973) *J. Cell Biol. 59*, 28–44.
Jick, H., & Shuster, L. (1966) *J. Biol. Chem. 241*, 5366–5369.
Kasper, C. B. (1971) *J. Biol. Chem. 246*, 577–581.
Khandwala, A. S., & Kasper, C. B. (1973) *Biochem. Biophys. Res. Commun. 54*, 1241–1246.
Kruppa, J., & Sabatini, D. D. (1977) *J. Cell Biol. 77*, 414–427.
Kuriyama, Y., Omura, T., Siekevitz, P., & Palade, G. E. (1969) *J. Biol. Chem. 244*, 2017–2026.
Laemmli, V. K. (1970) *Nature (London) 277*, 680–685.
Lam, K., & Kasper, C. B. (1979a) *Biochemistry 18*, 307–311.
Lam, K., & Kasper, C. B. (1979b) *J. Biol. Chem. 254*, 11713–11720.
Levin, D., & London, I. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1121–1125.
Lindell, T. J., Ellinger, R., Warren, J. T., Sundheimer, D., & O'Malley, A. F. (1977) *Mol. Pharmacol. 13*, 426–434.
Lowe, D., & Hallinan, T. (1973) *Biochem. J. 136*, 825–828.
Lu, A. Y. H., Junk, W., & Coon, A. J. (1969) *J. Biol. Chem. 244*, 3714–3721.
Mancini, G., Carbonara, A. O., & Heremans, J. F. (1965) *Immunochemistry 2*, 235–254.
Masters, B. S. S., Baron, J., Taylor, W. E., Isaacson, E. L., & LoSpalluto, J. (1971) *J. Biol. Chem. 246*, 4143–4150.
Moore, R. E., Cardelli, J. A., & Pitot, H. C. (1977) *Biochemistry 16*, 3037–3044.
Mueller, G. C., Vonderhaar, B., Kim, U. H., & LeMahieu, M. (1971) *Recent Prog. Horm. Res. 28*, 1–49.
Nebert, D. W., & Gielen, J. E. (1971) *J. Biol. Chem. 246*, 5199–5206.
O'Malley, B. W., Towle, H. C., & Schwartz, R. J. (1977) *Annu. Rev. Genet. 11*, 239–275.
Omura, T., & Takesue, S. (1970) *J. Biochem. (Tokyo) 67*, 249–257.
Orrenius, S., & Ericksson, J. L. E. (1966) *J. Cell Biol. 28*, 181–198.
Orrenius, S., Ernster, J. L. E., & Ernster, L. (1965) *J. Cell Biol. 25*, 627–639.
Parke, D. V. (1976) *Basic Life Sci. 6*, 207–271.
Pelham, H. R. B., & Jackson, J. (1976) *Eur. J. Biochem. 67*, 247–256.
Perry, R. P., LaTorre, J., Kelley, E. W., & Greenburg, J. R. (1972) *Biochim. Biophys. Acta 262*, 220–226.
Poland, A. P., & Glover, E. (1976) *J. Biol. Chem. 251*, 4936–4946.
Ragnatti, G., Lawlord, G. R., & Campbell, P. N. (1969) *Biochem. J. 112*, 139–147.
Ramsey, J. C., & Steele, W. J. (1976) *Biochemistry 15*, 1704–1712.
Remmer, H., & Merker, H. J. (1963) *Science 142*, 1657–1658.
Robash, M., & Penman, S. (1971a) *J. Mol. Biol. 59*, 227–241.
Robash, M., & Penman, S. (1971b) *J. Mol. Biol. 59*, 242–253.
Roberts, B. E., Payne, P. I., & Osborne, D. J. (1973) *Biochem. J. 131*, 275–286.
Roewekamp, W. G., Hofer, E., & Sekeris, C. E. (1976) *Eur. J. Biochem. 70*, 259–268.
Taylor, J. M., & Schimke, R. T. (1973) *J. Biol. Chem. 248*, 7661–7668.
Venkatesan, N., & Steele, W. J. (1975) *Chem.-Biol. Interact. 11*, 41–54.
Villa-Komaroff, L., McDowell, M., Baltimore, D., & Lodish, H. F. (1974) *Methods Enzymol. 30*, 711–723.
Wickner, W. (1979) *Annu. Rev. Biochem. 48*, 23–45.
Zimmerman, J. J., & Kasper, C. B. (1978) *Arch. Biochem. Biophys. 190*, 726–735.