AN ABSTRACT OF THE THESIS OF

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Title: ANALYSIS OF CYTOCHROME P-450 ISOZYMES FROM RAT LIVER AND STUDIES ON THE MECHANISM OF INDUCTION

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In order to determine the total number of cytochrome P-450 isozymes present in a given species following various treatments, mature male rats (Sprague-Dawley) were injected intraperitoneally with the classic mixed-function oxidase inducers phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN), 3-methylcholanthrene (3-MC), or the appropriate control vehicle.

Hepatic microsomes were solubilized and P-450 separated from the majority of other microsomal proteins by hydrophobic chromatography on ω-aminooctyl agarose (AOA). The P-450 isozymes were then separated by either sequential chromatography on hydroxyapatite and DEAE at 4°C or by DEAE at room temperature. The degree of P-450 purity during the procedure was monitored by determining the specific content (nmoles P-450 per mg protein) and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Isoelectric focusing (IF) was also used to determine the homogeneity of final preparations.

P-450, partially purified by AOA, was resolved into three to six peaks by DEAE. The pattern of elution obtained differed widely between the four groups. Although the specific content of most peaks was quite high, only the major P-450 isozyme from the PB induced

group appeared homogenous. SDS-PAGE and IF, although useful in determining the homogeneity of a particular preparation, were too limited in resolving power to distinguish isozymes from two or more preparations.

A recently developed model for the molecular mechanism by which chemicals induce the synthesis of P-450 was examined by analyzing the binding of the above inducers (radiolabeled) to cytosol from uninduced rats.

Using modifications of techniques for analyzing ligand-receptor binding, the existence of a specific, high affinity receptor for ^{3}H-3-MC was demonstrated and the binding parameters compared to published results.

The existence of high affinity receptors for ^{14}C-PCN and ^{3}H-PB was suggested, but the low radiospecific activity of the former and the very small percent bound of the latter, prevented any significant conclusions.

Analysis of Cytochrome P-450 Isozymes from Rat Liver and Studies on the Mechanism of Induction

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INTRODUCTION

The microsomal mixed-function oxidase system which has been found in mammals (1), fish (2), insects (3), bacteria (4), and even plants (5) functions in the oxidative metabolism of a wide variety of exogenous xenobiotics as well as endogenous compounds such as steroids and fatty acids. The activity of this system in mammals has been found to vary with intrinsic factors such as species (6), strain (7), sex (8), and age (9) and can be altered by extrinsic factors including diet and exposure to foreign chemicals (10). The importance of this system in the duration and effectiveness of therapeutic drugs, the detoxification or activation of toxins and carcinogens and the regulation of steroid metabolism has lead to a great deal of effort over the last 15-20 years to isolate and characterize individual components; establish the structural arrangement within the functional endoplasmic reticulum; reveal the kinetic parameters of each of the multiple steps leading to oxidized product; and discover the mechanism by which certain inducers can qualitatively and quantitatively alter the activity of the system.

Components Of The Mixed-Function Oxidase System

Most of the research discussed below was done utilizing the mammalian liver although the microsomal mixed-function oxidase system has also been found in many other tissues including lung, testes,

kidney, adrenal, and intestinal tissue (11).

The enzyme functioning as the terminal oxidase in this system was first discovered (12,13) in 1958 as a pigment containing protein in liver. The hemoprotein nature of this pigment was not established until 1964 (14). The protein was termed cytochrome P-450 as it exhibited a characteristic absorbance at 450 nm in its reduced state upon binding carbon monoxide. Early attempts to purify this protein resulted in the loss of enzymatic activity which was reflected by a shift in the absorbance maximum of the reduced CO-bound state to 420 nm. This denatured form was hence, termed cytochrome P-420. In subsequent experiments (15) it was found that polyols served to stabilize P-450 in the presence of the ionic detergents needed to solubilize P-450 from the microsomal membrane.

In recent years increasing evidence for the existence of multiple forms of P-450 has accumulated (16-28). These forms of P-450 are distinguishable by molecular weight (17,21,23,25,26,29,30), isoelectric point (31), amino acid composition (25,27,29,32), substrate specificity (16,21-23,27,30), spectral and immunochemical properties (11,19, 20,23,25,30,33) and response to various inducers in vivo (16,18,21-23, 27,29,30,33) as well as in cell cultures (34).

The heterogenity of P-450 must be kept in mind during the discussions of the morphology and functions of the mixed-function oxidase below.

The cytochrome P-450 species characterized to date have monomeric molecular weights of 45,000-60,000 daltons as shown by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and are embedded in the microsomal membrane. The amino acid composition is similar in

hydrophobic content to other membrane proteins. Studies on two different highly purified P-450s (29) have shown the presence of two moles of mannose and one of glucosamine per mole of P-450. In addition, partial sequencing of the N-terminus of these same two forms gave results very similar to signal sequence peptides (35) seen in many secretory proteins such as the pancreatic zymogens and the immunoglobulin light chains, the difference being that P-450 retains its signal sequence while proteins destined for export have their N-terminus cleaved.

Furthermore, all P-450 forms examined to date contain one heme, 0.3-0.5 phospholipid residues per polypeptide chain and in the absence of detergents will form aggregates with apparent molecular weights of 300,000-500,000 daltons.

The protein which serves to transfer electrons from NADPH to P-450 is termed NADPH-cytochrome P-450 reductase. Earlier work utilizing lipases or proteases to solubilize the reductase lead to the isolation of a protein capable of reducing cytochrome c but inactive towards P-450. This protein was termed NADPH-cytochrome c reductase and the P-450 active reductase is still sometimes referred to by this name. The molecular weight of the reductase is about 74,000 as shown by SDS-PAGE, but as with P-450, will aggregate under non-denaturing conditions to give an apparent molecular weight of 450,000 daltons. The reductase contains one molecule of FMN and one of FAD which serve to transfer one electron at a time to P-450.

The ratio of P-450:reductase can vary from 20-40:1 depending upon the animal examined and the history of exposure to inducers (36), however, kinetic studies have shown that the transfer of electrons from

NADPH to the oxidized system requires phospholipid to function in reconstituted systems. Although the exact function of the lipid moiety has not been demonstrated, phospholipid has been shown to alter the α-helical content of rabbit P-450 (but not the reductase) (38) and to decrease the K_s of substrate for P-450 and the apparent K_d of the reductase for P-450, as well.

Recent work utilizing ^{31}P-NMR for the analysis of microsomal lipid (39) suggests that the incorporation and formation of the mixed-function oxidase system is regulated by the phosphatidylethanolamine: phosphatidylcholine ratio. The degree of acyl saturation and the occurrence of peroxidation could also be expected to effect interactions between P-450 substrate and the reductase.

In addition to the components above, cytochrome b_5 and NADH-cytochrome b_5 reductase may serve to transfer electrons to oxycytochrome P-450 in the second reduction step. The actual importance of this pathway *in vivo* is still a matter of some controversy (37). Cytochrome b_5 serves as the electron carrier in the desaturation of long chain fatty acyl coenzyme A compounds and it has been suggested that this is its main *in vivo* function. However, cytochrome b_5 is also found in microsomal membranes with P-450 in tissues that are devoid of any desaturase activity.

Reaction Sequence Of The Mixed-Function Oxidase System

A schematic representation of the multi-step reaction sequence is shown in Figure 1. In step one the substrate is bound to the ferric form of P-450. The binding of the substrate is accompanied by a change in the heme configuration from a low to a high spin state and

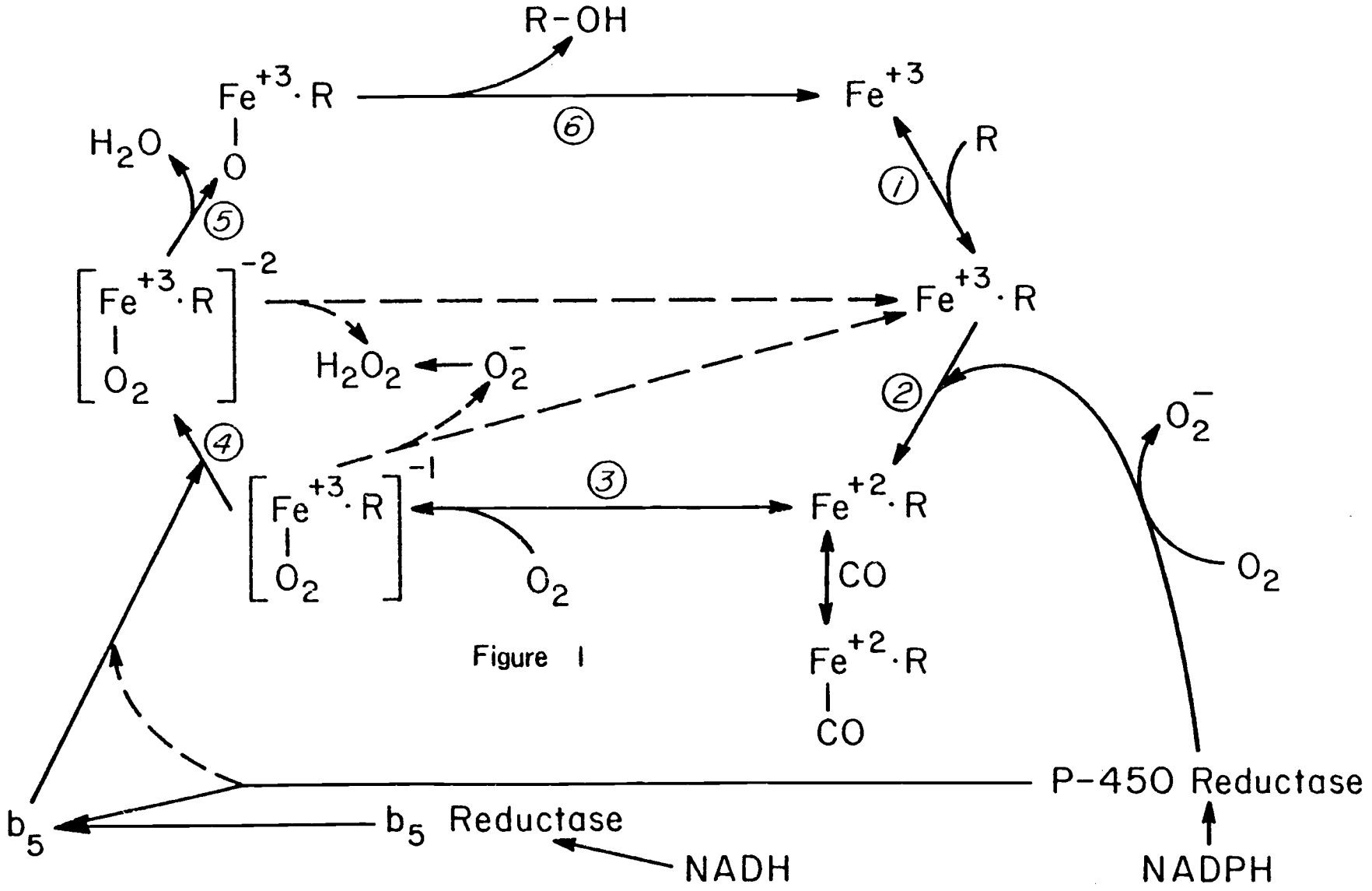

R-OH
$Fe^{+3}\cdot R$
⑥
Fe^{+3}
H_2O
O
R
①
⑤
$[Fe^{+3}\cdot R]^{-2}$
O_2
$Fe^{+3}\cdot R$
H_2O_2
O_2^-
②
O_2^-
④
$[Fe^{+3}\cdot R]^{-1}$
③
$Fe^{+2}\cdot R$
O_2
O_2
CO
$Fe^{+2}\cdot R$
CO
P-450 Reductase
b_5
b_5 Reductase
NADH
NADPH

Figure 1

the occurrence of a Type I pattern in the optical absorbance spectrum. Little is known about the actual location or configuration of the substrate binding site or the properties which enable it to interact with such a wide variety of substrates. Again, multiple P-450s may explain some of this "non-specificity".

In the second step, one electron is transferred from NADPH to P-450 through its reductase, to reduce the heme to the ferrous state. This form may combine with either O_2 or CO in the sixth ligand position.

In the presence of O_2 the oxycytochrome P-450 substrate complex is rapidly formed (step three), and the bound oxygen becomes "activated". Two forms with equivalent electronic valence states are thought to exist, the ferrous P-450 combined with substrate and molecular oxygen and the ferric substrate form in which the oxygen is activated to a superoxide anion. The latter form is capable of decaying to regenerate the ferric substrate form with the release of superoxide anion (or ultimately hydrogen peroxide). Peroxide forming "uncoupling" of the P-450 system could have destructive effects on the heme moiety of P-450 and also could initiate lipid peroxidation. Microsomes from animals which have been treated with either phenobarbital (PB) or pregnenolone 16α-carbonitrile (PCN) show an increase in such an uncoupling during metabolism of drug and steroid substrates (40).

The oxycytochrome P-450 substrate complex is further reduced (step four) to peroxycytochrome P-450 in which the ferric heme has two electrons and can exist in a number of forms. If protonated, the peroxide anion could dissociate with the formation of H_2O_2 and the ferric

substrate complex again producting an uncoupling effect. The source of the electron in the second reduction is still controversial; evidence for the participation of both NADH-cytochrome b_5 reductase and NADPH-cytochrome P-450 reductase has been presented.

In the fifth step, peroxycytochrome is thought to lose a molecule of water, generating the highly reactive oxene P-450 which reacts with substrate producting the oxidized product (step six) and releasing the free low spin ferric P-450 which can now combine with a new substrate and repeat the cycle.

Purification of P-450

In the 12 years since P-450 was first successfully solubilized a great deal of work has been done toward purifying variously induced or control P-450 forms from a variety of tissues using a number of different species. These studies have been reviewed by Lu and Levin (41), Levin (42) and, more recently, by Guengerich (28), and Johnson (113).

Comparison of purified fractions from different laboratories has been difficult due to variations in purification schemes and the types of detergents, etc; used in the process. In addition, no standardized procedure has been established for the nomenclature of purified P-450 forms. Coon and co-workers (29) have used polyethylene glycol 6,000 fractionation with cholate, DEAE-cellulose chromatography with the non-ionic detergent Renex 690, and hydroxyapatite to purify various P-450 forms from control, PB and 3-methylcholanthrene (3-MC) treated rabbits. These forms were given the abbreviation LM and numbered one through seven according to the molecular weight as judged by SDS-PAGE. The major form induced by PB was LM-2 (49,000 daltons) and was obtained

in a final yield of 5% with a specific content of 17.4 nmoles of P-450 per mg protein. Purification of 3-MC induced rabbits microsomes yielded a fraction termed LM-4 (55,000 daltons) with a similar yield and a specific content of 17.0. Both of the above preparations appeared homogenous by a variety of tests, but amino terminal sequencing of LM-4 suggested the presence of four closely related polypeptides (35). Control rabbits were shown to have the additional forms LM-1, LM-3a, LM-3b and LM-7 (43).

Levin (44) has used ammonium sulfate fractionation followed by chromatography on DEAE and CM-cellulose to purify P-450 from 3-MC induced rabbits. The final preparation was obtained in a 1% yield with a molecular weight of 51,000 daltons and a specific content of 15-17 nmoles per mg protein. Johnson and Muller-Eberhard (33,45) purified three different P-450 forms from 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced rabbits using a modification of the Coon procedure. These forms were designed P-450a (48,000 daltons), P-450b (60,000) and P-450c (54,000). The specific contents were 18 (3% yield), 9.8 (2% yield) and 9.7 (2% yield), respectively.

Guengerich used a similar procedure (46) to isolate P-450 forms from rabbits after treatment with β-naphthoflavone (BNF). LM-4a and LM-4b were purified to homogeneity according to SDS-PAGE but had lower specific contents. These two forms were very similar with respect to molecular weight (53,000 daltons) and CO-reduced maxima (447 nm) but differed in substrate specificity. LM-1 (47,000 daltons) and LM-7 (60,000) were purified from untreated rabbits to a specific content of 6.8 and 4.3 nmoles per mg protein, respectively. Both forms had CO-reduced maxima of 449 nm. PB induced rabbits gave two fractions, LM-2

and LM-X, the former having properties similar to LM-2 isolated by Coon. LM-X had a molecular weight of 53,000 daltons, a specific content of 9.0 and SDS-PAGE showed other minor protein contamination. The CO-reduced maximum was at 448 nm.

Levin's group (18,47,48) have used similar procedures to purify two major forms from rat liver. PB induced P-450 was purified to 14-18 nmoles per mg protein (molecular weight 48,000 daltons), while the 3-MC induced form (53,000 daltons) had a value of 19-22 nmoles per mg. Both forms were obtained at only about 1% yield. The CO-reduced difference spectra had maxima of 450 and 448 nm, respectively. Induction with polychlorinated biphenyls (PCBs) yielded a mixture of both these forms (49). Although the high specific content and the presence of a single band with SDS-PAGE shows that these were homogenous preparations, immunochemical data suggested that the PB and 3-MC preparations actually consisted of four and two different forms, respectively.

All of the procedures discussed above using classical biochemical separation techniques have produced final preparations in yields of only 1-5%. The possibility exists that some P-450 forms may be lost during purification.

During recent years the application of affinity chromatography towards the purification of a great many proteins has been utilized. The use of a bio-specific ligand-protein interaction to retain selectively the desired enzyme or receptor has produced purifications (in units of activity per mg total protein) of several thousand fold with relatively high yields. Jenofe et al (50) first suggested the possible use of octylamine as a P-450 specific ligand for affinity chromatography. Imai and Sato (51,52) and Imai (53) have developed this

technique to purify P-450 from rabbits and rats. When cholate solubilized microsomes are filtered through an ω-aminooctyl Sepharose column, the solubilized P-450 is retained on the column. After washing with a buffer containing cholate, the P-450 can be eluted with the same buffer containing 0.06-0.08% non-ionic detergent. The yield of P-450 is about 30-50% with a two to four fold purification. The P-450 fraction not retained by the column appears to still be bound to unsolubilized microsomes. Hemoglobin, as well as P-420, elutes in the void volume with the microsomal aggregates. The two other main components of the microsomal mixed-function oxidase system are also retained by the ω-aminooctyl Sepharose column. In some cases they are bound even more tightly than P-450. Such observations have lead investigators to refer to this procedure as hydrophobic chromatography rather than affinity chromatography (53).

Guengerich has modified this procedure so that P-450, NADPH-cytochrome P-450 reductase and b_5 can be separated by sequential elution on a single column (25).

ω-Aminooctyl Sepharose, therefore, provides a rapid, convenient method for the separation of P-450 from contaminating P-420 and hemoglobin, and under optimal conditions, cytochrome b_5 and the P-450 reductase. The yields and the degree of purification obtained appear superior to other techniques for the initial isolation of P-450 from solubilized microsomes.

Rat liver P-450 purified by ω-aminooctyl Sepharose can be separated by stepwise elution on hydroxyapatite (25). Each hydroxyapatite fraction gives rise to two peaks on DEAE-cellulose, again using a stepwise elution system. These fractions were termed A, B, C, and D

according to their order of elution. Induction with PB gave rise to three peaks. The major PB induced P-450 was PB-B (molecular weight 53,000 daltons) and had a specific content of 18 nmoles per mg protein. PB-A was later determined to actually contain two separate forms. A third form, PB-D (54,000 daltons) had a specific content of 14. The CO-reduced maxima of PB-B and PB-D were 450 and 448 nm, respectively. The major form induced by 3-MC was 3-MC-B and had a molecular weight of 56,000 daltons, a specific content of 17 and a CO-reduced maximum of 448 nm.

Recently, DEAE chromatography at room temperature, as first suggested by Neims and Warner (54) has been utilized by a number of investigators (55,56) to obtain highly purified P-450 forms, rapidly and in high yield. Levin's group (30) separated partially purified P-450 from rat liver into three forms on DEAE-cellulose after treatment with PB, 3-MC or PCBs. These forms were designated a, b, and c according to their order of elution using a linear NaCl gradient. PB induced an increase in the b fraction, 3-MC induced form c, while the PCB produced increases in both forms. P-450a was present as a minor peak in all three preparations. Characterization of these forms included SDS-PAGE, CO-reduced maxima, substrate specificity, immunochemical reactivity and peptide mapping after limited proteolysis. A later publication (32) by the same laboratory analyzed the amino acid content and partial sequences, providing some of the strongest evidence to date that these P-450 forms are, indeed, different polypeptides and do not originate from some common precursor.

Techniques For Assaying P-450 Purity

The two most commonly used criteria for establishing the degree of purity of P-450 preparations are specific content and homogeneity when analyzed by SDS-PAGE. Specific content is usually given as nmoles of P-450 per mg of protein. The amount of P-450 is determined from the CO-reduced vs. reduced difference spectra using the extinction coefficient of 91 mM^{-1} cm^{-1} (14). Protein can be assayed by a variety of standard procedures including that of Lowry (57), Bradford (58) and the biuret method (59). Results from a number of laboratories suggest that the Lowry assay gives protein values that are too high when compared to quantitative amino acid analysis (48). Guengerich (25), however, did not observe this difference when he included possible interfering agents such as glycerol and detergent in the protein standard. The Bradford assay was found to give results comparable with the Lowry method with microsomes or the final purified detergent-free samples but were inconsistent when analyzed at points within the purification (25).

Results comparing the three common protein assays have shown a wide variation in response between different proteins for all three procedures (59). Therefore, one could expect the value obtained to depend upon the protein chosed as the reference standard. Ideally, the standard should be a purified preparation of the protein being assayed. To date, such a procedure has not been possible due to the lack of enough purified P-450.

When using the Lowry procedure, the color development will depend mainly upon the relative number of tryptophan and tryrosine residues. Amino acid analysis of different purified P-450 forms (29) have shown up to a seven fold difference in the number of tryptophan residues

present. This may explain why some P-450 forms purified to apparent homogeneity still have relatively low specific content values (46). Also, since specific content is only a reflection of the total amount of P-450 per mg protein, it provides no indication of how many P-450 forms may be present.

In addition to the above, the specific content could also be underestimated if a significant amount of apoprotein is present (28). Certain P-450 forms have been postulated to exist in vivo without a full complement of heme (28). Loss of heme during purification would also increase the amount of apoprotein and, thus, lower the specific content.

Determination of the purity of P-450 by SDS-PAGE is limited by the degree of resolution obtained. The system of Laemmli (60) has been shown by a number of studies to be superior to other, earlier, methods appearing in the literature (25,29,42). This system is capable of resolving about four to eight different P-450 forms in the molecular weight range of 45,000-60,000 daltons. Any P-450 forms which differ by 1,000 daltons or less are probably not resolved.

Two very recent publications provide examples of the inadequacy of SDS-PAGE in distinguishing P-450 forms of similar size. Agrosin et al (27) purified two forms of P-450 from uninduced rat liver which differed in molecular weight by only 200 daltons. Although the two forms showed a large degree of similarity in amino acid composition, they could be distinguished by their CO-reduced absorbance maxima (450.0 and 450.8 nm), ethyl isocyanide difference spectrum and substrate specificity.

Negishi and Nebert (61) separated two P-450 forms from 3-MC

induced mice which were designated P_1-450 and P-448. In contrast to the results with rabbits (62) or rats (63), these two forms have almost identical molecular weights (55,000 daltons), but differ in CO-reduced maxima (449.3 for P_1-450 and 448.0 for P-448), the peptide composition obtained upon limited proteolysis, substrate specificity and immunochemical reactivity.

Another complication arises when using SDS-PAGE. The molecular weight range in which P-450s are found contain a number of other proteins such as epoxide hydrase. A peroxidase stain has been employed by some investigators to establish the identity of a particular band as a hemoprotein (16). However, heme transfer to other proteins during electrophoresis has been observed and could cause false band identification (64). Although SDS-PAGE does have disadvantages, it is still the current method of choice when comparing microsomes from differently treated animals or when establishing the homogeneity of a purified fraction.

Isoelectric focusing has become a powerful technique for determining the hetergeneity of mixtures of isozymes. Depending upon the pH range chosen, single amino acid substitutions can be detected, and proteins differing in pI by as little as 0.01 of a pH unit can be resolved. Such a technique seems especially suited for detecting different P-450 forms which may not be separated by SDS-PAGE. Warner and Neims (65) fractioned P-450 from uninduced male rat liver into four separate peaks on DE-52. Isoelectric focusing of each peak in polyacrylamide over the pH range of five to seven showed two to eight different heme containing bands as well as other non-hemoproteins. Later work by the same authors (54) on a preparative scale utilizing

focusing in a Sephadex G10 column gave six to ten distinct peaks, all of which still had native spectral characteristics. Isoelectric focusing of the major PB induced rabbit hepatic P-450 (LM_2), which appeared homogenous on SDS-PAGE, produced four distinct P-450 containing bands (31). A much more detailed study was done by Guengerich (66). Highly purified (15-19 nmoles/mg) P-450 from PB induced rats was focused in either polyacrylamide or granulated gels (Sephadex G75). Although this preparation had been judged to be homogenous by a number of other criteria, (including focusing under nondenaturing conditions in the absence of ampholytes), similar complex band patterns were seen in both focusing systems. The band pattern was found to depend upon the protein load and the point of sample application. In addition, when a single band was recovered after focusing in G75 and re-run on polyacrylamide, a multiple banding pattern was again produced. These observations lead Guengerich to conclude that the appearance of multiple bands was an artifact due to either protein-protein interactions or, binding of ampholyte to P-450. Alterations in experimental procedures such as focusing time, temperature or pre-focusing was without effect. Inclusion of eight M urea, and/or 1% non-ionic detergent, or one mM dithiothreitol (DTT) did not change the banding pattern. Ampholytes from different sources (Bio-Rad, LKB or Guengerich) all produced the same pattern. Further experiments suggested that the heterogeneity observed was not due to either heme loss or some effect due to detergent. Multiple band patterns were seen with a bacterial P-450 (P-450 cam) which had never been exposed to detergent. In contrast another bacterial P-450 (P-450 meg) focused as a single band in polyacrylamide (pI 4.9) under slightly different

conditions (67). Protein-protein interactions with P-450 could be possible as hydrodynamic experiments have shown that P-450 can aggregate even in six M guanidine-HCl or 1% deoxycholate or non-ionic detergent. Binding of ampholytes to proteins has been observed in other systems (68), and Guengerich found that a significant amount of a 3% ampholyte solution remained bound to P-450 even after exhaustive dialysis (66).

The existence of high molecular weight aggregates may explain the finding that solubilized microsomes are unable to enter either gel completely even in the presence of 1-2% detergent. Partial purification eliminates this problem, although the presence of detergents is still necessary for focusing to occur.

Therefore, although isoelectric focusing, especially if combined with SDS-PAGE (two dimensional electrophoresis) (69), is a powerful technique for separating isozymes with very similar molecular properties, its application to the field of P-450 analysis is still not established. Before this technique can become a useful analytical tool in this field, a system must be developed which is consistently capable of preventing P-450 from interacting with itself, other proteins or ampholytes.

Induction Of The MFO System

One of the earliest characteristics of the mixed-function oxidase system to be investigated was its increase in activity following exposure to a great variety of chemical compounds (1). This inductive effect has been divided into two types. The barbiturates, specifically PB, constitute the first type. Exposure to rats at doses of 30 mg/kg

or greater produce an increase in liver weight due to hypertrophy. The most significant effect is on the smooth endoplasmic reticulum. Proliferation of this microsomal fraction is paralleled by an increase in the amount of phospholipid, protein, P-450 and NADPH-cytochrome P-450 reductase (70). The type II inducers are the polycyclic hydrocarbons such as 3-MC which do not appear to have an effect on liver weight, microsomal protein, phospholipid or P-450 reductase (70). In addition, the P-450 induced has catalytic and spectral properties which differ from the uninduced or PB induced P-450. The CO-reduced difference spectrum is shifted after 3-MC treatment to a maximum at 448 nm, hence this novel hemoprotein has been termed P-448. An increase in P-448 content parallels an increased rate of benzo[a]pyrene (BP) metabolism which is not seen after PB induction.

More recent evidence indicates that the situation is actually more complicated. There have been inducers found, such as PCN (26,70, 71) or safrole (26,72) which have characteristics differing from both the above types, while some chlorinated hydrocarbons (DDT, PCBs) have both type I and type II inducing properties (26,73,74). Therefore, it appears that there is probably at least three mechanisms involved in the induction of the microsomal mixed-function oxidase system. To date, a molecular model substantiated by experimental evidence exists only for the type II inducers (75), although hypothetical models for other types of induction have been presented. In the discussion below the molecular events known to occur upon induction, for each type of inducer, will be presented in more detail.

Induction Of MFO By Barbiturates

Comparison of different barbiturates (76) have shown that the degree of inductive potency is correlated to the plasma half-life. Phenobarbital ($t_{\frac{1}{2}} \sim$ three hours) is the most effective barbiturate and has become a classic agent in the study of MFO activity. Within a few minutes after its application PB becomes bound to SER, reaching a maximum after about three hours (77). At about the same time an increase in phospholipid turnover begins (78). After about six hours increases in P-450 and P-450 reductase content can be seen in the rough endoplasmic reticulum indicating an increase in protein synthesis (79). Increases in the MFO components of smooth endoplasmic reticulum begin at between 8-12 hours and after abour 24 hours the content in the smooth and rough fractions is about equal. During this same time period an increase in microsomal phospholipid content due to a decrease in catabolism is observed.

From 24 hours on, the smooth/rough ratio of MFO components increases and liver hypertrophy begins. The peak amounts of MFO components induced by PB (on a per/mg protein basis) is 200-500% for P-450 and P-450 reductase while liver weight is increased 30-80% and total microsomal protein increased about 50%. Smaller increases are seen in the amount of cytochrome b_5 and its reductase (80). In addition to its effect on the MFO system, PB increases UDP-glucuronyl transferases (81), epoxide hydrase (82), and enhances the biliary clearance of a number of drugs (83).

Studies with inhibitors of transcription and translation have shown that both of these events are required for PB induction. During the first few hours, actinomycin D is quite effective in inhibiting induction, however, if it is given prior to treatment and then

withdrawn, it is without effect (84). These results demonstrate that PB induction requires the synthesis of new mRNA and not the activation of any pre-existing species. Also, if actinomycin D is administered more than about 4 hours after dosing, it has no effect. Similarly, the effect of inhibitors of protein synthesis such as cycloheximide can also be shown to be temporal, having their maximum inhibitor action at about the same time that actinomycin D is no longer effective. To date, little is known about the mechanism(s) by which PB induces selective mRNA transcription. If PB were somehow acting at a specific site in the genome, how does this explain the very general nature of this type of induction?

Induction Of MFO By PCN

Pregnenelone-16α-carbonitrile (PCN) is a synthetic "catatoxic" steroid with no known additional hormonal or pharmacological effects (85). Catatoxic steroids, such as PCN or spironolactone, are able to provide protection against the toxic actions of a great number of compounds by stimulating their metabolism by the MFO system and increasing their rate of elimination. Extensive examination of more than 1,300 such steroids (86) have shown that PCN is probably the most potent. The induction observed resembles more the PB "type" rather than 3-MC, in that increases in P-450 content, liver weight, NADPH-P-450 reductase, phospholipid, and the hepatic smooth endoplasmic reticulum are seen (70,71,87). PCN induction produces a significant decrease in total hepatic cytosol protein, however, the glutathione S-aryl transferases (including Y protein, the major organic anion-binding protein), and Z protein levels are elevated (88). As is the case

with PB, pretreatment with PCN enhances bile flow and microsomal UDP-glucuronyl transferase activity (89).

The combined effect of all of the above, probably accounts for the potency of PCN as a prophylactic drug and has stimulated interest in its possible clinical application in the treatment or prevention of kernicterus (89).

Studies comparing PB, PCN and 3-MC induced microsomes have demonstrated that the P-450 forms induced differ with respect to substrate specificity, spectral properties and molecular weight as judged by SDS-PAGE (21). In contrast with experiments with PB or 3-MC induced microsomes, the major P-450 form(s) induced by PCN has not been obtained in purified form, and therefore, the question of whether or not PCN induction produces an increase in a pre-existing form, or involves the synthesis of a novel P-450 species, remains to be answered.

In order to form a working model of PB or other (PCN, safrole, steroids, etc.) types of induction, one must first examine closely one model that has been tested experimentally, the type II inducers typified by 3-MC.

Induction Of MFO By 3-MC

Induction of aryl hydrocarbon hydroxylase (AHH) by polycyclic aromatic hydrocarbons such as 3-MC, TCDD, BNF, and BP has been demonstrated to be a genetically controlled autosomal dominant trait (21, 75,90-94) which Nebert has termed the Ah locus. The Ah locus appears to regulate the activity of at least two different forms of P-450 and 20 microsomal monooxygenase reactions. Activation of the Ah locus does not result in increased activity of NADPH-cytochrome P-450

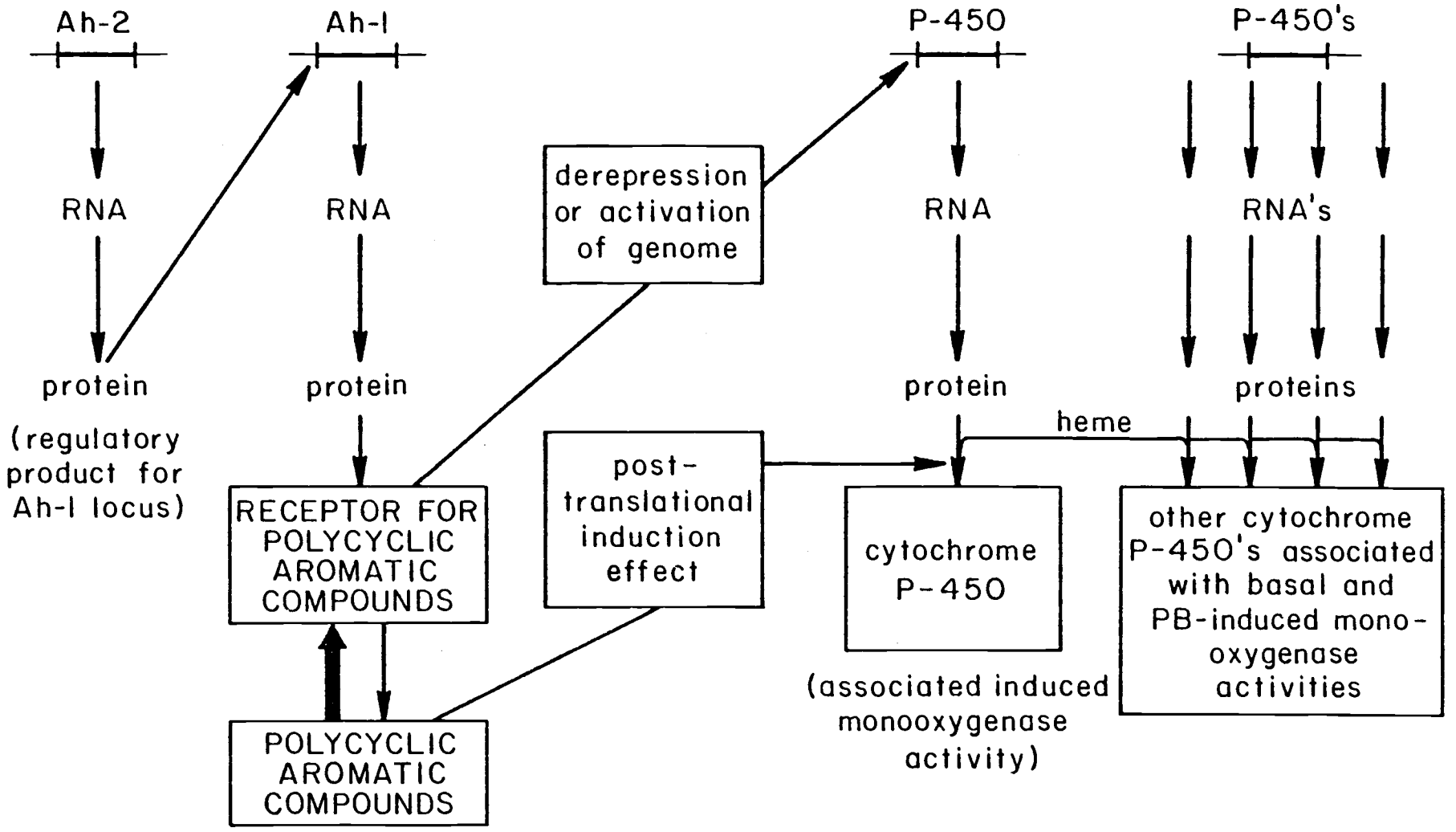

Ah-2
Ah-1
P-450
P-450's
RNA
RNA
RNA
RNA's
derepression or activation of genome
protein
(regulatory product for Ah-I locus)
protein
protein
proteins
heme
post-translational induction effect
RECEPTOR FOR POLYCYCLIC AROMATIC COMPOUNDS
cytochrome P-450
other cytochrome P-450's associated with basal and PB-induced mono-oxygenase activities
(associated induced monooxygenase activity)
POLYCYCLIC AROMATIC COMPOUNDS

Figure 2

reductase, epoxide hydrase or GSH S-transferase. In addition, other metabolic reactions which are not monooxygenases appear to be under the control of the Ah allele(s). These include UDP glucuronyl-transferase, menadione oxidoreductase, and ornithine decarboxylase. A diagram of the proposed control of the Ah locus is shown in Figure 2. In this model, Nebert (75) proposes the existence of at least two regulatory genes (Ah-2 and Ah-1) and a number of structural genes for multiple forms of P-450. The product of the Ah-1 locus is a cytosolic receptor which specifically binds the inducing ligand with high affinity, transports it into the nucleus where the inducer-receptor complex interacts with the genome, in an as yet unknown manner, to specifically induce or derepress structural genes involved in the synthesizing P-450. This model is nearly identical to that proposed for cytosolic steroid receptors (95).

The post-translational induction effect shown occurs in liver cell culture and involves a decrease in the rate of induction decay. Poland (96), as well as Nebert (97), have demonstrated the existence of a polycyclic aromatic hydrocarbon specific receptor with high affinity in both mouse and rat. The specificity of binding was quite thoroughly examined by Nebert. Using ^{3}H-TCDD as the ligand, it was found that only 3-MC could effectively act as a competitor for specific binding. Neither PB or PCN could displace TCDD from the receptor site even at concentrations as high as 10^{-6} M. Furthermore, in a series of binding assays using structural congeners of TCDD as competing ligands, a very good correlation was seen between receptor affinity and induction potency (98).

The two P-450s induced by TCDD in rabbits have been termed P_1-450

and P-448 and exhibit different mobilities in SDS-PAGE (57,000 and 54,000 daltons, respectively), and can also be distinguished by substrate specificity, and absorbance maxima in the CO-reduced difference spectra (62). The Ah locus is under temporal and developmental control as well. In rabbits, induction with 3-MC in the fetal or neonatal state up to 12 days induced P_1-450 but older animals respond with an increase in the P-448 form (63). Studies in inbred strains of mice have shown that about half the strains are genetically "responsive" and half are "non-responsive". The ED_{50} is about 100 times lower and maximum activity induced is about 100 times greater in "responsive" animals when compared to "non-responsive". This lack of response was initially postulated to be due to either a decrease in receptor binding sites or a lowering of their affinity for 3-MC and TCDD. However, experiments with "responsive" and "non-responsive" tissue culture lines (97) indicate similar binding parameters. Non-responsiveness could be due to a defect in the receptor such that the inducer-receptor complex is unable either to translocate to the nucleus or to interact with components in the genome to initiate transcription. Although "non-responsive" mice are not induced by 3-MC, this lack of response can be overcome by using TCDD at levels slightly higher than needed to produce an equivalent response in "responsive" mice. In the rat, TCDD is 30,000 times more potent than 3-MC (99) while the binding constants for the receptor are similar. Thus, although the existence of a specific, high affinity receptor appears necessary in order for an inducer to produce its effect, the molecular mechanisms involved in steps subsequent to the initial binding of the ligand are of great importance and are, as yet, not well defined.

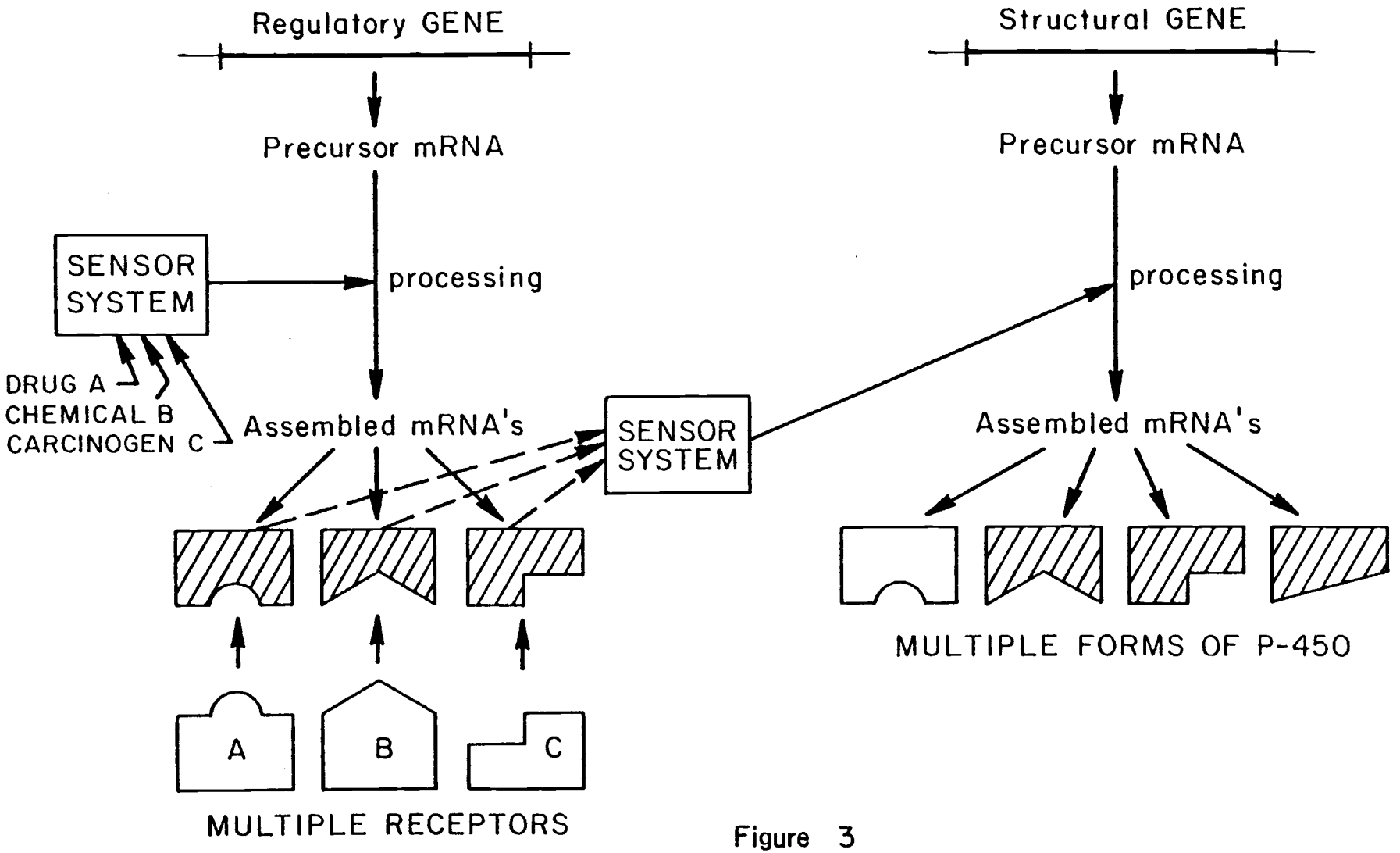

Regulatory GENE
Precursor mRNA
SENSOR SYSTEM
processing
DRUG A
CHEMICAL B
CARCINOGEN C
Assembled mRNA's
SENSOR SYSTEM
A
B
C
MULTIPLE RECEPTORS
Structural GENE
Precursor mRNA
processing
Assembled mRNA's
MULTIPLE FORMS OF P-450

Figure 3

The question also arises as to how many receptors are there? As early as 1967, there were over 200 known MFO inducers with great structural and chemical diversity (1). Evidence has been presented above stating that MFO inducers such as PCN and PB do not interact with the Ah receptor. Do these synthetic compounds, as well as the more than 200 other known inducers, (in addition to numerous compounds yet to be developed) each have a specific high affinity receptor? A more likely scheme is known in Figure 3 in a model by Nebert (75). It is postulated that some sensor system exists which interacts with an inducer in a manner similar to antigen imprinting of lymphocytes prior to the production of specific antibodies. The sensor system then interacts in some way with precursor mRNA from a regulatory gene to produce specific mRNAs capable of producing as one of the products, a receptor specific for the inducing compound. The receptor-inducer complex then reacts with a second sensor system responsible for processing precursor P-450 mRNA into a final specific form. One very intriguing aspect of this model is that it allows for the existence of dozens, even hundreds of different P-450 forms (perhaps having variable and constant regions similar to the immunoglobulins). Nebert (75) suggests that this could explain the ever increasing number of P-450 forms being isolated and characterized, as well as the immunochemical cross-reactivity observed in some laboratories using highly purified P-450 forms.

The aim of this research was to investigate two aspects of Neberts model, the existence of multiple micro-heterogenic P-450 forms, and high affinity, specific, cytosolic binding sites for 3-MC, PB and PCN.

MATERIALS AND METHODS

Purification Of Cytochrome P-450

Sprague-Dawley male rats (200-300 g) were induced by i.p. injections once a day for four days with either PB (75 mg/kg/day in saline), PCN (69 mg/kg/day in an aqueous micro-suspension of about two drops Tween 80 per 10 mls of water), or 3-MC (20 mg/kg/day in corn oil). Control animals received the appropriate vehicle alone. The animals were starved overnight before sacrificing (24 hours after the last injection) and the livers were immediately perfused with ice cold buffer containing KCl (100 mM), ethylenediamine tetraacetic acid (EDTA) (1 mM), butylated hydroxytoluene (BHT) (0.02 mM), phenylmethylsulfonylfluoride (0.1 mM), and Tris-acetate (100 mM, pH 7.4). The livers were then removed, weighed and homogenized with three volumes of the above buffer using a loose-fitting glass homogenizer.

The following method for the isolation and solubilization of microsomes is from Van der Hoeven and Coon (102), and the purification procedure is from Guengerich (25,46). The homogenate was centrifuged at 10,000 g for 30 minutes. The supernatant was set aside and the pellet re-homogenized in a minimal amount of buffer, centrifuged again and the supernatant combined with the original supernatant. This mixture was then centrifuged at 105,000 g for 90 minutes. The pellet was re-suspended, in a volume equal to that of the original homogenate, using a buffer containing EDTA (1 mM), BHT (0.02 mM) and potassim pyrophosphate (100 mM, pH 7.4) and treated with four 30 second bursts of a 200 W ultra tip sonicator (Wave Energy Systems) at full output.

Following centrifugation at 105,000 g for 60 minutes, the supernatant was discarded and the microsomal pellet suspended in buffer containing potassium phosphate (100 mM, pH 7.25), sodium cholate (0.6%, w/v), glycerol (20%, v/v), dithiothreitol (DTT) (0.1 mM) and EDTA (1 mM) to a protein concentration of about 4 mg/ml. Solubilization was allowed to proceed for about 30 minutes and then the mixture was centrifuged at 77,000 g for 120 minutes. The reddish, slightly turbid supernatant containing the solubilized microsomal protein was then applied to a ω-aminooctyl (2.5 x 30 cm) agarose column (about one ml of packed gel per five mg protein) obtained from Sigma (3.5 μmoles diaminooctane per ml packed gel). After washing with three to four column volumes of the above buffer, in which the cholate concentration had been reduced to 0.42%, the P-450 was eluted with the same buffer in which the cholate concentration had been further reduced to 0.33% and Triton N 101 (Sigma) added to 0.06% (v/v). Fractions were collected and the appearance of hemoprotein monitored by the absorbance at 417 nm.

Fractions containing P-450 were pooled, concentrated by ultrafiltration using an Amicon PM 30 membrane, diluted to 33 mM phosphate with 20% glycerol and then applied to columns of hydroxyapatite (Bio-Rad, Bio-Gel HT, 1.9 x 5 cm).

Cytochrome P-450 was eluted with a stepwise gradient of potassium phosphate buffer (pH 7.25) containing Triton N 101 (0.2%, v/v), glycerol (20%, v/v) and EDTA (0.1 mM). Fractions were collected and analyzed for absorbance at 417 nm.

Peak fractions were pooled, the amount of protein determined by the method of Lowry (57, using appropriate corrections for interfering

detergents and glycerol) and the P-450 content determined according to Omura and Sato (14), using a Hitachi 100-80 spectrophotometer to record the CO-reduced versus reduced difference spectra.

Peaks from the hydroxyapatite column were concentrated about four fold using an Amicon PM 30 membrane and then dialyzed twice against 30 volumes of buffer containing potassium phosphate (5 mM, pH 7.7), glycerol (20%, v/v) and EDTA (1 mM). After diluting two fold with the above buffer minus the phosphate, the samples were applied to a DEAE Sephacel (Pharmacia) column (1.2 x 15 cm) pre-equilibrated in the dialysis buffer containing Triton N 101 (0.2%, v/v). The P-450 was eluted with the equilibration buffer followed by the same buffer in which the phosphate concentration had been raised to 35 mM. The stepwise elution was continued by doubling the phosphate concentration until no more P-450 could be eluted. Fractions were collected and the absorbance at 417 nm monitored. P-450 containing fractions were pooled and analyzed for specific content as before.

An alternate procedure suggested by Guengerich (personal communication) was adopted after the PB induced P-450 had been purified as described above. This scheme omits the hydroxyapatite step (which was found to be time consuming and inconsistent) and employs DEAE Sepacel chromatography at room temperature. Pooled fractions containing P-450 from ω-aminooctyl agarose were concentrated as before, and then dialyzed against 10 mM phosphate pH 7.7, 20% glycerol and 0.1 mM EDTA. Sodium cholate was added to the dialyzed samples to a final concentration of 0.2% (w/v), and Lubrol PX (Sigma) was added to 0.1% (v/v). Lubrol PX consists of ethylene oxide condensates of fatty alcohols and, unlike the phenoxy polyethoxy nonionic detergents such as Triton,

has no significant UV absorbance which allows for monitoring the column eluate for protein at 280 nm. In addition, preliminary hydrodynamic experiments by Guengerich (personal communication) indicate the Lubrol PX may be more effective in preventing P-450 aggregation than other detergents used to date.

After partial purification by ω-aminooctyl agarose chromatography, samples from control or induced rats were loaded separately onto a DEAE Sephacel column (1.2 x 30 cm) which had been equilibrated with sample buffer and then eluted with the same buffer followed by a linear gradient of NaCl from 0-0.25 M. Fractions were collected and analyzed for absorbance at 417 nm and 280 nm and NaCl was measured by a conductivity meter (Radiometer, Copenhagen). P-450 containing fractions were pooled, concentrated, dialyzed against 20% glycerol, 0.1 mM EDTA, 0.06 M Tris-HCl pH 6.8 and analyzed for specific content prior to electrophoretic experiments.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis was performed using a modification of the procedure of Laemmli (60), in which miniature slab gels (70 x 100 x 0.5 mm) were employed. The advantages of this "mini-gel" system, when compared to standard sized slab gels, include a significant saving on materials, amount of sample needed, set-up and run time and ease of handling during staining and destaining procedures; all of which can be obtained with little or no loss in resolving power.

The separating gel, consisting of 10% acrylamide (Bio-Rad), 0.27% N,N-bis-methylene acrylamide (BIS, Bio-Rad), 0.375 M Tris-HCl (pH 8.8), 0.1% sodium dodecyl sulfate (SDS) (Sigma), 0.025% tetramethylethylene-

diamine (TEMED, Eastman), and 0.1% ammonium persulphate (APS, Sigma) was degassed for 15 seconds and then poured to a height of six cm. After overlayering with distilled water, polymerization was allowed to continue approximately two to three hours.

The stacking gel, consisting of 3% acrylamide, 0.08% BIS, 0.125 M Tris-HCl pH 6.8, 0.1% SDS, 0.2% TEMED and 0.1% APS was prepared as above and allowed to polymerize for one to two hours. Samples were made up to 2% SDS, 5% β-mercaptoethanol and 10% glycerol and immersed in boiling water for two minutes before being carefully applied to preformed wells in the stacking gel. The anode buffer was 0.025 M Tris pH 8.3, 0.192 M glycine and 0.1% SDS while all components were doubled in the cathode buffer to optimize resolution of multiple forms of P-450 as suggested by Guengerich (25).

Electrophoresis was performed at 4°C using constant voltage (200 V) from a Gelman power supply. The current reached a maximum of about 55 mamp per gel and then constantly dropped to a final value of about 25 mamp per gel. Termination of each run occurred when the bromophenol blue tracking dye reached the end of the gel. Such a system allows for the rapid analysis (average run time 50 minutes) of up to 30 samples each containing 0.5 µg or less of protein.

The gels were stained by shaking in a solution of 45% ethanol, 10% acetic acid, and 0.1% Coomassie Blue R (Sigma) for one hour at 20°, and destained in 10% ethanol with 7.5% acetic acid. Although banding patterns could be visualized after about one hour, the gels were destained overnight with two changes of solution in order to obtain the clearest background possible prior to drying and photographing.

Molecular weights were determined by comparing the relative mobility of unknowns to the logarithm of the molecular weight of several standards (Sigma).

Isoelectric Focusing (IF) In Polyacrylamide Gel Tubes

The procedure below was a modification of O'Farrell (69). To a stock solution of 4% acrylamide, 0.2% BIS, 9.2 M urea (Bio-Rad), 0.07% TEMED, and 1% Lubrol PX was added ampholines (1% Bio-lyte 3-10, 1% Bio-lyte 5-7, Bio-Rad) and the protein sample. The mixture was degassed for one minute, made 0.1% with respect to APS, poured into six mm I.D. glass tubes to a height of 120 mm and overlayered with 20 µl of distilled water. After the gels polymerized about one hour, they were transferred to a gel electrophoresis cell (Bio-Rad Model 750), the top end overlayered with 20 µl of six M urea, 1% ampholine and then 0.01 M phosphoric acid to the top of the tube. The bottom reservoir (cathode) was filled with 500 mls of freshly prepared 0.02 M sodium hydroxide and the top (anode) with 500 mls of 0.01 M phosphoric acid. Constant voltage was maintained at 350 V for 30 minutes followed by 500 V for four hours. The gels were gently extruded from the tubes and stained according to Guengerich (104). In this procedure, protein was first fixed in a solution of 30% methanol, 3% sulfosalicylic acid and 10% trichloroacetic acid (TCA) with gentle shaking for 40 minutes. Proteins were stained by shaking the gels in the same solution plus 0.1% Coomassie Blue R at 37°C for 40 minutes. Destaining was done by shaking the gels overnight in 25% ethanol and 10% acetic acid. Protein banding patterns were recorded at 550 nm using a Beckman DU spectrophotometer (Model 2400) and a Gilford gel scanner.

The pH gradient was determined by focusing a gel which contained no protein, as described above, sectioning the gel into 0.5 cm pieces and measuring the pH directly using a flat membrane combination electrode (Corning 476216).

Macromolecular Binding Of PB, PCN and 3-MC To Rat Liver Cytosol

3β-Hydroxy-20-oxo-5-pregnene-16α-^{14}C-carbonitrile (^{14}C-PCN) was synthesized from 16-dehydropregnenolone acetate (Sigma) and H^{14}CN (ICN) by a modification of the method of Romo (100). The product was purified by TLC, stored at -70°C in 95% ethanol under nitrogen and assayed periodically for radiochemical purity. At no time was the amount of total impurities found to be greater than 1%. The maximum specific activity obtained was about 55 Ci mole^{-1}. Unlabelled PCN was synthesized in an identical procedure and purified by crystallization from methanol and water. The final product (m.p. 238-239°C) identity was confirmed to be PCN by NMR, IR and MS.

Ethyl-5-phenyl barbituric acid, 5-[^{3}H(G)] (^{3}H-PB) was purchased from New England Nuclear Co. and had a specific activity of 8.1 Ci mmole^{-1}. Unlabelled PB was a gift from Dr. Robert Larson of the Pharmacy School at Oregon State University.

[G-^{3}H] 3-Methylcholanthrene (^{3}H-3-MC) was purchased from Amersham. The specific activity was given as 27 Ci mmole^{-1}. The unlabelled material was purchased from Tridom-Fluka.

Male Sprague-Dawley rats (Simonsons of Gilroy, California) were starved overnight, sacrificed and the livers immediately perfused with buffer containing sucrose (300 mM), KCl (500 mM), EDTA (1 mM), DTT (0.1 mM) and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

(HEPES) (20 mM), pH 7.8 at 4. A 1:1 liver homogenate was prepared in this buffer using a loose-fitting glass homogenizer and was centifuged successively at 12,000 g for 15 minutes and at 234,000 g for two hours. The 234,000 g supernatant (care being taken to exclude any lipid) after centrifugation constituted the cytosol.

When binding of ^{14}C-PCN to macromolecular cytosol components was measured by gel filtration, aliquots of ^{14}C-PCN in dimethylsulfoxide (DMSO) were added to cytosol (1.2 ml) at 0°C and incubated for 90 minutes. The DMSO concentration did not exceed 0.1% (v/v) of the reaction mixture. After incubation, 0.5 ml aliquots of the mixture were applied to columns (8 x 150 mm, 4.8 ml bed volume) of either Sephadex LH-20 or G-25 (Pharmacia) equilibrated in a buffer of KCl (50 mM), EDTA (1 mM), DTT (0.1 mM) and HEPES (20 mM) pH 7.8. Each column was eluted with the same buffer 0.2 ml min^{-1} at a constant flow rate using a peristaltic pump); fractions (0.4 ml) were collected and the A_{280} and radioactivity of each fraction was measured. Recovery of radioactivity from each column was essentially quantitative (>96%).

The procedure employed in the measurement of ^{14}C-PCN, ^{3}H-PB and ^{3}H-3-MC binding using dextran-coated charcoal was a modification of that of Poland et al (96). Cytosol was incubated as above, following which, dextran (Sigma, average molecular weight 500,000) coated charcoal (MCB, Norit A, acid washed) was added to each sample to a final concentration of 0.5% (w/v). Samples were shaken 10 minutes at 4°C and then centrifuged at 23,000 g for five minutes. The supernatant was removed for determination of radioactivity. Ligand binding dissociation constants were calculated from measurements of the bound and total ligand at a variety of ligand concentrations by the method of

Scatchard (101). Competition binding experiments were performed by repeating the above in the presence or absence of an excess of unlabelled ligand.

RESULTS

Hepatic Morphological Changes Associated With Induction

Upon dissection, the livers of approximately 75% of both the PCN and the 3-MC treated rats were observed to have what appeared to be lipid deposits. These discrete spots were white and varied in size and number from animal to animal, and seemed to originate in the capsule tissue and in more advanced stages, became deeply embedded in the liver. Multiple samples of tissue containing these spots were submitted to Dr. N. M. Patton, Director, Laboratory Animal Resources and Pathology, for histopathological examination. His report stated that the spots were "....focal areas of necrosis with debris and surrounding inflammatory cells. PAS and Worthens Starry stains were negative for bacteria or parasites." The official finding was listed as focal necrotizing hepatitis. Injections of the 3-MC vehicle (corn oil) produced necrotic tissue but the occurrence and extent observed were lower than with the experimental group. No gross morphological changes in the livers of the PB induced or controls injected with the PB vehicle (saline) or the PCN vehicle (aqueous Tween 80) were observed.

The Effect Of PB, 3-MC And PCN On Liver Weight, Total Protein And P-450 Levels

Data obtained on the effect of these MFO inducers on various liver parameters are listed in Table 1. The differences between groups in body weight reflect slight age variations (up to one week) and are not thought to be due to any effect of the inducers themselves. The relative liver size was significantly increased ($p > 0.005$) in all

Table 1. Hepatic Changes Following Induction

Inducer	Body weight	Percent liver	Solubilized microsomal protein (mg/g liver)	P-450 (nmoles/mg protein)	Difference spectrum λ-max (nm)
None	261 ± 26	3.10 ± 0.18	4.63	1.35	449.5
PB	276 ± 21	3.83 ± 0.35	6.48	2.51	450.0
PCN	253 ± 17	3.84 ± 0.21	4.49	1.23	449.5
3-MC	238 ± 13	4.14 ± 0.29	6.21	2.57	448.0

three induced groups. Although this was expected with PB and PCN, liver hypertrophy is usually not observed following 3-MC induction. This could be due to the smaller size of this youngest group.

Increases in the amount of total solubilized microsomal protein were seen in the PB and 3-MC induced rats. The latter inducer usually has little effect on total microsomal protein levels.

The levels of P-450 in control rats (Table 1) was slightly higher than the average for unsolubilized microsomes (about 0.8 nmoles per mg protein) cited in the literature for this species (6), but there are reports of similar values (71). Furthermore, the value given here is for the solubilized P-450, the cholate treatment may have produced a slight increase in specific content. Induction with PB produced a 86% increase in solubilized microsomal P-450 specific content and shifted the CO maximum difference spectra of reduced P-450 to slightly longer wavelengths. PCN did not significantly alter P-450 content or the difference spectrum. Pretreatment with 3-MC produced the expected hypochromic shift in the CO-reduced spectrum (P-448) and increased P-450 content to levels slightly greater than the PB group.

Purification Of P-450 On ω-Aminooctyl Agarose

Elution profiles of control and induced solubilized microsomes on ω-aminooctyl agarose are shown in Figure 4. The eluate containing the broad hemoprotein peak eluting in the void volume was quite turbid and contained a substantial percentage of P-420. A second hemoprotein peak, with was not turbid and only slightly retarded, was quite prominent with solubilized PB microsomes, but appeared only as a shoulder on the other chromatograms. Partially purified P-450 was eluted from

Figure 4. Purification of P-450 from solubilized microsomes on ω-aminooctyl agarose. C. uninduced; PB. phenobarbital induced; PCN. 16α-cyanopregnenolone induced; 3-MC. 3-methylcholanthrene induced. Microsomes, (200 mls, 750-2500 nmoles P-450, 500-1000 mg protein) which had been solubilized with cholate, were applied directly to 100 mls of ω-aminooctyl agarose. The washing and elution of P-450 were as described in the Materials and Methods section.

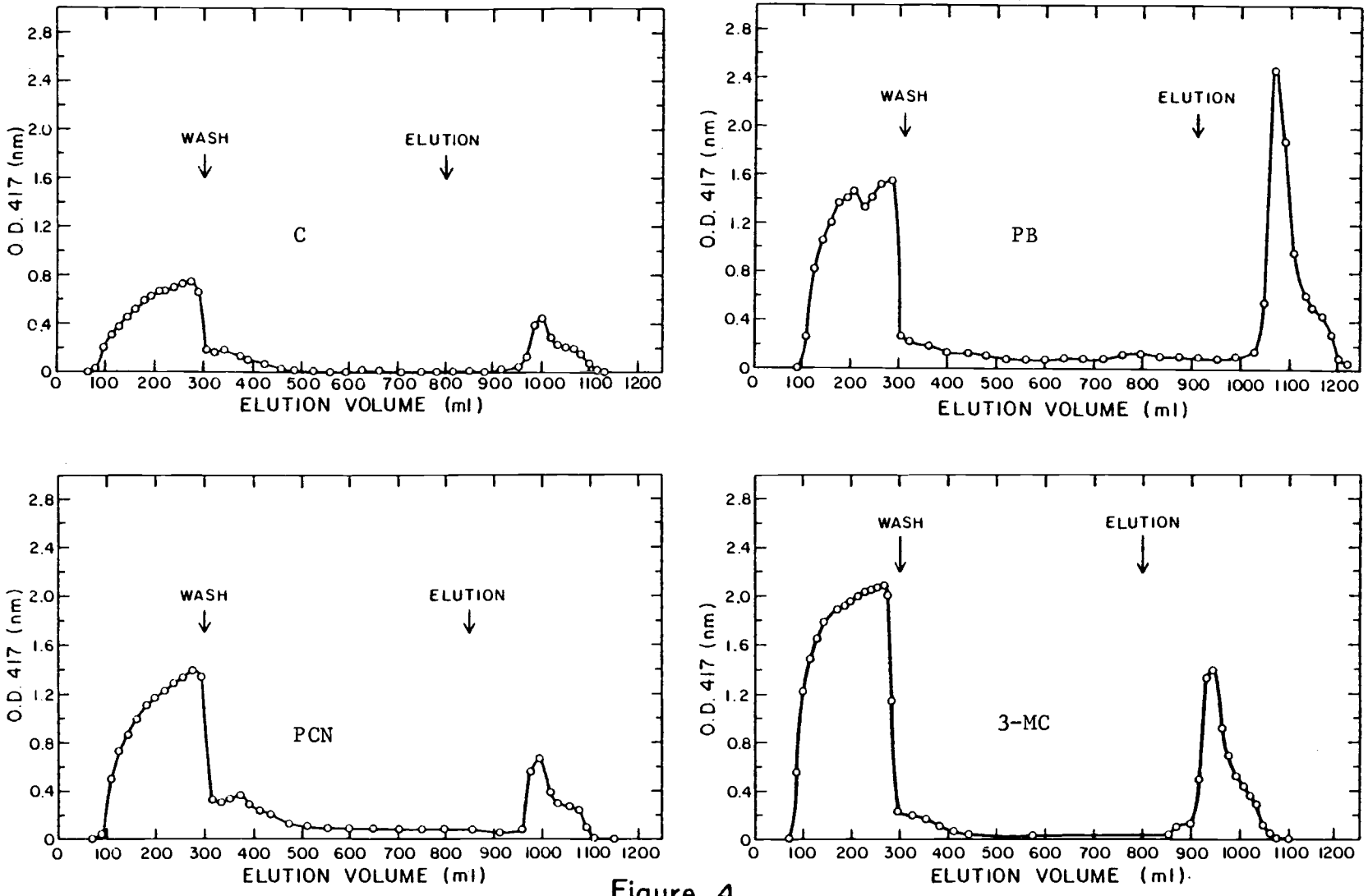

C
PB
PCN
3-MC
WASH
ELUTION
O.D. 417 (nm)
ELUTION VOLUME (ml)
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
100
200
300
400
500
600
700
800
900
1000
1100
1200

Figure 4

Figure 5. SDS-PAGE of solubilized microsomal protein and P-450 partially purified by ω-aminooctyl agarose (AOA) chromatography. Samples from right to left were, C. uninduced microsomes, 4 µg; PB. phenobarbital induced microsomes, 4 µg; PCN. 16α-cyanopregnenolone induced microsomes, 4 µg; 3-MC. 3-methylcholanthrene induced microsomes, 4 µg; S. protein standards, bovine serum albumin (67,000 daltons), purified P-450 (54,400), and ovalbumin (45,000), each 1 µg; C. uninduced P-450 after AOA, 2 µg; PB. phenobarbital induced P-450 after AOA, 2 µg; PCN. 16α-cyanopregneneolone induced P-450 after AOA, 2 µg; 3-MC. 3-methylcholanthrene induced P-450 after AOA, 2 µg. Electrophoresis was performed as described in Materials and Methods.

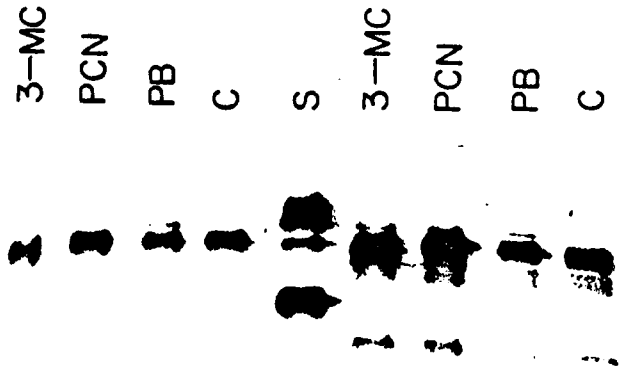


Figure 5

the column by the addition of 0.06% Triton N 101. Specific contents and yields of combined P-450 fractions are given in Table 2.

Comparison of banding patterns in SDS-PAGE between solubilized microsomes and ω-aminooctyl agarose purified fractions (Figure 5) confirmed the increases in the relative concentration of protein in these fractions which was found to be in the P-450 molecular weight range (45,000-60,000 daltons).

Table 2. Partial Purification on a ω-Aminooctyl Agarose Column

Inducer	P-450 (nmoles/mg protein)	Purification (fold)	Yield (%)	Difference spectra λmax (nm)
None	3.44	2.5	34.6	449.5
PB	7.10	2.8	45.0	449.5
PCN	4.46	3.6	34.2	449.0
3-MC	6.15	2.4	33.1	447.5

Chromatography Of Partially Purified PB Induced P-450 On Hydroxyapatite And DEAE-Sephacel Columns At 4°C

When PB induced P-450, which had been partially purified by ω-aminooctyl agarose, was eluted from hydroxyapatite with a stepwise gradient of phosphate, (46) two main peaks were obtained (Figure 6). Although the pattern of elution is nearly identical to that reported by Guengerich (46), the specific contents of each peak are reversed (Table 3), and the yields for both are lower.

The last step in this purification scheme involves stepwise elution from a DEAE-Sephacel column with phosphate buffer. The results obtained with the 90 mM and 150 mM hydroxyapatite fractions are shown

Figure 6. Purification of PB induced P-450 by stepwise phosphate elution from a hydroxyapatite column at 4°C. Fractions containing P-450 which were eluted by AOA chromatography (800 nmoles P-450, 110 mg protein) were concentrated by ultrafiltration, diluted to 33 mM phosphate with 20% glycerol and applied to a hydroxyapatite column (1.9 x 5 cm) and eluted as described in Materials and Methods.

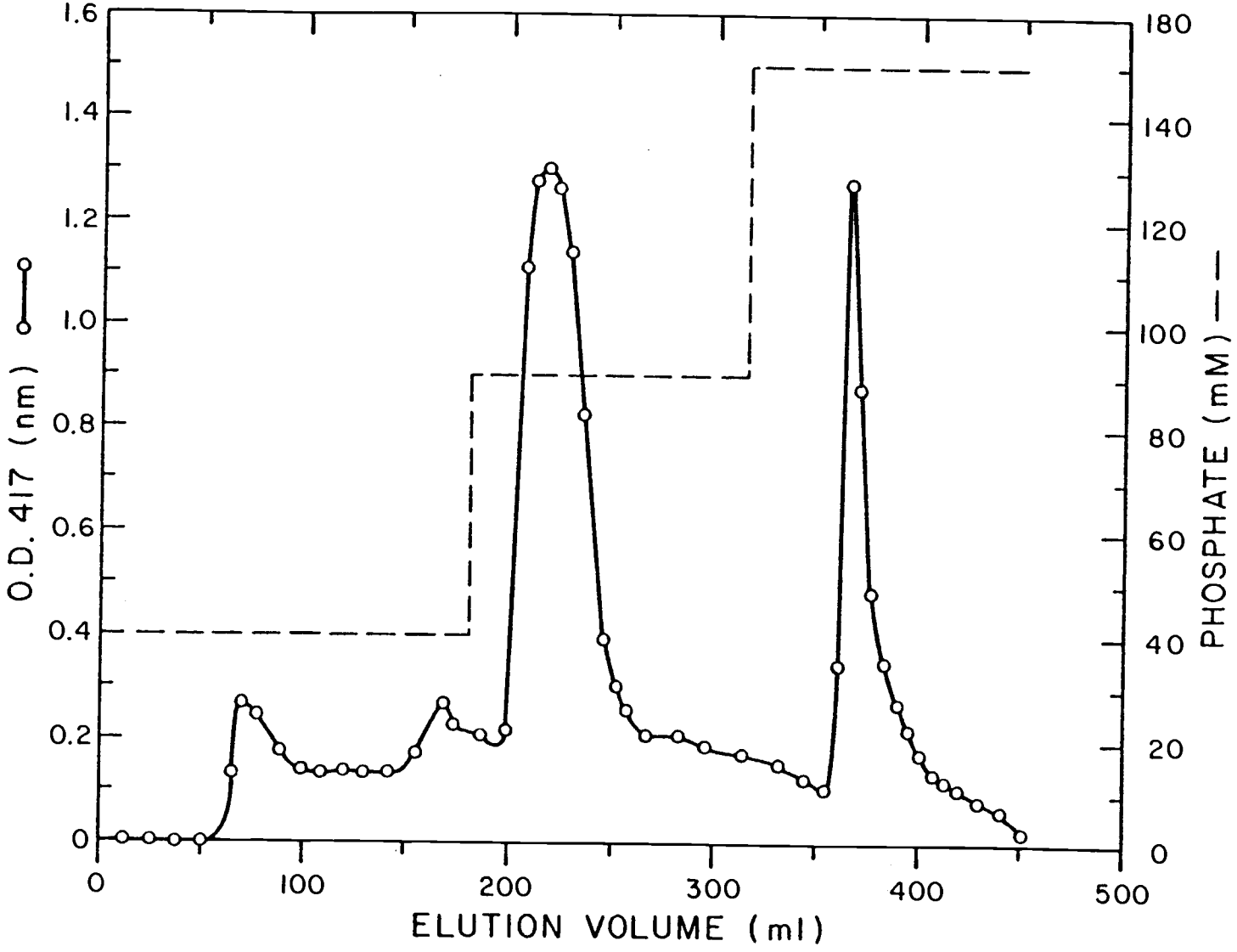

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
O.D. 417 (nm)
180
140
120
100
80
60
40
20
0
PHOSPHATE (mM)
0
100
200
300
400
500
ELUTION VOLUME (ml)

Figure 6

Table 3. PB Induced P-450 Purified by Hydroxyapatite (HA) Chromatography

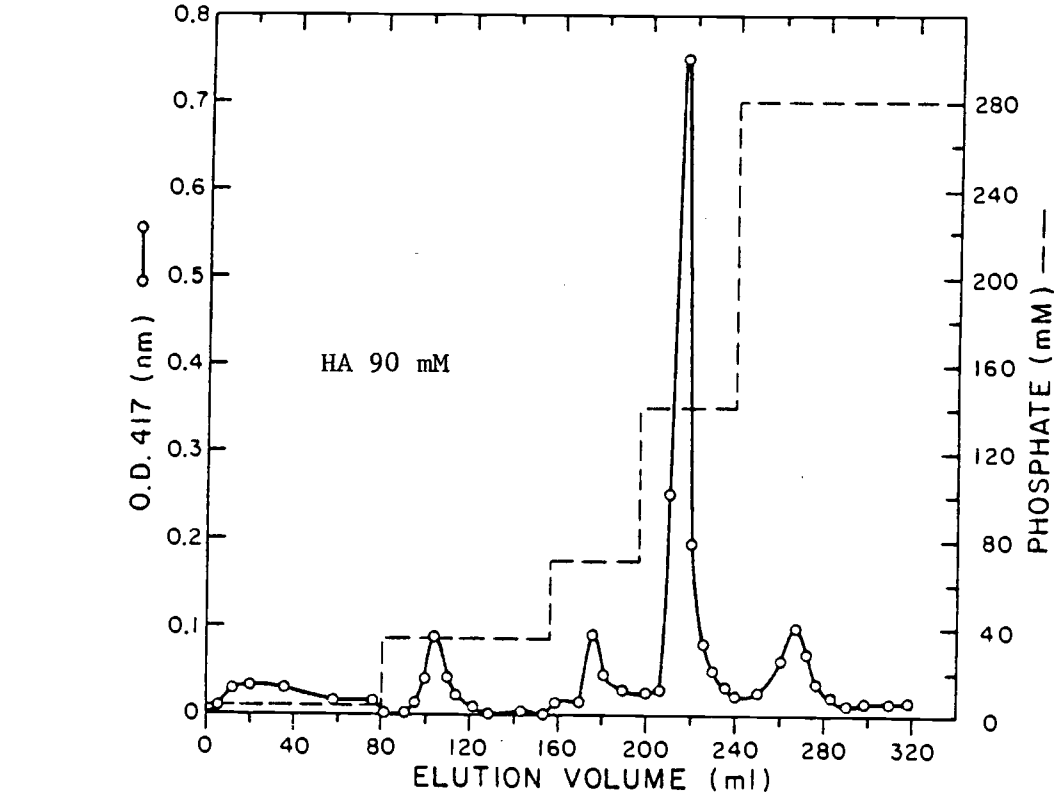
	P-450 (nmoles/mg)	Yield (%)	Difference spectra λmax (nm)
Guengerich			
HA- 90 mM	13.3	33	—
HA-150 mM	6.0	13	--
Williams			
HA- 90 mM	8.3	16	450.5
HA-150 mM	13.4	10	449.5

in Figure 7, and the specific contents and yields of each DEAE-Sephacel fraction are listed in Table 4.

Table 4. PB Induced P-450 Purified by DEAE-Sephacel Chromatography at 4°C

Fraction	P-450 (nmoles/mg)	Yield (%)
HA- 90 mM		
DEAE- 5 mM	—	—
DEAE- 35 mM	0.3	0.5
DEAE- 70 mM	5.8	2.1
DEAE-140 mM	5.7	3.3
DEAE-280 mM	4.6	1.6
		Total Yield 7.5%
HA-150 mM		
DEAE- 5 mM	1.4	1.2
DEAE- 35 mM	17.1	3.1
DEAE- 70 mM	13.5	3.8
DEAE-140 mM	14.2	4.1
DEAE-280 mM	14.6	2.7
		Total Yield 14.9%

Figure 7. Purification of PB induced P-450 by stepwise elution with phosphate from DEAE Sephacel columns at 4°C. HA 90 mM. P-450 fractions (135 nmoles P-450, 16 mg protein) which eluted from hydroxyapatite with 90 mM phosphate were combined, concentrated, dialyzed and applied to a DEAE Sephacel column (1.2 x 15 cm); HA 150 mM. P-450 fractions (86 nmoles P-450, 6 mg protein) were combined, treated as above, and applied to a DEAE Sephacel column (1.2 x 13 cm). Elution of both columns was as described in Materials and Methods.


HA 90 mM
O.D. 417 (nm)
PHOSPHATE (mM)
ELUTION VOLUME (ml)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
280
240
200
160
120
80
40
0
0
40
80
120
160
200
240
280
320

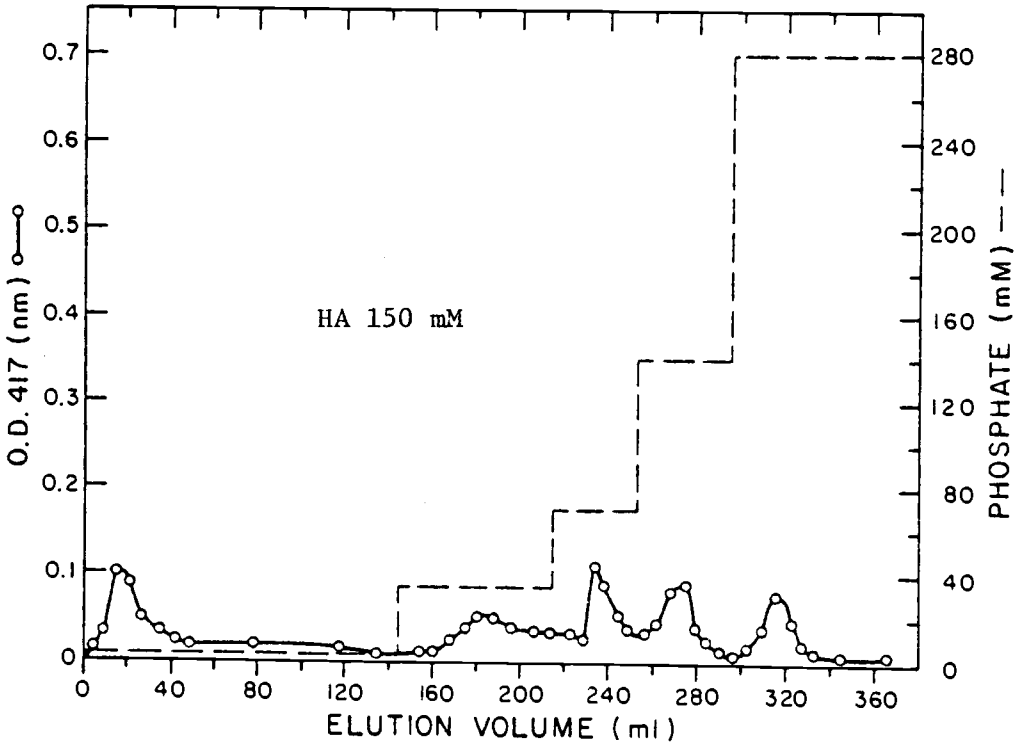

HA 150 mM
O.D. 417 (nm)
PHOSPHATE (mM)
ELUTION VOLUME (ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
280
240
200
160
120
80
40
0
0
40
80
120
160
200
240
280
320
360

Figure 7

Figure 8. SDS-PAGE of PB induced P-450 purified by hydroxyapatite and DEAE Sephacel chromatography at 4 °C. Samples, from right to left were, S. protein standards, same as in Figure 5; M. PB induced microsomes, 5 µg; AOA. PB induced P-450 partially purified by ω-aminooctyl agarose, 3 µg; HA 90 mM. PB induced P-450 purified by elution from hydroxyapatite with 90 mM phosphate, 1 µg; HA 150 mM. PB induced P-450 purified by elution from hydroxyapatite with 150 mM phosphate, 1 µg; next four samples (HA 90 mM-DEAE 35-280) were PB induced P-450 (eluted from hydroxyapatite with 90 mM phosphate) further purified by stepwise phosphate elution from DEAE Sephacel at 4 °C, 1 µg each; next four samples (HA 150 mM-DEAE 5-280) were PB induced P-450 (eluted from hydroxyapatite with 150 mM phosphate) further purified by stepwise phosphate elution from DEAE Sephacel at 4 °C, 1 µg each. Electrophoresis was as described in Materials and Methods.

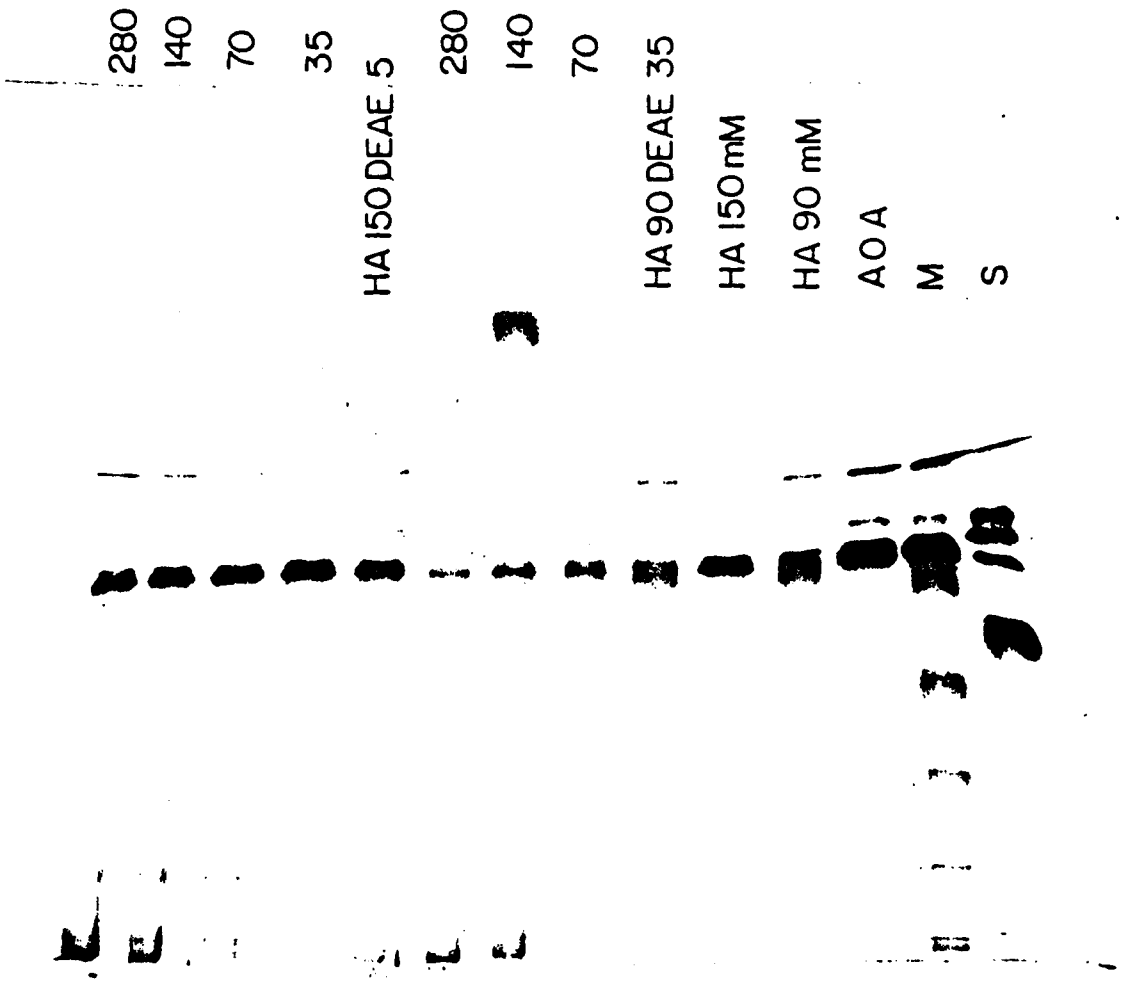

280
140
70
35
HA 150 DEAE .5
280
140
70
HA 90 DEAE 35
HA 150 mM
HA 90 mM
AOA
M
S

Figure 8

Analysis of the hydroxyapatite and DEAE fractions by SDS-PAGE is pictured in Figure 8. The specific contents of the DEAE-fractions from P-450 eluted from the hydroxyapatite column at 90 mM are about three fold lower than DEAE fractions from P-450 eluted at 150 mM (Table 4). Speculation that this may be due to an overestimation of the protein content by the Lowry method in the former DEAE fractions is supported by Figure 8 in which equal loads of protein stain less intensely in DEAE fractions from the hydroxyapatite 90 mM eluate compared to the DEAE fractions from the hydroxyapatite 150 mM eluate. Low specific content could also be due to a greater degree of heme loss in the HA-90 mM fractions, as the total yield of P-450 was 50% of the HA-150 mM fractions.

Chromatography Of Partially Purified P-450 On DEAE-Sephacel At Room Temperature

Advantages gained by elimination of the hydroxyapatite step and performing the DEAE chromatography at 25°C instead of 4°C include: (1) a reduction in the total time required to obtain purified fractions from solubilized microsomes (two or three days compared to two weeks); (2) the increased ease of equipment set-up; and (3) a reduction in the degree of interference from contaminating lipiphilic substances. Replacement of Triton N101 with Lubrol PX as the nonionic detergent allows for the monitoring of total protein in the eluate by measuring the absorbance at 280 nm. DEAE-Sephacel chromatograms of partially purified P-450 from control, PB, PCN, and 3-MC induced rats are shown in Figure 9. Although the ratio of P-450 to DEAE was kept fairly constant (except in the PCN group), there were differences in the shape of the NaCl

Figure 9. Purification of P-450 by elution from DEAE Sephacel columns using a linear NaCl gradient at room temperature. C. uninduced P-450 partially purified by AOA chromatography (200 nmoles P-450, 60 mg protein); PB. phenobarbital induced P-450 partially purified by AOA chromatography (300 nmoles P-450, 50 mg protein); 3-MC. 3-methylcholanthrene induced P-450 partially purified by AOA chromatography (620 nmoles P-450, 100 mg protein); PCN. 16α-cyanopregnenolone induced P-450 partially purified by AOA chromatography (215 nmoles P-450, 48 mg protein). Preparation of samples and conditions for chromatography are described in Materials and Methods.

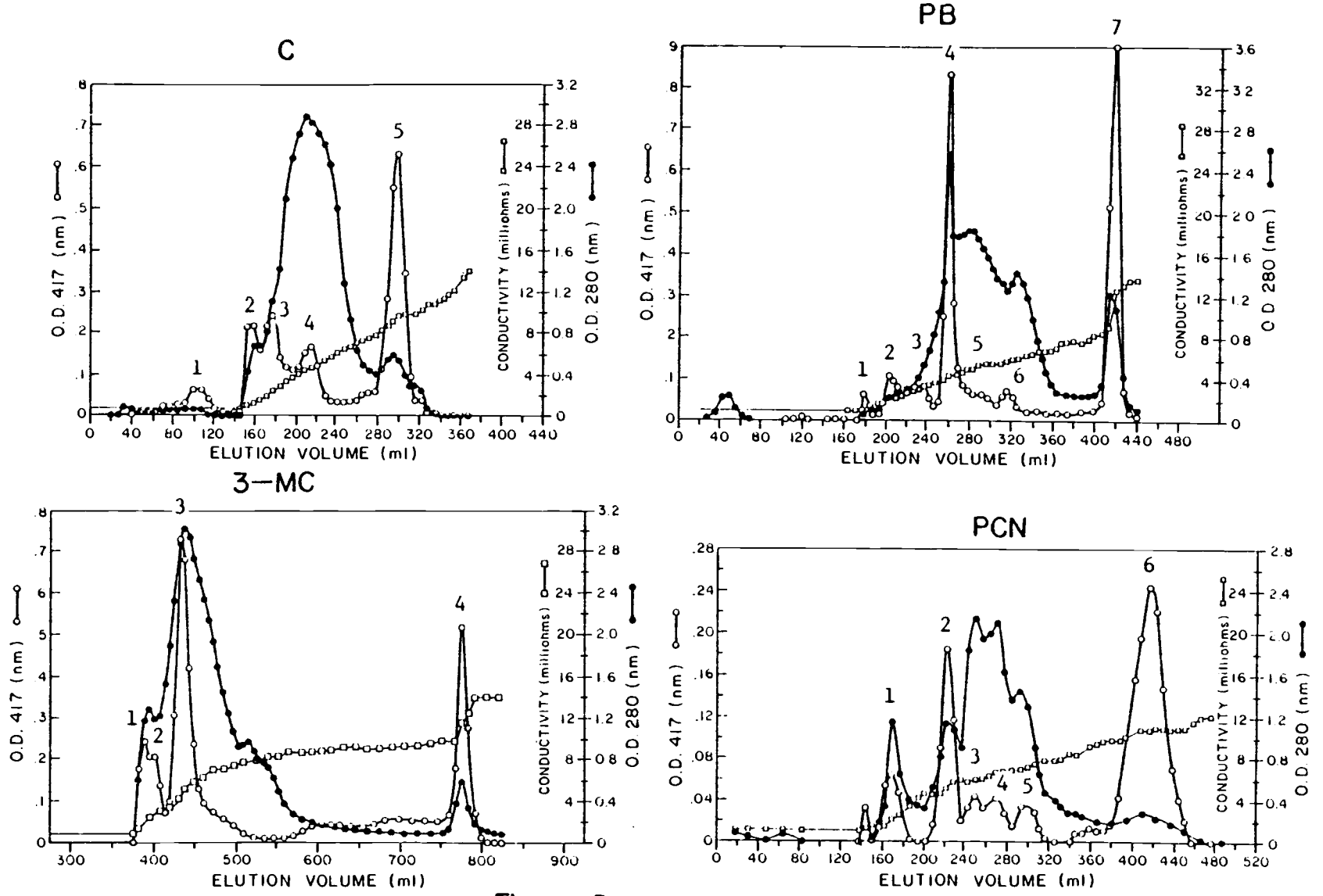

C
PB
3-MC
PCN
O.D. 417 (nm)
O.D 280 (nm)
CONDUCTIVITY (millionms)
ELUTION VOLUME (ml)

Figure 9

gradient between runs. Partially purified P-450 from each group was resolved into four to seven hemoprotein peaks by DEAE-Sephacel. Fractions from each peak were pooled and analyzed with respect to the concentration of salt at which the peak eluted, the specific content and yield of the P-450 and the λmax of the CO difference spectrum of reduced P-450 (Table 5).

Table 5. P-450 Purified by DEAE-Sephacel Chromatography at 25°C

Inducer	Peak	NaCl (mohms)	P-450 (nmoles/mg)	Yield (%)	Difference spectra λmax (nm)
None	1	0.7	25.9	4.9	450.0
	2	1.2	9.3	13.6	450.0
	3	2.4	7.2	15.9	450.5
	4	4.7	4.8	13.8	448.5
	5	9.7	—	—	415.5
				48.2	
PB	1	1.2	18.0	0.9	449.0
	2	1.9	24.8	4.7	449.5
	3	3.1	12.6	2.7	451.0
	4	4.3	11.3	8.7	450.0
	5	5.3	5.9	1.8	448.5
	6	5.7	3.8	1.2	448.5
	7	12.5	--	--	414.5
				20.0	
PCN	1	2.2	--	--	420.0
	2	5.1	—	—	420.0
	3	5.9	3.0	1.9	449.5
	4	6.7	4.4	2.1	448.5
	5	7.0	2.0	0.9	448.0
	6	10.8	1.2	2.1	415.5
				10.6	
3-MC	1	2.1	4.3	4.2	449.0
	2	2.8	6.1	2.3	450.5
	3	4.2	11.6	16.0	447.0
	4	11.6	—	—	415.0
				22.5	

Yields of total P-450 are still low but are improved relative to

chromatography at 4°C. Interestingly, the best yields were obtained with P-450 from non-induced animals. The lowest yield was with PCN-induced P-450, but this could be due to the fact that the ratio of DEAE to P-450 was three to four times greater than the other fractions. Other relationships between the four groups exist. For instance, the λmax of difference spectra has the same general relationship from group to group with respect to the order of elution. The first peak or two has an intermediate value of 449.0-450.0 nm. The next peak(s) are slightly higher (450.5-451.0), while the last to elute are the lowest (447.0-448.5). A similar pattern was seen by Levin (30) in which the three peaks were lettered according to order of elution and were termed a (450), b (452), and c (448).

A second observation is that, for the most part, the specific content decreases in fractions which elute later. Increased heme loss could be related to the time required for elution. An increase in apoprotein is also suggested by the peak ratio of A_{280}/A_{417}. Guengerich (personal communication) states that this ratio should be close to unity, but as can be seen in Figure 9, this ratio is greater than one, especially for the later eluting fractions.

Another possibility may be an overestimation of protein by the Lowry procedure due to the amino acid composition of different P-450s or an increase in some interfering agent such as NaCl or detergent eluting from the column. The 3-MC chromatogram does not follow this pattern, possibly due to the fact that, unlike the others, the main 3-MC peak contains the majority of P-450 eluted.

A third observation is that the last hemoprotein eluted, which except in the case of the 3-MC group, is the largest single hemoprotein

peak, contains little or no P-450. The subunit molecular weight as determined by SDS-PAGE (Figures 10-13), suggests that this fraction is either cytochrome b_5 or hemoglobin. Beaune (56), using P-450 partially purified by n-octyl agarose from human liver microsomes found cytochrome b_5 eluting at a similar location in the NaCl gradient. However, cytochrome b_5 should be reduced in both the sample and reference cuvette and have no absorbance in the difference spectrum.

Chromatographic patterns of PB, PCN and 3-MC induced P-450s differed from the control and each other. Pretreatment with PB or 3-MC produced significant increases in peaks eluting at conductivities of 4.3 and 4.2, respectively. The induction produced a less dramatic increase in peaks eluting at the low end of the salt gradient. Interestingly, the major peak induced by 3-MC was very similar in position of elution and the difference spectra λmax to control DEAE peak four (Figure 9), while no corresponding control peak for the major PB induced form is observed. Such results contrast with conclusions drawn from other studies comparing control versus PB or 3-MC induced P-450s in which other experimental evidence (molecular weight, substrate specificity, etc.) suggested that 3-MC induced a "novel" P-450 while PB increased the relative amount of a pre-existing form.

Re-chromatography of purified fractions to establish the reproducibility of the elution pattern was not done, partly due to the low yields obtained.

SDS-PAGE And IF Of P-450s Purified By DEAE-Sephacel Chromatography At Room Temperature

Purified P-450 fractions from control animals were electrophoresed

Figure 10. SDS-PAGE of uninduced P-450 purified by DEAE Sephacel chromatography at room temperature. Samples from right to left were, S. protein standards, same as in Figure 5; M. uninduced microsomes, 5 µg; AOA. uninduced P-450 partially purified by chromatography on ω-aminooctyl agarose, 3 µg; next five fractions (1 through 5) were uninduced P-450 (partially purified by AOA chromatography) further purified by DEAE Sephacel chromatography. Peaks were numbered according to their order of elution (Figure 9C), 1 µg each; 1 through 5. uninduced P-450 from all DEAE peaks combined, 0.5 µg each. Conditions for electrophoresis as described in Materials and Methods.

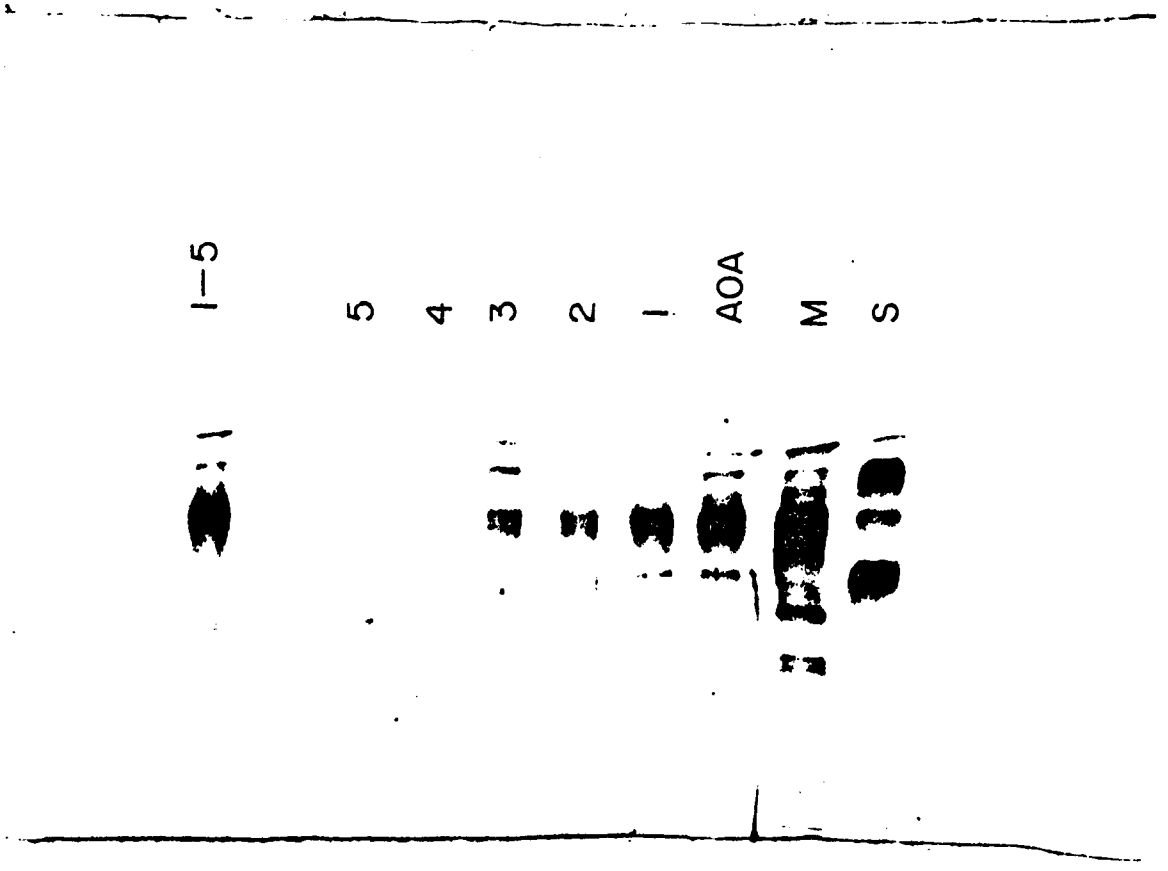


Figure 10

Figure 11. pH profile of IF gels containing 1% Lubrol PX, 9.2 M urea, 2% Biolytes (1:1 pH 3-10; pH 5-7), 4% acrylamide, 0.2% BIS, 0.07% TEMED and 0.1% APS in the absence of protein. Preparation of gels, focusing and measurement of the gel pH are described in the Materials and Methods.

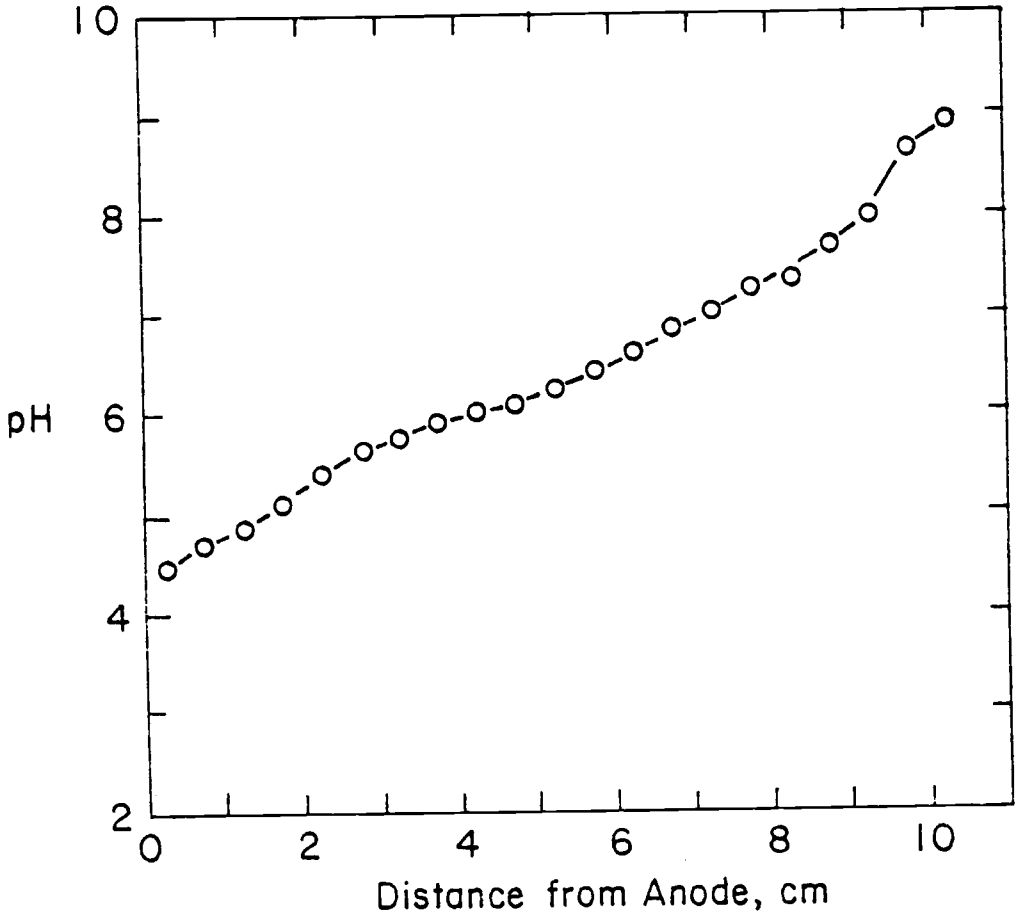


Figure 11

on SDS-PAGE (Figure 10). None of the fractions appeared homogeneous. Peak one contained small amounts of two high molecular weight proteins (>67,000 daltons). Only one band in the molecular weight range of P-450 (45,000-60,000) appeared to be present (54,700). The second DEAE peak was resolved into two bands (57,800 and 55,200), again, with some minor high molecular weight contaminants. Peak three had three bands (57,100, 54,700 and 45,400) with little or no other impurities. The last P-450 containing peak was quite faint with the prominent band at 53,900. Finally, the hemoprotein which eluted at high salt had a major band at 15,700 daltons.

The heterogeneity seen with peaks two through four could be due to slight peak overlaps (Figure 9). Although a constant amount of protein was applied, band intensities were quite varied, the lighter bands perhaps indicative of an overestimation of protein by the Lowry method and, hence, an underestimation of the specific content (Table 5).

The pH at which proteins banded in IF gels was determined by comparison to the pH gradient shown in Figure 11. Isoelectric focusing banding patterns of purified P-450 from control rats (Figure 12) were consistent with the heterogeneity seen on SDS-PAGE. For instance, peak two had two major focused bands (pH 7.40 and 7.85), while peak three had three and possibly four major focused bands (7.68, 7.80, and 8.00). Peak five focused at pH 5.15 with little or no banding elsewhere. Except for the last peak the pI of focused P-450 fractions seemed to increase with the salt gradient, contrary to what would be expected with anion-exchange chromatography.

The first three DEAE peaks (Figure 9) from PB induced rats each contain 4-6 bands (Figure 13) in the molecular weight range of 48,000-

Figure 12. Gel scans of focused, uninduced, P-450 from peaks eluted by DEAE Sephacel chromatography at room temperature. Peak numbers correspond to pooled fractions as shown in Figure 9C. Each sample was focused by polymerization of 20 µg of protein in the gel. Conditions for polymerization, focusing, staining and destaining were as described in the Materials and Methods. The pH of focused bands (see text) was determined by measuring the distance from the anode to the focused peak and comparing to Figure 11.

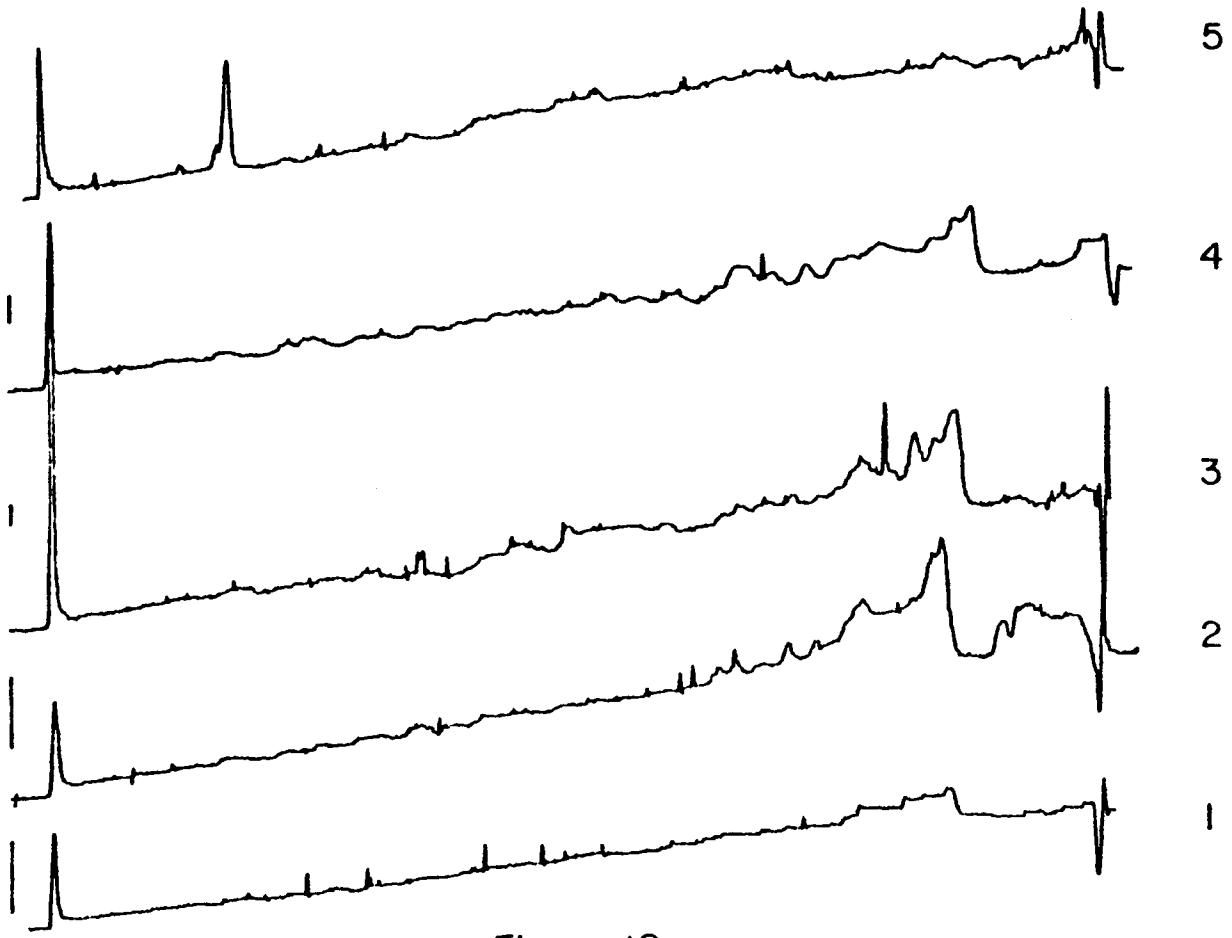


Figure 12

Figure 13. SDS-PAGE of PB induced P-450 purified by DEAE Sephacel chromatography at room temperature. Samples from right to left were, S. protein standards, same as in Figure 5; M. PB induced microsomes, 5 µg; AOA. PB induced P-450 partially purified by chromatography on ω-aminooctyl agarose, 3 µg; next seven fractions (1 through 7) were PB induced P-450 (partially purified by AOA chromatography) further purified by DEAE Sephacel chromatography. Peaks were numbered according to their order of elution (Figure 9PB), 1 µg each; 1 through 7. PB induced P-450 from all DEAE peaks combined, 0.5 µg each. Conditions from electrophoresis were as described in Materials and Methods.

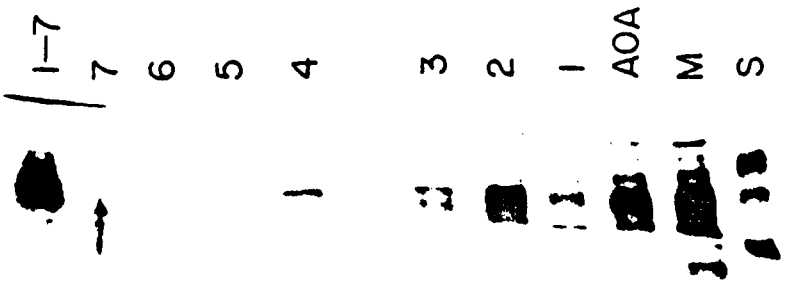


Figure 13

Figures 14 & 15. Gel scans of focused, PB induced, P-450 from peaks eluted by DEAE Sephacel chromatography at room temperature. Peak numbers correspond to pooled fractions as shown in Figure 9PB. Each sample was focused by polymerization of 20 µg of protein in the gel. Conditions for polymerization, focusing, staining and destaining were as described in the Materials and Methods. The pH of focused bands (see text) was determined by measuring the distance from the anode to the focused peak and comparing to Figure 11.

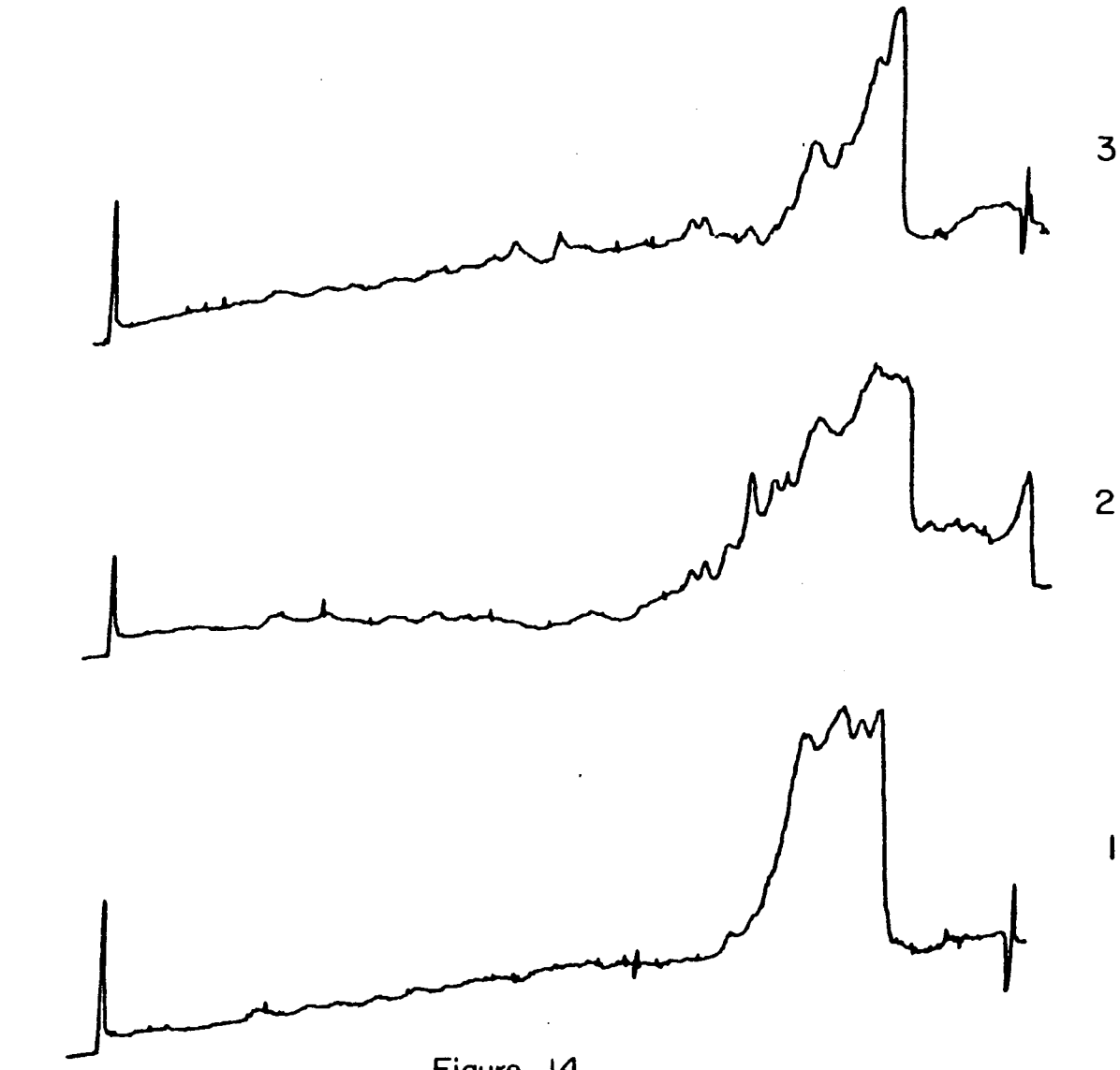


Figure 14

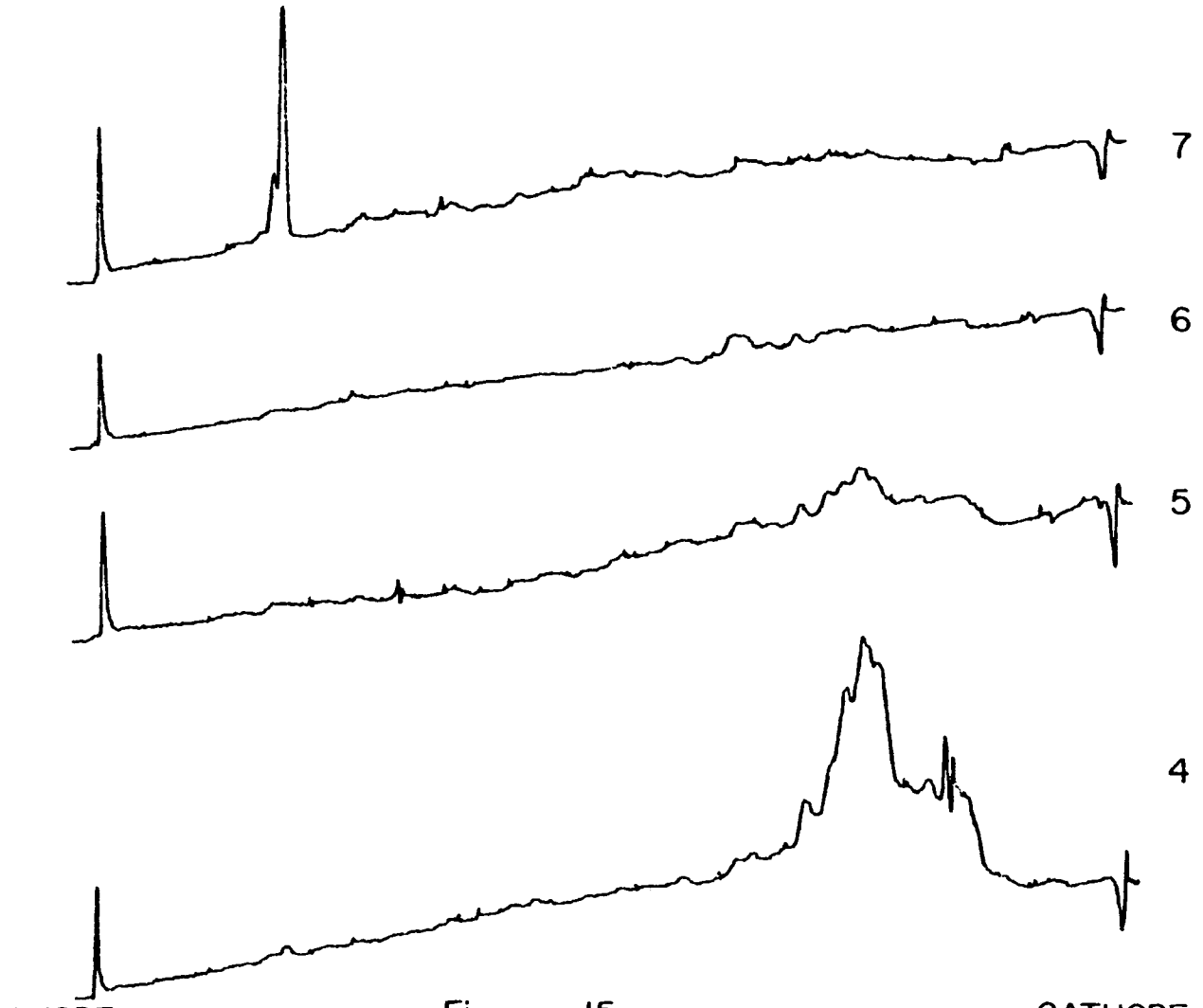


Figure 15

63,000 daltons. Although the relative intensities differ, the similarities in molecular weight suggest that the banding patterns may have been due to varying amounts of the same P-450s due to incomplete resolution on DEAE-Sephacel (Figure 9). Peak four represented the major PB induced P-450 and came the closest to homogeneity of any P-450 fraction examined. The molecular weight (54,800) was in reasonable agreement with the major PB induced form purified by Guengerich (54,400) who was kind enough to supply this form (specific content 17.4) for use as an electrophoretic standard.

As was the case with control P-450, the number of bands obtained with isoelectric focusing of purified PB induced P-450 (Figures 14 and 15) corresponded to the number of bands observed on SDS-PAGE. For example, peaks one through three gave 4-6 major focused bands of varying intensity within the same pH range (7.43-8.22). The PB fraction which was nearly homogenous on SDS-PAGE focused into one major band (pH 7.38) with two shoulders (7.35 and 7.45) and one minor peak (7.20). The recorder deflection at slightly higher pH was due to a break in the gel. Again, the last peak eluted focused at the acidic end (pH 5.24) of the IF gel.

DEAE fractions of P-450 purified from PCN induced rats were also quite heterogenous when analyzed by SDS-PAGE (Figure 16) or IF (Figure 17 and 18). Peak one exhibited three prominent peaks on SDS-PAGE (53,700, 51,900, and 48,400 daltons) and also on IF gels (pH 8.12, 7.44 and 7.24). The second and largest hemoprotein peak to elute had an additional low molecular weight form (42,300 daltons) which was present in diminishing amounts in later DEAE peaks (Figure 16). The last peak to elute was, again, a hemoprotein (probably cytochrome b_5

Figure 16. SDS-PAGE of PCN induced P-450 purified by DEAE Sephacel chromatography at room temperature. Samples from right to left were, S. protein standards, same as in Figure 5; M. PCN induced microsomes, 5 µg; AOA. PCN induced P-450 partially purified by chromatography on ω-aminooctyl agarose, 3 µg; next six fractions (1 through 6) were PCN induced P-450 (partially purified by AOA chromatography) further purified by DEAE Sephacel chromatography. Peaks were numbered according to their order of elution (Figure 9PCN), 2 µg each; 1 through 6. PCN induced P-450 from all DEAE peaks combined, 1 µg each. Conditions for electrophoresis were as described in Materials and Methods.

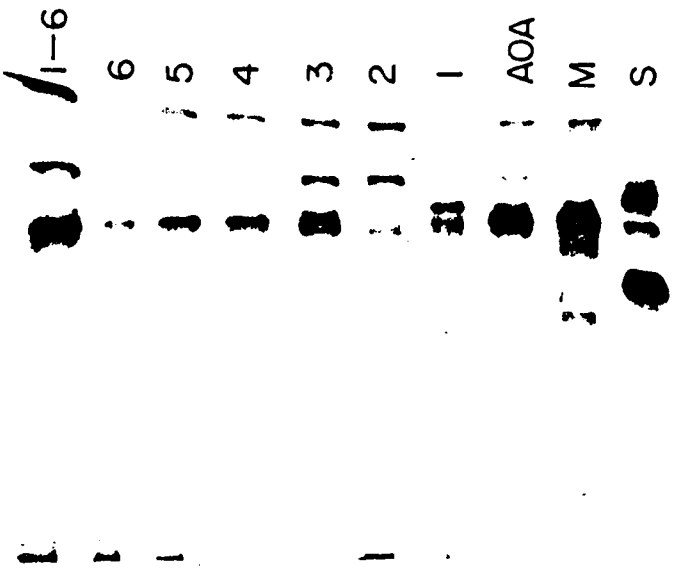


Figure 16

Figures 17 & 18. Gel scans of focused, PCN induced, P-450 from peaks eluted by DEAE Sephacel chromatography at room temperature. Peak numbers correspond to pooled fractions as shown in Figure 9PCN. Each sample was focused by polymerization of 40 µg of protein in the gel. Conditions for polymerization, focusing, staining and destaining were as described in the Materials and Methods. The pH of focused bands (see text) was determined by measuring the distance from the anode to the focused peak and comparing to Figure 11.

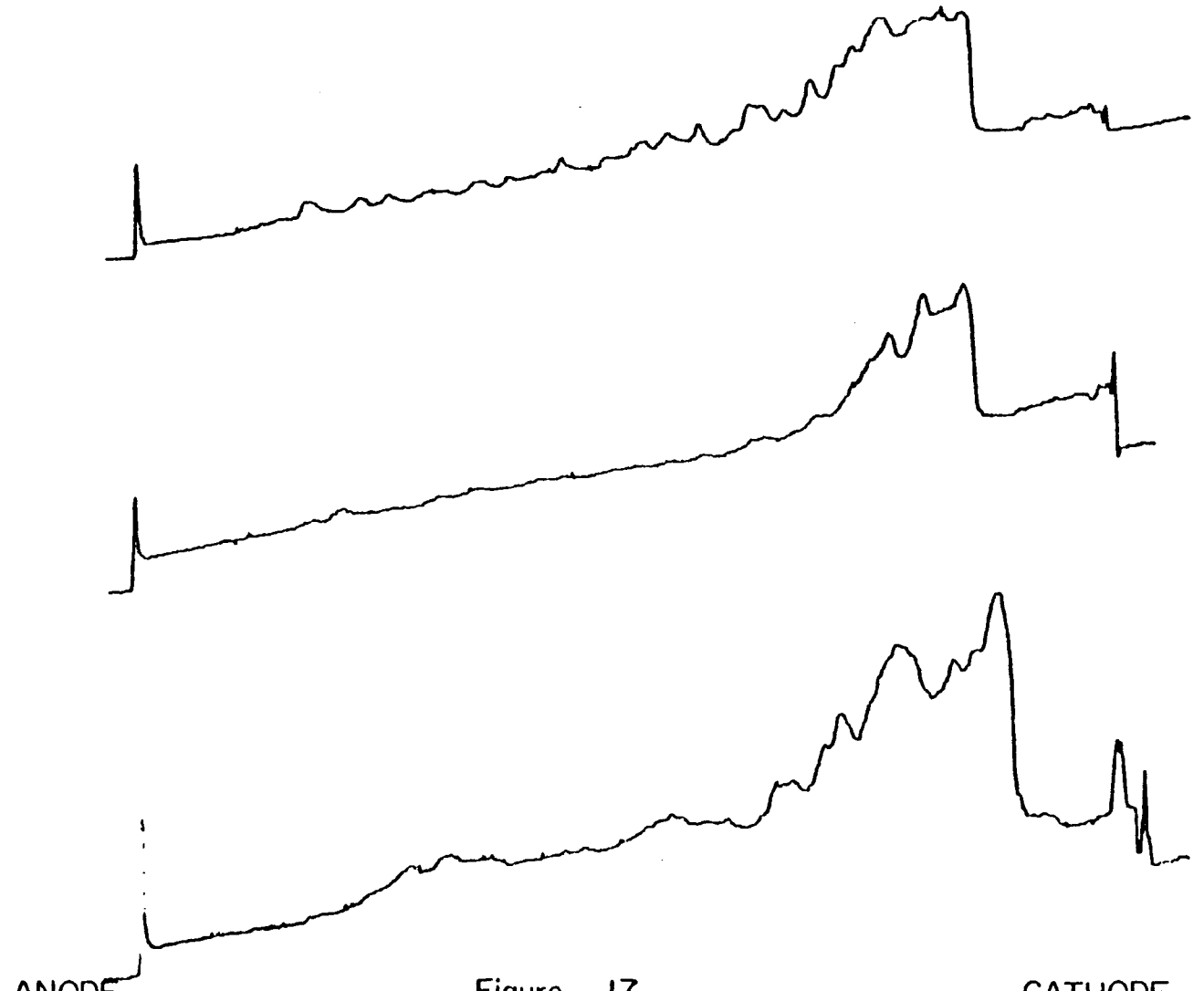


Figure 17

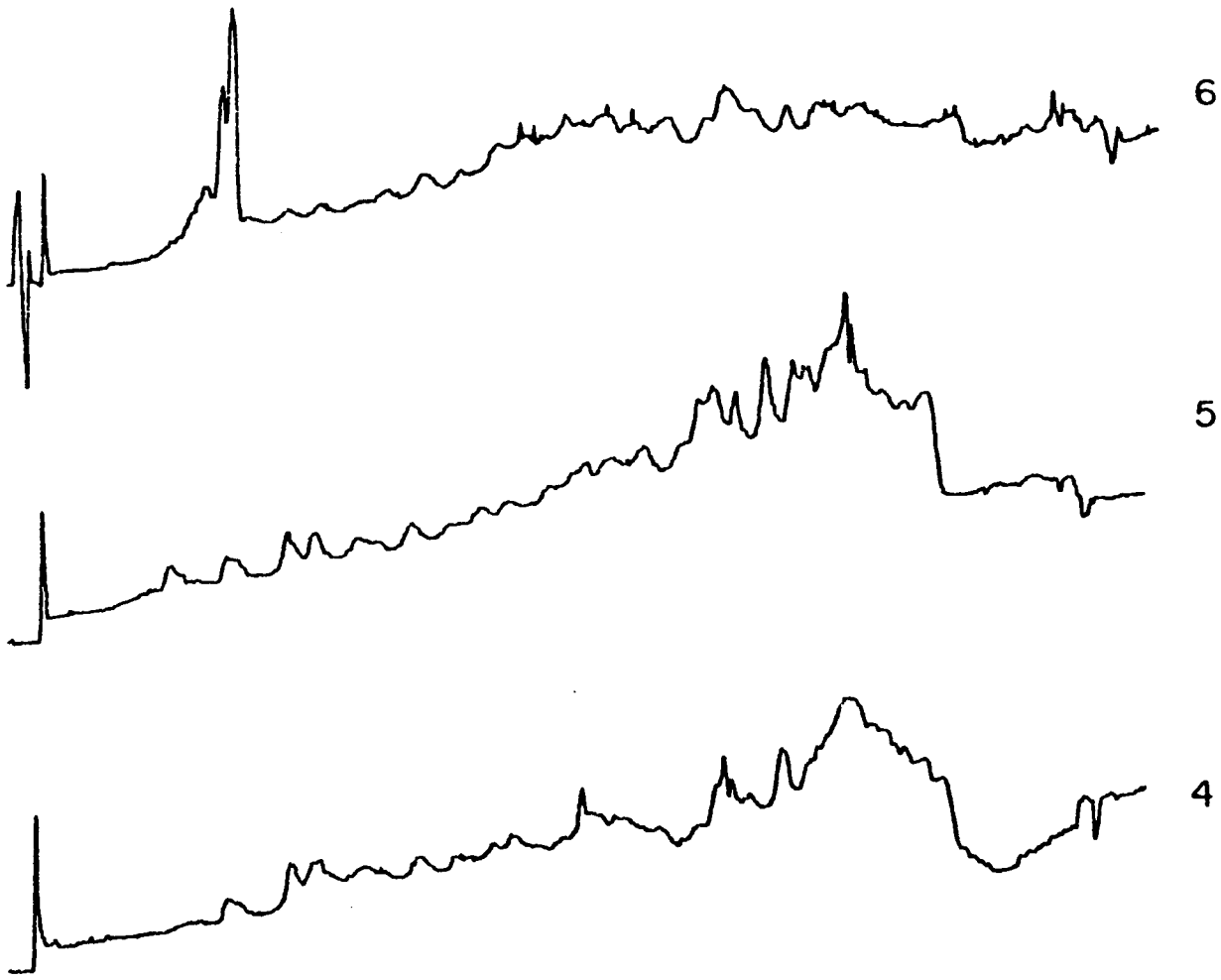


Figure 18

or hemoglobin) which banded at 14,300 daltons, and focused at pH 5.22 (Figure 18), consistent with the above results with control and PB fractions.

SDS-PAGE of 3-MC DEAE peak one showed four bands in the molecular weight range of P-450 (65,200, 63,500, 58,100, and 49,500 daltons) and a fairly prominent band of molecular weight 72,100. The identity of the latter protein is not known but it could be NADPH cytochrome P-450 reductase (Figure 19). As would be expected from the poor resolution between peaks one and two (Figure 9), the SDS-PAGE banding pattern of the second DEAE peak was very similar to the first, except for the appearance of a new band at 53,600 daltons. The IF banding patterns of peaks one and two (Figure 20) was also very similar, except that the latter contained a band at pH 7.62. Most of the 3-MC induced P-450 eluted from DEAE in peak three and appeared to have a molecular weight of about 57,800 daltons. Two other bands at 63,000 and 44,300 could be seen. The majority of this fraction focused at pH 7.42 but there appeared to be 4-5 additional impurities which banded at positions very close to focused bands from peaks one and two. Again, there was a low molecular weight (17,900 daltons), acidic (pH 5.15) protein eluted at high salt believed to be either cytochrome b_5 or hemoglobin. Comparison of IF patterns of this fraction (pI $\sim$ 5.2) to IF gels of beef cytochrome b_5 (pI $\sim$ 5.2) and human hemoglobin (pI 6.5-8) suggest that this is, indeed, rat cytochrome b_5.

Close inspection of the focused protein fractions revealed the presence of a slightly more acidic shoulder on each peak. Scanning of the IF gels on an expanded scale (Figure 21) clearly demonstrated the existence of this second peak. Interestingly, the fractional

Figure 19. SDS-PAGE of 3-MC induced P-450 purified by DEAE Sephacel chromatography at room temperature. Samples from right to left were, S. protein standards, same as in Figure 5; M. 3-MC induced microsomes, 5 µg; AOA. 3-MC induced P-450 partially purified by chromatography on ω-aminooctyl agarose, 3 µg; next four fractions (1 through 4) were 3-MC induced P-450 (partially purified by AOA chromatography) further purified by DEAE Sephacel chromatography. Peaks were numbered according to their order of elution (Figure 9 3-MC), 1 µg each; 1 through 4. 3-MC induced P-450 from all DEAE peaks combined, 0.5 µg each. Conditions for electrophoresis were as described in Materials and Methods.

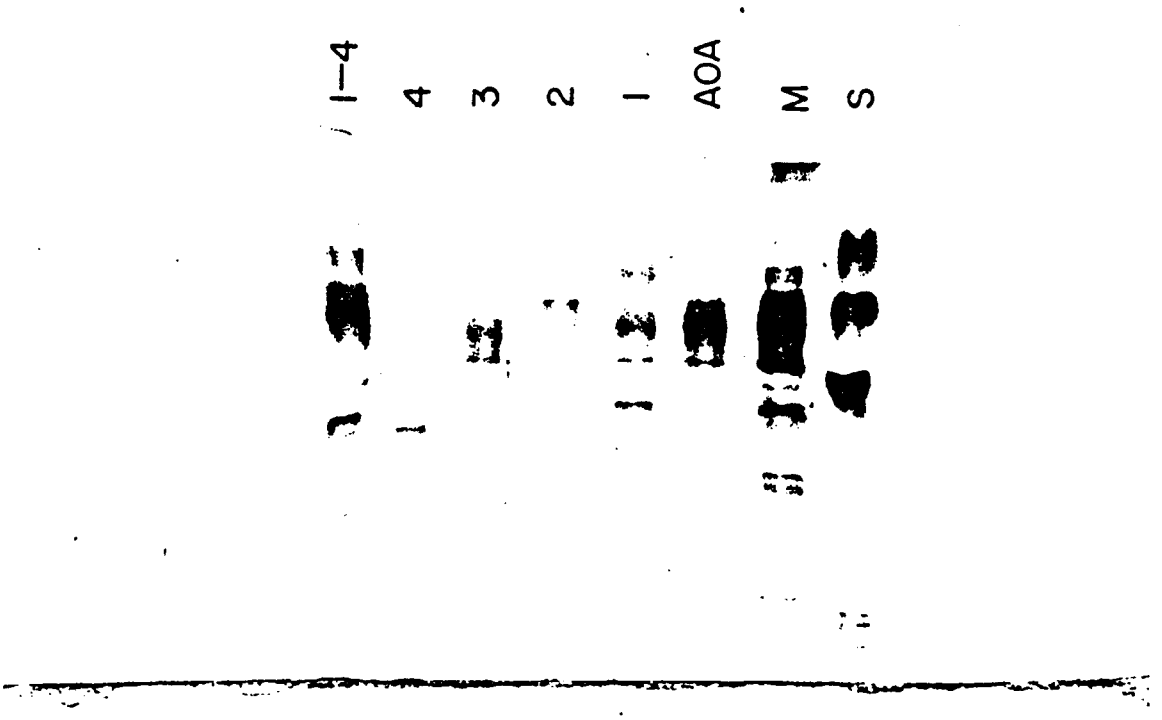


Figure 19

Figure 20. Gel scans of focused, 3-MC induced, P-450 from peaks eluted by DEAE Sephacel chromatography at room temperature. Peak numbers correspond to pooled fractions as shown in Figure 3 3-MC. Each sample was focused by polymerization of 20 µg of protein in the gel. Conditions for polymerization, focusing, staining and destaining were as described in the Materials and Methods. The pH of focused bands (see text) was determined by measuring the distance from the anode to the focused peak and comparing to Figure 11.

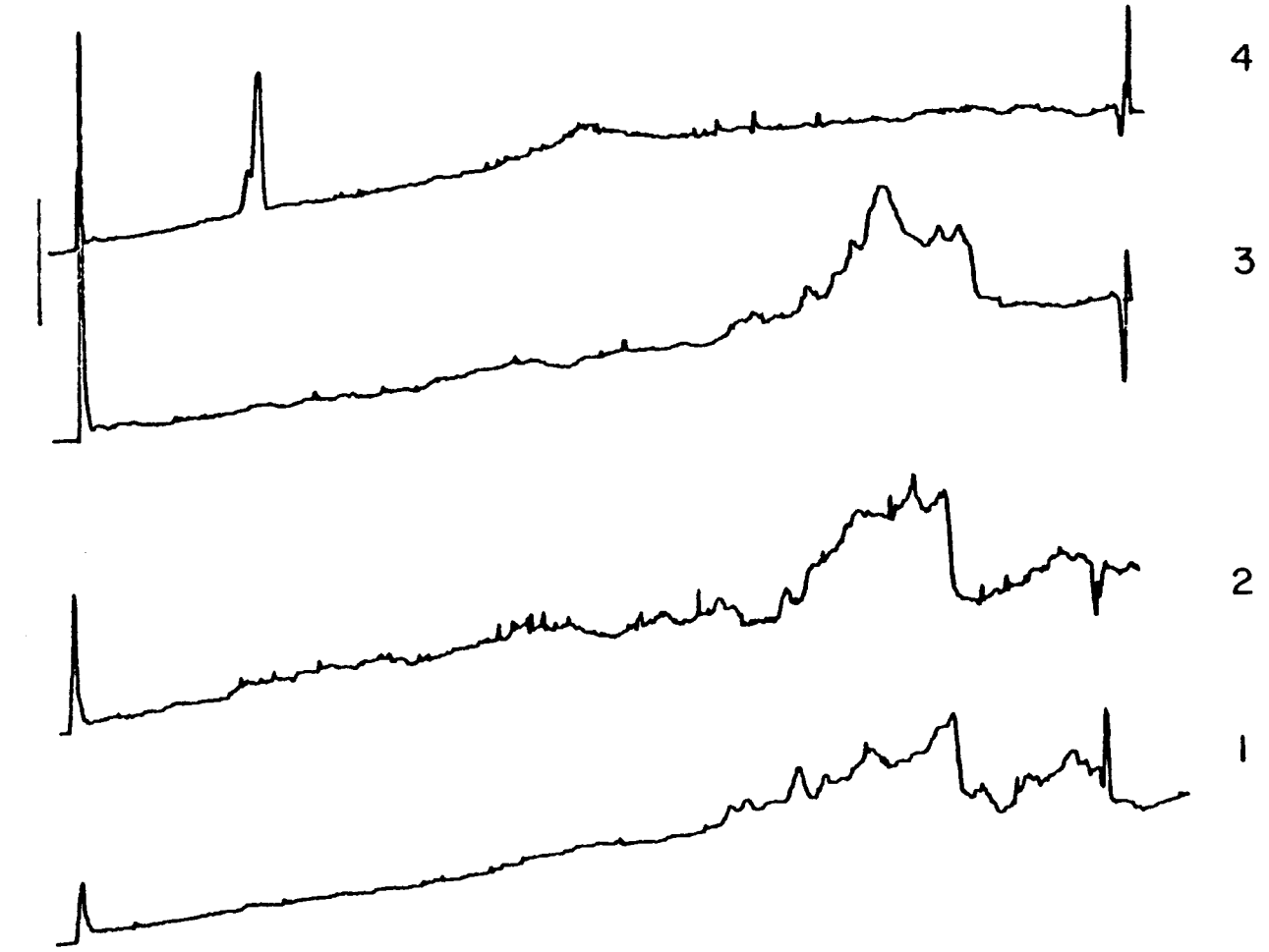


Figure 20

amount of the more acidic form increased after induction with PB or 3-MC from 15% to 20% and after PCN treatment to greater than 30%. To my knowledge, no evidence has been presented to suggest the existence of multiple forms of cytochrome b_5, which is not surprising given their nearly identical size and charge. Alternatively, the multiple banding pattern seen could have been due to artifacts caused by charge modification. In order to insure that such modification was not occurring, standards of cytochrome b_5 from rat and beef liver and human hemoglobin were focused by applying the protein at the anode and/or by polymerizing in the gel. The pattern observed (data not shown) was independent of the method of application. Interestingly, the bovine b_5 also focused as a doublet at about pH 5.2 with the peaks staining at almost equal intensities.

The validity of previously observed multiple IF banding patterns obtained by Guengerich (66) of highly purified P-450 (determined to be homogenous by other methods) has been questioned. The band "artifacts" could be due to protein-protein and protein-ampholyte interactions. In order to minimize such associations, the IF gels contained 1% Lubrol PX, a non-ionic detergent which may be more effective in preventing P-450 aggregation than previously used detergents (Guengerich, personal communication). In addition to the detergent, the gels contained 9.2 M urea. This combination should have maintained P-450 in a denatured, non-associated state, as much as possible. Another change made was in the manner of sample application. Instead of layering a concentrated sample on the end of the gel where it could be exposed to extremes of pH, the protein was added to the final gel mixture just prior to polymerization.

Figure 21. Gel scans of focused protein, eluted from DEAE Sephacel at high salt, presumed to be cytochrome b_5. C. DEAE peak 5 (Figure 9C) from uninduced rats, 20 µg; PB. DEAE peak 7 (Figure 9PB) from PB induced rats, 20 µg; PCN. DEAE peak 6 (Figure 9PCN) from PCN induced rats, 40 µg; 3-MC. DEAE peak 4 (Figure 9 3-MC) from 3-MC induced rats, 20 µg. Conditions for polymerization, focusing, staining and destaining were described in the Materials and Methods.

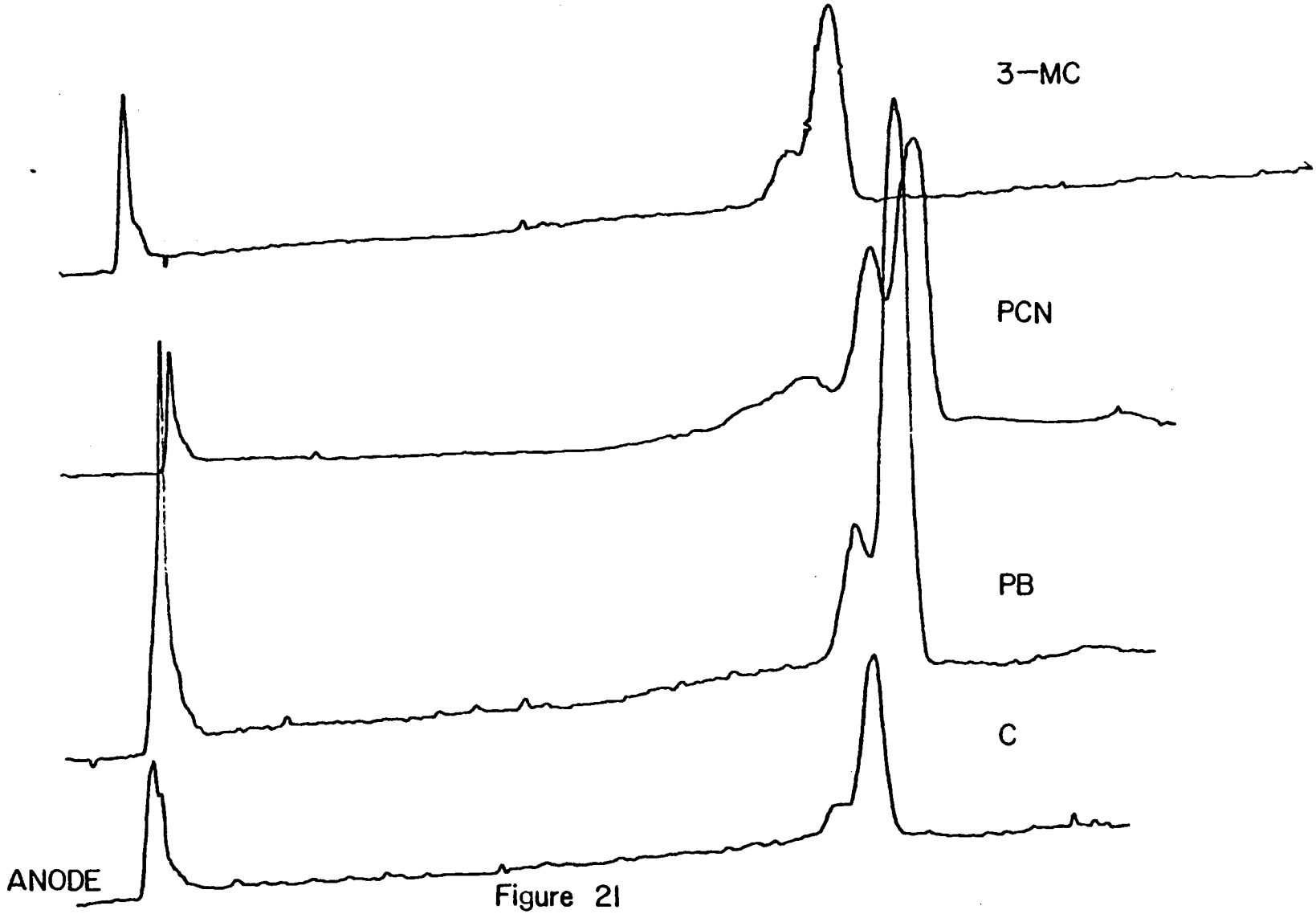


Figure 21

Figure 22. Gel scans of focused P-450 (P-450 B from Guengerich) demonstrating the effect of increasing the sample load (2-20 µg) on the banding patterns obtained. All samples were polymerized in the gel and focused, stained and destained as described in the Materials and Methods.

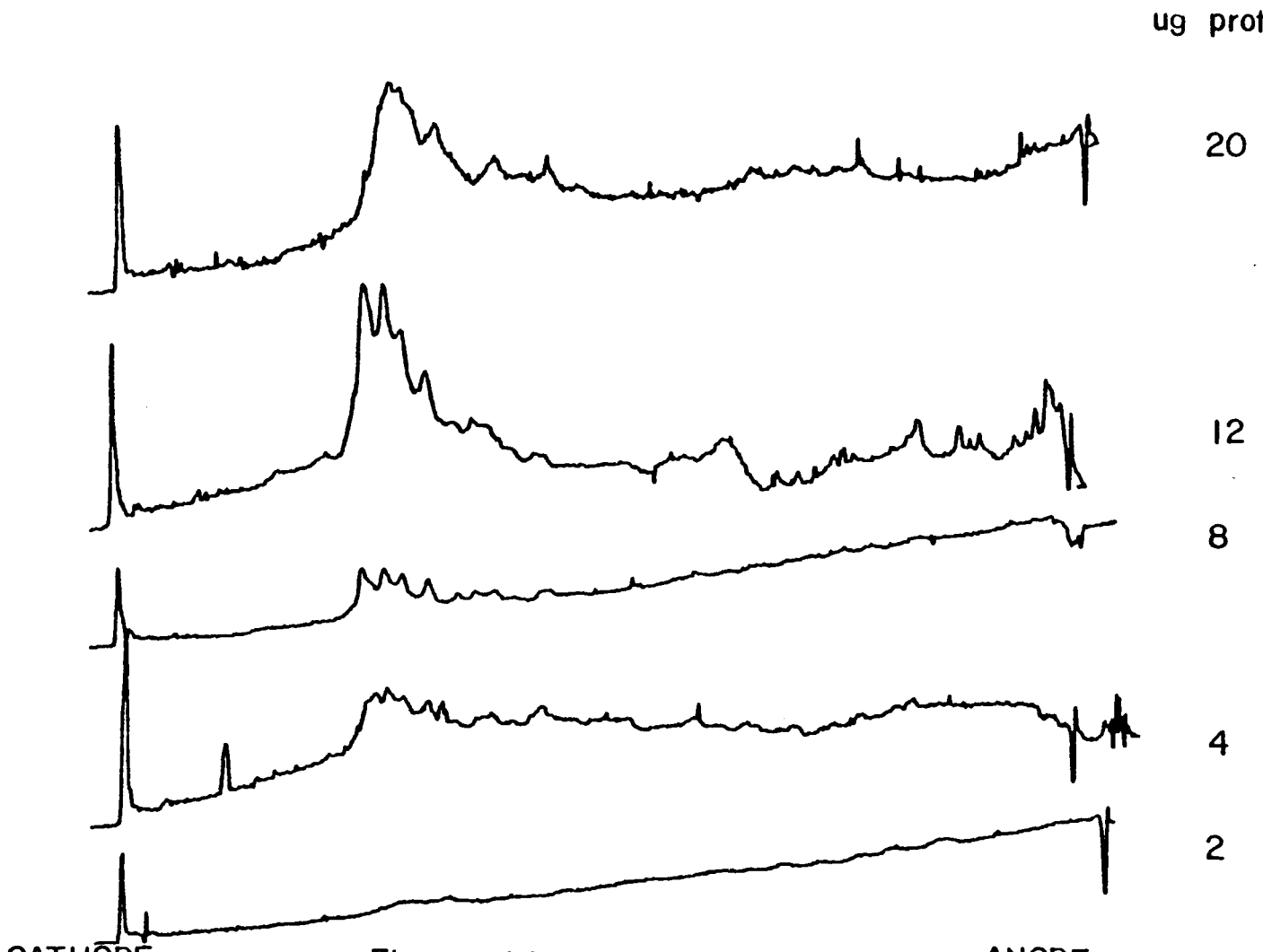


Figure 22

Banding patterns obtained by IF should, theoretically, be independent of sample load or position of application. The failure of focused PB-B P-450 to meet these criteria was, in part, the reason Guengerich concluded that the charge heterogeneity observed was an artifact (66). The effect of increasing amounts of PB-B P-450 (obtained from Guengerich) applied at the anode on the banding pattern in gels containing 1% Lubrol and 9.2 M urea is shown in Figure 22. Number, position and relative peak intensities do not appear to change appreciably with the amount of P-450 applied except, perhaps, with the 20 µg sample in which incomplete resolution of the three major peaks was observed. The position of focused bands (pH 6.90-7.40) compare with the PB-B P-450 banding patterns obtained by Guengerich (66).

Changes in the relative intensity of focused peaks with the point of sample application is shown in Figures 23 and 24. Increasing amounts of purified P-450 from non-induced rats (DEAE peak 1) was focused starting from either the anode (Figure 23) or evenly distributed throughout the gel (Figure 24). Multiple bands were observed in both cases, but the pattern was noticeably different. Focused bands from samples applied at the anode were not quite as sharp, and were shifted towards the acid end of the gradient when compared to protein polymerized in the gel. Also, the major peak in the pattern was towards the cathode (Figure 23) in one case and the anode in the other Figure 24). Such differences in the pattern obtained between sample applied to the gel and sample polymerized in the gel has been observed before with plasma proteins (105). Although protein charge modification due to polymerization conditions is possible, the absence of an increase in the number of peaks obtained compared to the applied

Figure 23. Gel scans of focused P-450 (DEAE peak 1 from uninduced rats) demonstrating the effect of increasing the sample load (10-100 µg) on the banding pattern obtained for samples applied at the anode. Preparation of gels, focusing, staining and destaining were as described in the Materials and Methods.

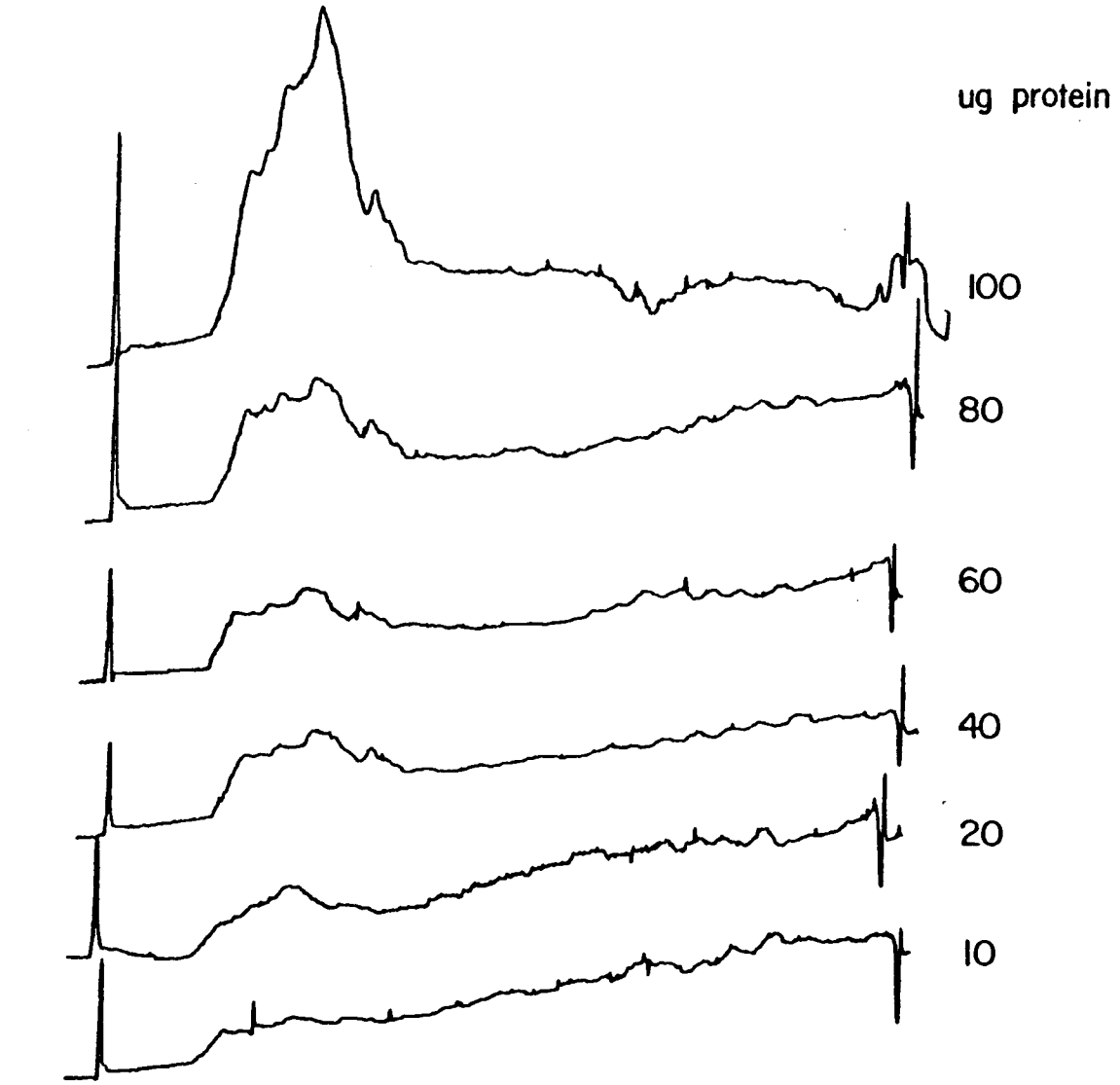


Figure 23

Figure 24. Gel scans of focused P-450 (DEAE peak 1 from uninduced rats) demonstrating the effect of increasing the sample load (7.5-75 µg) on the banding pattern obtained for samples polymerized in the gel. Preparation of gels, focusing, staining and destaining were as described in the Materials and Methods.

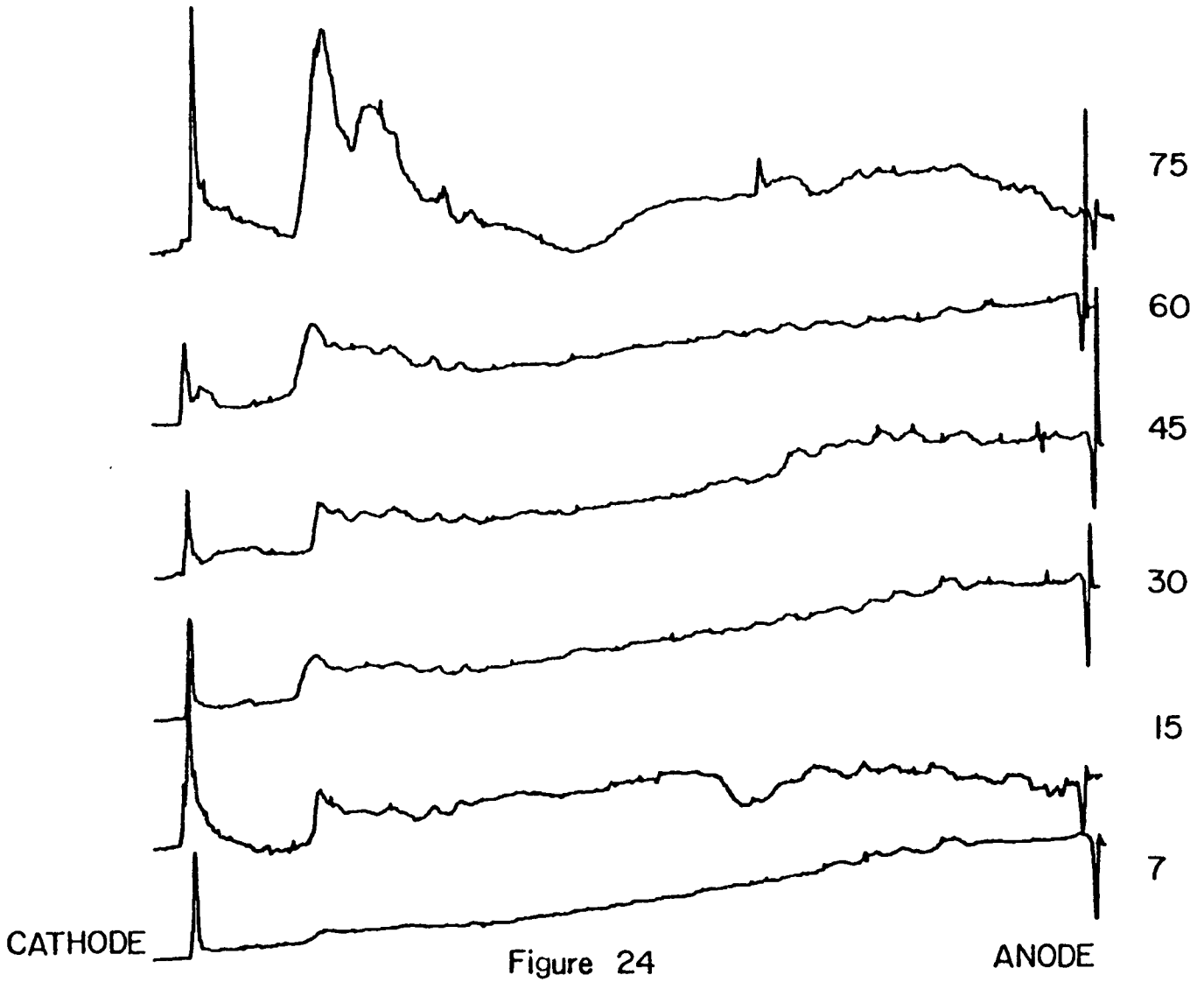


Figure 24

sample suggests that for this to be the case, the modifying reaction would have to be very specific. Therefore, Figures 23 and 24 demonstrate that P-450 polymerized in the acrylamide gel matrix was not subject to indiscriminate charge modification and focused with a better degree of resolution than samples applied at the anode and focused under identical conditions. Although no time course studies were performed, the poorer resolution of applied samples and the slight shift toward the acid end may have been caused by individual bands not quite reaching their respective pIs. This could be due to the further migration distance required for protein samples applied at the anode. The close band proximity of most focused P-450s and the difficulty involved in eluting protein from polyacrylamide and separating it from dye or ampholines, made attempts at refocusing P-450 impractical. Nevertheless, the independence of banding patterns and protein load seen with purified P-450s focused utilizing the conditions described above and the correlation with heterogeneity observed with SDS-PAGE, suggests that these multiple IF bands were not artifacts but very closely related P-450 forms. Conclusive proof of this could only be obtained by confirmation using other techniques. Small differences in pI and molecular weight could be even better exploited in the separation of multiple P-450 forms by using two-dimensional electrophoresis. To date such an analysis has not been done, due to doubts about the validity of patterns obtained in the first dimension.

Binding Of ^{3}H-PB, ^{14}C-PCN, And ^{3}H-3-MC To Hepatic Cytosol Macromolecules

Numerous techniques have been used to characterize the binding

parameters of steroid hormone receptors. The most common method, used clinically in the analysis of estrogen (106), progesterone and glucocorticoid (107) receptors involves adsorption of the free steroid in a steroid-cytosol mixture to charcoal or dextran-coated charcoal. Other methods include equilibrium dialysis, adsorption of steroid-receptor complexes to DEAE-paper, ultracentrifugation through sucrose gradients, gel electrophoresis, protamine precipitation or filtration through Sephadex G-25 (95,108,109). Each technique possesses its own particular advantages and disadvantages. For example equilibrium dialysis, although in principle providing the most accurate measurement of equilibrium constants, involves conditions of time and temperature which may promote receptor degradation. On the other hand, adsorption to charcoal has the advantage of rapidity and ease of handling multiple samples, but may differentially strip steroids from both non-specific and specific binding sites (109,110), with consequent disturbance of equilibrium conditions.

Analysis Of ^{14}C-PCN Binding To Hepatic Cytosol Macromolecules By Gel Filtration Using Either Sephadex G-25 Or LH-20

The degree of resolution obtained between the amount of ligand bound to macromolecules (eluting in the void volume) and the remaining free ligand when using gel filtration will be dependent on sample size, column size and flow rate. As the sample time on the column is increased, the possibility that some ligand will be dissociated (depending upon the k_{off}) during filtration also increases. Substitution of LH-20 for G-25 provides a more hydrophobic gel matrix, which should enhance the retardation of the small free hydrophobic ligand. This,

Figure 25. Chromatographic fractionation on G-25 and LH-20 of ^{14}C-PCN bound to rat liver cytosol. A. G-25; B. LH-20. The load for each column was 0.5 ml of cytosol (18 mg protein) incubated for 90 min at 0°C with 0.8 nmoles ^{14}C-PCN. (O——O) A_{280}; (Δ——Δ) radioactivity.

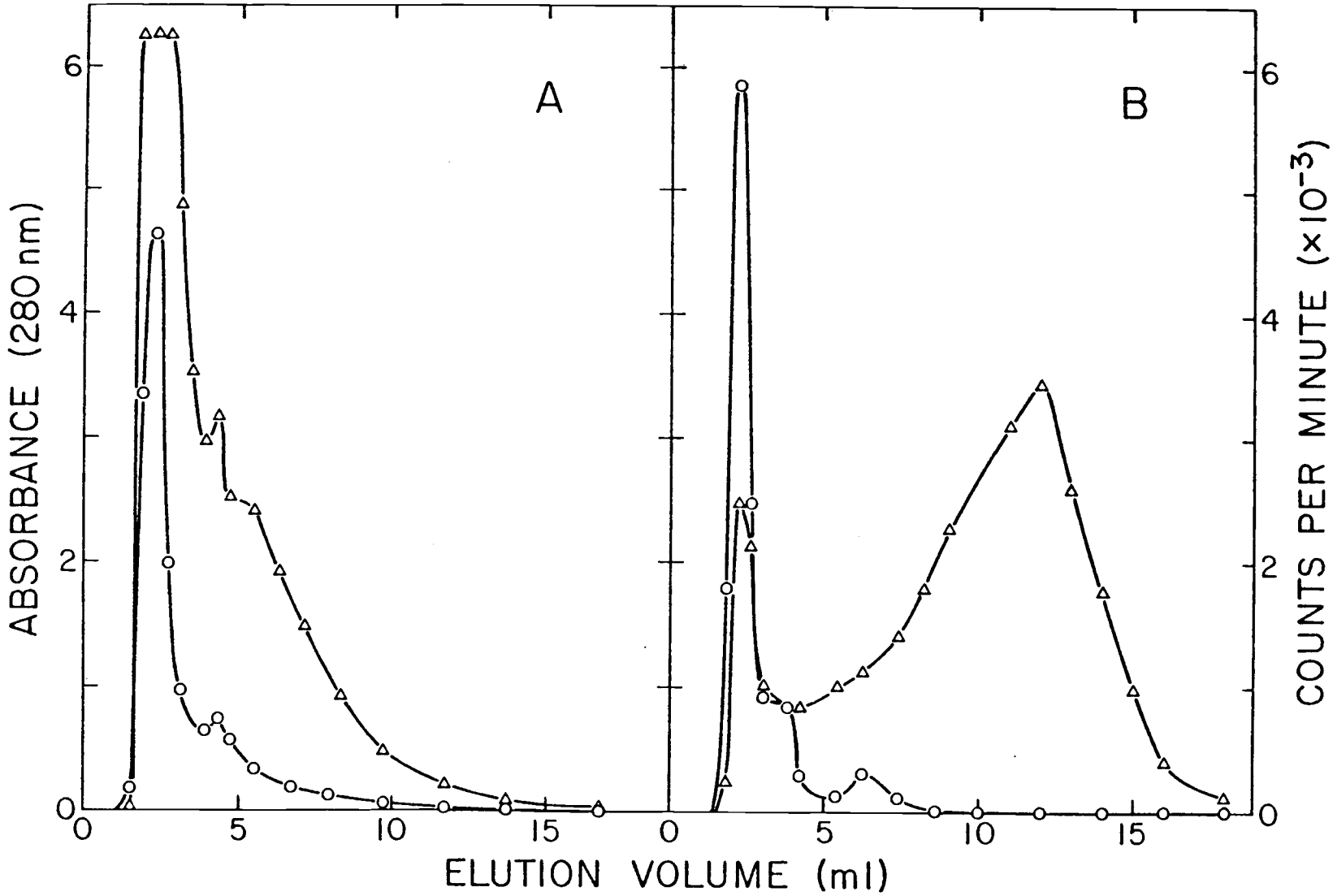

A
B
ABSORBANCE (280 nm)
COUNTS PER MINUTE (×10⁻³)
ELUTION VOLUME (ml)
0
2
4
6
0
5
10
15

Figure 25

in turn, would allow for more accurate quantitation of free and bound fractions and would enable larger samples (containing smaller concentrations of ligand), smaller columns and faster flow rates to be used. The superior resolution between free and macromolecular bound ^{14}C-PCN obtained with LH-20, when all other parameters are identical, is shown in Figure 25. Usage of G-25 would have led to a large overestimation of the percentage of ligand bound. Control experiments (not shown) confirmed that free PCN was eluted from LH-20 in the same fractions as the trailing peak of Figure 25B and further, that in the absence of cytosol, no PCN appeared in the void volume of either column. No observed tailing of bound PCN into the free PCN fraction on LH-20 strongly suggests that "stripping" of PCN from receptor sites by LH-20 during filtration does not occur.

In order to determine the binding parameters for the PCN-receptor interaction, cytosol was incubated with much lower ^{14}C-PCN concentrations (1.6-28 nM), bound PCN was measured on both G-25 and LH-20 and the data were plotted by Scatchard's method (Figure 26). Experimental scatter was generally much larger with the data from G-25 owing to the difficulty of separation bound and free PCN on this substrate. Both G-25 and LH-20, however, gave comparable K_d values ($1.3 \cdot 10^{-8}$ M and $9.8 \cdot 10^{-9}$ M, respectively) for the PCN-receptor dissociation but the levels of receptor as measured on G-25 (550 femtomoles/mg protein) were much higher than those obtained on LH-20 (50 femtomoles/mg). The latter value is comparable to known concentrations of the mouse hepatic TCDD receptor (96).

The finding that chromatography on G-25 gave, even at low concentrations, improbably high values for the putative PCN receptor is

Figure 26. Scatchard plots of ^{14}C-PCN binding to rat liver cytosol. A. G-25; B. LH-20. ^{14}C-PCN was incubated with 1.2 mls of cytosol at levels ranging from 1.9 pmoles to 34 pmoles at 0°C for 90 min. Samples of 0.5 ml (18 mg protein) were loaded onto columns and eluted as described in the Materials and Methods. Bound ^{14}C-PCN was collected directly into scintillation vials and counted (S_b). Aliquots of 0.5 ml were also counted from the original incubation mixture to determine the total steroid concentration (S_t). Unbound steroid (S_u) was calculated as the difference between S_t and S_b.

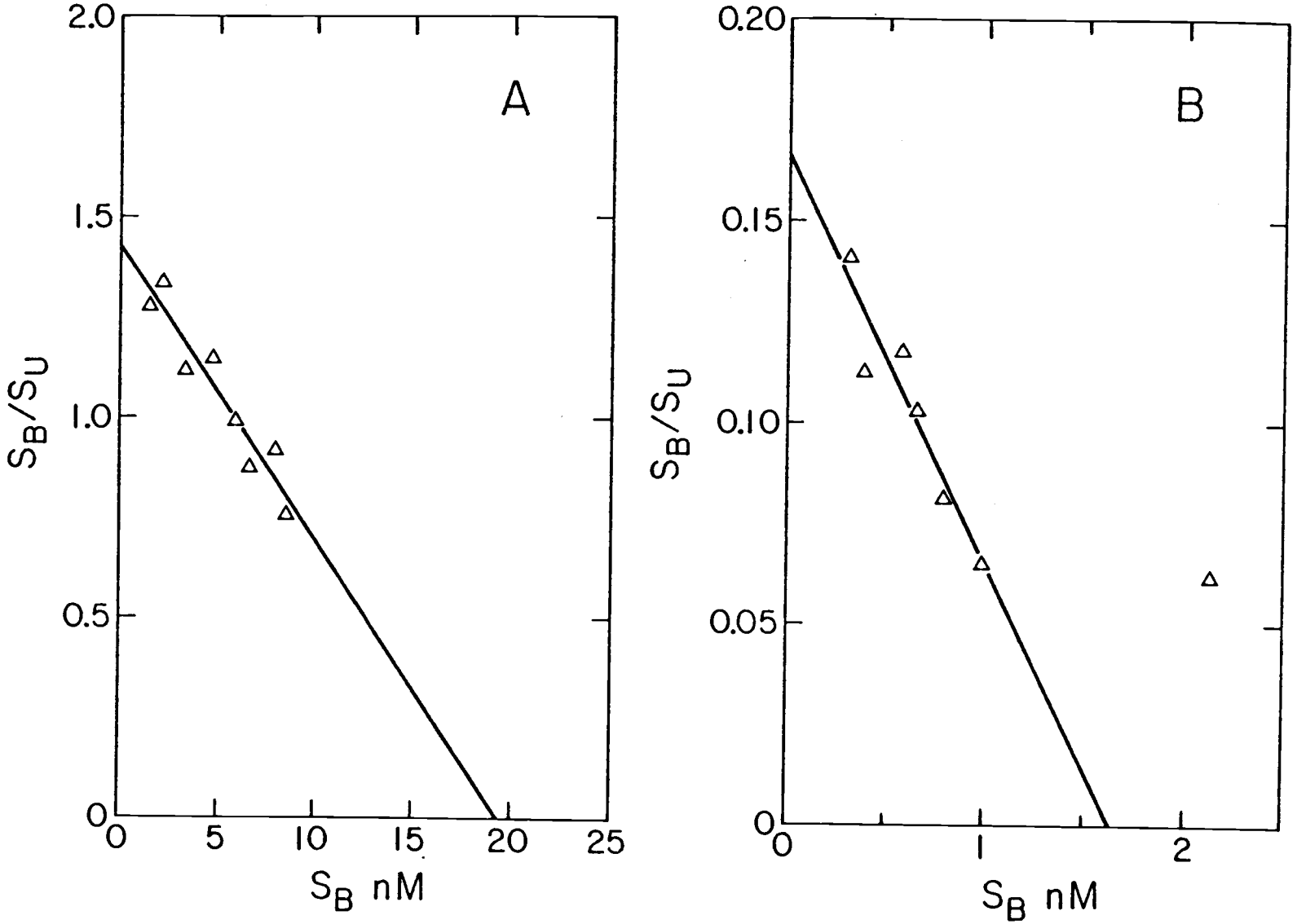


Figure 26

supported by recent theoretical and practical studies of Richard-Foy et al (111). They showed that at high concentrations of protein, adsorption to charcoal-dextran or hydroxyapatite was far superior to chromatography on G-25 for accurate measurement of calf uterine estradiol receptor concentrations.

Although LH-20 gel filtration was demonstrated to be precise and reliable in separating free and bound PCN, it proved to be impractical when multiple samples and competition experiments were attempted.

Analysis Of ^{14}C-PCN Binding To Non-Induced Rat Hepatic Cytosol Macromolecules By Adsorption To Dextran-Coated Charcoal

Before dextran-coated charcoal could be employed in the analysis of ^{14}C-PCN binding it was necessary to obtain some information with regard to the degree to which a number of experimental variables would affect the results. It was found that the two factors, which most critically determined the amount of ^{14}C-PCN removed from the supernatant, were the time of incubation of the steroid cytosol mixture with the dextran-coated charcoal (Figure 27) and the percent of dextran on the charcoal (Figure 28). Adsorption of ^{14}C-PCN to charcoal decreases rapidly with increasing dextran in the range 0-0.1% and then plateaus at 0.25-0.5%. Presumably, dextran is making certain high affinity sites on the charcoal, which may be stripping ^{14}C-PCN from receptor sites, unavailable for binding. The amount of ^{14}C-PCN adsorbed with time decreases at a rapid rate initially and then plateaus between 10-15 minutes. The increase with time may be due to competition between the charcoal and a number of low affinity sites, such as albumin. In order to provide the maximum amount of reproducibility

Figure 27. Effect of the length of incubation with dextran coated charcoal on the amount of ^{14}C-PCN left in the supernatant. Cytosol (1.2 mls) from uninduced rats was incubated with ^{14}C-PCN (50 pmoles) at 0°C for 90 min, following which, 1 ml of charcoal (1%) coated with 1% dextran was added. Samples were shaken at 4°C for 0-15 min, centrifuged at 23,000 g for 5 min and the radioactivity of the supernatant determined.

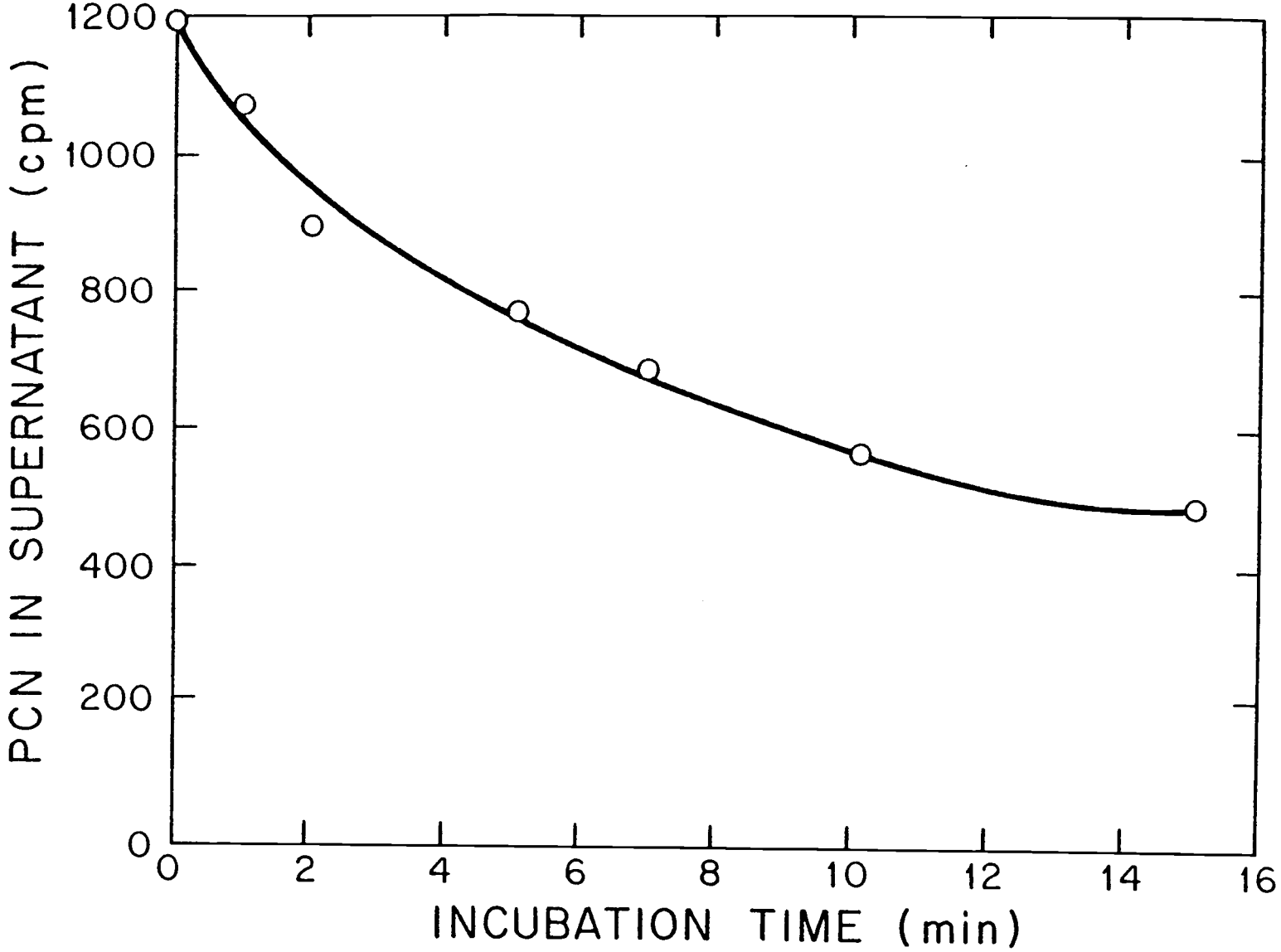

PCN IN SUPERNATANT (cpm)
1200
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
INCUBATION TIME (min)
Figure 27

Figure 28. Effect of the percentage of dextran coated onto the charcoal on the amount of ^{14}C-PCN left in the supernatant. Cytosol (1.2 mls) from uninduced rats was incubated with ^{14}C-PCN (50 pmoles) at 0°C for 90 min, following which, 1 ml of charcoal (1%) coated with varying amounts of dextran (0-2%) was added. Samples were shaken at 4°C for 10 min, centrifuged at 23,000 g for 5 min and the radioactivity of the supernatant determined.

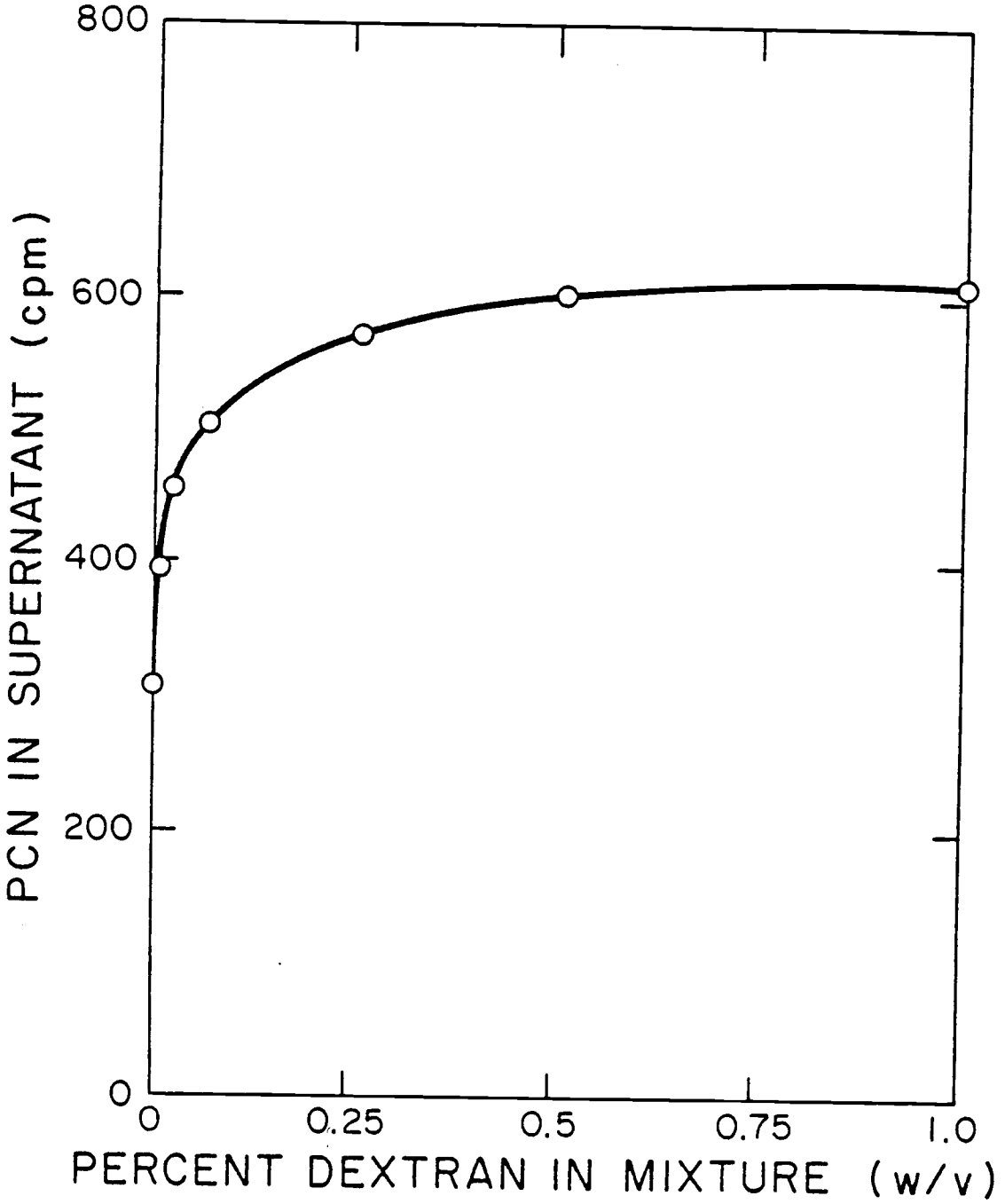

800
600
400
200
0
PCN IN SUPERNATANT (cpm)
0
0.25
0.5
0.75
1.0
PERCENT DEXTRAN IN MIXTURE (w/v)

Figure 28

between experiments, subsequent analysis was performed using 0.5% dextran-coated charcoal incubated with the ligand-cytosol mixture for 10 minutes at 0-4°C.

When concentrations of ^{14}C-PCN similar to those used with LH-20 (1-30 nM) were analyzed by dextran-coated charcoal, comparable binding parameters were obtained (K_d=6.8•10^{-9} M and a receptor concentration of 145 femtomoles/mg). However, larger sample volumes used in dextran-coated charcoal experiments made it possible to reduce the concentration of ^{14}C-PCN used by about three fold without any reduction in the total cpms obtained. When these lower concentrations (0.3-15 nM) were used, a lower concentration (20 femtomoles/mg) for a second binding species of increased affinity (K_d=1•10^{-10} M) was observed. Although the relatively low radioactivity present in the bound fraction (as little as 5-10 cpm above background at the lowest concentrations of ^{14}C-PCN used) prevent any conclusive findings, the values cited were obtained repeatedly. The reliability of the data was enhanced by counting the samples for prolonged periods. When a wider concentration range of ^{14}C-PCN was used, both binding components were observed (Figure 29B). Inability to completely displace ^{14}C-PCN from its binding site by an excess of unlabelled PCN indicated that neither of these two species was a PCN specific receptor. Again, data obtained at the lower ^{14}C-PCN concentrations were questionable. The lower affinity binding of ^{14}C-PCN could have been due to a nonspecific association with one of the many known intracellular steroid receptors in liver. Competition experiments to determine which receptor this might be were not done.

Analysis Of ^{3}H-PB And ^{3}H-3-MC Binding To Hepatic Cytosol Macromolecules By Adsorption To Dextran-Coated Charcoal

The high specific activity of tritiated ligands made it possible to perform accurate binding assays at even lower total ligand concentrations. Results with ^{3}H-PB would suggest the existence of a high affinity ($4 \cdot 10^{-11}$ M) receptor (Figure 29D). However, the fraction of bound ^{3}H-PB and the estimated number of binding sites were so low that accurate determination of S_B values at low ^{3}H-PB concentrations was difficult even with the increase in ligand specific activity. Attempts to displace ^{3}H-PB with an excess of unlabelled PB were unsuccessful (data not shown). A second low affinity binding ($K_D = 1 \cdot 10^{-6}$ M) component was also observed for ^{3}H-PB (data not shown).

Repeated dextran-coated charcoal binding assays with ^{3}H-3-MC demonstrated the presence of a high affinity binding component ($K_D = 8 \cdot 10^{-11}$ M). Initial attempts at demonstrating binding specificity by competition with 3-MC were unsuccessful. It was found that, unlike the case with ^{14}C-PCN or ^{3}H-PB, the ability of the dextran-coated charcoal to adsorb free ^{3}H-3-MC was rapidly reduced with increasing ^{3}H-3-MC concentration and this effect was even more pronounced in the presence of excess unlabelled 3-MC (Figure 29A). When the data were replotted using values to correct for the observed decrease in adsorption with increasing ^{3}H-3-MC or ^{3}H-3-MC plus 3-MC, the Scatchard plot in Figure 29C obtained. There was essentially no ^{3}H-3-MC bound in the presence of excess of 3-MC, strongly suggesting that this was a receptor specific for 3-MC. Both the affinity ($K_D = 8 \cdot 10^{-11}$ M) and the concentration of binding sites (1 femtomole/mg protein) differ from the 3-MC (TCDD) receptor

Figure 29. Background binding of ^{3}H-3-MC and Scatchard plots of ^{14}C-PCN, ^{3}H-3-MC and ^{3}H-PB binding to rat liver cytosol measured by dextran-coated charcoal. Figure 29A. ^{3}H-3-MC buffer blank. One ml aliquots of buffer containing no cytosol protein were incubated for 90 min at 0°C with 0.05-0.7 pmoles of ^{3}H-3-MC in the presence (open circles) or absence (closed circles) of 100 pmoles unlabelled 3-MC. Following the incubation, 1 ml of 1% Norit A coated with 1% Dextran was added, the samples shaken for 10 min at 4°C, centrifuged at 23,000 g for 5 min and the radioactivity of the supernatant determined; Figure 29B. Scatchard plot of ^{14}C-PCN binding to rat liver cytosol. Cytosol (1.5 mls) from uninduced rats (9.3 mg protein) was incubated with ^{14}C-PCN (0.4-8.0 pmoles) in the presence (open circles) or absence (closed circles) of 100 pmoles of unlabelled PCN. The conditions for incubation, treatment with dextran-coated charcoal and the isolation of bound ^{14}C-PCN in the supernatant was as described above, except that 1.5 mls of dextran-coated charcoal was used; Figure 29C. Scatchard plot of ^{3}H-3-MC binding to rat liver cytosol. Cytosol (1 ml) from uninduced rats (8.0 mg) was incubated with ^{3}H-3-MC (0.03-3.3 pmoles) in the presence or absence of 100 pmoles of unlabelled 3-MC. When the values of S_B were corrected for background binding using data from Figure 29A, there was no binding of ^{3}H-3-MC in the presence of excess 3-MC. The corrected Scatchard plot obtained in the absence of excess 3-MC is

shown here (open circles). The conditions for incubation and separation of bound and unbound ligand by dextran-coated charcoal was as described above; Figure 29D. Scatchard plot of ^{3}H-PB binding to rat liver cytosol. Cytosol (1 ml) from uninduced rats (7.0 mg) was incubated with ^{3}H-PB (0.01-0.8 pmoles). Incubation, addition of dextran-coated charcoal and determination of bound and unbound ligand was as described above.

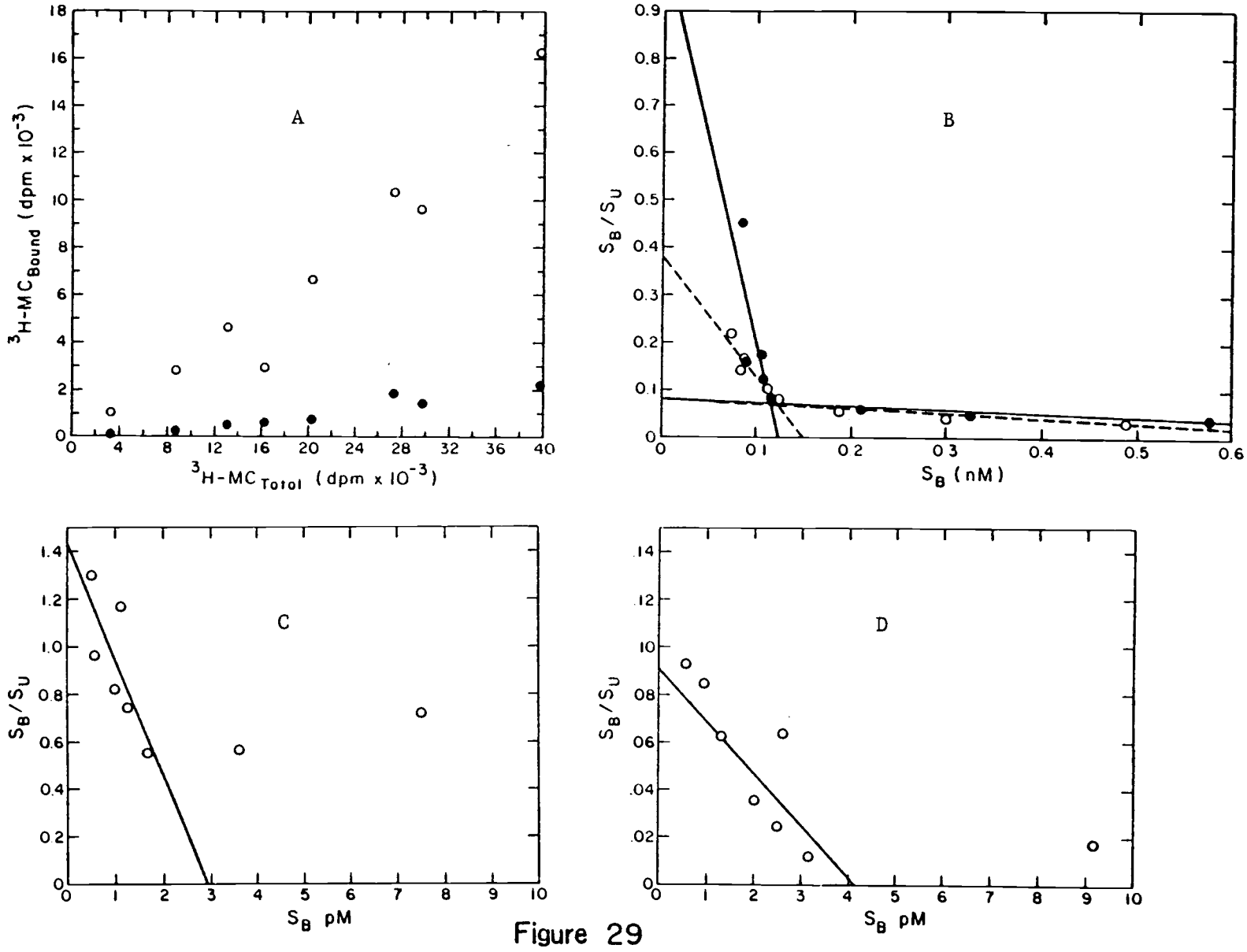

A
^{3}H-MC$_{Bound}$ (dpm x 10^{-3})
^{3}H-MC$_{Total}$ (dpm x 10^{-3})
B
S_B/S_U
S_B (nM)
C
S_B/S_U
S_B pM
D
S_B/S_U
S_B pM

Figure 29

of mouse liver. However, if values of specifically bound 3-MC are calculated without taking into account the relationship seen in Figure 29A, overestimation of the concentration of receptor sites and an underestimation of binding affinity would result.

Analysis of hepatic cytosol binding of these three MFO inducers established the existence of a specific, high affinity receptor present for binding 3-MC in rat. Although the results were suggestive for the existence of high affinity macromolecular components binding PB and PCN, acquistion of definitive data was not possible due to the low percentage bound in the former and the low specific activity of the latter.

DISCUSSION

Purification of cytochrome P-450 from variously induced mammals has usually focused on obtaining the major form present in a homogenous state (28). Fewer attempts have been made to purify minor induced or constitutive forms to a high specific content (115,116). The relatively low yield of final product (1-10%) in most studies suggests the possibility that other P-450 forms could be lost during purification. Due to the limited resolving power of the commonly used method of SDS-PAGE, it is not possible to be confident of how many P-450 forms are initially present in microsomes. This research was, in part, an attempt to purify as many forms of P-450 as possible from microsomes of control rats as well as rats treated with the classic inducers PB, PCN and 3-MC. Analysis of the composition of these purified fractions using the high resolving power of isoelectric focusing (IF) should provide a better answer to a question that is currently generating much controversy and speculation. How many P-450 forms are there?

Further comparison of the pattern of P-450 forms from control and induced rats may help to answer other questions. Are induced P-450 forms novel hemoproteins or constitutive? Do the relative concentration of some forms increase, while other decrease and is the pattern unique for each inducer? These latter questions are tied into the more complex question of the molecular mechanism(s) of induction. One widely accepted model for induction by polycyclic aromatic hydrocarbons (PAHs) has been presented (75,94). A very important feature of this model involves a specific, high affinity, cytosolic receptor. The other section of this thesis involved an attempt to establish the

presence of such a binding component for each of the classic inducers PB, PCN and 3-MC. The discussion which follows is basically a critique of how well the above questions were answered.

The original procedure adopted for purification of P-450 was that of Guengerich (25,46) and involved sequential chromatography on three different matrices. In the first step, P-450 is separated from other solubilized microsomal proteins by adsorption onto a ω-aminooctyl agarose (AOA) column, and is subsequently eluted with a non-ionic detergent. The advantages of AOA chromatography when compared to other currently used initial steps (23) include high yields (33-45%), a two to four fold increase in specific content and the elimination of hemoglobin and the enzymatically inactive P-420 in the column void volume. Using this procedure, one should also be able to remove NADPH-dependent-P-450 reductase and cytochrome b_5, both of which should elute after the P-450. Although comparison of SDS-PAGE results before and after AOA chromatography (Figure 5) suggests that such was the case, bands which correspond to the molecular weights of the reductase and b_5 were obtained following electrophoresis (Figures 10, 13, 16 and 19) of peaks eluting from DEAE Sephacel at high salt concentration (Figure 9). Figure 4 shows the presence of a shoulder on each P-450 peak which was included in the pooled P-450 fraction. As this paper was being prepared, it was learned that the flow rate used was about twice that recommended for optimal resolution (112). Therefore, the shoulder observed in Figure 4 is probably the source of b_5 and the reductase as well. In addition, Guengerich has very recently (112) replaced Triton N101 with Lubrol PX as the eluting detergent with no loss in performance and is, thus, able to monitor total protein

(A_{280}) as well as hemoprotein (A_{417}). Although the results obtained with AOA were satisfactory, the P-450 specific content could, presumably, have been increased had a slower flow rate been employed.

The second step in the original purification scheme called for stepwise elution of a hydroxyapatite column. Although both P-450 fractons obtained had a fairly high specific content, the extremely slow flow rate and low yield prompted the elimination of this step in subsequent experiments. Very recently Guengerich (114) utilized a special form of hydroxyapatite, Hypatite C from Clarkson Chemical Co. He found that inclusion of a hydroxyapatite step was necessary only if one began with a very low specific content, as is the case with human liver microsomes (0.18 nmoles per mg protein), but was not required to obtain pure P-450 in high yields from induced rats.

The final step in the original scheme involved stepwise elution from DEAE Sephacel at 4°C. The yields obtained were poor and the specific content of some fractions (especially the 90 mM hydroxyapatite peak) were low. Due to the poor performance of hydroxyapatite and DEAE Sephacel chromatography at 4°C, a modification (Guengerich, personal communication) of the original procedure involving DEAE Sephacel chromatography at room temperature was utilized. Depending upon the source of P-450, four to seven hemoprotein peaks were obtained. The last peak to elute in each case had a molecular weight of about 15,000 daltons and a pI of about 5.2 and is thought to be cytochrome b_5, even though no peak should have been observed in the CO-reduced versus reduced difference spectrum. Analysis of each of the remaining peaks by SDS-PAGE showed that only peak four from PB induced rats could be regarded as homogenous (Figure 13). The remaining

peaks had multiple bands in the molecular weight range of P-450 (45,000-60,000 daltons). Isoelectric focusing results (assuming no artifacts were produced) confirmed the heterogeneity observed. Part of the heterogeneity could be due to peak overlap. A shallower salt gradient may further separate the peaks, but regardless, the resolution observed is superior to published chromatograms (30,56).

The poorest results obtained from DEAE Sephacel chromatography were with the PCN induced P-450. Both of the first two peaks consisted solely of P-420. Peaks three through five were obtained as P-450 but the specific content was quite low. Subsequent to the completion of this work, Elshourbagy and Guzelian (117) found that PCN induced P-450 was too unstable to be chromatographed at room temperature without conversion to P-420. Even when chromatography was performed at 4°C, the specific content of the major peak obtained was only 5.0. It is interesting to speculate if this decrease in stability is due to altered properties of some unique PCN induced P-450 forms, or if PCN induction somehow causes a decrease in the stability of all P-450 forms.

Isoelectric focusing could be a powerful tool for studying the isozymic forms of P-450. If, indeed, many forms exist, two dimensional electrophoretic fingerprints (using IF as the first and SDS-PAGE as the second dimension) could be used to compare P-450 patterns from a variety of sources. Although not conclusive, the IF results comparing the effect of protein load and point of application suggest that the heterogeneity observed was not caused by artifacts. Further suggestive evidence comes from the observation that the degree of heterogeneity (number of IF bands) observed for each fraction could

be related to the SDS-PAGE banding pattern. However, the molecular weight and pI range of P-450 forms on SDS-PAGE and IF, respectively, was too narrow under the conditions employed to accurately distinguish the various P-450 forms. An expanded SDS-PAGE system and a narrower pH IF range (pH 6-8) utilized separately or together, should be employed in any future attempts to examine P-450 heterogeneity.

Therefore, the electrophoretic data obtained was not able to provide accurate answers to most of the questions proposed regarding the multiplicity of P-450. However, the DEAE chromatograms observed following induction, suggest that pretreatment with PB, PCN or 3-MC produced distinct isozymic patterns which involved not only large increases in the relative amounts of certain forms, but also decreases in the amount of other forms. The identification of these induced or repressed forms as either novel or constitutive hemoproteins was not possible. It was not possible to establish the absolute numbers of forms present; however, the large degree of heterogeneity observed in these purified fractions would be consistent with the model of Nebert.

One of the interesting observations in this study was the multiplicity of cytochrome b_5. As previously discussed, the exact role of b_5 in the MFO reaction scheme is still uncertain. One current idea (118) is that b_5, in concert with NADH and NADH dependent cytochrome b_5 reductase, could provide an electron sparing effect when NADPH is being consumed during 'uncoupling' prior to the second reduction (Figure 1). The degree of uncoupling of the MFO system is thought to increase following induction by PB and PCN. Although the exact mechanism by which uncoupling occurs is not known, PB could enhance the rate of uncoupling by increasing the level of NADPH-

dependent-P-450 reductase activity and by acting as a slowly metabolized substrate. The net result would be an increase in the level of the P-450-oxygen-substrate complex. Induction of b_5 would help to increase the rate of the second reduction (which many researchers regard as the probable rate limiting step) and prevent the build-up of the above intermediate. Protection against high levels of this intermediate would be advantageous to the cell as not only are NADPH and O_2 consumed during uncoupling, but the very toxic superoxide radical is also probably generated. Perhaps the minor form of b_5 which is increased following induction (especially with PB and PCN) in this study is a form which is more active in feeding electrons into the P-450 cycle compared to the major form which is more active in the other reactions commonly associated with b_5 such as desaturase activity. Such a dual function of b_5 would help to explain the conflicting results workers have seen with respect to the role of b_5 in the MFO reaction cycle. On the other hand, without further analysis, it is entirely possible that the minor component observed was b_5 which had somehow been altered either during purification or isoelectric focusing.

An essential component of the model of MFO induction proposed by Nebert is a high affinity, specific receptor, presumed to be located in the cytosol, unique for each type of inducer. The existence of such a receptor for PAHs has been demonstrated. Although attempts have been made, no such receptor has, as yet, been found for other inducers including PB and PCN. One possible explanation for this is the fact that all work to date has utilized non-induced animals. According to Nebert's model (Figure 3) a previous exposure to the drug or chemical would be necessary for the synthesis of the receptor. Thus,

the presence of a PAH receptor may simply be due to exposure of animals to some unknown PAH inducer in, for instance, the diet. This is not surprising given the wide environmental distribution of PAHs compared to the synthetic inducers such as PB or PCN. The difficulty in analyzing the specific binding of induced animals in vitro involves the high background due to endogenous inducer still present.

Analysis of cytosolic binding of ^{3}H-PB (Figure 29D) by dextran-coated charcoal (DCC) suggested that a high affinity receptor may be present in very low amounts. However, the very low percentage bound (2-8%), reflecting very few CPMs above background and the failure to be eliminated by an excess of PB, make these findings suspect. Recently, Poland (personal communication) has synthesized a much more potent PB agonist which may be more useful in establishing whether or not a PB receptor exists.

Cytosolic binding of ^{14}C-PCN was shown to be present as at least two components, one of fairly high affinity and low concentration as well as a low affinity moiety present in fairly high concentration. The latter binding species was demonstrated using gel filtration and DCC but was found to be unsaturable using an excess of PCN. The high affinity species, seen only with DCC, gave equivocal results when analyzed in the presence of excess PCN (Figure 29B); usually only about one-half the ^{14}C-PCN bound was eliminated by excess PCN. Accurate analysis at low ^{14}C-PCN concentrations was difficult due to the relatively low specific activity of ^{14}C.

Results obtained when the specific binding of ^{3}H-MC was analyzed by DCC were more conclusive (Figure 29C). Once the high non-specific background in the presence of excess MC (Figure 29A) was corrected

for, a high affinity ((K_d = 0.08 nM) receptor specific for ^{3}H-MC, and present in low amounts (one femtomole per mg protein) was repeatedly demonstrated. These binding parameters are quite different from published values. The mouse liver cytosolic receptor had a K_d of 0.2 nM for ^{3}H-TCDD (DCC) and was present at a concentration of 84 femtomoles per mg protein. Very recently, Tierney et al. (119), using gel filtration through Sephadex G200, found a receptor specific for ^{3}H-MC in rat liver cytosol with a K_d of 2.8 nM and a concentration of 770 femtomoles per mg protein. The differences observed probably relate to the methodologies employed. As demonstrated in this thesis, separation of bound and free ^{14}C-PCN using Sephadex G25 and LH20 was useful only in demonstrating the lower affinity binding species. Furthermore, G25, due to its failure to separate bound and free ^{14}C-PCN as efficiently as LH20 (Figure 25), led to erroneously high values of receptor concentration (550 femtomoles per mg protein). The use of Sephadex G200 (119) showed the presence of two binding components for ^{3}H-MC (one is probably ligandin); however, as no free ^{3}H-MC peak of radioactivity is shown and a significant amount of ^{3}H-MC eluted in the same region when protein was absent, the binding parameters may be inaccurate. This study did however, demonstrate that whatever the actual binding parameters are, only PAHs, such as BP could effectively compete for binding sites, while other inducers such as PB and PCN were much less effective. Furthermore, the ^{3}H-MC-receptor complex was demonstrated to be translocated into the nucleus. In this same paper, attempts to characterize the binding of ^{3}H-MC by DCC were unsuccessful due to high specific backgrounds.

Specific binding in both of the above studies is calculated as

the difference between total binding (^{3}H-MC only) and non-specific binding (^{3}H-MC plus excess MC). The much larger increase in DCC background binding with excess MC would lead to an increasing overestimation of bound ligand with increasing total concentration, which would result in a Scatchard plot of lower slope and, thus, an underestimation of K_d and an overestimation of receptor concentration.

Therefore, the results of this study confirm the existence of a high affinity rat liver cytosolic receptor for ^{3}H-MC. Differences between the values presented here and those of two previously published reports may be due to the failure of the latter to accurately determine absolute concentrations of free and bound ^{3}H-MC. No conclusive evidence for the existence of PB or PCN receptors was found; however, such negative evidence does not eliminate the existence of such receptors in rats pretreated with these inducers.

BIBLIOGRAPHY

1. Conney, A. H. (1967). Pharmacological implication of microsomal enzyme induction. Pharmacol. Rev. 19, 317-366.

2. Buhler, D. R. and Rasmusson, M. E. (1968). The oxidation of drugs by fishes. Comp. Biochem. Physiol. 25, 223-239.

3. Casida, J. E. (1969). Insect microsomes and insecticide chemical oxidations. in Microsomes and Drug Oxidations. Pp. 517-532. Gillette, J.R., Conney, A.H., Cosmides, G.J., Estabrook, R.W., Fouts, J.R. and Mannering, G.J. (eds.), Academic Press, N.Y.

4. O'Keeffe, D. H., Ebel, R. E. and Peterson, J. A. (1978). Purification of bacterial cytochrome P-450. in Methods in Enzymology. Vol. LII, Part C, Pp. 151-157. Fleischer, S. and Packer, L. (eds.), Academic Press, N.Y.

5. Reichhart, D., Salaun, J., Benveniste, I. and Durst, F. (1979). Induction by manganese, ethanol, phenobarbital, and herbicides of microsomal cytochrome P-450 in higher plant tissues. Arch. Biochem. Biophys. 196, 301-303.

6. Kato, R. (1979). Characteristics and differences in the hepatic mixed function oxidases of different species. Pharmac. Therap. 6, 41-98.

7. Jori, A., Pugliatti, C. and Santini, V. (1972). Differences in microsomal enzyme induction between Sprague-Dawley and Long-Evans rats. Pharmacology 7, 296-304.

8. Kato, R. (1974). Sex-related differences in drug metabolism. Drug Metab. Rev. 3, 1-32.

9. Iba, M. M., Soyka, L. F. and Schulman, M. P. (1977). Characteristics of the liver microsomal drug-metabolizing enzyme system of newborn rats. Molec. Pharmacol. 13, 1092-1104.

10. Alvares, A. P. (1978). Interactions between environmental chemicals and drug biotransformation in man. Clin. Pharmacokinet. 3, 462-477.

11. Guengerich, F. P. and Mason, P. S. (1979). Immunological comparison of hepatic and extrahepatic cytochrome P-450. Molec. Pharmacol. 15, 154-164.

12. Garfinkel, D. (1958). Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions. Arch. Biochem. Biophys. 77, 493-509.

13. Klingenberg, M. (1958). Pigments of rat liver microsomes. Arch. Biochem. Biophys. 75, 376-386.

14. Omura, T. and Sato, R. (1964). The carbon monooxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370-2378.

15. Ichikawa, Y. and Yamano, T. (1967). Reconversion of detergent and sulfhyl aryl reagent-produced P-420 to P-450 by polyols and glutathione. Biochim. Biophys. Acta 131, 490-497.

16. Welton, A. F. and Aust. S. D. (1974). Multiplicity of cytochrome P-450 hemoproteins in rat liver microsomes. Biochem. Biophys. Res. Commun. 56, 898-906.

17. Haugen, D. A., van der Hoeven, T. A. and Coon, M. J. (1975). Purified liver microsomal cytochrome P-450. Separation and characterization of multiple forms. J. Biol. Chem. 250, 3567-3570.

18. Ryan, D., Lu, A. Y. H., West, S. and Levin, W. (1975). Multiple forms of cytochrome P-450 in phenobarbital and 3-methylcholanthrene-treated rats. Separation and spectral properties. J. Biol. Chem. 250, 2157-2163.

19. Welton, A. F., O'Neal, F. O., Chaney, L. C. and Aust, S. D. (1975). Multiplicity of cytochrome P-450 hemoproteins and specificity of an antibody to the hemoprotein induced by phenobarbital. J. Biol. Chem. 250, 5631-5639.

20. Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J. and Levin, W. (1976). Multiple forms of rat liver cytochrome P-450. Immunochemical evidence with antibody against cytochrome P-448. J. Biol. Chem. 251, 1385-1391.

21. Haugen, D. A., Coon, M. J. and Nebert, D. W. (1976). Induction of multiple forms of mouse liver cytochrome P-450. Evidence for genetically controlled de novo protein synthesis in response to treatment with β-naphthoflavone or phenobarbital. J. Biol. Chem. 251, 1817-1827.

22. Powis, G., Talcott, R. E. and Schenkman, J. B. (1977). Kinetic and spectral evidence for multiple species of cytochrome P-450 in liver microsomes. in Microsomes and Drug Oxidation. Pp. 127-135. Ullrich, V., Routs, I., Hildebrandt, A., Estabrook, R.W. and Conney, A.H. (eds.), Pergamon Press, N.Y.

23. Levin, W., Ryan, D., Huang, M.-T., Kawalek, J., Thomas, P. E., West, S. B. and Lu, A. Y. H. (1977). Characterization of multiple forms of highly purified cytochrome P-450 from the liver microsomes of rats, mice and rabbits. in Microsomes and Drug Oxidation. Pp. 185-191. Ullrich, V., Routs, I., Hildebrandt, A., Estabrook, R.W. and Conney, A.H. (eds.), Pergamon Press, N.Y.

24. Ullrich, V. and Kremers, P. (1977). Multiple forms of cytochrome P-450 in the microsomal monooxygenase system. Arch. Toxicol. 39, 41-50.

25. Guengerich, F. P. (1978). Separation and purification of multiple forms of microsomal cytochrome P-450. Partial characterization of three apparently homogenous cytochrome P-450 prepared from livers of phenobarbital- and 3-methylcholanthrene-treated rats. J. Biol. Chem. 253, 7931-7939.

26. Sharma, R. N., Cameron, R. G., Farber, E., Griffin, M. J., Joly, J.-G. and Murray, R. K. (1979). Multiplicity of induction patterns of rat liver microsomal mono-oxygenases and other polypeptides produced by administration of various xenobiotics. Biochem. J. 182, 317-327.

27. Agosin, M., Morello, A., White, R., Repetto, Y. and Pedemonte, J. (1979). Multiple forms of non-induced rat liver cytochrome P-450. Metabolism of 1-(4'-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-trans-2-octene by reconstituted preparations. J. Biol. Chem. 254, 9915-9920.

28. Guengerich, F. P. (1979). Isolation and purification of cytochrome P-450, and the existence of multiple forms. Pharmac. Therap. 6, 99-121.

29. Haugen, D. A. and Coon, M. J. (1976). Properties of electrophoretically homogenous phenobarbital-inducible and β-naphthoflavone-inducible forms of liver microsomal cytochrome P-450. J. Biol. Chem. 251, 7929-7939.

30. Ryan, D. E., Thomas, P. E., Korzeniowski, D. and Levin, W. (1979). Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. J. Biol. Chem. 254, 1365-1374.

31. Ingelman-Sundberg and Gustafsson, J.-A. (1977). Resolution of multiple forms of phenobarbital-induced liver microsomal cytochrome P-450 by electrofocusing on granulated gels. FEBS Lett. 74, 103-106.

32. Botelho, L. H., Ryan, D. E. and Levin, W. (1979). Amino acid compositions and partial amino acid sequences of three highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, or 3-methylcholanthrene. J. Biol. Chem. 254, 5635-5640.

33. Johnson, E. F. and Muller-Eberhard, U. (1977). Resolution of two forms of cytochrome P-450 from liver microsomes of rabbits treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. J. Biol. Chem. 252, 2838-2845.

34. Bridges, J. W. and Fry, J. R. (1979). The use of isolated liver cell preparations for studying the induction of mixed function oxidase. in The Induction of Drug Metabolism. Pp. 343-354. Estabrook, R.W. and Lindenlaub, E. (eds.), F.K. Schattauer Verlag, Stuffgart, N.Y.

35. Haugen, D. A., Armes, L. G., Yasunolov, K. T. and Coon, M. J. (1977). Amino-terminal sequence of phenobarbital-inducible cytochrome P-450 from rabbit liver microsomes. Similarity to hydrophobic amino-terminal segments of pre-proteins. Biochem. Biophys. Res. Commun. 77, 967-973.

36. Estabrook, R. W. and Werringloer, J. (1979). The microsomal enzyme system response for the oxidative metabolism of many drugs. in The Induction of Drug Metabolism. Pp. 187-199. Estabrook R.W. and Lindenlaub, E. (eds.), F.K. Schattauer Verlag, Stuttgart, N.Y.

37. Björkhem, I. (1977). Rate limiting step in microsomal cytochrome P-450 catalyzed hydroxylations. Pharmac. Therap. A, 1, 327-348.

38. Chiang, Y.-L. and Coon, M. J. (1979). Comparative study of two highly purified forms of liver microsomal cytochrome P-450: circular dichroism and other properties. Arch. Biochem. Biophys. 195, 178-187.

39. Stier, A., Kühnle, W., Bösterling, B. and Finch, A. E. (1979). The integrated system of drug metabolism and its perturbation on induction. in The Induction of Drug Metabolism. Pp. 225-241. Estabrook, R.W. and Lindenlaub, E. (eds.), F.K. Schattauer Verlag, Stuttgart, N.Y.

40. Estabrook, R. W., Matsubara, T., Mason, J. I., Wrringloer, J. and Baron, J. (1973). Studies on molecular function of cytochrome P-450 during drug-metabolism. Drug Metab. Dispos. 1, 98-110.

41. Lu, A. Y. H. and Levin, W. (1974). The resolution and reconstitution of the liver microsomal hydroxylation system. Biochim. Biophys. Acta 344, 205-240.

42. Levin, W. (1977). Purification of liver microsomal cytochrome P-450: hopes and promises. in Microsomes and Drug Oxidation. Pp. 735-747. Ullrich, V., Roots, I., Hildebrandt, A., Estabrook, R.W. and Cooney, A.H. (eds), Pergamon Press, N.Y.

43. Coon, M. J., Vermilion, J. L., Vatsis, K. P., French, J. S., Dean, W. L. and Gaugen, D. A. (1977). Biochemical studies on drug metabolism: isolation of multiple forms of liver microsomal cytochrome P-450. in Drug Metabolism Concepts. American Chemical Symposium, Series No. 44. Pp. 46-71. Jerina, D.M. (ed.), American Chemical Society, Washington, D.C.

44. Kawalek, J. C., Levin, W., Ryan, D., Thomas, P. E. and Lu, A. Y. H. (1975). Purification of liver microsomal cytochrome P-448 from 3-methylcholanthrene-treated rabbits. Molec. Pharmacol. 11, 874-878.

45. Johnson, E. F. and Muller-Eberhard, U. (1977). Multiple forms of cytochrome P-450: Resolution and purification of rabbit liver aryl hydrocarbon hydroxylase. Biochem. Biophys. Res. Commun. 76, 644-651.

46. Guengerich, F. P. (1977). Separation and purification of multiple forms of microsome cytochrome P-450 activities of different forms of cytochrome P-450 towards several compounds of environmental interest. J. Biol. Chem. 252, 3970-3979.

47. Levin, W., Ryan, D., West, S. and Lu, A. Y. H. (1974). Preparation of partially purified, lipid-depleted cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase from rat liver microsomes. J. Biol. Chem. 249, 1747-1754.

48. Ryan, D., Lu, A. Y. H., Kawalek, J., West, S. B. and Levin, W. (1975). Highly-purified cytochrome P-448 and P-450 from rat liver microsomes. Biochem. Biophys. Res. Commun. 64, 1134-1141.

49. Ryan, D., Thomas, P. E. and Levin, W. (1977). Properties of purified liver microsomal cytochrome P-450 from rats treated with the polychlorinated biphenyl-mixture Aroclor 1254. Molec. Pharmacol. 13, 521-532.

50. Jefcoat, C. R. E., Gaylor, J. L. and Calabreso, R. L. (1969). Ligand interactions with cytochrome P-450. I. Binding of primary amines. Biochemistry 8, 3455-3463.

51. Imai, Y. and Sato, R. (1974). An affinity column method for partial purification of cytochrome P-450 from phenobarbital-induced rabbit liver microsomes. J. Biochem. 75, 689-697.

52. Imai, Y. and Sato, R. (1974). A gel-electrophoretically homogenous preparation of cytochrome P-450 from liver microsomes of phenobarbital-pretreated rabbits. Biochem. Biophys. Res. Commun. 60, 8-14.

53. Imai, Y. (1976). The use of 8-aminooctyl Sepharose for the separation of some components of the hepatic microsomal electron transfer system. J. Biochem. 80, 267-276.

54. Warner, M., LaMarca, M. V. and Neims, A. H. (1977). Preparative isoelectric focusing of solubilized cytochromes P-450 from untreated male rat liver. Federation Proc. 36, 991.

55. West, S. B., Huang, M.-T., Miwa, G. T. and Lu, A. Y. H. (1979). A simple and rapid procedure for the purification of phenobar-

bital-inducible cytochrome P-450 from rat liver microsomes. Arch. Biochem. Biophys. 193, 42-50.

56. Beaune, P. H., Dansett, P., Flinois, J. P., Columelli, S., Mansuy, D. and Leroux, J. P. (1979). Partial purification of human liver cytochrome P-450. Biochem. Biophys. Res. Commun. 88, 826-832.

57. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

58. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

59. Bio-Rad Laboratories. (1979). Bio-Rad Protein Assay instruction manuel.

60. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227, 680-685.

61. Negishi, M. and Nebert, D. W. (1979). Structural gene products of the Ah locus. Genetic and immunochemical evidence for two forms of mouse liver cytochrome P-450 induced by 3-methylcholanthrene. J. Biol. Chem. 254, 11015-11023.

62. Atlas, S. A., Boobis, A. R., Felton, J. S., Thorgeirsson, S. S. and Nebert, D. W. (1977). Ontogenic expression of polycyclic aromatic compound-inducible monooxygenase activities and forms of cytochrome P-450 in rabbit: evidence for temporal control and organ specificity of two genetic regulatory systems. J. Biol. Chem. 252, 5712-5721.

63. Guenther, T. M. and Nebert, D. W. (1978). Evidence in rat and mouse liver for temporal control of two forms of 2,3,7,8-tetrachlorodibenzo-ρ-dioxin-inducible cytochrome P-450. Eur. J. Biochem. 91, 449-456.

64. Thomas, P. E., Ryan, D. and Levin, W. (1976). An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. Anal. Biochem. 75, 168-176.

65. Warner, M. and Neims, A. H. (1976). Resolution of multiple forms of solubilized cytochrome P-450 by isoelectric focusing. Pharmacologist 18, 242.

66. Guengerich, R. P. (1979). Artifacts in isoelectric focusing of the microsomal enzymes cytochrome P-450 and NADPH-cytochrome P-450 reductase. Biochem. Biophys. Acta 577, 132-141.

67. Berg, A., Ingelman-Sundberg, M. and Gustafsson, J.-A. (1979). Purification and characterization of cytochrome P-450$_{meg}$. J. Biol. Chem. 254, 5264-5271.

68. Wallevik, K. (1973). Isoelectric focusing of bovine serum albumin. Influence of binding of carrier ampholytes. Biochim. Biophys. Acta 322, 75-87.

69. O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250, 4007-4021.

70. Lu, A. Y. H., Somogyi, A., West, S., Kuntzman, R. and Conney, A. H. (1972). Pregnenolone-16α-carbonitrile: A new type of inducer of drug-metabolizing enzymes. Arch. Biochim. Biophys. 152, 457-462.

71. Birnbaum, L. S., Baird, M. B. and Massie, H. R. (1976). Pregnenolone-16α-carbonitrile-inducible cytochrome P-450 in rat liver. Res. Commun. Chem. Path. Pharm. 15, 553-562.

72. Fennell, T. R., Dickins, M. and Bridges, J. W. (1979). Interaction of isosafrole in vivo with rat hepatic microsomal cytochrome P-450 following treatment with phenobarbitone or 20-methylcholanthrene. Biochem. Pharm. 28, 1427-1429.

73. McLean, A. E. M. and Driver, H. E. (1977). Combined effects of low doses of DDT and phenobarbital on cytochrome P-450 and aminopyrine demethylation. Biochem. Pharm. 26, 1299-1302.

74. Alvares, A. P. and Kappas, A. (1977). Heterogeneity of cytochrome P-450s induced by polychlorinated biphenyls. J. Biol. Chem. 252, 6373-6378.

75. Nebert, D. W. (1979). Genetic aspects of enzyme induction by drugs and chemical carcinogens. in The Induction of Drug Metabolism. Pp. 419-452. Estabrook, R. W. and Lindenbaub, E. (eds.), F.K. Schattauer Verlag, Stuttgart, N.Y.

76. Parke, D. V. (1979). The responsiveness of cells to various drug inducers. in The Induction of Drug Metabolism. Pp. 101-111. Estabrook, R.W. and Lindenbaub, E. (eds.), Schattauer Verlag, Stuttgart, N.Y.

77. Ernster, L. and Orrenius, S. (1965). Substrate-induced synthesis of the hydroxylating enzyme system of liver microsomes. Federation Proc. 24, 1190-1199.

78. Orrenius, S., Ericsson, J. L. E. and Ernster, L. (1965). Phenobarbital-induced synthesis of the microsomal drug-metabolizing enzyme system and its relationship to the proliferation of endoplasmic membranes. J. Cell Biol. 25, 627-639.

79. Orrenius, S. (1965). Further studies on the induction of the

drug-hydroxylating enzyme system of liver microsomes. J. Cell Biol. 26, 725-733.

80. Omura, T. (1979). Biosynthesis and drug-induced increase of microsomal enzymes. in The Induction of Drug Metabolism. Pp. 161-175. Estabrook, R.W. and Lindenbaub, E. (eds.), F.K. Schattauer Verlag, Stuttgart, N.Y.

81. Lake, B. G., Longland, R. C., Harris, R. A., Severn, B. J. and Gangolli, S. D. (1978). The effect of prolonged sodium phenobarbitone treatment on hepatic xenobiotic metabolism and the urinary excretion of metabolites of the D-glucuronic acid pathway in the rat. Biochem. Pharm. 27, 2357-2361.

82. Hasani, S. and Burchell, B. (1979). Regulation of rat liver epoxide hydratase activity by phenobarbital and 3-methylcholanthrene. Biochem. Pharm. 28, 2175-2179.

83. Roberts, R. J. and Plaa, G. L. (1967). Effect of phenobarbital on the excretion of an exogenous bilirubin load. Biochem. Pharm. 16, 827-835.

84. Bresnick, E. (1978). The molecular biology of the induction of the hepatic mixed function oxidases. Pharmac. Therap. A. 2, 319-335.

85. Kouroumakis, P., Selye, H. and Tache, Y. (1977). Catatoxic steroids. Adv. Steroid Biochem. Pharm. 6, 35-57.

86. Selye, H. (1971). in Hormones and Resistance. 1400 p. Springer-Verlag, Heidelberg, Germany.

87. Solymoss, B., Werringloer, J. and Troth, S. (1971). The influence of pregnenolone-16α-carbonitrile on hepatic mixed-function oxidases. Steroids 17, 427-433.

88. Lykissas, E. D., Kouroumakis, P. and Brookman, S. (1978). Effect of pregnenolone-16α-carbonitrile on bilirubin- and sulfobromophthalein-binding to hepatic Y and Z proteins in the rat. Pharmacology 16, 217-220.

89. Solymoss, B. and Zsigmond, G. (1973). Effect of various steroids on the hepatic glucuronidation and biliary excretion of bilirubin. Can. J. Physiol. Pharmacol. 51, 319-323.

90. Bielen, J. E., Goujon, F. M. and Nebert, D. W. (1972). Genetic regulation of aryl hydrocarbon hydroxylase induction. II. Simple mendelian expression in mouse tissue *in vivo*. J. Biol. Chem. 247, 1125-1137.

91. Nebert, D. W. and Gielen, J. E. (1972). Genetic regulation of aryl hydrocarbon hydroxylase induction in the mouse. Federation Proc. 31, 1315-1325.

92. Nebert, D. W. and Felton, J. S. (1976). Importance of genetic factors influencing the metabolism of foreign compounds. Federation Proc. 35, 1133-1141.

93. Nebert, D. W. (1978). Genetic differences in microsomal electron transport: The Ah locus. Method Enzymol. LII (Part C), 226-240. Fleischer, S. and Packer, L. (eds.), Academic Press, N.Y.

94. Nebert, D. W. (1979). Multiple forms of inducible drug-metabolizing enzymes: a reasonable mechanism by which any organism can cope with adversity. Molec. Cell. Biochem. 27, 27-46.

95. McCarty, K. S., Jr. and McCarty, K. S., Sr. (1977). Steroid hormone receptors in the regulation of differentiation. Am. J. Path. 86, 705-744.

96. Poland, A., Glover, E. and Kende, A. S. (1976). Stereospecific high affinity binding of 2,3,7,8-tetrachlorodibenzo-ρ-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. J. Biol. Chem. 251, 4936-4946.

97. Guenthner, T. M. and Nebert, D. W. (1977). Cytosolic receptor for aryl hydrocarbon hydroxylase induction by polycyclic aromatic compounds. Evidence for structural and regulatory variants among established cell lines. J. Biol. Chem. 252, 8981-8989.

98. Poland, A., Glover, E., Kende, A. S., DeCamp, M. and Giandomenico, C. M. (1976). 3,4,3',4'-Tetrachloroazoxybenzene and azobenzene: Potent inducers of aryl hydrocarbon hydroxylase. Science 194, 627-630.

99. Poland, A. and Glover, E. (1974). Comparison of 2,3,7,8-tetrachloridibenzo-ρ-dioxin, a potent inducer of aryl hydrocarbon hydroxylase, with 3-methylcholanthrene. Molec. Pharm. 10, 349-359.

100. Romo, J. (1958). The addition of hydrocyanic acid to Δ16-pregnen-20-ones. Tetrahedron 3, 37-42.

101. Scatchard, G., Acheinberg, I. H. and Armstrong, S. H., Jr. (1950). Physical chemistry of protein solutions. IV. The combination of human serum albumin with chloride ion. J. Am. Chem. Soc. 72, 535-540.

102. van der Hoeven, T. A. and Coon, M. J. (1974). Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. J. Biol. Chem. 249, 6302-6310.

103. Ogita, Z.-I. and Marker, C. L. (1979). A miniaturized system

for electrophoresis on polyacrylamide gels. Anal. Biochem. 99, 232-241.

104. Guengerich, F. P. (1977). Preparation and properties of highly purified cytochrome P-450 and NADPH-cytochrome P-450 reductase from pulmonary microsomes of untreated rabbits. Molec. Pharm. 13, 911-923.

105. Aron, C. G. (1973). A new method for the detection of isoelectrically focused proteins in polyacrylamide gels. ISCO Applications Research Bulletin No. 12.

106. McGuire, W. L. (1973). Estrogen receptors in human breast cancer. J. Clin. Invest. 52, 73-77.

107. Lippman, M., Huff, K. and Bolan, G. (1977). Progesterone and glucocorticoid interactions with receptor in breast cancer cells in long-term tissue culture. Ann. N.Y. Acad. Sci. 286, 101-115.

108. Baulieu, E. E., Raynaud, J. P. and Milgrom, E. (1979). Measurement of steroid binding proteins. 2nd Karolinska Symposia on Research Methods in Reproductive Endocrinology, Pp. 104-121.

109. Schrader, W. T. (1975). Methods for extraction and quantification of receptors. Methods Enzymol. IIIVI, Part A, 197-211. O'Malley, B.W. and Hardman, J.G. (eds.), Academic Press, N.Y.

110. Peck, E. J., Jr. and Clark, J. H. (1977). Effect of ionic strength on charcoal adsorption assays of receptor-estradiol complexes. Endocrinology 101, 1034-1043.

111. Richard-Roy, H., Redeuilh, G. and Richard-Foy, R. (1978). A simple, rapid, and precise method for calculating the steroid hormone-receptor complex concentration in the presence of saturating levels of hormone by using "differential dissociation" techniques. Anal. Biochem. 88, 367-381.

112. Guengerich, F. P. and Martin, M. V. (1980). Purification of cytochrome P-450, NADPH-cytochrome P-450 reductase, and epoxide hydratase from a singe preparation of rat liver microsomes. Biochem. Biophys. Acta, in press.

113. Johnson, E. F. (1979). Multiple forms of cytochrome P-450: criteria and significance. Rev. Biochem. Toxicol. 2, 1-26.

114. Wang, P., Mason, P. S. and Guengerich, F. P. (1980). Purification of human liver cytochrome P-450 and comparison to the enzyme isolated from rat liver. Arch. Biochem. Biophys. 199, 206-219.

115. Koop, D. R. and Coon, M. J. (1979). Purification and properties of P-450$_{LM\ 3b}$, a constitutive form of cytochrome P-450, from rabbit liver microsomes. Biochem. Biophys. Res. Commun. 91,

1075-1081.

116. Johnson, E. F. (1980). Isolation and characterization of a constitutive form of rabbit liver microsomal cytochrome P-450. J. Biol. Chem. 255, 304-309.

117. Elshourbagy, N. A. and Guzelian, P. S. (1980). Separation, purification, and characterization of a novel form of hepatic cytochrome P-450 from rats treated with pregnenolone-16α-carbonitrile. J. Biol. Chem. 255, 1279-1285.

118. Ullrich, V. (1979). Cytochrome P-450 and biological hydroxylation reactions. Topics Current Chem. 83, 67-104.

119. Tierney, B., Weaver, D., Heintz, N. H., Schaeffer, W. I. and Bresnick, E. (1980). The identity and nuclear uptake of a cytosolic binding protein for 3-methylcholanthrene. Arch. Biochem. Biophys. 200, 513-523.