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AGE RELATED CHANGES IN THE INDUCTION OF HEPATIC
MIXED-FUNCTION MONOOXYGENASE SYSTEM IN MINIATURE PIGS:
EFFECTS OF PENTACHLOROANISOLE,
PENTACHLOROPHENOL AND PHENOBARBITAL

BY

ALAN PAUL AGINS

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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1982

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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ABSTRACT

Agins, Alan P., Ph.D., University of Rhode Island, 1982. Age Related Changes in the Induction of the Hepatic Mixed-Function Monooxygenase System in Miniature Pigs: Effects of Pentachloroanisole, Pentachlorophenol and Phenobarbital. Major Professor: Dr. George C. Fuller.

Pentachloroanisole (PCA), an environmental degradation product of the biocide pentachlorophenol (PCP), has been detected in the food chain. The metabolic fate of PCA was examined in miniature pig hepatic microsomes, in vitro. The compound was shown to be a substrate for a cytochrome P450-dependent demethylation reaction, which results in the regeneration of the parent compound, PCP. A disproportionately large increase in PCA demethylase activity (PCADM) following pretreatment with phenobarbital suggests that the compound is preferentially metabolized by specific-inducible form(s) of cytochrome P450.

A comparison of the effects of PCA and purified PCP on the hepatic MFO system of miniature pigs was conducted at various stages of postnatal development. Phenobarbital was utilized as a positive control for induction. PCA, PCP and phenobarbital (10 mg/kg/day X 4 days, P.O.) were administered to piglets at 1, 4, and 8 weeks of age and the levels of cytochromes P450 and b_5 , and the activities of NADPH-Cytochrome c reductase, aniline hydroxylase (ANOH), p-nitroanisole demethylase (NADM), and PCA demethylase were determined. In one week old piglets, PCA produced significant increases in all parameters measured, with the greatest effect (300% of control) on its own in vitro metabolism. The pleiotropic response evoked by PCA

was similar to that of phenobarbital, but of lesser magnitude. PCP produced small increases in only P450 and nitroanisole demethylase. The qualitative differences in the induction patterns produced by PCA and PCP suggests that the two compounds exert different effect on MFO. By eight weeks of age, the magnitude of induction by PCA was diminished. Furthermore, although specific activities for ANOH, NADM, and PCADM in phenobarbital treated pigs were similar at 1 and 8 weeks of age, examination of catalytic activity profiles suggested an age dependent decrease in the induction of specific forms of cytochrome P450. On further investigation, Eadie-Hofstee plots from kinetic experiments with ANOH and PCADM exhibited biphasic patterns suggestive of multiple forms of P450 catalyzing the same reaction. By integrating the effects of age and treatment on the various kinetic species for each substrate, a minimum of four forms of cytochrome P450 are suggested to exist in miniature pig hepatic microsomes. Of the four forms, two are inducible by phenobarbital and one of these forms appears to display age-dependency in the magnitude of induction. These data indicate that MFO induction by exogenous chemicals varies qualitatively as well as quantitatively with age.

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INTRODUCTION

In recent years, research in perinatal toxicology has gained substantial impetus and is now considered by many an important facet for the complete evaluation of chemical hazards to man. The increased interest in developmental toxicity studies has grown from both theoretical considerations and practical experience. In pharmacology, it has long been recognized that an effect in the infant cannot always be predicted by extrapolation of adult response. Yet, with the exception of teratology studies, the vast majority of toxicological research is conducted in relatively mature animals. Since the human infant is often unavoidably exposed to the same chemical milieu as the adult population, responses of the neonate need to be examined and defined.

It appears that within this framework, perinatal enzymology may play a central role. Chemically induced perturbations of enzyme ontogenetic profiles, alterations in isoenzyme patterns, or other modifications to key metabolic pathways may result in subtle biochemical lesions in the absence of apparent morphological or functional abnormalities. Furthermore, such changes may have profound effects on maturational processes or future health.

One enzyme system, the microsomal mixed-function monooxygenase (MFO) system, plays a critical role in the metabolism of foreign compounds such as drugs, pesticides and carcinogens. The enzyme system also has important homeostatic functions through its metabolism of steroids, heme, fatty acids and a number of other endogenous substrates.

Additionally, the multicomponent enzyme system, in many cases, is extremely sensitive to induction by exogenous chemicals. This adaptive mechanism, once thought to be strictly beneficial for the detoxification of xenobiotics, has received much attention over the last decade with the knowledge that many chemicals, particularly carcinogens, require metabolic activation prior to exhibiting their detrimental effects.

This study attempts to examine temporal changes in the response of hepatic microsomal enzymes to xenobiotic induction during early postnatal life in miniature pigs. The effects of the environmental contaminants, pentachlorophenol (PCP) and pentachloroanisole (PCA) on the levels and activity of various MFO parameters are investigated with emphasis on age dependent differences in the response to induction. The in vitro metabolism of PCA is characterized and subsequently utilized in activity measurements. Due to its well established potent, comprehensive inductive effects, phenobarbital is used as a positive control for comparisons. Finally, alterations in cytochrome P450 subpopulations are examined as a determinant for age and treatment effects.

LITERATURE SURVEY

Historical perspectives of Microsomal Metabolism

The metabolism of foreign compounds by hepatic microsomes was first described by Mueller and Miller (1949). These investigators showed that both the oxidative N-demethylation and the reduction of the azo linkage of aminoazo dyes were catalyzed in vitro by microsomes derived from rat liver homogenates. In 1955, Brodie and coworkers extended these initial studies by utilizing various drugs as substrates, and after compiling the research efforts from numerous laboratories (Brodie et al., 1958) proposed that the microsomal fraction of cells was responsible for a vast number of reactions involved in drug metabolism. The reactions had a strict requirement for both molecular oxygen and pyridine nucleotides as reducing agents, which lead to the classification of this enzyme system as a mixed-function oxidase (Mason, 1957) or monooxygenase (Hayaishi, 1962). Further direct support for this terminology was provided by Posner et al. (1961) who showed that the oxygen molecule inserted into a hydroxylated product was derived from $^{18}\text{O}_2$ and not water.

Although investigators in the early 1950s had successfully isolated and characterized various individual components of microsomes, a

direct connection to drug metabolizing capacity remained obscure. In the latter part of the decade, Klingenberg (1958) and Garfinkel (1958) reported that an additional component, a cytochrome not yet accounted for, existed in microsomes. The ability of this cytochrome to undergo unique spectral changes in the presence of carbon monoxide led to the early name of "CO Binding Pigment". Omura and Sato (1964) further characterized this new cytochrome and labeled it Cytochrome P450 due to the location of the Soret peak of the reduced, carbon monoxide complexed material. The reactivity of Cytochrome P450 with carbon monoxide provided a powerful tool for experimentation linking microsomal electron transport to drug metabolism. Estabrook et al., (1963) provided firm evidence that Cytochrome P450 was a crucial component for hydroxylation reactions in adrenal cortex particles and subsequently established its role as the "terminal oxidase" of the microsomal drug-metabolizing enzyme system (Cooper et al., 1965).

In 1968, Lu and Coon successfully solubilized and resolved the enzyme system into fractions containing Cytochrome P450, NADPH-Cytochrome P450-Reductase, and a heat stable factor, subsequently shown to be phosphatidylcholine. The ability to reconstitute catalytic activity toward a variety of drug, steroid and fatty acid substrates was a major milestone and opened the way for more refined approaches to mechanistic and functional studies.

In the two decades following the initial studies of microsomal mediated metabolism an extremely large body of literature has been generated from research in the areas of pharmacology, toxicology, biochemistry, endocrinology, molecular biology and genetics.

Components of the Microsomal MFO System

The microsomal fraction of liver contains at least three flavo-proteins; NADPH-Cytochrome P450-Reductase, NADH-Cytochrome b_5 -Reductase and Amine Oxidase, two heme proteins; Cytochrome P450 and Cytochrome b_5 , and a non-heme iron protein; Stearyl-CoA Desaturase. In the presence of the microenvironment of the smooth endoplasmic reticulum these components work either independently or in concert in an electron transfer capacity. The following discussion will be limited to those components investigated in the present study.

NADPH-Cytochrome P450-Reductase

NADPH-Cytochrome P450-Reductase (NADPH Dehydrogenase. EC 1.6.2.4) was first observed in whole liver extracts by Horecker (1950). Phillips and Langdon (1962) and Williams and Kamin (1962) identified the microsomal fraction as the origin of this enzyme and upon purification revealed that the protein is capable of reducing a wide variety of both one and two electron acceptors. The nomenclature for this enzyme varies between laboratories and is dependent on both the acceptor used and the condition for the assay. Artificial acceptors such as cytochrome c and ferricyanide are routinely utilized due to the ease of measurement, the extended linearity and the higher turnover in these assays. Although Cytochrome P450 is known to be the native acceptor, the term NADPH-Cytochrome c Reductase is often used interchangeably. One major difference is that phosphatidylcholine is required for

reduction of Cytochrome P450 in reconstituted systems but not for electron transfer to artificial acceptors (Strobel and Digman, 1978).

The mechanism by which the enzyme transfers electrons to Cytochrome P450 has been extensively investigated. It has been demonstrated that the protein contains equimolar amounts of FAD and FMN (Yasukochi and Masters, 1976). Vermillion and Coon (1978), using FMN-depleted reductase demonstrated that the enzyme containing only FAD remains capable of being readily reduced by NADPH and suggested that the FMN moiety probably interacts directly with Cytochrome P450. Although conclusive evidence for the exact mechanism is still lacking, the overall sequence of electron transfer from NADPH to FAD to FMN to P450 is consistent with the observed biphasic reduction kinetics of the enzyme. Furthermore, this scheme appears to be thermodynamically favorable as a function of reduction potential differences of the two flavin moieties (Oprian et al., 1979).

In addition to its essential role in transferring reducing equivalents to Cytochrome P450, the reductase has also been reported to have P450-independent catalytic activity. Hernandez et al., (1967) showed that Cytochrome c Reductase catalyzed the reduction of azo dyes without benefit of P450. Furthermore, the participation of this flavo-protein in the initiation of microsomal NADPH-dependent lipid peroxidation has been reported (Pederson et al., 1973).

Cytochrome B₅

Cytochrome b₅ was originally isolated from microsomes by Strittmatter and Velick (1956). In addition to purifying and

characterizing this cytochrome the investigators also purified a flavo-protein specific for NADH which transferred electrons to Cytochrome b_5 (Strittmatter and Velick, 1956a). Although the physiological function of this enzyme system was not apparent at that time, approximately a decade later Holloway and Wakil (1970) implicated Cytochrome b_5 in microsomal fatty acid desaturation reactions. It is now well established that this microsomal electron transport system is composed of NADH-Cytochrome b_5 -Reductase, Cytochrome b_5 and a cyanide-sensitive terminal desaturase which functions to convert Steryl-CoA to Oleyl-CoA (Prasad and Joshi, 1979).

Less well established has been the role of these components with respect to the "drug-metabolizing" enzyme system. The synergistic effect of NADH on NADPH-dependent reactions led early investigators to postulate a permissive role for Cytochrome b_5 . With the knowledge that Cytochrome P450 mediated oxidations required two separate electron transfers, it was suggested that the second electron may be denoted via Cytochrome b_5 (Estabrook and Cohen, 1969). Lu and coworkers (1974) and Imai and Sato (1977) demonstrated that in reconstituted metabolic systems, Cytochrome b_5 was not an obligatory component for activity, although in both studies synergistic effects were seen. It was also suggested by Lu et al. (1974) that the role of Cytochrome b_5 may be dependent on such factors as tissue, sex, age and the particular substrate utilized. More recently, Imai (1979), using various purified forms of Cytochrome P450, showed that Cytochrome b_5 was required for maximal activity with some forms of P450, but had little or no effect

on other forms in reconstituted systems. Sugiyama (1979) reported the purification of a unique form of Cytochrome P450 from rabbit liver. This form of P450 had a high affinity for Cytochrome b_5 and required its presence for reconstitution of catalytic activity with nitroanisole as substrate. Further support for the role of Cytochrome b_5 in P450-dependent reactions has come from the finding that in addition to NADH-Cytochrome b_5 -Reductase, NADPH Cytochrome P450 Reductase can efficiently reduce Cytochrome b_5 (Enoch and Strittmatter, 1979). Thus it appears that Cytochrome b_5 serves a central role in microsomal electron transport by interacting with two separate reductases, a desaturase and some forms of Cytochrome P450.

Cytochrome P450

Cytochrome P450 is the dominant heme protein in microsomes. The mammalian liver is the richest source of the hemoprotein, however, Cytochrome P450 is also found in kidney, lung, skin, intestinal tract, adrenal gland, placenta, ovary and blood platelets (Hodgson and Dauterman, 1980). cytochrome P450 as the terminal oxidase of the MFO system displays functional heterogeneity in the various tissues, yet although many of the extrahepatic tissues are capable of supporting low levels of xenobiotic metabolism, empirically, the liver is the major site for such metabolism. The Cytochrome P450 in kidney, for example, appears to be quite active in the omega oxidation of fatty acids (Masters et al., 1980), while the P450 in adrenal gland mitochondria is mainly responsible for the metabolism of steroid hormones (Sih, 1969).

The ability of Cytochrome P450 to serve as the terminal oxidase of the MFO system lies in its ability to bind substrate, undergo reversible oxidation state transitions, bind molecular oxygen and activate it for subsequent insertion into the substrate molecule. The binding of substrate by P450 results in measurable spectral perturbations. Remmer et al. (1966) and Schenkman et al. (1967) described characteristic absorbance changes upon addition of various compounds to microsomal suspensions. There are primarily two major and one minor types of spectra observed dependent on the compound utilized. A Type I difference spectrum is characterized by an absorption maximum between 385 - 390 nm and a minimum at approximately 420 nm. A Type II spectrum is characterized by a peak at about 430 nm and a broad trough between 390 - 410 nm. A third type of difference spectrum was also described as a reverse Type I. This spectrum is characterized by a peak at 420 nm and a trough between 385 - 390 nm and thus appears to be a mirror image of a Type I spectrum.

Whereas Type I producing compounds represent a large, structurally diverse group including drugs, pesticides and steroids, Type II compounds tend to be primary amines, pyridines and imidazole compounds (Mailman et al., 1974). Gigon and coworkers (1968) found that the reduction of Cytochrome P450 by NADPH-Cytochrome P450-Reductase was accelerated in the presence of Type I compounds while unchanged or decreased in the presence of Type II compounds. These results correlated well with the fact that Type I compounds were generally better substrates for P450 dependent oxidation than Type II compounds and

further indicated that the substrate binding preceded reduction in the dominant reaction pathway.

In conjunction with optical difference spectra studies, electron paramagnetic resonance (EPR) spectroscopy had shown that Cytochrome P450 can exist in a high-spin (Fe^{3+} heme: $S = 5/2$), a low-spin (Fe^{3+} heme: $S = 1/2$) or most commonly in a mixed spin state (Jefcoate, 1978). A Type I change correlates with an increase in high-spin character upon binding of the substrate, while a Type II change reflects a conversion of native high-spin P450 to a low-spin complex of the heme and the ligand. In this respect, two modes of compound interaction with P450 are now recognized as "substrate" binding and "ligand" binding (Testa and Jenner, 1981).

From the integration of diverse physical and chemical studies, a structural model of the active site of Cytochrome P450 has been proposed (Lipscomb and Gunsalus, 1973; Rein et al., 1976).

In these models, the active site contains an iron protoporphyrin IX moiety in a large, relatively accessible hydrophobic pocket in the apoprotein. The heme is loosely anchored to the site by a combination of hydrophobic forces and covalent bonds to the central iron ion. The fifth iron ligand has been reported to be a thiolate anion contributed by a cysteine residue of the protein (Dolphin et al., 1979). This anionic mercaptide linkage has been implicated for both the unique spectral properties of this cytochrome and in having important electronic effects on oxygen activation in the normal cycle of catalysis (Collman and Sorrell, 1975). The sixth axial ligand

has been reported to be either oxygen, in the form of water or a hydroxyl group from a proximal amino acid (Griffin and Peterson, 1975) or nitrogen in the form of histidine. Although conclusive evidence is lacking, it is generally accepted that the sixth linkage is relatively weak, but functions to hold the heme iron in a square octahedral, hexacoordinated configuration. Displacement of the weak bond by stronger ligands such as amines and pyridines results in a heme-ligand complex typified by a Type II spectrum and low-spin cytochrome. The "locking" of Cytochrome P450 into a low-spin state appears to be responsible for some of the inhibitory actions exerted by such compounds (Testa and Jenner, 1981).

In contrast to ligand binding, substrate binding involves mainly hydrophobic interactions between non-polar regions of the protein and the substrate. This binding results in a dissociation of the native sixth ligand linkage and a change in the configuration of the heme molecule from octahedral, hexacoordinated to square pyramidal, pentacoordinated (White and Coon, 1980). This change also results in a conversion to a high-spin state, which increases the redox potential of the system and creates a more favorable electron flow sequence to Cytochrome P450 (Slingar et al., 1979).

The mechanism of binding and subsequent activation of molecular oxygen by Cytochrome P450 is quite complex and has recently been reviewed (White and Coon, 1980). Essentially, following the binding of oxygen to the reduced P450-substrate complex, a second electron transfer results in the creation of peroxide anion. This reactive

protein-complexed intermediate then reacts with substrate to form a hydroxylated compound and water. In addition to this mechanism, evidence has accumulated indicating that in some cases, Cytochrome p450 acts in an oxidase or peroxygenase capacity resulting in the production of hydrogen peroxide or other peroxy compounds. The homolytic cleavage of the oxygen-oxygen bond may lead directly to insertion of a hydroxyl group into a substrate independent of the NADPH/O₂ pathway (Coon, 1981).

Induction of Monooxygenase Activity

Conney and Burns (1959) first demonstrated the phenomenon of drug induced synthesis of liver microsomal enzymes. This finding served to substantiate a number of earlier assumptions concerning the MFO system and became a powerful tool for the study of induced enzyme synthesis in general. Extensive reviews on MFO induction have been published (Conney, 1967; Mannering, 1968).

Historically, inducers of MFO have fallen into one of two categories. One group, containing numerous drugs and xenobiotics of diverse chemical structure, is best typified by phenobarbital. The other class consists primarily of polycyclic aromatic hydrocarbons of which 3-methylcholanthrene is generally recognized as the prototype (Conney, 1967). The effects of these two types of inducers are similar in some respects in that they lead to increased protein synthesis, as determined by amino acid incorporation both in vivo and

in vitro (Kato et al., 1965; Gelboin, 1964). The increase in protein synthesis can be blocked by the metabolic inhibitor, ethionine (Conney et al., 1956), and at the level of translation by puromycin, however, the inhibition by Actinomycin D indicates that induction by both types of inducers involves DNA-dependent RNA synthesis (Nebert and Gelboin, 1969). There are however, major differences between the two classes of inducers.

Phenobarbital is a much more comprehensive inducer of MFO components than 3-MC. Among the effects produced by phenobarbital are an increase in both the total liver protein and the specific protein content in microsomes (Conney et al., 1960), increased levels of Cytochrome P450, Cytochrome b_5 , NADPH-Cytochrome P450 Reductase, and numerous Cytochrome P450-dependent reactions (Kuriyama et al., 1969; Conney, 1967). In addition, a marked proliferation of the smooth endoplasmic reticulum can be observed by electron microscopy (Fouts and Rogers, 1965) which is consistent with the observed increase in microsomal protein and phospholipid content following induction (Orrenius and Ericsson, 1966).

In contrast to phenobarbital, 3-MC and congeners induce fewer components of microsomes. There are, however, two major qualitative changes observed in microsomes following 3-MC treatment. A shift in the wavelength of the reduced CO peak to 448 nm and a substantial increase in the activity of Aryl Hydrocarbon Hydroxylase, have been associated with the de novo synthesis of a new hemoprotein, Cytochrome P448 (Alvares et al., 1967; Kuntzman et al., 1969).

A third class of inducers, the polychlorinated biphenyls, came to interest during the 1970s as a result of their increasing contamination of the environment (Alvares et al., 1973). These compounds, typified by Aroclor 1254, exhibited potent inductive effects which were consistent with both phenobarbital and 3-MC effects. On further examination, it was concluded that this combined effect was probably due to the complex mixture of isomers in various preparations (Stonard and Greig, 1976).

Although most inducers of MFO usually require multiple administrations with fairly high doses to produce their maximal effect, the extremely toxic compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was found to be an inducer of the 3-MC type and was more potent than any other inducer on a dose-per-unit body weight basis (Poland and Glover, 1974). Lucier et al. (1975) demonstrated that a single oral dose of TCDD at 3 µg/kg to pregnant rats caused marked elevations in both maternal and progeny hepatic MFO parameters. These effects were still present months later.

Although the number of xenobiotics capable of induction is quite large they have in the past been conveniently placed into one of the two classes. It is, however, becoming evident as assay capabilities and separation techniques expand, that many compounds exhibit unique induction properties. The effects of pregnenolone-16- α -carbonitrile (Elshourbagy and Guzelian, 1980) and isosafrole (Ryan et al., 1980) cannot be definitively categorized as either 3-MC or phenobarbital type of induction.

The mechanism(s) by which inducers lead to increased synthesis of MFO components remains somewhat obscure. The documented increase in DNA-dependent RNA synthesis during the induction process has led investigators to propose that inducers play some role as derepressors of regulatory or other genes (Nebert et al., 1981). One early theory to account for the ability of such a diverse structural group of compounds to invoke similar effects was proposed by Marshall and McLean (1971). These authors suggested that induction was mediated through an "endogenous factor", which was normally inactivated by Cytochrome P450. Compounds capable of binding to P450 would block inactivation, resulting in increased cellular levels of the factor and subsequent induction. This theory is supported by the empirically derived evidence that a very large proportion of inducers are either substrates or inhibitors of P450 capable of producing Type I binding spectra (Mannering, 1969). Identification of a specific "endogenous factor" however, has yet to be accomplished.

The discovery of a hepatic cytosolic "species" in mice, which stereospecifically and reversibly bound TCDD, led to the postulation of a specific receptor for the induction of Aryl Hydrocarbon Hydroxylase (Poland et al., 1976). This receptor was shown to bind a variety of halogenated dibenzo- dioxins and furans with different affinity which closely correlated with their potencies as inducers. Furthermore, although the inductive polycyclic aromatic hydrocarbons competed with TCDD for binding, phenobarbital, pregnenolone-16- α -carbonitrile and steroid hormones displayed no specific binding. This cytosolic

species, now termed the Ah receptor, is recognized as the major product of regulatory gene(s) in the murine Ah locus (Nebert et al., 1981). The receptor has also been detected in rats and rabbits (Kahl et al., 1980). Excellent reviews of the research on the Ah locus and genetic control of induction have recently been published (Nebert et al., 1981, 1982). Of great interest has been Nebert's comparison of the Cytochrome P450 and immune systems and his hypothesis that mammalian tissues have the genetic capacity to produce hundreds or thousands of inducible forms of P450 in response to different chemical stimuli.

Developmental Aspects of Monooxygenase Activity

Since the first reports by Jondorf et al. (1959) and Fouts and Adamson (1959), investigators of the perinatal development of MFO activity have flourished in the literature. Extensive reviews by Short et al. (1976), Neims et al. (1976) and most recently Klinger et al. (1981) have indicated that although variations exist between laboratories with respect to species, sex and substrates utilized, the general patterns of development are similar in most mammalian species. Essentially the monooxygenases are apparently absent or barely detectable in fetal organs, especially in the fetal liver, until just before birth. After birth, in most species, development of specific microsomal components and metabolic pathways generally follow one of three profiles: (1) activity rises rapidly from birth and

plateaus at maturity, (2) activities peak shortly after birth, often exceeding adult levels, and subsequently decrease to adult levels, or (3) activity remains low during early postnatal development and rises rapidly coincident with the onset of sexual maturity.

A number of factors have been postulated to explain the deficiency of MFO activity during late fetal and early postnatal life. Wilson (1969) suggested that high levels of somatotropin during development inhibited MFO. Soyka and Long (1972) found that progesterone inhibited MFO in vitro and suggested that this maternally derived inhibitor was responsible for decreased activity in fetal and neonatal animals. Short et al. (1976) and Kuenzig et al. (1975) implicated the immaturity of hepatocellular morphology, especially the absence of smooth endoplasmic reticulum in the fetus and neonate, as a primary determinant for low MFO activity. Both groups demonstrated a good correlation between age related development of smooth endoplasmic reticulum and increases in hepatic drug metabolic activity. Other factors, including the presence of endogenous P450 ligands or age related phospholipid differences in microsomal membranes, have recently been discounted as responsible for the age differences in biotransformation (Klinger et al., 1981). Furthermore, these authors suggested that qualitative differences in Cytochrome P450, or in the ratio of different P450 subpopulation may be responsible for the many variations observed in developmental profiles.

Induction of monooxygenase activity during perinatal development appears to be highly dependent on both the inducer and species utilized. In laboratory animals with long gestation periods, such as the

guinea pig, phenobarbital type inducers are effective in increasing MFO activity in the late gestational fetus (Kuenzig et al., 1975). Species less developed at birth, such as the rat, are generally refractory to phenobarbital induction in utero (Guenther and Mannering, 1977; Cresteil et al., 1979), but rapidly become responsive after birth. Shubert and Netter (1981) have further demonstrated that the onset of inducibility is independent of parturition, but is largely determined by the time passed since conception. In contrast, inducers of Cytochrome P448, such as TCDD and 3-MC, are quite effective trans-placental inducers (Guenther and Mannering, 1977; Lucier et al., 1975).

Early studies on the postnatal inducibility of MFO activity indicated that younger animals were more responsive to phenobarbital induction when measured as percent increase of specific activity over controls (Basu et al., 1971). Although such studies did not receive a great deal of attention at that point in time, more recently interest has been rekindled with the knowledge of the heterogeneity of Cytochrome P450. Atlas et al. (1977) showed that various P450 (P448) subspecies follow different developmental patterns in rabbit, as well as altered sensitivity to induction. Cresteil and coworkers (1979) reported age dependent changes in the catalytic activity of aniline hydroxylation and nitroanisole demethylation as a function of induction by either phenobarbital or 3-MC. Klinger et al. (1981) has summarized a number of reports showing greater enhancement of MFO by phenobarbital in young rats. The conclusion that is beginning to

emerge from these studies is that the neonate is not only quantitatively different from the adult in its capacity to metabolize compounds, but may be qualitatively different in its response to induction.

Heterogeneity of Cytochrome P450

Species differences in the rates of metabolism of several narcotics (Axelrod, 1956) and the induction of specific metabolic pathways at the expense of others in a single species (Conney et al., 1959) led to the early proposal of more than one liver microsomal drug metabolizing system. With the discovery that Cytochrome P450 was the terminal oxidase of the enzyme system (Cooper et al., 1965) numerous investigations focused on determining whether the broad substrate specificity, the equally diverse number of metabolic reactions, and the induced alterations in specific catalytic activities could be accounted for by a single Cytochrome P450.

Early approaches to resolving the question of multiple forms of P450 were based on manipulations of various biochemical and biophysical properties of the enzyme in microsomal suspensions. The different, selective effects of phenobarbital and 3-MC induction have been well documented (Conney, 1967) and previously discussed. Similarly, in vitro inhibition studies have centered around the use of selective inhibitors of various reactions and forms of P450. SKF 525A, a potent inhibitor of drug metabolism, was utilized to demonstrate differences between the forms of Cytochrome P450 in control and phenobarbital

induced microsomes and that in 3-MC treated animals (Sladek and Mannering, 1969). It was generally accepted during this period that phenobarbital caused only a quantitative increase in the form of Cytochrome P450 normally present in uninduced microsomes, however, Grasdalen et al. (1975) demonstrated that phenobarbital induced microsomes displayed different characteristics from controls and indicated that metyrapone was more selective for the former while SKF 525A was more selective for uninduced form(s). Jonen et al. (1974) also indicated that metyrapone had a greater affinity for phenobarbital induced P450. Thus the early evidence indicated that phenobarbital induction led to qualitative as well as quantitative changes in microsomes. Naphthoflavone (7,8-Benzoflavone) strongly inhibits many reactions induced by 3-MC (Burke et al., 1977), yet stimulates the activity of native and phenobarbital induced Cytochrome P450 (Cinti, 1978). Tetrahydrofuran has recently been shown to be a potent inhibitor of a form of P450 found in uninduced microsomes, but has little or no effect in phenobarbital induced forms of P450 (Hultmark et al., 1979). An extremely thorough review of the types of mechanisms of P450 inhibitors was recently published (Testa and Jenner, 1981).

Further evidence for the presence of multiple forms of Cytochrome P450 in liver microsomes has been deduced from kinetic studies with various substrates. Although the metabolism of many compounds appears to follow normal Michalis-Menten kinetics, with resulting linear double reciprocal plots, a number of substrates such as aniline (McCoy, 1980), aminopyrine (Kotake, 1981) 7-ethoxycoumarin (Greenlee

and Poland, 1978; Boobis et al., 1981) and phenacetin (Boobis et al., 1981) have been shown to exhibit biphasic kinetic profiles indicative of multiple enzymes acting on the same substrate (Segel, 1975). Multiple forms have also been implicated (Shiverick and Neims, 1979) for the developmental changes observed in the hydroxylation of testosterone at the 6 β , 7 α and 16 α positions in rats (Conney et al., 1969).

The ability to solubilize and resolve microsomal enzyme components (Lu and Coon, 1968), has enabled a finer approach to studying the multiplicity of Cytochrome P450. A number of criteria have been utilized and include determination of molecular weights by SDS-PAGE, differences in spectral characteristics, differences in catalytic activities of purified forms, immunological properties, peptide mapping, and amino acid sequencing (Lu and West, 1980). By assimilating the research from numerous laboratories, the authors have indicated that thus far, depending on treatment, there are five to seven forms of P450 that have been isolated from rabbit, five to six forms from rat, four to six forms from mice, and at least two forms from pigs. An undetermined number may be present in human liver microsomes.

Pentachlorophenol and Pentachloroanisol

Pentachlorophenol (PCP) and the lower chlorinated phenols, tetra- and tri- chlorophenol have been used as fungicides, herbicides, insecticides, and precursors in the synthesis of other pesticides since the early 1930s. The literature on PCP is abundant. A review of the toxicology and occurrence of PCP in the environment up to 1967 was

published by Bevenue and Beckman (1967). More recently, Ahlborg and Thunberg (1980) compiled an extensive review of the literature from 1967 on, including some aspects not covered by the previous authors.

The acute toxicity of PCP has generally been attributed to the uncoupling effect of the compound on oxidative phosphorylation (Weinbach, 1954). The clinical symptoms associated with acute poisoning, including increased respiratory rate and volume, progressive neuromuscular weakness and increased body temperature are consistent with such a mechanism of action. Of greater concern, however, are the potential chronic effects of chlorinated phenols. The occurrence of PCP in water and the food chain (Ahlborg and Thunberg, 1980) coupled with the detection of this compound in the urine of a diverse population of non-occupationally exposed persons, suggests that PCP is quite ubiquitous in the environment.

Subacute and chronic toxicity studies (Knudsen et al.; Schwetz et al., 1977) have led to variable and inconsistent results, which have now been attributed to differences in the purity of the PCP preparations utilized (Ahlborg and Thunberg, 1980). Specifically, the presence of chlorinated dibenzodioxins and dibenzofurans in technical grades of PCP, and the documented wide spectrum of toxic manifestations of these agents (Kimbrough, 1972) precludes analysis of specific cause and effect relationships. Goldstein and coworkers (1977) demonstrated that technical grade PCP produced hepatic porphyria and dramatic increases in microsomal drug metabolizing activity in an eight month feeding study in female rats. The qualitative and quantitative

nature of these effects were consistent with the effects of a number of chlorinated dioxins. In the same study, purified PCP was shown to be devoid of any hepatic effects with the exception of a moderate increase in glucuronyl transferase activity at the highest dosage level. This study implied that many of the hepatic and extrahepatic effects reported in earlier studies were probably due to contaminants. Kimbrough and Linder (1975) reported that purified PCP, in addition to increasing liver size, produced an enlargement of hepatocytes, a slight increase in smooth endoplasmic reticulum and lipid vacuoles. The cause of these effects, however, is not known.

In contrast to studies on enzyme induction, a number of investigators have examined the potential for PCP and other chlorinated phenols to inhibit MFO activity. Arrhenius et al., (1977) showed that in vitro, PCP selectively inhibited the C-oxygenation of dimethylaniline (P450-dependent) thus favoring the N-oxygenation (P450-independent). The author concluded that these results were due to either a specific attack on the P450 enzyme or a disturbance in the transfer of electrons to P450. Carlson (1978) reported inhibition of EPN detoxification and nitroanisole demethylation in vitro by various trichlorophenol isomers. The observed inhibition appeared to be non-competitive and was not demonstrated in microsomes obtained from treated animals. It thus appears that PCP and congeners may be similar to various other phenols and alcohols in their interaction with Cytochrome P450 (cf Testa and Jenner, 1981). Furthermore, a competitive aspect for inhibition may be consistent with the finding that PCP is

a substrate for Cytochrome P450 (P448) mediated dechlorination (Ahlborg, 1978).

Pentachloroanisol (PCA), the methyl ether of PCP, has been found in lake sediments and fish tissues (Kuehl et al., 1978), in shellfish (Miyazaki, 1981) and in the blood and milk of cows exposed to commercial grades of PCP (Firestone et al., 1979). The occurrence of PCA in the environment has been attributed solely to the degradation of PCP by microorganisms in soil and wood (Kaufman, 1978). Cserjesi and Johnson (1972) reported the capacity of three species of *Trichoderma* to methylate PCP in liquid cultures. Curtis et al. (1972) reported that feeding chickens maintained on shavings from wood treated with chlorophenols produced meat and eggs displaying a musty taint. The same group (Curtis et al., 1974) established that the methylating action of certain fungi present in poultry litter was responsible for the occurrence of chlorinated anisoles.

Despite these findings, pentachloroanisole has not received much attention in the toxicological literature. Glickman and coworkers (1977) studied the uptake, distribution and elimination of PCP and PCA in rainbow trout. Their results indicated that PCA is more readily bioaccumulated, more persistent, and that the presence of PCP-glucuronide in the bile of PCA treated fish indicated some demethylation of the compound in vivo. The same group (Vodicnik et al., 1980) undertook similar experiments in female mice. The results of this study, however, indicated that the pattern of distribution and

elimination of PCA was similar to that for PCP in rodents. They concluded that PCA must be demethylated prior to excretion and that this step was probably rate limiting for clearance.

Miniature Pigs

The development of a strain of genetically small pigs was initiated at the Hormel Institute of the University of Minnesota in 1949 (England, 1954). Since then, other breeds of miniature pigs have been established in the United States and abroad.

The use of the pig in biomedical research received much attention in the 1960s by virtue of its similarities to the human in renal, cardiovascular, and digestive tract anatomy and physiology, dental characteristics, eye structure, and skin morphology. In addition to these traits, the pig is capable of developing many human pathological conditions including atherosclerosis, gastric ulcer and obesity (Pond and Houpt, 1978). The young pig has found greatest utility in studies of nutrition. Since the digestive physiology and nutrient requirements of newborn pigs is remarkably similar to human infants, baby pigs have been used to develop and evaluate some human infant formulas (Book and Bustard, 1974). Although the pig has received increasing popularity as a laboratory model, housing and handling constraints continue to restrict its use to larger, well equipped facilities.

The use of the swine in drug toxicity studies was advocated by Earl et al. (1964). Investigators of drug metabolism pathways in miniature pigs, however, have been extremely limited. In an attempt to

find a suitable "metabolic" replacement for the dwindling supply of rhesus monkeys, Litterst et al. (1976) conducted a comparative study using the miniature pig as one of five species. Based on an arbitrary scale, the authors concluded that the miniature pig was the most comparable species. Freudenthal et al. (1976) further characterized some parameters of the MFO system in miniature pigs ranging in age from two to eight months. They concluded that the two month old pig demonstrated adult levels of activity. Early postnatal development of the MFO system has been reported for the domestic, Duroc pig (Short and David, 1970; Short and Stith, 1973), however to date similar studies in the miniature pig have not been found in the literature.

EXPERIMENTAL

Materials

Pentachlorophenol (99%) was purchased from Aldrich Chemical Company (Milwaukee, WI). The stock material was subjected to further purification as described below. Pentachloroanisole was synthesized from the purified PCP as below. Sodium Phenobarbital was purchased from Mallinkrodt Chemical Works (St. Louis, MO).

NADP, NADPH, NADH, Glucose-6-Phosphate and Glucose-6-Phosphate Dehydrogenase were purchased from PL Biochemicals, Inc. (Milwaukee, WI). Aniline hydrochloride was purchased from Fisher Scientific (Silver Spring, MD). Sigma Chemical Company (St. Louis, MO) was the supplier of cytochrome c (Type III) and p-nitrophenol standard solution. Eastman-Kodak (Rochester, NY) was the supplier of p-nitroanisole and p-aminophenol.

All other reagents utilized throughout the investigation were analytical grade or better.

Preparation of PCP and PCA

Pentachlorophenol (Aldrich 99%) was further purified in an attempt to remove trace residual of non-phenolic contaminants. Twenty grams of PCP, Aldrich 99% were dissolved in 100 mls methanol in a 1.0 liter

separatory funnel. Fifty mls of 2.5 N NaOH were added, followed by 200 mls distilled water. The contents were extracted four separate times with 100 mls petroleum ether, which was discarded after each extraction by aspiration. The aqueous layer was then acidified with 20 mls concentrated HCl. The resulting white precipitate was transferred to a Buchner funnel and vacuum filtered. The remaining material was washed copiously with distilled water and then transferred to a clean container and dried overnight in a vacuum oven. The procedure decolorized the starting material (yellow to white) and the final product gave a melting point of 187-190^o, consistent with a purified standard.¹ Further analysis for the presence of contaminating materials was kindly provided by Mr. Ron Thomas of the Office of Pesticide Programs, US EPA, Beltsville, MD. A summary of this analysis is provided in Appendix A.

Pentachloroanisole was synthesized by methylating PCP using diazomethane (P. Sapienza and G. Ikeda, personal comm.). Ten grams of purified PCP were dissolved in a small volume of methanol. A three-fold molar excess of diazomethane, prepared using a Diazald Kit (Aldrich Chemical Company, Milwaukee, WI) was added to the PCP-methanol solution. The solution was then evaporated under nitrogen. The resulting material was then recrystallized with hot 95% ethanol. Activated charcoal was added to aid in decolorizing. The solution was filtered

¹Purified standards of pentachlorophenol and pentachloroanisole were provided by the Division of Chemistry, Bureau of Foods, Food and Drug Administration.

while hot to remove the charcoal and again after cooling. The resulting PCA was dried overnight in a vacuum oven. The newly synthesized PCA had a melting point of 106-107° which agreed with a purified standard. Thin layer chromatographic analysis using Benzene:MeOH (95:5) showed a single spot (Rf 0.91) identical to the PCA standard. No PCP (Rf 0.47) was detected in the sample. Further analysis for contaminants was conducted as above.

Animals

The miniature pigs utilized in this study were a cross of Hanford and Hormel strains belonging to FDA's Beltsville Research Facility. Sows were bred at a swine breeding facility operated by Environmental Consultants, Inc., Suffolk, Virginia. Bred sows were transferred to the Beltsville Research Facility at approximately the beginning of their third trimester (70 days of gestation), where they were maintained until farrowing. From the 109th day of gestation, sows were housed in individual pens containing sterilized corn cob bedding and equipped with an overhead heat lamp. Within 24 hours of birth, piglets were assigned a unique identification number and received 1 cc iron dextran (Nomemic; Burns-Biotec, Omaha, NB). Piglets were maintained with the sow for 96 ± 12 hours after birth at which time they were removed to individual stainless-steel metabolism cages in a specially designed neonatal nursery. The temperature and humidity in the nursery were maintained at approximately 90°F and 40%, respectively, during the first few weeks and thereafter the temperature was reduced at

a rate of 2-5°F per week to a minimum of approximately 72°F. A twelve hour light/dark cycle was maintained through the entire study.

Weaned piglets were fed a diet of whole cow's milk obtained from the USDA facility (Beltsville, MD). Milk diet was supplemented with 0.4% of a multivitamin mixture (Dawes STRESSEZ). During the first few weeks in the nursery, piglets received amounts of milk equal to approximately 20% of their body weight, divided into three equal portions at 8:00 A.M., Noon and 4:00 P.M. Milk was heated to room temperature prior to feeding. As animals increased in size and weight, diet was adjusted accordingly so that by eight weeks of age, piglets received approximately 30% of their body weight. Piglets that failed to thrive, as determined by weight gain and overall condition, were excluded from the study. Piglets were assigned to treatment groups based on a random division of littermates across the four experimental treatments. Each treatment group had equal numbers of male and female piglets.

Animal Treatments

Piglets were treated at 1 week, 4 weeks, or 8 weeks of age with either PCP (10 mg/kg in corn oil), PCA (10 mg/kg in corn oil), Phenobarbital (10 mg/kg in water) or corn oil alone. Phenobarbital treated piglets received an equivalent volume of corn oil immediately after dosing. All dosages were adjusted such that 1 ml of solution was administered per kilogram of body weight. Dosing was done by oral administration using a syringe fitted with a 19 ga stainless-steel stomach

tube. Piglets were held securely with their mouths open and the tip of the stomach tube was placed to the rear of the oral cavity. After the contents of the syringe were ejected, the piglet was held until complete swallowing of the dose was evidenced.

Piglets were treated once a day at 8:00 A.M. for four consecutive days and sacrificed on the fifth day, approximately 24 hours after the final dose.

Preparation of Microsomes

Piglets were sacrificed by electrocution and then exsanguinated. Livers were removed and immediately placed into ice cold 0.02 M Tris-HCL (pH 7.4) containing 1.15% KCl. After removing the gall bladders, the livers were blotted dry and weighed.

A portion of the liver, between five and seven grams, was thoroughly minced with scissors, and transferred to a glass Potter-Elvehjem homogenizing tube. Three volumes of 0.02 M Tris-HCL were added and the contents homogenized with a teflon pestle attached to a variable speed motor. The homogenate was centrifuged for 15 minutes at 10,000 RPM in a Sorvall RC2-B refrigerated centrifuge at 4°C. The resulting supernatant was filtered through one layer of sterilized gauze to remove the floating fat layer and then centrifuged at 105,000 x g in a Beckman L2-65B ultracentrifuge. The supernatant was removed by aspiration and the microsomal pellet was resuspended in buffer and centrifuged again at 105,000 x g for 30 minutes. The

"washed" microsomal pellet was then gently resuspended with sufficient 0.02 M Tris-HCl buffer to give a final protein concentration of approximately 4-6 mg per ml. Microsomal suspensions were kept on ice until utilized.

Pentachloroanisole Demethylation

The in vitro metabolism of pentachloroanisole was determined by measuring the formation of the demethylated by-product, formaldehyde, according to the method of Nash (1953). Assays were performed in 25 ml erlenmeyer flasks and consisted of the following in 0.1 M Tris-HCl (pH 7.4): NADP (0.8 mM); glucose-6-phosphate (5.0 mM), $MgCl_2$ (5.0 mM), glucose-6-phosphate dehydrogenase (1.9 Units) and semicarbazide (1.0 mM).

Stock substrate was prepared by suspending PCA in 0.1 M Tris-HCl which contained 2.5 mg/ml Tween 80 and the mixture was sonicated for 5 minutes. The resulting milky white suspension appeared evenly dispersed and was vortexed prior to each addition to incubation mixtures. Substrate blanks were run for each individual sample and received an equal volume of the Tween 80 solution without PCA. Final PCA concentration in the assay was 0.5 mM.

Flasks containing cofactors and substrate (or blank) were preincubated for 10 minutes at 37°C to allow temperature equilibration. The reaction was initiated by the addition of microsomes (approximately 2-3 mg protein) and the mixtures (in a total volume of 2.5 ml)

were incubated aerobically, with vigorous shaking in a Dubenoff Metabolic shaking incubator for 10 minutes. The reaction was terminated with 0.5 mls of 50% TCA and the protein precipitated by centrifugation. A 1.0 ml aliquot of the clear supernatant was transferred to a clean test tube and 0.5 mls of Nash reagent (3.9 M Ammonium acetate; 0.039 M acetylacetone) was added. The resulting colored product was measured in an Abbott Bichromatic Analyzer (ABA-100) using a peak wavelength of 415 nm and sideband wavelength of 450 nm. An internal calibration factor, previously determined from formaldehyde standards, was utilized and results were obtained directly as nMoles HCHO/ml. Activity was calculated by subtracting substrate blank values from their corresponding sample value, which was then converted to nMoles HCHO/min/mg protein.

Determination of PCP Formation

Proof of the formation of pentachlorophenol (PCP) as a result of the demethylation of PCA was established qualitatively as follows: The PCA demethylation assay (above) was modified slightly such that the final volume was 1.0 ml. Assays were conducted in screw cap culture tubes and contained 0.2 mM PCA and microsomes from phenobarbital treated piglets. The reaction was terminated after 20 minutes by the addition of 1.0 ml 6 N HCl, followed by 5.0 mls hexane. Tubes were vortexed for 1 minute and then centrifuged to separate the aqueous and organic layers. The hexane phase was removed to a clean glass vial and the extraction was repeated. The combined hexane extracts

were evaporated to dryness under a stream of nitrogen at room temperature. Analysis for PCP (and PCA) was performed using a Waters Associates Liquid Chromatograph. The HPLC parameters were as follows: Solvent - $\text{MeOH:H}_2\text{O:Acetic Acid}$ (80:19:1), Flow Rate - 1.3 ml/min, Column - C-18 (reverse phase), UV Detector - 254 nm (P. Sapienza, personal communication). The residue was taken up in 0.1 mls of solvent and 20 μl were injected into the chromatograph. A standard containing both PCA and PCP was run immediately prior to the sample.

Aniline Hydroxylation

The para-hydroxylation of aniline was determined by measuring the formation of the product, p-aminophenol, according to the method of Imai et al. (1965). Incubation conditions were identical to those for PCA demethylation except semicarbazide was omitted. Aniline-HCl, dissolved in 0.1 M Tris-HCl, was added to give a final concentration of 5.0 mM. Blanks received buffer alone. Reactions were terminated after 10 minutes by the addition of 1.5 mls 50% TCA and the protein precipitated by centrifugation. To a 1.0 ml aliquot of the clear supernatant, 0.5 mls of 10% Na_2CO_3 was added to neutralize the acid. One ml of 2% phenol in 0.2 N NaOH was then added to each tube and the color allowed to develop at 37°C for 40 minutes. The resulting colored product was read in an Abbott Bichromatic Analyzer using a 650 nm primary and 550 nm sideband wavelength filter. An internal calibration factor, previously determined from p-aminophenol standards was utilized and

results were obtained directly as nMoles pAP/ml. Activity was converted to nMoles pAP/min/mg protein.

p-Nitroanisole Demethylation

The activity of p-Nitroanisole demethylase was determined by a modification of the procedure described by Netter and Seidel (1964) in which the production of p-nitrophenol is followed directly in the incubation mixture at 420 nm at pH 7.8.

The modified assay was performed in an Abbott Bichromatic Analyzer as follows: The reagent vial contained MgCl_2 , glucose-6-phosphate and glucose-6-phosphate dehydrogenase in 50 mM Tris-HCl (pH 7.6). Sample cups contained microsomal suspension, and multicuvette compartments contained either buffer or NADP (20 μl). The contents of the reagent vial and the multicuvette were maintained at 37°C. Reactions were initiated by an automated sampling system. At timed intervals, 10 μl aliquots of sample (microsomes) were drawn and dispensed together with 250 μl cofactor mix (reagent) into individual compartments of a multicuvette. The final concentrations in a total volume of 0.28 mls were: NADP (0.62 mM), glucose-6-phosphate (5.0 mM), glucose-6-phosphate dehydrogenase (0.9 Units), MgCl_2 (5.0 mM) and p-nitroanisole (1.2 mM). The total amount of microsomal protein in each assay was approximately 40-60 μg .

Each individual sample was run in the presence and absence of NADP and the absorbance differences recorded at 5 minute intervals at

415 nm and 450 nm. Activity was determined by subtracting the NADP blank value from its corresponding sample value and comparing that value to a p-nitrophenol standard curve generated along with the assay. Activity was converted to nMoles pNP/min/mg protein.

Cytochromes P450 and B₅

The content of Cytochrome P450 in microsomes was determined according to the method of Omura and Sato (1964). Microsomes were diluted in 0.1 M Potassium Phosphate Buffer (pH 7.5) to a protein concentration of about 1-2 mg/ml. A few milligrams of solid sodium dithionite were added, the contents mixed and 3 mls of the microsomal suspension were pipetted into each of two cuvettes. The cuvettes were placed into a Beckman Acta CII Split-beam Spectrophotometer and a baseline of equal light absorbance was recorded by scanning from 500 to 400 nm. The contents of the sample cuvette were then gently bubbled with carbon monoxide for 40 seconds and returned to the spectrophotometer. The difference spectra was recorded after 3 minutes (500-400 nm) and again after 5 minutes. The amount of Cytochrome P450 was calculated by using a millimolar extinction coefficient of $91 \text{ mM}^{-1} \text{ cm}^{-1}$ for the absorbance difference at 450-490 nm.

The content of Cytochrome b₅ was determined by the method outlined by Estabrook and Werringloer (1978). Fresh microsomes, at the same concentration used for P450 determinations, were placed into each of two cuvettes. After establishing a baseline, 10 ul of a 15 mM

solution of NADH were added to the sample cuvette and the contents mixed well. The reference cuvette received 10 μ l of buffer. Cytochrome b_5 was calculated from the absorbance difference between 426 nm and 409 nm using the millimolar extinction coefficient of $185 \text{ mM}^{-1} \text{ cm}^{-1}$.

NADPH - Cytochrome c - Reductase

The rate of reduction of cytochrome c was measured, as outlined by Mazel (1969) by following the increase in absorbance at 550 nm due to the appearance of reduced cytochrome c. Cuvettes contained in a final volume of three mls; 0.15 μ moles cytochrome c, 3.0 μ moles KCN, and approximately 0.2-0.4 mg microsomal protein in 0.1 M Potassium Phosphate buffer (pH 7.6). The reaction was initiated by the addition of 0.45 μ moles NADPH to the sample cuvette and was followed for approximately 1 minute at room temperature in a Beckman Acta CII Spectrophotometer. Activity was calculated from the change in absorbance for 1 min using the extinction coefficient of $18.5 \text{ mM}^{-1} \text{ cm}^{-1}$ of reduced cytochrome c at 550 nm (Gigon et al., 1968).

Substrate Binding Spectra

Microsomes, in 0.1 M potassium phosphate buffer, were pipetted into each of two cuvettes and placed into a Beckman Acta CII Spectrophotometer. PCA, dissolved in methanol (20 mg/ml) was added to the sample cuvette in 1 μ l increments. Equal volumes of methanol were added to the reference cuvette. After each addition, the difference

spectra was recorded by scanning from 460 nm to 360 nm. The spectral dissociation constant (K_s) and maximum absorbance change were determined from double-reciprocal plots of $1/S$ versus $1/ABS$ (386–420 nm).

Determination of Kinetic Parameters

Kinetic profiles for aniline hydroxylation and PCA demethylation were determined in control and phenobarbital treated pigs at 1, 4, 8, and 16 weeks of age. Piglets were treated with phenobarbital (10 mg/kg) for four days by gavage. Controls received equal volumes of saline. Within each age and treatment group, microsomes were derived from a pool of three livers, with the exception of the 16 week old groups where microsomes were obtained from a pool of two pigs.

Assays for PCA demethylation and aniline hydroxylation were conducted as previously described, modified to be conducted in 16 x 125 mm disposable test tubes in a final volume of 1.0 ml. Substrate concentrations ranged from 0.01 to 4.0 mM for PCA and 0.005 to 5.0 mM for aniline. Generally, 9–11 different concentrations were run in duplicate in addition to pooled blanks. Assays were run for either 10 minutes (control) or 5 minutes (phenobarbital) and contained approximately 1 mg microsomal protein from either source. Further, at all ages, one high and one low substrate concentration were run concurrently to establish linearity with time. In all cases where substrate consumption exceeded 5%, average velocities were utilized (Segel, 1975). Reactions were terminated by the addition of 200 μ l of 50% TCA

and determination of formaldehyde (PCA demethylation) and p-aminophenol (Aniline hydroxylation) was conducted as before.

Kinetic parameters were determined using Eadie-Hofstee plots (V vs. V/S). After graphing the data, linear regression analysis was conducted using those points corresponding to low substrate concentration. The slope and intercept of the line represent the apparent K_m and V_{max} , respectively, for the high affinity component. The velocities due to the low affinity component were obtained by subtracting the V_{max} for the high-affinity component from the total velocity at high substrate concentrations. Linear regression analysis for the new values (V_2 vs. V_2/S) gave the apparent K_{m2} and V_{max2} for the low affinity component.

Protein Determination

Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the protein standard. The intensity of the characteristic blue color was read using an Abbott Bichromatic Analyzer with a primary wavelength of 650 nm.

Electron Microscopy

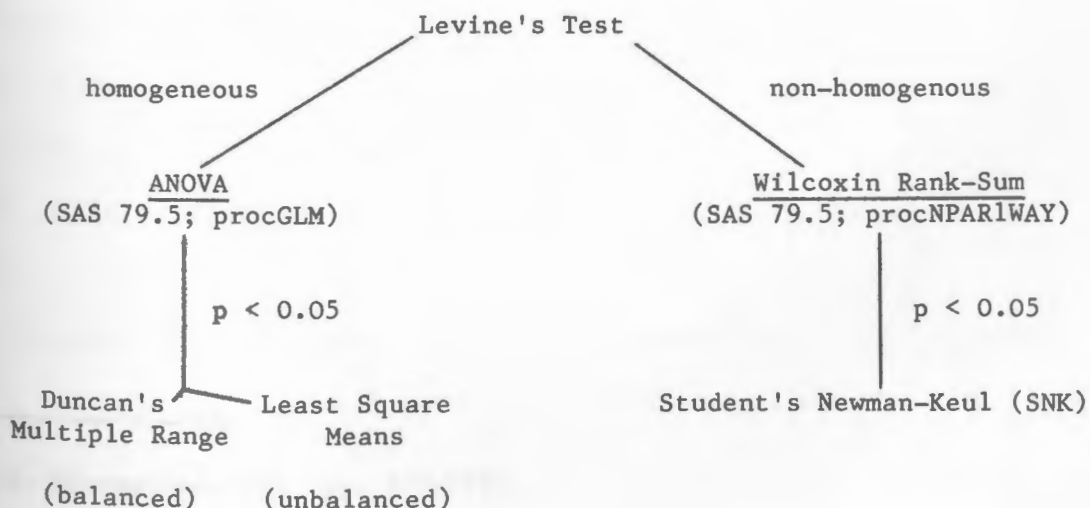
The effects of PCA, PCP, and phenobarbital on hepatic ultrastructure were determined by electron microscopy in 1 week old piglets. At the time of sacrifice, thin (approximately 4 mm) sections of liver were obtained from one animal in each treatment group. The liver

sections were blotted and placed into a formaldehyde-glutaraldehyde fixative (Karnovsky, 1965). Samples were immediately transferred to Dr. Werner Seibel of the Department of Anatomy, University of Maryland Dental School, Baltimore, MD. All further steps in the processing of tissue and electron microscopy were kindly done by Dr. Seibel. The methods utilized appear in Appendix B.

Statistical Methods

The statistical analyses for this study were performed using the Statistical Analysis System (SAS), of the SAS Institute, Inc. Cary, NC. The following analyses were performed: Levine's Test for Homogeneity of Variance, Analysis of Variance (one-way classification; balanced and unbalanced design), Duncan's Multiple Range test, Least Square Means, Wilcoxin Rank-Sums Analysis, Non-parametric Student's Newman-Keuls test, Students t test (balanced and unbalanced design - Control vs. Phenobarbital).

The scheme depicted below was followed:



Additional statistical methods

Simple Linear Regression

$$y = a + bx \quad (\text{regression equation})$$

$$r^2 = \frac{(\sum xy)^2}{\sum x^2 \sum y^2} \quad (\text{regression coefficient})$$

Outlier Analysis¹

$$G_1 = \frac{(y_2 - y_1)}{(y_N - y_1)}$$

¹U.S. Pharmacopeia XVI (pp. 873-874).

RESULTS

In Vitro Metabolism of Pentachloroanisole

Since pentachlorophenol has been detected in biological fluids of fish (Glickman et al., 1977) and of mice (Vodicnik et al., 1980) treated with pentachloroanisole, PCA was investigated as a substrate for the microsomal mixed-function monooxygenase system. The demethylation reaction, one of the many diverse pathways involved in xenobiotic metabolism, is easily measured since the by-product, formaldehyde, can be conveniently quantitated colorimetrically. In the case of PCA, the quantity of formaldehyde formed should represent the stoichiometric conversion of the substrate to PCP.

In order to establish monooxygenase involvement, cofactor requirements were determined as well as the effects of various MFO inhibitors. The results of these experiments are shown in Table 1. In the absence of NADPH, microsomes were totally incapable of demethylating PCA. Furthermore, the presence of carbon monoxide in the reaction mixture resulted in strong inhibition (95%) of activity. NADH as the sole electron donor was capable of supporting a low level of activity and produced a synergistic effect when added to the complete system. At equimolar concentrations, metyrapone produced the strongest inhibition (91%) while SKF 525A and 7,8-Benzoflavone (ANF) inhibited the reaction 70% and 20%, respectively.

TABLE 1. Characterization of PCA demethylation by miniature pig hepatic microsomal enzymes.^a

Reaction Mixture	% Total Activity
Complete ^b	100%
- NADPH	0
- NADPH + NADH (0.5 mM)	8.2
Complete + NADH	105
Complete + Carbon Monoxide	4.6
+ SKF 525A ^c	30
+ Metyrapone	9
+ 7,8-Benzoflavone (ANF)	80

^aActivity was measured as the rate of formaldehyde production in microsomes from phenobarbital induced minipigs as detailed in Materials and Methods.

^bActivity in the complete system was 5.45 nmoles/min/mg protein.

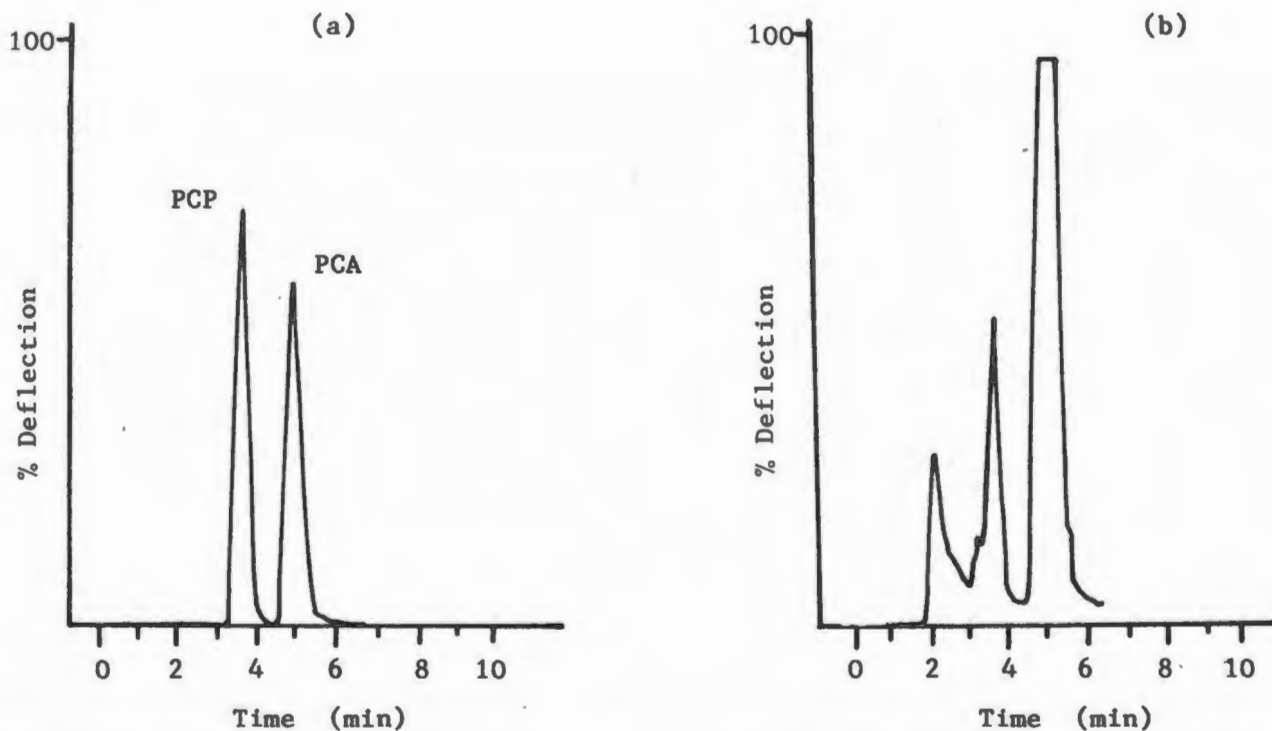
^cThe final concentration of all inhibitors was 5×10^{-5} M.

In order to verify that the formaldehyde produced in the assay was a result of the oxidative demethylation of PCA to PCP, some incubation mixtures were subjected to organic extraction and the residues were analyzed by HPLC. Using the HPLC parameters described in the Methods section, PCA had a retention time of 5.2 minutes and PCP had a retention time of 3.8 minutes. A typical chromatogram of a mixed PCA/PCP standard is shown in Figure 1(a), while the results from an incubation extract are shown in Figure 1(b). An additional peak at 2.3 minutes was not identified, however, it may represent additional metabolism of PCP.

Preliminary kinetic analysis of PCA demethylation was conducted using microsomes from uninduced and phenobarbital treated, mature minipigs. Kinetic parameters were determined from Lineweaver-Burke plots shown in Figure 2(a & b). The apparent K_m for both uninduced and phenobarbital induced microsomes, 0.052 and 0.047 mM, respectively, are relatively similar. The maximum velocities, however, differ by a factor of 10. The V_{max} for phenobarbital induced microsomes is 4.9 nmoles/min/mg protein compared to 0.45 nmoles/min/mg protein in controls.

In conjunction with catalysis of PCA, the interaction of the substrate with cytochrome P450 was investigated using difference spectroscopy. Pentachloroanisole when added to microsomal suspensions produced a typical Type I binding spectrum with a peak absorbance at 385 nm and a trough at 419 nm (Figure 3). Of particular interest, however, is the inability of the compound to produce discernable spectral changes when added to uninduced microsomal preparations.

FIGURE 1. High-Pressure Liquid Chromatograms for the Analysis of PCP and PCA.

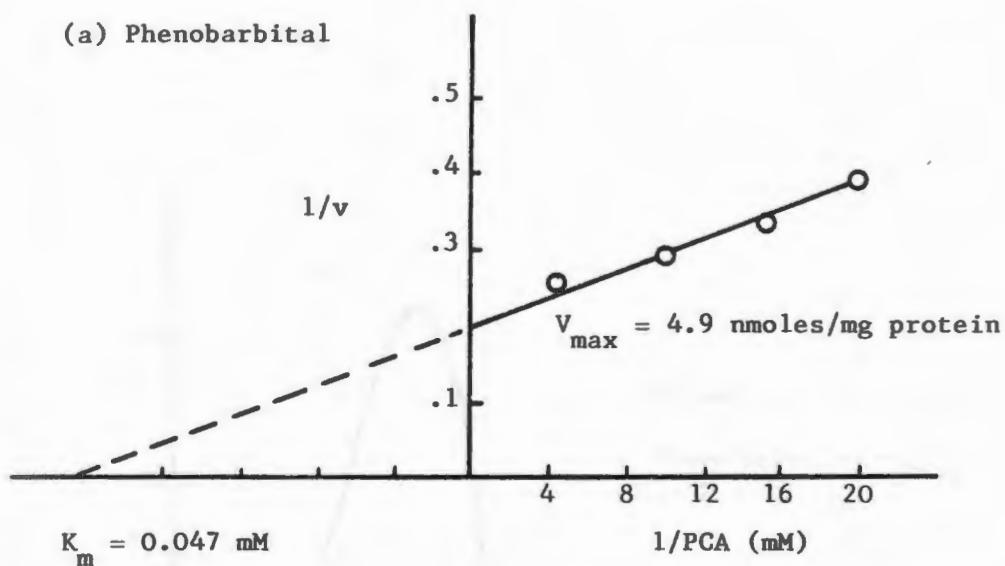


- a) HPLC parameters and incubation extraction procedures are described in Materials & Methods.
b) Retention times are 3.8 minutes (PCP) and 5.2 minutes (PCA).
c) Typical chromatograms depicted are for (a) standard solution and (b) incubation extracts.

FIGURE 2. Lineweaver-Burke Plots of PCA Demethylation in Hepatic Microsomes from Phenobarbital (a) and Uninduced (b) Minipigs.

- a) Incubation mixtures for PCA demethylase were identical to that described in Materials and Methods with the exception that PCA was solubilized in acetone prior to addition to the Tween 80 mixture. Final PCA concentrations ranged from 0.05 to 1.0 mM.
- b) Microsomes were obtained from a pool of three livers from mature (16-17 weeks old) miniature pigs. Phenobarbital was administered at 10 mg/kg P.O. for four consecutive days.

(a) Phenobarbital



(b) Uninduced

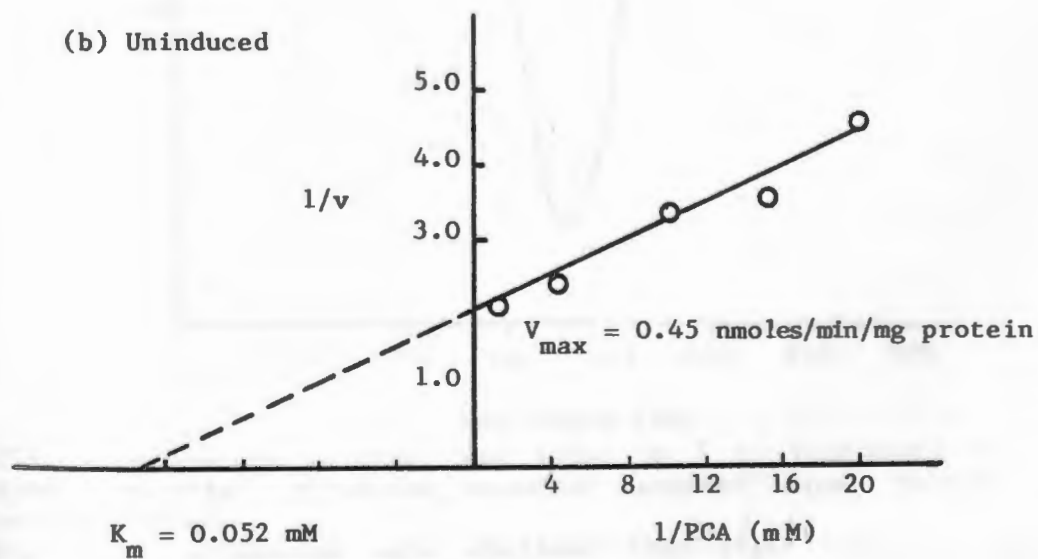
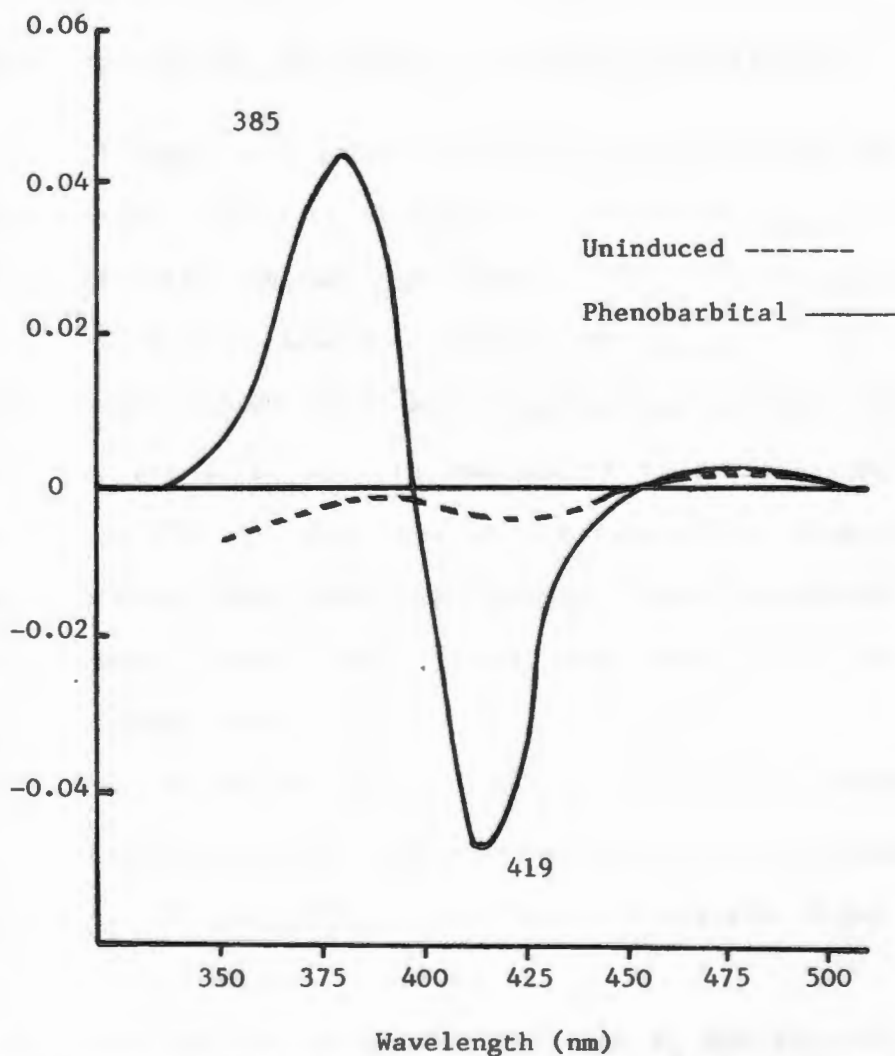


FIGURE 3. Typical Substrate Binding Spectra Observed Following Addition of PCA to Miniature Pig Microsomal Suspensions.



- PCA, dissolved in methanol was added in 1 ul increments to the sample cuvette. Reference cuvette received equal volumes of methanol alone.
- Microsomal suspension were obtained from eight week old pigs. Phenobarbital was administered as previously described.
- The observed spectra is characteristic of "Type I" binding with λ_{max} at 385 nm and λ_{min} at 419 nm.

Using phenobarbital induced microsomes, PCA was readily titrated to apparent saturation. Double reciprocal plots of $1/\{PCA\}$ vs. $1/\Delta Abs.$ (peak to trough) yielded values of 0.014 mM for the spectral dissociation constant (K_s) and a maximum peak to trough absorbance change (A_{max}) of 0.035 per milligram of protein (Figure 4).

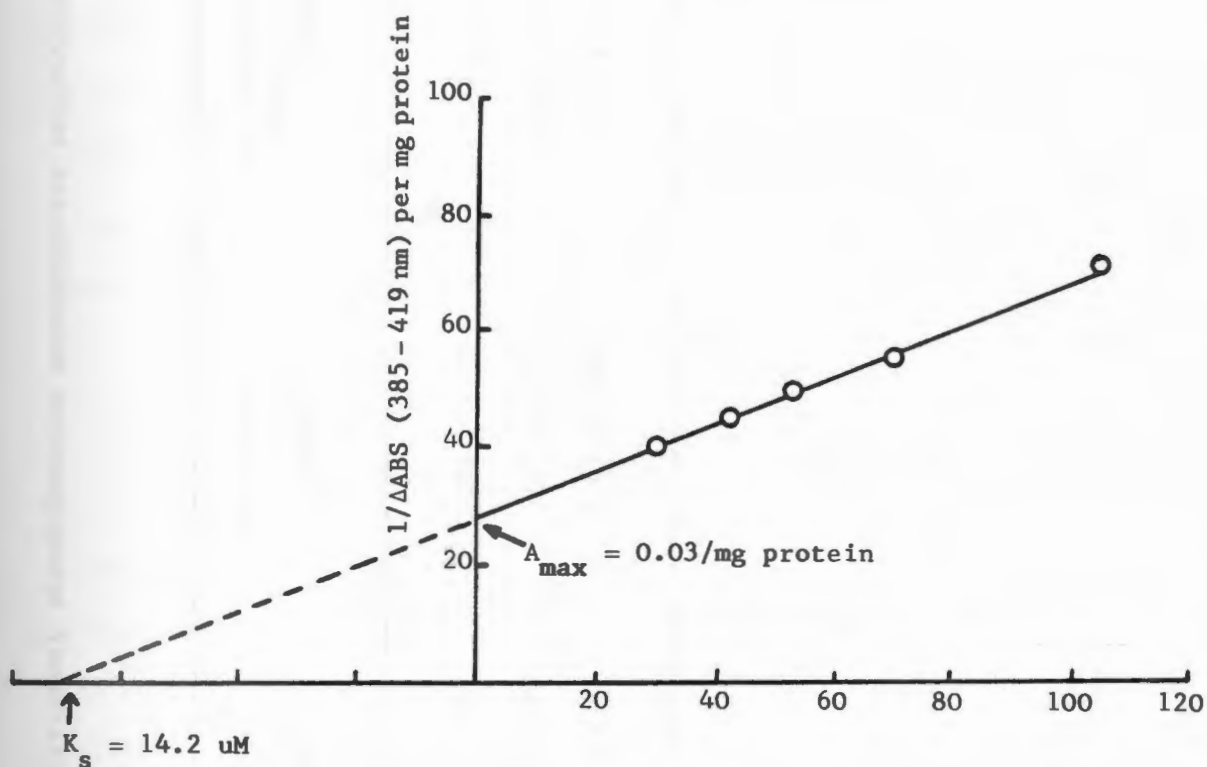
Development of the MFO System in Growing Miniature Pigs

In order to assess the pattern of postnatal development of the hepatic MFO system, a variety of parameters were measured in 1, 4, and 8 week old minipigs. Two reaction pathways, the aromatic hydroxylation of aniline and the O-demethylation of p-Nitroanisoie were investigated since these pathways have well characterized in other laboratory species and are often used in assessments of MFO function. The O-demethylation of PCA was also used as a parameter for comparison. In all cases, optimal assay conditions were previously determined and the reactions were within linear ranges with respect to time and protein concentrations used.

The specific activities as a function of age are shown in Table 2. In all cases maximal levels are attained by four weeks of age. Furthermore, the activities in one week old piglets ranged from 65 to 80% of the eight week old activity.

The concentrations of cytochromes P450 and b_5 and the activity of NADPH cytochrome-c Reductase were also determined in developing minipigs. The concentration of microsomal cytochrome P450 increased in linear manner from 0.57 nmoles/mg protein in one week old piglets

FIGURE 4. Double-Reciprocal Plot for the Determination of the Spectral Dissociation Constant (K_s) for PCA in Phenobarbital Induced Microsomal Suspensions.



- Each point represents the reciprocal of the peak-to-trough absorbance difference (as shown in Figure 3) as a function of PCA concentration.
- Values of K_s and A_{max} are for eight week old, phenobarbital induced minipigs.

TABLE 2 Postnatal development of three microsomal mixed-function monooxygenase activities in miniature pigs.

Age (weeks)	N	Aniline ^a Hydroxylation (ANOH)	Nitroanisoie ^b Demethylation (NADM)	Pentachloroanisoie ^c Demethylation (PCADM)
1	(6)	0.39 \pm 0.03 ^d	1.56 \pm 0.10	0.24 \pm 0.02
4	(3)	0.48 \pm 0.04	2.24 \pm 0.55	0.37 \pm 0.04
8	(6)	0.47 \pm 0.05	2.15 \pm 0.08	0.29 \pm 0.02

^anMoles p-aminophenol formed/min/mg protein.

^bnMoles p-nitrophenol formed/min/mg protein.

^cnMoles formaldehyde formed/min/mg protein.

^dValues are mean \pm S.E.M.

to 1.00 nmoles/mg protein in eight week old piglets (Table 3). Cytochrome b_5 concentration was equal to P450 concentration in one week old piglets, however the concentration of cytochrome b_5 reached maximum by four weeks of age and remained constant to eight weeks. The activity of NADPH cytochrome-c Reductase appears to follow a similar pattern as the activities shown in Table 2. There is an increase from one to four weeks of age, followed by a slight decrease in activity at eight weeks of age.

Effects of PCA, PCP and Phenobarbital on
MFO Parameters in Developing Miniature Pigs

Since a vast number of compounds metabolized by the hepatic mixed-function monooxygenase system are also capable of inducing the enzyme system, pentachloroanisole was investigated for its induction potential. Furthermore, since pentachlorophenol is the product of microsomal demethylation, this compound was also investigated in order to determine if the parent compound (PCA) or the metabolite (PCP) was responsible for induction. Phenobarbital was utilized in this study as a positive control.

At the dose of 10 mg/kg/day for four days, PCA and PCP produced no overt signs of toxicity in any of the age groups. Similarly, phenobarbital treated piglets remained alert and active. All piglets, during treatment situations, consumed their normal ration of milk diet and either maintained or gained weight. At the time of the sacrifice, livers from treated pigs were inspected grossly and appeared normal with respect to color and texture.

TABLE 3 Postnatal development of cytochrome P450, cytochrome b₅ and NADPH-cytochrome-c-Reductase in miniature pigs.

Age	N	Cytochrome P450 ^a	Cytochrome b ₅ ^b	NADPH-Cytochrome-c ^c Reductase
1	(6)	0.57 ± 0.04 ^d	0.56 ± 0.04	110.3 ± 5.1
4	(3)	0.81 ± 0.05	0.76 ± 0.003	165.5 ± 1.8
8	(6)	1.00 ± 0.04	0.74 ± 0.01	151.6 ± 6.8

^anMoles P450/mg protein.

^bnMoles b₅/mg protein.

^cnMoles cytochrome-c reduced/min/mg protein.

^dValues are mean ± S.E.M.

One Week Old Piglets

The effects of PCA, PCP and phenobarbital on MFO activity in one week old piglets varied with respect to the parameter measured and the degree of induction. A comparison of the specific activities for aniline hydroxylase (ANOH), nitroanisole demethylase (NADM) and PCA demethylase (PCADM) is shown in Table 4. PCA produced a small, yet significant 41% increase in ANOH activity and a somewhat larger (67%) increase in NADM activity. Of particular interest, however, is the disproportionate three-fold increase in the activity of the enzyme catalyzing PCA's own metabolism. In contrast to PCA's effects, microsomes from PCP treated piglets showed no change in PCADM and ANOH activities. PCP treatment did have a minor effect on NADM (50% over controls). The effects of phenobarbital on the three enzyme activities were consistent with its strong comprehensive inductive effects. ANOH and NADM activities were increased 5.5- and 9-fold, respectively, over controls. A dramatic 14-fold increase in PCADM was observed.

The observed increases in the different enzyme activities are paralleled by an increase in the concentration of cytochrome P450 in microsomes from treated pigs. Phenobarbital produced a three-fold increase in P450 (Table 5), while PCA and PCP produced much smaller, but significant increases in the concentration of the hemoprotein. The wavelength of maximum absorbance in all microsomal preparations was 450 nm. The microsomal concentration of cytochrome b_5 and the activity of NADPH-cytochrome-c Reductase are also shown in Table 5.

Table 4. Effects of PCA, PCP, and phenobarbital on microsomal enzyme activities in one week old miniature pigs.^{a,b}

Treatment	Aniline ^c Hydroxylase	Nitroanisole ^d Demethylase	Pentachloroanisole ^e Demethylase
Control (6)	0.39 \pm 0.03	1.56 \pm 0.10	0.24 \pm 0.02
PCA (6)	0.55 \pm 0.03*	2.60 \pm 0.24*	0.72 \pm 0.09*
PCP (5)	0.44 \pm 0.04	2.35 \pm 0.21*	0.27 \pm 0.03
Phenobarbital (6)	2.15 \pm 0.23**	13.96 \pm 1.99**	3.37 \pm 0.28**

^aPCA, PCP, and phenobarbital were administered at 10 mg/kg/day P.O. for four consecutive days.

^bMiniature pigs were seven days of age at the onset of treatment.

^cnMoles p-aminophenol formed/min/mg protein.

^dnMoles p-nitrophenol formed/min/mg protein.

^enMoles formaldehyde formed/min/mg protein.

Numbers in parentheses are the number of values used to calculate the mean \pm S.E.M.

* Significantly different from control ($p < 0.05$) Duncan's Multiple Range Analysis.

**Significantly different from control ($p < 0.05$) Student's t test.

TABLE 5. Effects of PCA, PCP, and phenobarbital on cytochromes P450, b₅, and cytochrome-c Reductase in one week old miniature pigs.^a

Treatment	Cytochrome ^b P450	Cytochrome ^c b ₅	Cytochrome-c ^d Reductase
Control (6)	0.57 ± 0.01 (450nm) ^e	0.56 ± 0.04	110.3 ± 5.1
PCA (6)	0.76 ± 0.04* (450nm)	0.75 ± 0.05*	144.2 ± 12.1*
PCP (6)	0.85 ± 0.06* (450nm)	0.70 ± 0.03	120.6 ± 4.5
Phenobarbital (6)	1.67 ± 0.12** (450nm)	0.77 ± 0.03**	145.2 ± 11.7**

^aTreatments were as described in legend of Table 4.

^bnMoles/mg protein.

^cnMoles/mg protein.

^dnMoles cytochrome-c reduced/min/mg protein.

^eObserved wavelength of maximum absorbance in the reduced-CO difference spectra.

All values are the mean ± S.E.M. for the number of observations in parentheses.

* Significantly different from control (p < 0.05) Duncan's Multiple Range Analysis.

**Significantly different from control (p < 0.05) Student's t test.

Phenobarbital produced similar increases in b_5 and the reductase (37% and 32%, respectively) over controls. It is interesting to note that PCA produced almost identical increases in both parameters and these correlated with the increase in P450 concentration in that treatment group.

Treatment Effects on Hepatocellular Morphology

Since induction of cytochrome P450 and related metabolic activities by phenobarbital has been associated with hepatocellular changes in smooth endoplasmic reticulum content, electron microscopy was used in an attempt to correlate biochemical and morphological effects. Furthermore, qualitative similarities in the induction profiles of phenobarbital and PCA suggested that the latter compound may be a "phenobarbital type" of inducer.

Figure 5 shows a low power electron micrograph of a liver parenchymal cell from a one week old control piglet. The liver cells are characterized by a round nucleus, rough endoplasmic reticulum and an abundance of glycogen. A high power micrograph (Figure 6) better illustrates that the endoplasmic reticulum in these cells is primarily RER although vesicles of smooth ER occur intermittantly (insert).

In contrast, phenobarbital results in a marked proliferation of smooth endoplasmic reticulum (Figure 7). Although RER is present, vesicles of agranular endoplasmic reticulum (SER) appear to predominate in the cytoplasm. Similarly, PCP and PCA appear to shift the

FIGURE 5. Electron Photomicrograph of Hepatic Parenchymal Cells from a One Week Old Control Miniature Pig.

Details of the fixation and staining procedures appear in Appendix B.

Legend: Nucleus (N), Mitochondria (M), Rough Endoplasmic Reticulum (R), Smooth Endoplasmic Reticulum (S), Glycogen (G).

Magnification X6,000.

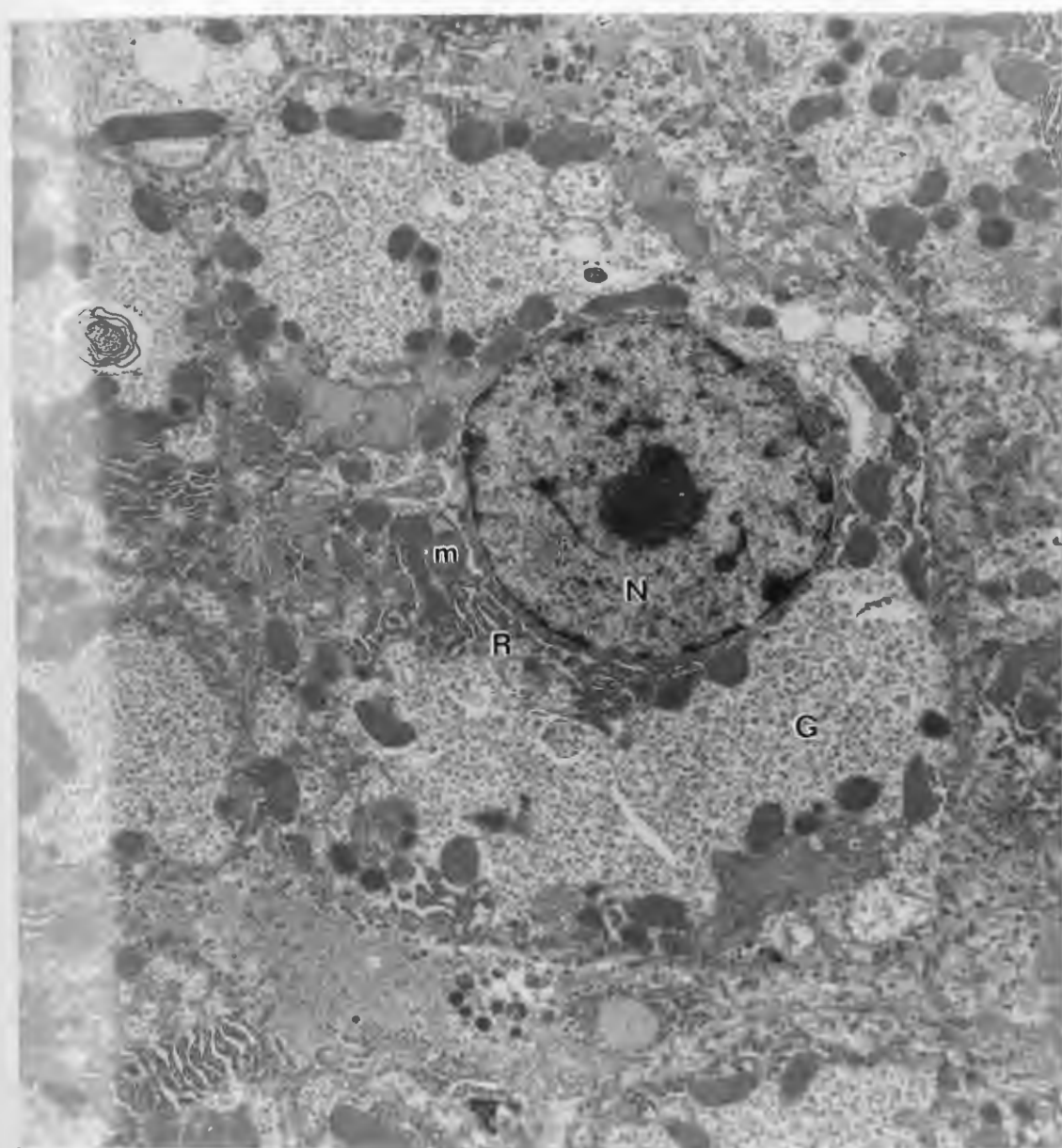


FIGURE 6. Higher Power Electron Photomicrograph of an Hepatic Cell
from a One Week Old Control Miniature Pig.

Legend: see Figure 5.

Magnification X22,500 (Insert: X15,000).

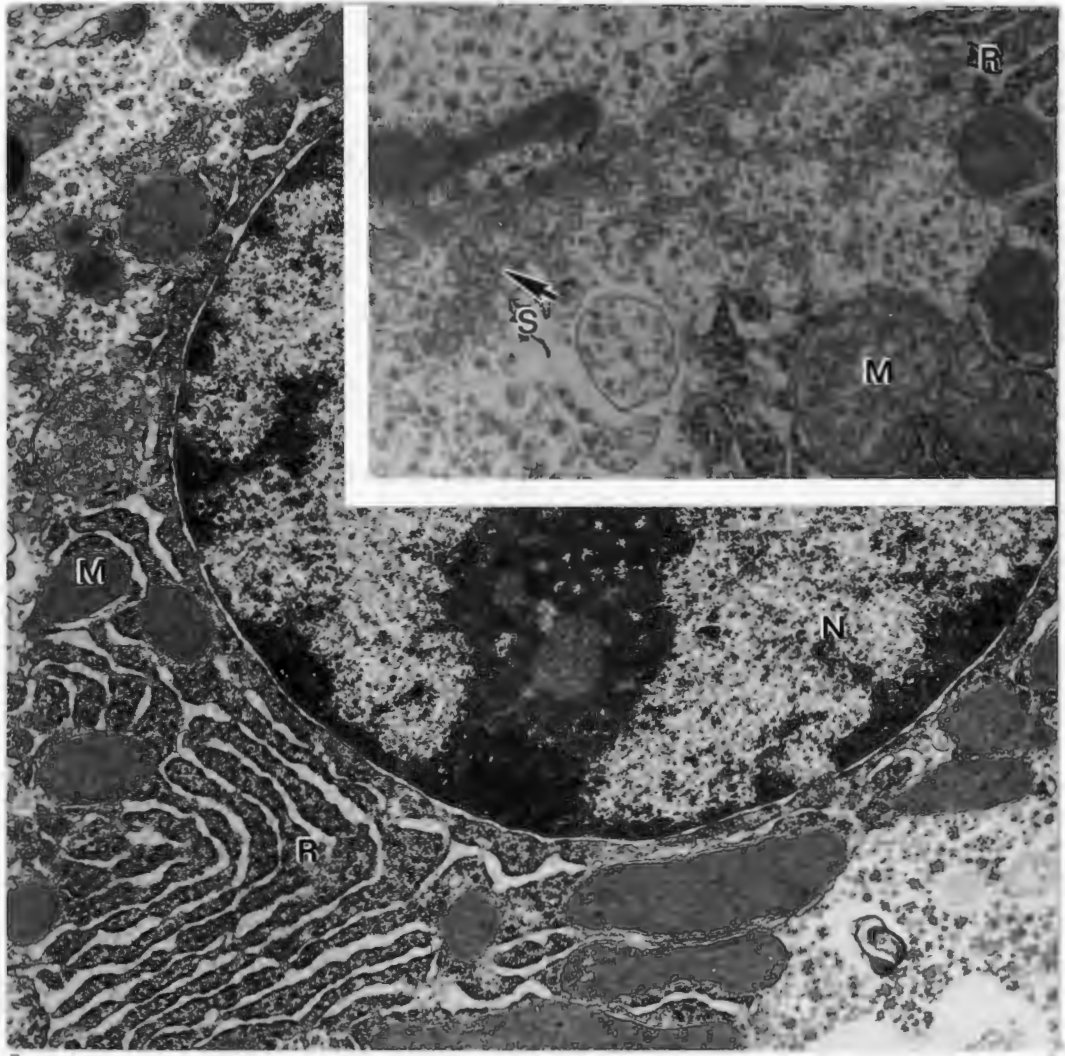
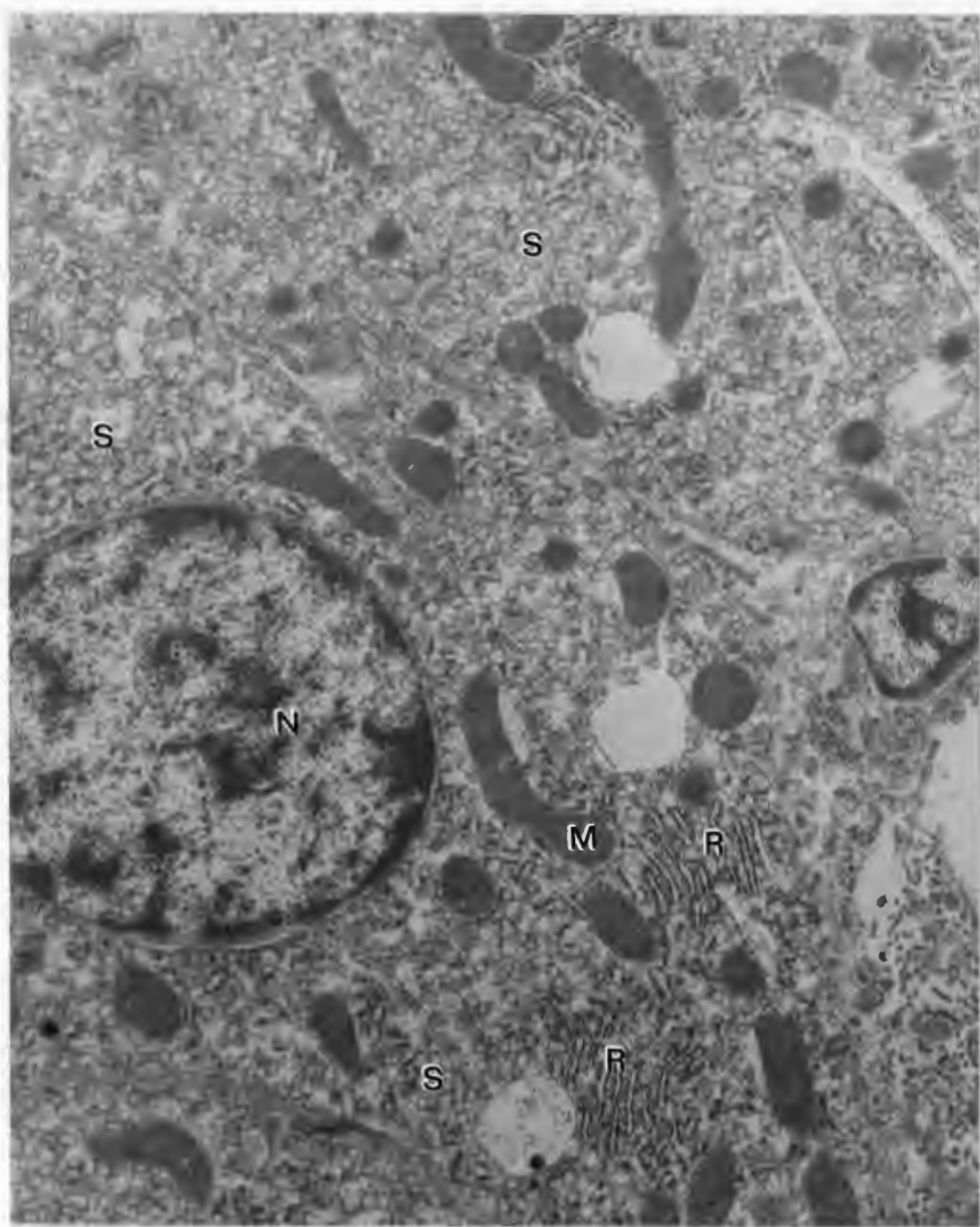


FIGURE 7. Electron Photomicrograph of Hepatic Cells from a Phenobarbital Treated One Week Old Miniature Pig.

Legend: see Figure 5.

Magnification X12,500.



predominance of endoplasmic reticulum from rough to smooth (Figures 8 & 9). It should be noted that in the latter two cases, regions of RER appear more dispersed in the cytoplasm rather than arranged in parallel stacks as observed in controls and phenobarbital treated piglets.

Biochemical Effects in Four Week Old Piglets

The effects of the various treatments in four week old piglets are shown in Tables 6 and 7. Due to the smaller number of animals used per treatment group and the occurrence of aberrant values in some parameters, statistical significance could not be demonstrated for the relatively small changes produced by either PCA or PCP. A similar trend in the induction patterns of the two compounds, however, is apparent, with PCA producing small increases in ANOH and NADM activity and its major effect on its own metabolism (Table 6). PCP, as in one week old piglets, appears to increase only NADM activity.

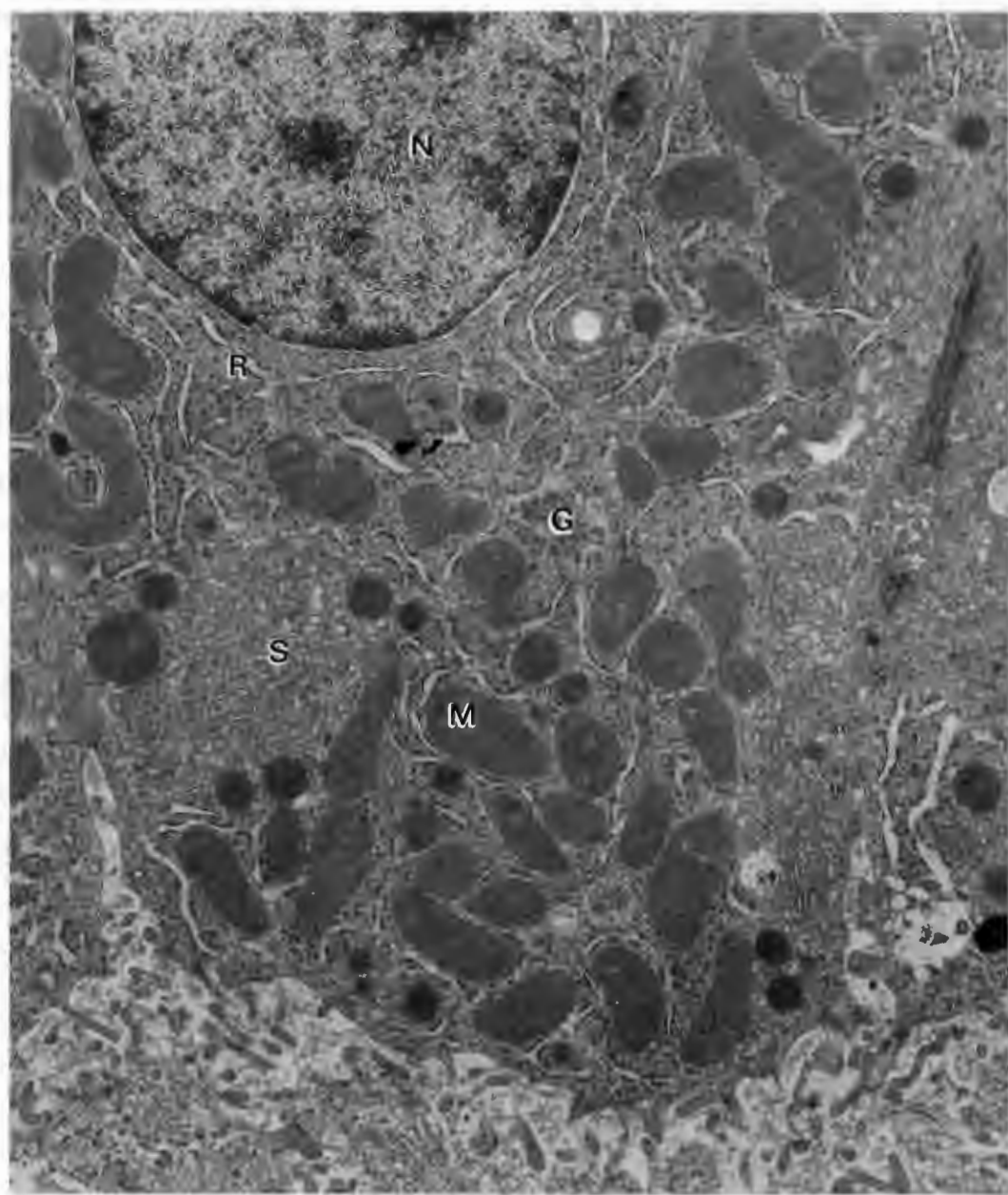
Phenobarbital treatment in four week old piglets results in significant increases in all six parameters measured. ANON, NADM, and PCADM were 4.5, 5.4 and 15-fold, respectively, greater than control activities (Table 6).

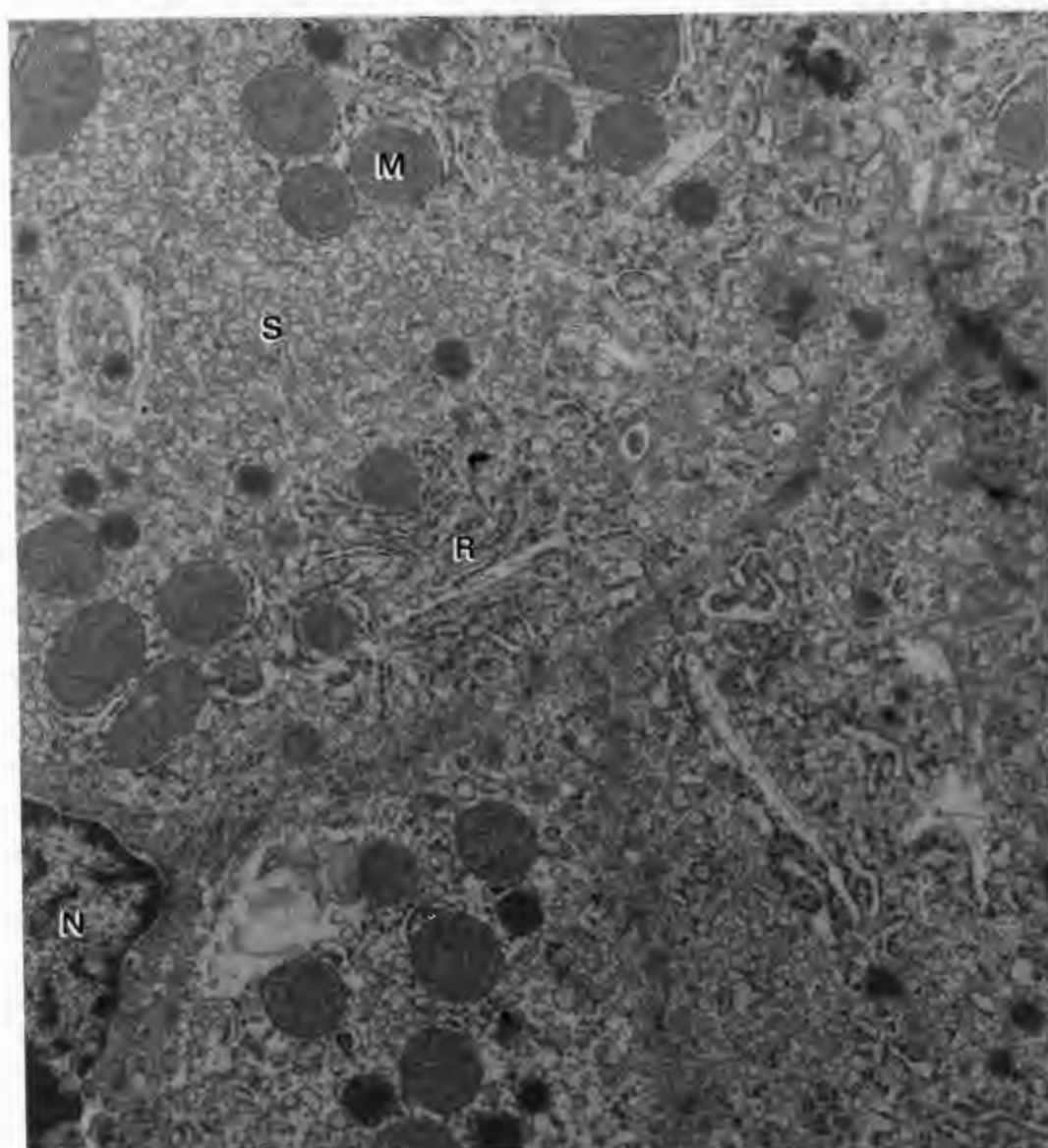
The levels of cytochrome P450, b_5 and cytochrome c Reductase were not significantly changed by PCA or PCP, however, phenobarbital again produced a 3-fold increase in P450 and significant, albeit smaller increases in the other two parameters (Table 7).

FIGURE 8. Electron Photomicrograph of an Hepatic Cell from a PCP
Treated One Week Old Miniature Pig.

Legend: see Figure 5.

Magnification X11,250.





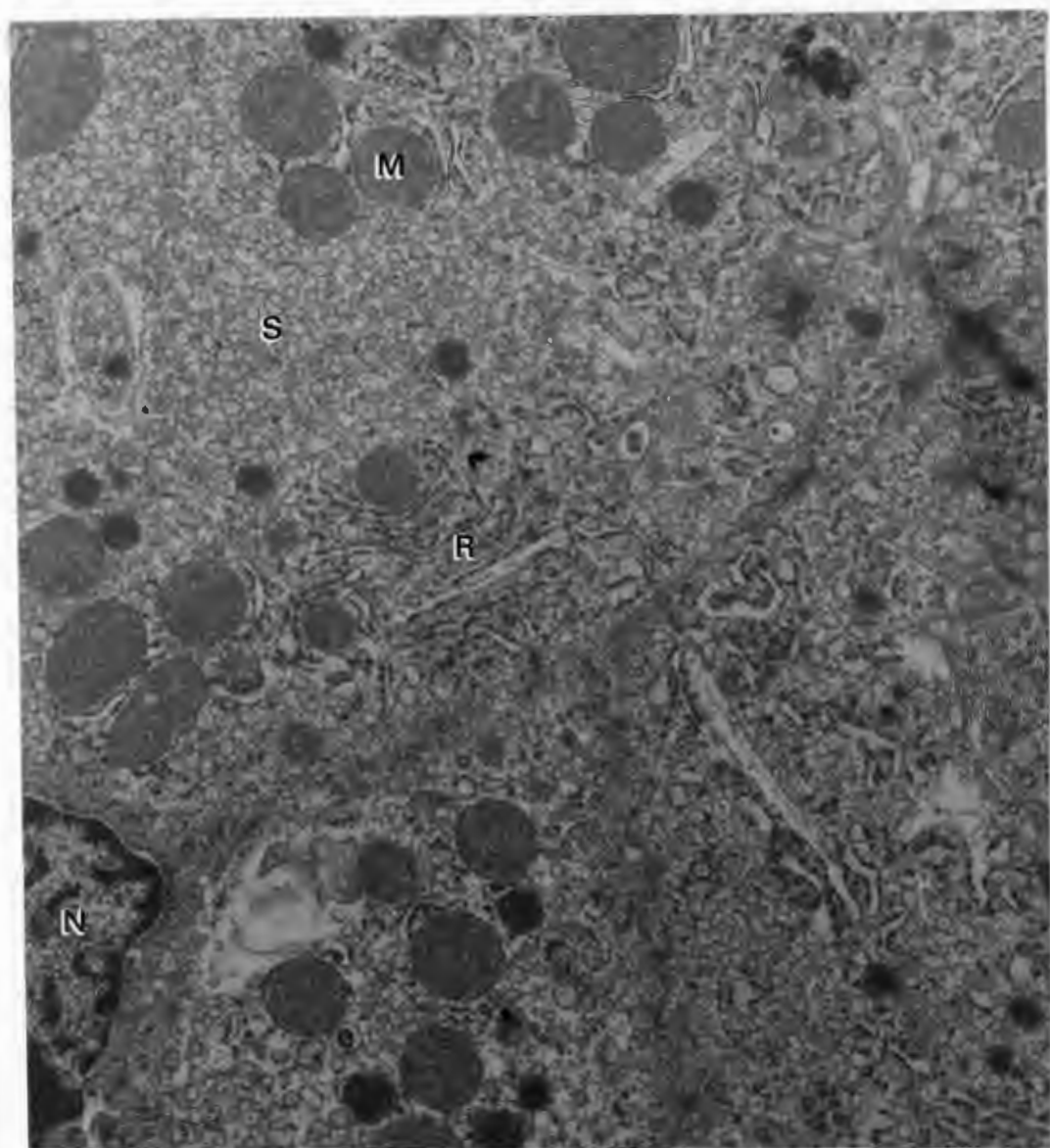


TABLE 6. Effects of PCA, PCP, and phenobarbital on microsomal enzyme activities in four week old miniature pigs.^a

Treatment	Aniline ^b Hydroxylase	Nitroanisole ^c Demethylase	Pentachloroanisole ^d Demethylase
Control (3)	0.48 \pm 0.04	2.24 \pm 0.55	0.37 \pm 0.04
PCA (3)	0.67 \pm 0.03	3.09 \pm 0.28	0.80 \pm 0.16
PCP (3)	0.56 \pm 0.07	3.25 \pm 0.20	0.46 \pm 0.14
Phenobarbital (3)	2.16 \pm 0.32**	12.09 \pm 1.57**	5.60 \pm 0.32**

^aTreatments are described in Materials and Methods section.

^bnMoles p-aminophenol formed/min/mg protein.

^cnMoles p-nitrophenol formed/min/mg protein.

^dnMoles formaldehyde formed/min/mg protein.

All values are the mean \pm S.E.M. for the number of observations in parentheses.

**Significantly different from control (p < 0.05) Student's t test.

TABLE 7. Effects of PCA, PCP, and phenobarbital on cytochromes P450, b₅, and cytochrome-c Reductase in four week old miniature pigs.^a

Treatment	Cytochrome ^b P450	Cytochrome ^c b ₅	Cytochrome-c ^d Reductase
Control (3)	0.81 ± 0.04	0.76 ± 0.003	165.5 ± 1.8
PCA (3)	0.92 ± 0.05	0.74 ± 0.03	148.5 ± 6.8
PCP (3)	1.10 ± 0.02	0.85 ± 0.04	152.7 ± 4.7
Phenobarbital (3)	2.63 ± 0.06**	0.93 ± 0.05**	187.3 ± 5.4**

^aTreatments are described in the Materials and Methods section.

^bnMoles/mg protein.

^cnMole/mg protein.

^dnMoles cytochrome c reduced/min/mg protein.

All values are the mean ± S.E.M. for the number of observations in parentheses.

**Significantly different from control (p < 0.05) Student's t test.

Biochemical Effects in Eight Week Old Piglets

The results in eight week old piglets were for the most part similar to those in younger animals (Tables 8 & 9), however, the extent of induction by PCA appears to be less than that in one week old piglets. Although PCA produces a significant 35% increase in NADM activity, this is approximately half the increase (67%) observed in the younger animals. Similarly, PCA treatment produced only a 75% increase in its own metabolism (PCADM) as compared to a 3-fold increase in the one week old group. ANOH activity is no longer significantly different from controls. PCP treatment, as with the earlier groups, had no significant effect on ANOH, however, unlike the younger animals the compound did produce a small increase in PCADM.

The effects of phenobarbital compared relatively well with those seen in the two previous age groups. PCADM activity was approximately 13-fold greater and ANOH 4.6-fold greater than controls (Table 8). NADM activity, however, was increased 4.7-fold by phenobarbital which is approximately half the 9-fold increase observed in one week old piglets.

As with the four week old piglets, the only significant increases in cytochrome P450, b_5 and Reductase in eight week old piglets were produced by phenobarbital (Table 9).

Catalytic Activities

The relative ease by which cytochrome P450 can be quantitated in microsomal suspensions imparts the ability to analyze data from

TABLE 8. Effects of PCA, PCP, and phenobarbital on microsomal enzyme activities in eight week old miniature pigs.^a

Treatment	Aniline ^b Hydroxylase	Nitroanisole ^c Demethylase	Pentachloroanisole ^d Demethylase
Control (6)	0.47 \pm 0.05	2.15 \pm 0.08	0.29 \pm 0.02
PCA (6)	0.60 \pm 0.03	2.94 \pm 0.24*	0.51 \pm 0.03**
PCP (6)	0.53 \pm 0.07	2.92 \pm 0.31*	0.41 \pm 0.02**
Phenobarbital (6)	2.14 \pm 0.18***	10.13 \pm 0.43***	3.85 \pm 0.40***

^aTreatments are described in Materials and Methods section.

^bnMoles p-aminophenol formed/min/mg protein.

^cnMoles p-nitrophenol formed/min/mg protein.

^dnMoles formaldehyde formed/min/mg protein.

All values are the mean \pm S.E.M. for the number of observations in parentheses.

* Significantly different from control ($p < 0.05$) Student Newman-Keuls test.

** Significantly different from control ($p < 0.05$) Duncan's Multiple Range test.

***Significantly different from control ($p < 0.05$) Student's t test.

TABLE 9. Effects of PCA, PCP, and phenobarbital on cytochromes P450, b₅, and cytochrome-c Reductase in eight week old miniature pigs.^a

Treatment	Cytochrome ^b P450	Cytochrome ^c b ₅	Cytochrome-c ^d Reductase
Control (6)	1.00 ± 0.04	0.74 ± 0.01	151.6 ± 6.8
PCA (6)	1.08 ± 0.06	0.83 ± 0.03	163.9 ± 8.3
PCP (6)	1.15 ± 0.06	0.75 ± 0.04	142.9 ± 5.0
Phenobarbital (6)	3.71 ± 0.24*	1.04 ± 0.05*	201.7 ± 7.0*

^aTreatments are described in Materials and Methods section.

^bnMoles/mg protein.

^cnMoles/mg protein.

^dnMoles cytochrome c reduced/min/mg protein.

All values are the mean ± S.E.M. for the number of observations in parentheses.

* Significantly different from control (p < 0.05) Student's t test.

enzyme assays in terms of "molecular" or "catalytic" activity. This method is particularly useful for determining changes in the specificity of induced form(s) of cytochrome P450. Catalytic activities, expressed as nmoles product/min/nmole P450, for PCADM as a function of age and treatment are shown in Table 10. Consistent with its relatively large effect on PCA metabolism, phenobarbital produced a 5.2-fold increase in catalytic activity in one week old piglets. PCA treatment also produced a significant 2-fold increase in catalytic activity in the youngest age group. Although there are no significant changes in controls as a function of age, an age related decrease in catalytic activity was observed in PCA and phenobarbital induced pigs. Catalytic activity in eight week old piglets is less than half the one week old activity for each respective group.

Similar, yet less pronounced alterations in catalytic activity were observed for ANOH (Table 11) and NADM (Table 12). In both cases, however, only phenobarbital produced any significant effects.

Kinetic Analysis

Changes in various catalytic activity profiles following chemical induction of the MFO system have generally been associated with either an increase in the concentration of a substance specific form of cytochrome P450 or alterations in the relative proportions of various subpopulations of the hemoprotein. To further investigate the age related changes in catalytic activities as well as the nature of the large effect produced by phenobarbital on PCA metabolism, kinetic

TABLE 10. Catalytic activity of PCA demethylase as a function of age and treatment.^a

Treatment	AGE		
	1 Week	4 Week	8 Week
Control	0.41 \pm 0.04 (5)	0.46 \pm 0.06 (3)	0.29 \pm 0.01 (6)
PCA	0.94 \pm 0.08 (6) [†]	0.88 \pm 0.20 (3)	0.48 \pm 0.03 (6) ^{†¶}
PCP	0.32 \pm 0.05 (5)	0.42 \pm 0.12 (3)	0.35 \pm 0.02 (6)
Phenobarbital	2.15 \pm 0.28 (5) [§]	2.12 \pm 0.10 (3) [§]	0.91 \pm 0.04 (5) ^{§¶}

^aCatalytic activity is expressed as nMoles formaldehyde formed/min/nMole cytochrome P450.

Values are the mean \pm S.E.M. for the number of observations in parentheses.

[†]P < 0.05 vs. age matched controls (Duncan's Multiple Range).

[§]P < 0.05 vs. age matched controls (Student's t test).

[¶]P < 0.05 vs treatment matched one week olds (Least Square Means).

TABLE 11. Catalytic activity^a of aniline hydroxylase as a function of age and treatment.

Treatment	<u>AGE</u>		
	1 Week	4 Week	8 Week
Control	0.66 \pm 0.06 (5)	0.59 \pm 0.09 (3)	0.48 \pm 0.06 (6)
PCA	0.75 \pm 0.03 (6)	0.74 \pm 0.07 (3)	0.56 \pm 0.03 (6)
PCP	0.54 \pm 0.05 (5)	0.51 \pm 0.05 (3)	0.46 \pm 0.05 (6)
Phenobarbital	1.28 \pm 0.06 (6) [†]	0.82 \pm 0.06 (3) [§]	0.60 \pm 0.07 (6) ^{§†}

^aCatalytic activity is expressed as nMoles p-aminophenol formed/min/nMole cytochrome P450.

Values are the mean \pm S.E.M. for the number of observations in parentheses.

[†]P < 0.05 vs. age matched control (Student's t test).

[§]P < 0.05 vs. treatment matched one week olds (Least Square Means).

[†]P < 0.05 vs. treatment matched four week olds (Least Square Means).

TABLE 12. Catalytic activity of nitroanisol demethylase as a function of age and treatment.^a

Treatment	AGE		
	1 Week	4 Week	8 Week
Control	2.79 \pm 0.27 (5)	2.74 \pm 0.55 (3)	2.17 \pm 0.14 (6)
PCA	3.42 \pm 0.25 (6)	3.39 \pm 0.46 (3)	2.75 \pm 0.26 (6)
PCP	2.87 \pm 0.16 (5)	2.96 \pm 0.15 (3)	2.52 \pm 0.20 (6)
Phenobarbital	8.29 \pm 1.05 (6) [†]	4.57 \pm 0.55 (3) [§]	2.78 \pm 0.21 (6) ^{†§¶}

^aCatalytic activity is expressed as nMoles p-nitrophenol formed/min/nMole cytochrome P450.

Values are the mean \pm S.E.M. for the number of observations in parentheses.

[†]P < 0.05 vs. age matched controls (Student's t test).

[§]P < 0.05 vs. treatment matched one week olds (Least Square Means).

[¶]P < 0.05 vs. treatment matched four week olds (Least Square Means).

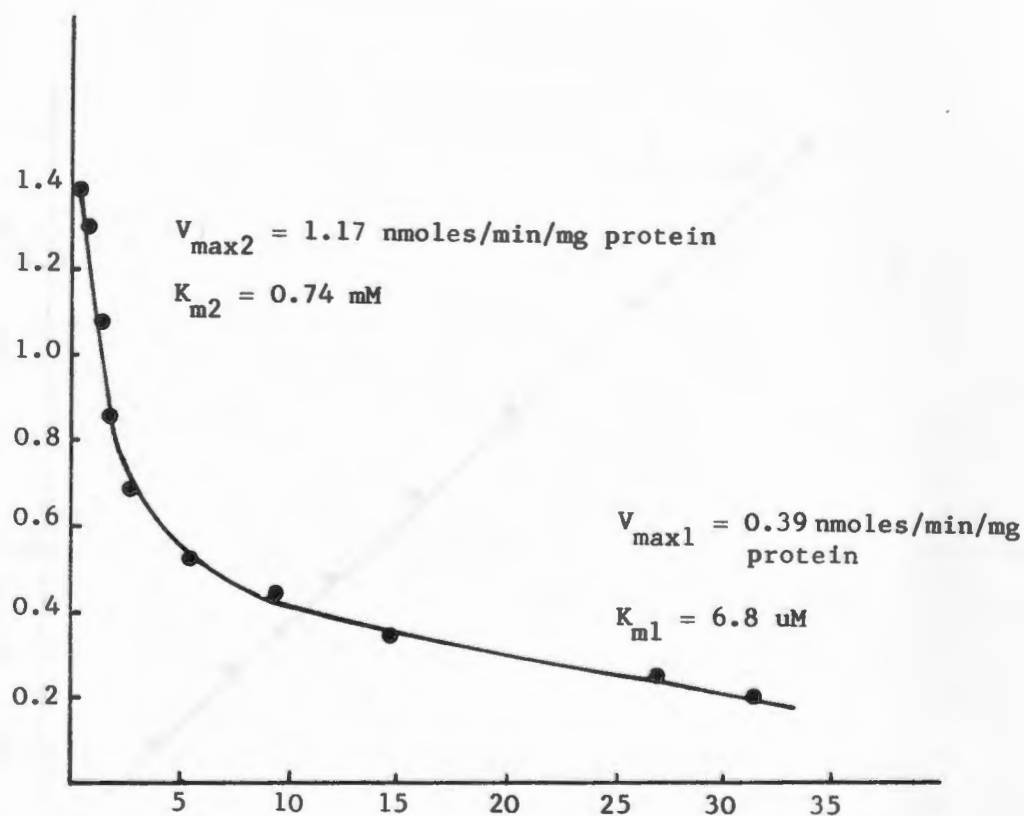
analyses were conducted. Based on the assumption that there are multiple forms of cytochrome P450, which may differ in their affinities toward a particular substrate, it should be possible to detect the activities of different forms by kinetic analysis providing the affinity differences are large enough.

When aniline concentrations were varied from 5 μ M to 5 mM, a biphasic pattern was observed when plotted by the Eadie-Hofstee method (Figure 10). Kinetic constants for the two phases were determined as described in the Methods section. In adult (4 month old) uninduced pigs, the high affinity component had an apparent K_m of 6.8 μ M and a V_{max} of 0.39 nmoles/min/mg protein. The low affinity component had an apparent K_m of 0.74 mM and a V_{max} of 1.17 nmoles/min/mg protein. Using these four kinetic parameters, theoretical values for velocity were calculated for the substrate concentrations utilized. The calculated velocities correlated extremely well ($r^2 = .99$) with the experimentally derived velocities (Figure 11).

Similar analyses were conducted with microsomes from 1, 4, and 8 week old uninduced piglets. A summary of the kinetic constants are listed in Table 13. In uninduced microsomes, the high affinity component, designated Form I, remains relatively constant with respect to affinity and maximum velocity over the four ages. In contrast, the low affinity component, Form II, appears to undergo age dependent changes in both parameters.

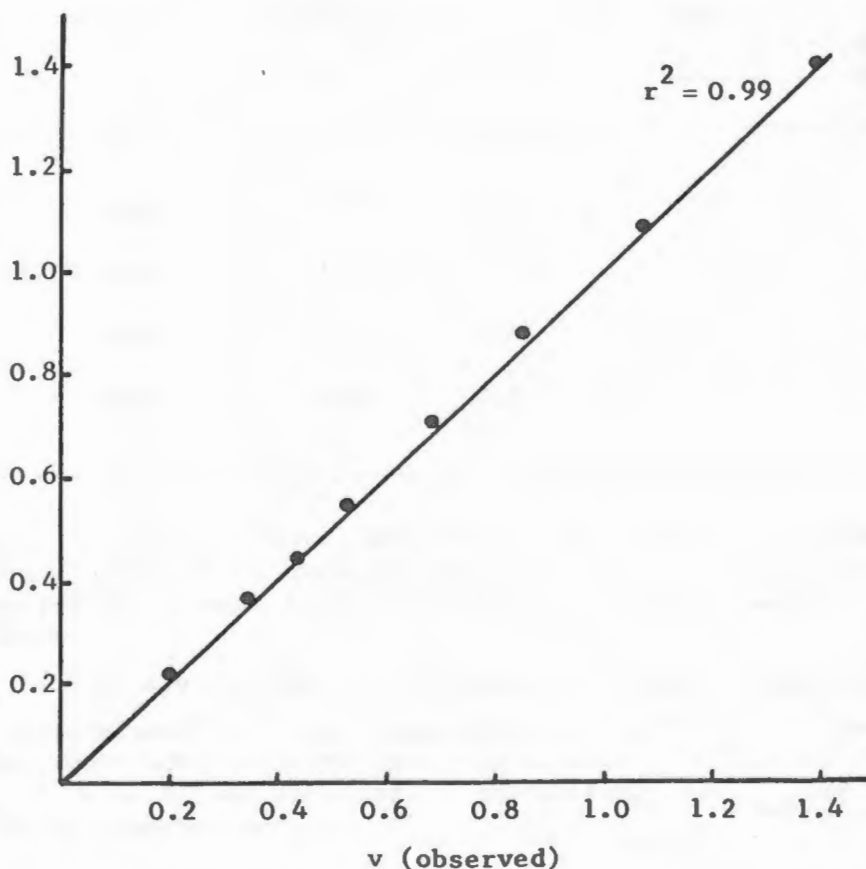
The developmental profiles of the two forms of ANOH are depicted in Figure 12. In one week old piglets, Form I constitutes approximately 65% of the total activity. Between one and four weeks

FIGURE 10. Eadie-Hofstee Plot of Aniline Hydroxylase Activity in Microsomes from Uninduced, Adult Miniature Pigs.



- b) Details of the assay conditions and derivation of kinetic constants are described in Materials & Methods.
- b) Miniature pigs were 16 weeks old at the time of sacrifice. Microsomes were obtained from a pool of two livers.
- c) Each point represents the average of duplicate determinations for the substrate concentration utilized.

FIGURE 11. Plot of v (observed) Against v (calculated) Based on Biphasic Kinetic Analysis of Aniline Hydroxylase Activity.



- a) The observed velocity represents the total velocity (v_T) at each aniline concentration from the experimental data shown in Figure 10.
 b) Theoretical values of v_T and v_T/S were calculated using the estimates for the apparent K_m and V_{max} of each component (Figure 10) and the substrate concentrations used in the assay. The values were substituted into the equation:

$$v_T = v_1 + v_2 = \frac{V_{max1}S}{K_{m1} + S} + \frac{V_{max2}S}{K_{m2} + S}$$

TABLE 13. Summary of the kinetic parameters for the two forms of aniline hydroxylase in developing minipigs.^a

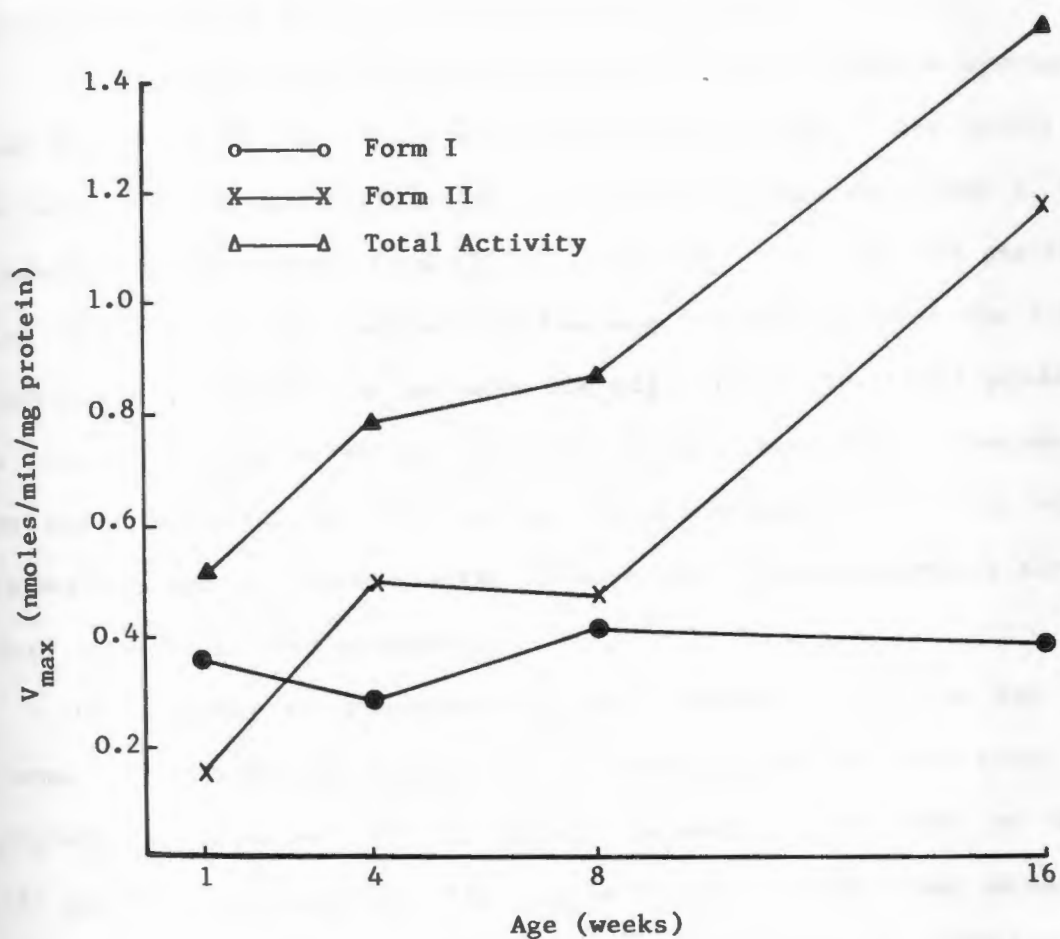
Age	FORM I		FORM II	
	Apparent <u>K_m</u> (uM)	<u>V_{max}</u>	Apparent <u>K_m</u> (mM)	<u>V_{max}</u>
1 Week	8.2	0.36	ND ^c	0.16
4 Week	3.6	0.29	0.52	0.50
8 Week	5.4	0.41	0.56	0.47
16 Week	6.8	0.39	0.74	1.17

^aKinetic parameters were determined as detailed in Materials and Methods. Experimental data was obtained from duplicate determinations on pooled microsomes from three pigs (1, 4 and 8 weeks) or two pigs (16 weeks).

^bV_{max} is expressed as nMoles p-aminophenol formed/min/mg protein.

^cThe predominance of the high-affinity activity in one week old piglets precluded accurate analysis of the K_m value for Form II. The V_{max} of Form II was obtained by subtracting^m the V_{max} of Form I from the total observed velocity (V_{max2} = V_T - V_{max1}).

FIGURE 12. Developmental Profiles for the Two Forms of Aniline Hydroxylase in Uninduced Miniature Pigs.



a) Points represent the V_{\max} values shown in Table 13.

b) Total aniline hydroxylase activity is the sum of the V_{\max} values for Form I and Form II.

of age, the maximum velocity of Form II approximately triples while Form I remains relatively constant. At this point, Form II contributes to 65% of the total activity. There appears to be a leveling off in both components to eight weeks followed by a further 2.5-fold increase in the activity of Form II by sixteen weeks of age.

The effects of phenobarbital on the two forms of aniline hydroxylase are shown in Table 14. At all ages, phenobarbital has little or no effect on the activity of the high-affinity component, Form I. The low-affinity component, Form II, is increased at all ages. Of particular interest is the inverse relationship between age and the total increase in activity. In one week old piglets, phenobarbital produced a 12.8-fold increase in the activity of the low-affinity component. By eight weeks of age, the increase is approximately half that value (6.4-fold) and by sixteen weeks phenobarbital produces only a three-fold increase in this component.

In addition to phenobarbital, the effects of PCA on the two forms of aniline hydroxylase were investigated in four week old piglets. A comparison of the kinetic parameters are shown in Table 15. As with phenobarbital, the high-affinity component was unchanged while the low-affinity component was slightly increased in PCA treated piglets. Furthermore, while phenobarbital does not appear to alter the apparent K_m of the low-affinity component, PCA treatment reduced the apparent K_m by approximately 50%.

Similar kinetic analyses were conducted using PCA demethylase. As with aniline hydroxylase, Eadie-Hofstee plots appeared biphasic

TABLE 14. Summary of the kinetic parameters of the two forms of aniline hydroxylase in phenobarbital induced, developing minipigs.^a

Age	FORM I			FORM II		
	Apparent K_m (uM)	V_{max}	V_{pb}/V_c	Apparent K_m (mM)	V_{max}	V_{pb}/V_c
1 Week	11.3	0.35	.97	0.93	2.04	12.8
4 Week	6.9	0.24	.83	0.58	2.26	4.5
8 Week	6.2	0.29	.71	0.55	2.99	6.4
16 Week	19.5	0.35	.90	0.94	3.40	2.9

^aKinetic parameters were determined as detailed in Materials and Methods. Experimental data was obtained from duplicate determinations on pooled microsomes from three pigs (1, 4, and 8 weeks) or two pigs (16 weeks).

^bPhenobarbital was administered at 10 mg/kg P.O. for four consecutive days.

^c V_{pb}/V_c is the ratio of the V_{max} for phenobarbital induced pigs to the V_{max} for uninduced pigs (Table 13).

TABLE 15. Comparison of the kinetic parameters for the two forms of aniline hydroxylase in uninduced, PCA and phenobarbital treated minipigs at four weeks of age.^a

Treatment	FORM I		FORM II	
	Apparent K_m (uM)	V_{max}	Apparent K_m (mM)	V_{max}
Control	3.6	0.29	0.52	0.50
PCA ^c	3.0	0.30	0.27	0.78
Phenobarbital ^b	6.9	0.24	0.58	2.26

^aKinetic parameters were determined as detailed in Materials and Methods. Experimental data was obtained from duplicate determinations on pooled microsomes from three piglets in each treatment group.

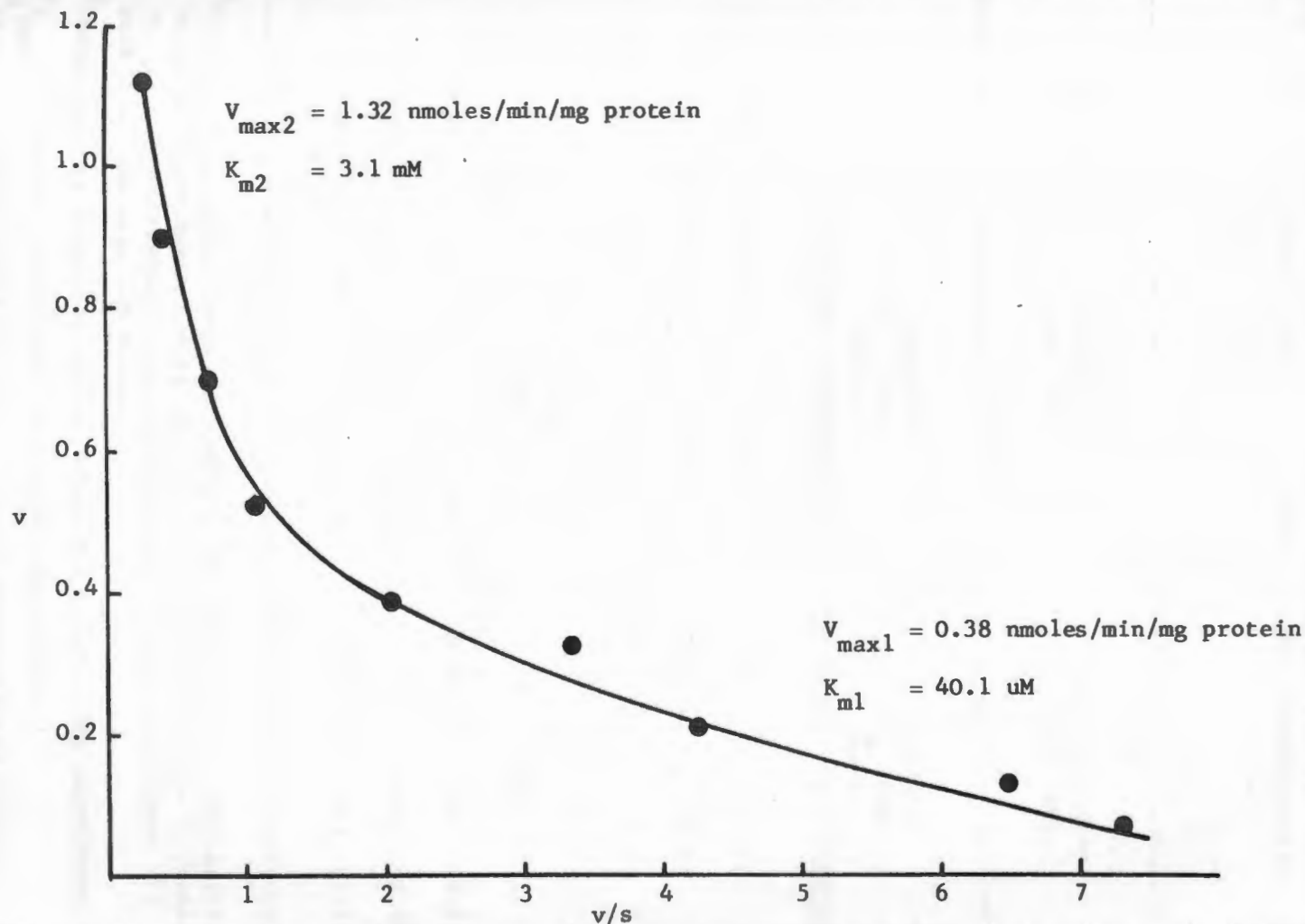
^bPhenobarbital was administered at 10 mg/kg P.O. for four consecutive days.

^cPCA was administered at 10 mg/kg P.O. for four consecutive days.

(Figure 13). In sixteen week old uninduced pigs, the high affinity component had an apparent K_m of 40.1 μ M and a V_{max} of 0.38 nmoles/min/mg protein. The low affinity component had an apparent K_m of 3.1 mM and a V_{max} of 1.32 nmoles/min/mg protein. A summary of the kinetic constants for uninduced and phenobarbital induced microsomes at various ages is shown in Table . In the case of PCA demethylation, both the high and low-affinity components are increased by phenobarbital. Furthermore, while the apparent K_m of the high-affinity component appears relatively unchanged by treatment, the apparent K_m of the low-affinity form is markedly reduced.

When the data are arranged to illustrate age related increases in the two components as a result of phenobarbital induction (Table 16), it is interesting to note that while the low-affinity form is increased to a greater extent over basal levels, the magnitude of this increase remains relatively constant with age. In contrast, the magnitude of the increases in the high-affinity component produced by phenobarbital appears to decrease with age. In this respect, an interesting correlation exists between these data and that seen in Table 10 for catalytic activity of PCA demethylase. In Table 16, there appears to be a greater than 50% decrease in the magnitude of phenobarbitals effect between one and eight weeks of age (5.4-fold vs. 2.5-fold). Similarly, the catalytic activity of PCADM (Table 10) decreases from 2.15 to 0.91 between one and eight weeks of age in phenobarbital treated pigs.

FIGURE 13. Eadie-Hofstee Plot of PCA Demethylase Activity in Microsomes from Uninduced, Adult Miniature Pigs.



- Details of the assay conditions and derivation of kinetic parameters are described in Materials & Methods.
- Miniature Pigs were 16 weeks old at the time of sacrifice. Microsomes were obtained from a pool of two livers.
- Each point represents the average of duplicate determinations for the substrate concentration utilized.

TABLE 16. Comparison of the kinetic parameters for the two forms of PCA demethylase in uninduced and phenobarbital induced developing miniature pigs.^a

Age	High Affinity Component		Low Affinity Component	
	Apparent K_m (uM)		Apparent K_m (mM)	
	<u>Uninduced</u>	<u>Phenobarbital</u>	<u>Uninduced</u>	<u>Phenobarbital</u>
1 Week	25.5	17.6	1.9	0.66
8 Week	18.0	12.9	2.6	0.65
16 Week	40.1	25.6	3.1	1.01
	V_{max}^c		V_{max}	
1 Week	0.14	0.75 (5.4) ^d	0.64	5.26 (8.2)
8 Week	0.22	0.55 (2.5)	0.55	4.92 (8.9)
16 Week	0.38	0.57 (1.5)	1.32	9.63 (7.3)

^aKinetic parameters were determined as detailed in Materials and Methods. Experimental data was obtained from duplicate determination on pooled microsomes from three pigs in each treatment (1 and 8 weeks) or two pigs (16 weeks).

^bPhenobarbital induction was as described in previous experiments.

^c V_{max} is nMoles formaldehyde formed/min/mg protein.

^dValues in parenthesis are the ratio of phenobarbital induced activity (V_{max}) to uninduced activity.

DISCUSSION

In support of those studies reporting the detection of pentachlorophenol in biological fluids following treatment with pentachloroanisole (Glickman *et al.*, 1977; Vodcnik *et al.*, 1980), the present investigation has shown that PCA is indeed a substrate for microsomal mixed-function oxidation. As evidenced by the requirement for NADPH and the inhibition by carbon monoxide and other documented inhibitors of MFO (Table 1), PCA is metabolized via a cytochrome P450-dependent demethylation reaction resulting in the formation of PCP (Figure 1). Since PCP has been shown to be metabolized to lower chlorinated compounds such as tetrachlorohydroquinone and trichlorophenol (Ahlborg and Thunberg, 1978), the peak at 2.3 minutes in Figure 1(b) may represent one of these metabolites, although further characterization is required.

These results present an interesting and ironic situation. Whereas it is often assumed that biodegradation of pesticides and other environmental contaminants renders them inactive, PCA represents one biodegraded product that can be reactivated to its parent compound, PCP, in species possessing the proper metabolic capacity. Furthermore, as indicated by Glickman *et al.* (1977), PCP exhibits a higher degree of bioaccumulation and is more persistent than PCA in fish. Thus, the compound may present a more difficult assessment with respect to contamination of the food chain.

Initial kinetic analysis of PCA demethylation provided some interesting results. The activity in uninduced microsomes (< 0.5 nmoles/min/mg protein) is much lower than that reported in the literature for other O-demethylated substrates such as nitroanisole in various laboratory species. The possibility of species differences, however, has been discounted since a similarly low rate of PCA metabolism has been observed in uninduced rat liver (Agins *et al.*, 1982). An unusually large increase in activity following phenobarbital induction, in this case greater than ten-fold, suggested the existence of a specific-inducible form of cytochrome P450 that preferentially metabolized PCA. In support of this assumption are the sensitivity of PCA demethylation in phenobarbital induced microsomes to metyrapone inhibition (Table 1) and the results from substrate binding spectra (Figure 3). The inability to detect discernable spectral changes in uninduced microsomes is most likely a function of the low concentration or absence of the PCA specific form of cytochrome P450. Following phenobarbital induction, a binding spectra becomes readily apparent, which correlates with the increase in demethylase activity. Furthermore, the spectral dissociation constant (K_s) of approximately 15 μ M indicates that PCA has a relatively high affinity for the inducible form of cytochrome P450 (Figure 4).

In conjunction with these findings, it is interesting to note that Hultmark and coworkers (1979) reported a similar situation for the metabolism of dichloro-p-nitroanisole in rats. Their results indicated that this chlorinated analogue of nitroanisole was demethylated quite poorly by uninduced microsomes and that phenobarbital

induction resulted in a 24-fold increase in activity as well as increased sensitivity to metyrapone inhibition. Using the selectivity of various MFO inhibitors, the authors concluded that the forms of cytochrome P450 metabolizing dichloro-p-nitroanisole in uninduced and phenobarbital induced microsomes were different. It would appear then, that the presence of chlorine atoms or the degree of chlorination alters the physio-chemical properties of these anisole in such a way that changes their interactions with various cytochrome P450 subpopulations.

The finding that PCA was a substrate for cytochrome P450-dependent metabolism prompted an investigation of the compound as a potential inducer of the MFO system. Furthermore, since it has been reported that younger animals, in many cases, respond to a greater extent than their adult counterparts (Basu et al., 1971; Klinger et al., 1981) the present investigation attempted to focus on age related differences in induction.

The choice of the miniature pig for this study was based on a number of factors. The minipig is the largest, non-altricious animal giving multiple births. As such it is well suited for perinatal studies, because unlike the rat and most laboratory species, the neonate is mobile minutes after birth. This imparts the ability to wean and raise piglets with minimal effort and handling. Furthermore, the size of the neonate permits easy, direct oral administration of test compounds, thereby avoiding indirect maternal influences.

Although a large number of studies have been conducted on the postnatal development of the hepatic MFO system in various laboratory

species, similar studies in the miniature pig have not appeared in the literature. Short and Davis (1970) reported that in the domestic Duroc pig, MFO activity rises rapidly and in a linear fashion from birth to approximately four weeks of age. From this point, activity either plateaus or continues to increase at a much reduced rate. Similar patterns have been reported for rabbits, rats and mice (Gram and Fouts, 1966). As seen in Tables 3 and 4, the developmental pattern of MFO in miniature pigs is consistent with those in other laboratory animals. Oxidative metabolism appears to reach maximum at four weeks of age. Furthermore, activities in the one week old piglet range from 65% to 80% of activity in eight week old pigs. Although the present investigation did not include newborns, values of 0.14 nmoles/mg protein for cytochrome P450 and 0.22 nmoles/min/mg protein for aniline hydroxylase were obtained from piglets less than 24 hours old in a previous study (Agins, unpublished results). Thus when compared to the respective values in Tables 3 and 4, it appears that the most rapid rate of development probably occurs between birth and one week of age.

Although early postnatal studies in minipigs are lacking, the results in eight week old pigs were able to be compared to two studies on MFO in Hanford Miniature pigs of similar age. Cytochrome P450 levels and aniline hydroxylase activity compared almost identically to values reported by Freudenthal et al. (1976) and Litterst et al. (1976). Furthermore, as reported in both studies, no sex differences in MFO activity were demonstrated.

In one week old piglets, PCA produced significant increases in all parameters measured. The greatest increase, a three-fold increase in PCADM, suggests that as with phenobarbital, PCA induces a specific form a cytochrome P450. The smaller increases observed for ANOH and NADM suggest that the induced form of P450 displays over-lapping substrate specificity in agreement with the literature. In this respect, it is generally accepted that some substrates are metabolized most efficiently by one form of cytochrome P450, yet can also be metabolized by other forms at much lower rates.

Although differing in magnitude, the qualitative nature of PCA's induction is quite similar to that of phenobarbital. In both cases, the greatest effect was seen in PCADM followed by NADM and then ANOH (Table 4). Similarly, PCA increased the microsomal concentration of cytochromes P450 and b_5 and the activity of cytochrome c reductase (Table 5). Interestingly, the increases in the latter two parameters were identical in both phenobarbital and PCA treated pigs. The increase in cytochrome P450 concentration in phenobarbital treated pigs, however, was much greater than in PCA treated animals. In explaining this apparent disparity (i.e. similar effects of the two compounds on some microsomal components and differences in others) an important issue requires clarification.

The increased concentration of microsomal proteins following treatment may be a result of an increased rate of synthesis, a decreased rate of degradation or a combination of both. In this respect, Kuriyama et al. (1969) showed that the net increase in cytochrome c reductase following phenobarbital treatment was a

function of both increased synthesis and decreased degradation. In contrast, the increased concentration of cytochrome b_5 seen after treatment was only due to a decrease in the rate of decay of this protein. The authors additionally showed that after a short lag, cytochrome P450 synthesis followed that of reductase.

Thus, it is likely that phenobarbital and PCA produce a true induction of reductase and P450. That differences exist in the magnitude of P450 induction would suggest that phenobarbital is a more efficient or stronger inducer. In view of the proposed heterogeneity of cytochrome P450 populations (Lu and West, 1980), however, the greater effect of phenobarbital may be a result of more than one form of cytochrome P450 induced.

In favor of this assumption, at least two forms of cytochrome P450 have been shown to be induced by phenobarbital in mice (Huang et al., 1976), rats (Bornheim and Franklin, 1982) and more closely related to the present study, pigs (Tsuji et al., 1980). In the latter study, the authors reported that at least two and possibly three forms of P450 were induced by phenobarbital in adult male pigs.

From the present results, it may be speculated that one form of phenobarbital inducible P450 may be intimately coupled with cytochrome c reductase (i.e. within the same genetic locus) while additional form(s) are not linked directly with this microsomal protein. Furthermore, the almost identical increases in P450 and reductase (33% and 31%, respectively) produced by PCA, coupled with the qualitative similarities in relation to phenobarbital's effects, suggests that PCA induces one of the phenobarbital inducible forms.

In addition to the biochemical effects produced by PCA and phenobarbital, morphological changes in hepatic ultrastructure were also comparable. While in the one week old control piglet, the major portion of endoplasmic reticulum is associated with ribosomes (Figures 5 and 6), both PCA and phenobarbital produced a substantial proliferation of smooth endoplasmic reticulum (Figures 7 and 8), consistent with the documented effect of phenobarbital on this organelle (Fouts and Rogers, 1965).

The effects of PCP are more difficult to explain. A significant increase in the cytochrome P450 content in one week old piglets is coupled only with a small increase in the activity of nitroanisole demethylase. A number of possibilities exist. Tsuji and coworkers (1980), in addition to demonstrating various phenobarbital forms of P450, reported that ethanol induced a form of P450 similar to a constitutive form. Furthermore, both forms exhibited the lowest activities toward the three substrates used in their study. In this respect, PCP may induce a similar form of P450 displaying limited activity towards the substrates used in this study.

A second possibility is that PCP, in view of its known membrane binding effects (Danner and Resnick, 1980), may interfere with the association of newly synthesized P450 within the endoplasmic reticulum, rendering it relatively inactive. Whatever the mechanism, the present results confirm earlier studies which indicated that purified PCP is relatively devoid of hepatic MFO effects (Goldstein et al., 1977).

On a morphological basis, PCP did produce an increase in the cellular content of smooth ER (Figure 9). This finding while consistent with a study by Kimbrough and Linder (1975), would not appear to be consistent in view of the correlation between the biochemical and morphological data for PCA and phenobarbital.

Cresteil et al. (1980), however, proposed three effects of exogenous chemicals on endoplasmic reticulum: (1) induction of metabolic activities without proliferation of membranes as produced by polycyclic hydrocarbons, (2) simultaneous induction of enzyme activities and proliferation of membranes as produced by the total sequence of events involving phenobarbital, and (3) proliferation of membranes without apparent enzyme induction as produced by the para-hydroxylated metabolite of phenobarbital. Therefore, the possibility exists that PCP produces the latter effect or some combination of the three. Caution should, however, be observed concerning the morphological results since the electron photomicrographs shown in Figures 5 through 9 are representative of only one animal per treatment group.

By eight weeks of age, the inductive effects of PCA were greatly diminished. However, in the absence of a significant increase in cytochrome P450, PCA demethylase activity continues to be significantly greater than controls. Although this would appear to be inconsistent with the necessary requirement for P450, Bornheim and Franklin (1982) warned that compounds might cause subtle changes within the subpopulations of cytochrome P450 not necessarily evidenced by increases in microsomal cytochrome P450 concentrations. Furthermore, it is likely that synthesis of one form of cytochrome P450 may

occur at the expense of others (Atlas et al., 1977). Thus, in eight week old pigs the rate of degradation of a constitutive form of P450 may be offset by a similar rate in the synthesis of an inducible form such that the overall concentration of P450 appears unchanged in PCA treated animals. Furthermore, if the induced form were specific for PCA demethylation it would explain the significant increase observed in this parameter.

In view of the concept of various "isoenzymatic" forms of cytochrome P450, each differing in substrate specificity, the expression of results in terms of catalytic activities is finding increasing popularity in the literature.

When the specific activities for PCADM, ANOH, and NADM were recalculated to reflect catalytic activities, a number of interesting effects were observed with respect to age and treatment. PCA and phenobarbital increased the catalytic activity of PCA demethylase to the greatest extent. Thus, as suggested earlier, these compounds induce one or more forms of cytochrome P450 that preferentially catalyze PCA. Phenobarbital also increased the catalytic activity of nitroanisole demethylase, but to a lesser extent than in PCADM. Furthermore, while statistical significance could not be demonstrated, PCA treatment tended to increase catalytic activity for this substrate. These results would suggest that while PCA may be most efficiently metabolized by phenobarbital inducible forms of P450, nitroanisole is probably metabolized by a combination of constitutive and inducible forms.

The finding that PCP does not significantly alter catalytic activities in any of the parameters, especially in nitroanisole demethylase where increases were observed in specific activities, lends some support to the suggestion that the compound may induce a constitutive form of P450.

The most striking effects, upon further examination of catalytic activity results, are the age related changes observed in induced pigs. While the catalytic activities for control animals remained relatively constant from one to eight weeks of age, the effects of PCA and phenobarbital appear to decrease in an age dependent manner. The eight week old values in all three parameters are less than 50% of the corresponding one week old catalytic activities in phenobarbital treated pigs. This would imply that the inducible, substrate specific form(s) of cytochrome P450 represent a smaller percentage of the total P450 concentration in microsomes of eight week old pigs. This may be the result of either a decrease in the synthesis of a particular form or a diluting effect of increased synthesis of non-specific forms.

In support of this proposal, Thomas and coworkers (1980) reported that the major phenobarbital inducible form of P450 (P450-b) in rats accounted for 70% of the total P450 in immature animals, while only 35% in adult microsomes. Furthermore, a minor form of P450 (P450-a) present in uninduced microsomes, was induced by both phenobarbital and 3-MC in immature, but not adult rats.

Since a number of investigations have suggested that biphasic Michaelis-Menten kinetics are indicative of more than one species of

P450 catalyzing the same reaction (Greenlee and Poland, 1978; Boobis et al., 1981; McCoy, 1980), attempts were made to further investigate the possibility of age dependent changes in the induction of P450 subpopulations utilizing kinetic experiments. Based on the assumption that sufficiently large differences in affinities toward a particular substrate may enable detection of the activities of different P450 forms, aniline concentrations were varied over three orders of magnitude. When plotted by the Eadie-Hofstee method, aniline hydroxylase activity could, in fact, be resolved into two kinetic components (Figure 10). While the affinities of the two forms differed by greater than 100-fold, the differences in maximum velocities varied by only three-fold. Furthermore, although the absolute values for the kinetic constants differ slightly, these results are in agreement with those reported by McCoy (1980) for aniline hydroxylase activity in hamster liver microsomes.

By conducting similar kinetic analyses at various ages, a developmental profile was obtained for the two forms of aniline hydroxylase activity (Figure 12). While the high-affinity component (Form I) remained relatively constant during development, the low affinity component (Form II) exhibited a biphasic nature, increasing from 1 to 4 weeks and then again between 8 and 16 weeks of age. Interestingly, between 1 and 4 weeks of age, there is a cross-over in the developmental pattern. Thus, there is an age dependent change in the contribution of the two forms to the total aniline hydroxylase activity. The high-affinity component constitutes 70% of total

activity in one week old piglets while the low-affinity component contribute to 75% in the sixteen week old animal.

Based strictly on the kinetic data from uninduced pigs, it would be impossible to establish the relationships between the two components of aniline hydroxylase and various, well characterized forms of cytochrome P450. The selective induction of only one component of aniline hydroxylase activity by phenobarbital (Table 14), however, provided some basis for comparisons. That the high-affinity component was essentially unaffected by phenobarbital treatment suggested that this form may be cytochrome P448. Indeed, this 3-methylcholanthrene inducible form, synonymous with arylhydrocarbon hydroxylase (AHH), is not induced by phenobarbital. Furthermore, studies with various purified forms of cytochrome P450 (448), isolated from both rat (Ryan et al., 1979) and pig (Tsuji et al., 1980) have shown that aniline is most efficiently metabolized by cytochrome P448.

In contrast to its lack of effect on the high-affinity component, phenobarbital does increase the low-affinity activity. In comparing the apparent K_m values observed in uninduced and induced microsomes (Tables 13 and 14), it would appear that phenobarbital's effect was strictly quantitative. In otherwords, phenobarbital induces the synthesis of a constitutive form of cytochrome P450. This, however, is contrary to the overwhelming evidence in the literature that phenobarbital induces forms of cytochrome P450 distinctly different from the major form in uninduced animals. Therefore, a more tenable explanation is that the phenobarbital induced form is in fact qualitatively different from the constitutive form, but exhibits a similar affinity

towards aniline. In this respect, Tsuji and coworkers (1980) reported that the major phenobarbital inducible form (P450 B) purified from pig liver displayed identical catalytic activity as the form isolated from uninduced animals (P450 Cent). The two forms, however, were determined to be uniquely different proteins by a variety of other criteria. Additionally, the authors reported that one minor form of P450 (P450 A), induced by phenobarbital, exhibited a two-fold greater catalytic activity towards aniline than the major inducible form.

At this point, it is important to mention that while two aniline hydroxylase activities could be resolved in control and induced animals in this study, the possibility exists that the detection of additional kinetic species may be obscured by the predominance of a low-affinity/high-capacity enzyme or similarities in enzyme affinity towards a particular substrate. Thus, the observed activity of the low-affinity component in phenobarbital induced pigs may be the cumulative activities of more than one form of cytochrome P450. This possibility may help to explain the changes in the magnitude of phenobarbital's effect as a function of age (Table 14). As previously discussed, Thomas and coworkers (1980) described a minor form of cytochrome P450 (P450a) which was induced by phenobarbital in immature, but not adult rats. If the minor inducible form in pig liver (P450 A) (Tsuji et al., 1980), which was more active towards aniline, is under similar temporal control, it could explain the greater effect of phenobarbital on one week versus older animals. Furthermore, the age-dependent induction of a more highly active form of aniline

hydroxylase would explain the significant increase in catalytic activity in one week old piglets (Table 11) and the absence of a significant effect in older animals.

In view of the qualitative similarities between PCA and phenobarbital induction, an attempt was made to further characterize the PCA inducible form using similar kinetic analysis. As with phenobarbital, PCA had no effect on the high-affinity component of aniline hydroxylase (Table 15). PCA did however appear to increase the activity of the low-affinity component slightly as well as decrease the apparent K_m . Based on the earlier suggestion that the low-affinity component in phenobarbital induced microsomes probably represents more than one form of P450, it is tempting to speculate that the difference in apparent K_m 's reflects the induction of only one of these forms by PCA.

The proposal by Hultmark et al. (1979) of at least three different forms of cytochrome P450 active in the demethylation of dichloro-p-nitroanisole in rat liver microsomes prompted an investigation to determine if PCA demethylation could also be resolved into kinetic components. Thus, while the Lineweaver-Burke plots of PCADM (Figure 2) appeared linear, two factors should be considered. First, the substrate concentration range used for these experiments varied by only twenty-fold. Secondly, in these early experiments, acetone was incorporated as a vehicle for PCA. Although the transition to Tween 80 alone, as substrate carrier followed shortly thereafter, it was later learned that acetone produced inhibitory effects similar to ethanol in uninduced microsomes (Hultmark et al., 1979). By widening the substrate concentration range and avoiding organic solvent

vehicles, PCA demethylase activity did in fact display a biphasic nature (Figure 13). The summary of the kinetic data for the two forms of PCA demethylase as a function of age and treatment (Table 16) provided some interesting correlations with a number of results throughout the entire study.

The close similarity in the apparent K_m values for the high-affinity component in uninduced and induced pigs would suggest that the same form of cytochrome P450 exists in both sources. Unlike the high-affinity form of aniline hydroxylase, however, this component is induced by phenobarbital. The small contribution of the high-affinity component to the total phenobarbital induced activity would further indicate that this form is a minor inducible form. Collectively, these assumptions compare favorably with the report by Thomas and coworkers (1980) of an identical form of cytochrome P450 (P450a) present in uninduced, phenobarbital and 3-methylcholanthrene induced rat liver microsomes. In all cases, this form accounted for a small fraction (3-7%) of the total cytochrome P450 concentration.

In contrast to the high-affinity component, the marked differences in the apparent K_m values of the low-affinity component would indicate that the induced and uninduced forms of cytochrome P450 are different. Hence, the predominance of the low-affinity component in the total phenobarbital induced PCADM activity would suggest that this form may be the major phenobarbital inducible form described by Tsuji et al. (1980).

The low-affinity component in uninduced microsomes represents, most likely, the P450-control form in pigs (Tsuji et al., 1980).

Whether this form is similar to the "ethanol sensitive" constitutive form in rats (Hultmark et al., 1979) remains to be determined. It may be worth noting, however, that the kinetic parameters in uninduced adult pigs from earlier experiments (Figure 2) are remarkably similar to those for uninduced adult pigs' high-affinity component (Table 16). Thus, the presence of acetone in the early PCA demethylase assay may have inhibited the expression of the low-affinity constitutive form.

In agreement with the age-dependent changes in the degree of phenobarbital's effect on aniline hydroxylase activity, a similar phenomenon was observed for PCA demethylase. The magnitude of phenobarbital's effect on the low affinity component of PCADM remains relatively constant with age. However, since the constitutive and phenobarbital inducible forms of P450 responsible for the low-affinity activity are probably different, these results may be misleading. Although uninduced microsomes may contain a small amount of the phenobarbital inducible form of P450 (Thomas et al., 1980; Tsuji et al., 1980), kinetic analysis was not able to separate out the contribution of the component in uninduced pigs. Therefore, the magnitude of phenobarbital's effect is most likely underestimated. For the same reasons above, age-dependent changes in the inducibility of this particular form would not be detectable.

In contrast, the high-affinity component does undergo age related changes in the degree of induction. The increase in activity over basal level in eight week old pigs is approximately 50% of the effect observed in one week old animals. This effect is consistent with that

seen in aniline hydroxylase activity where a similar 50% decrease in the inducibility of the low-affinity component was observed between one and eight weeks of age.

In summary, based on the biphasic kinetic characteristics of two substrates, as a function of age and treatment, it would appear that at least four forms of cytochrome P450 exist in hepatic microsomes of miniature pig. They are: (1) the high-affinity component of aniline hydroxylase, present in uninduced and induced microsomes, but not altered by phenobarbital or PCA induction, (2) the low-affinity components of aniline hydroxylase and PCA demethylase in uninduced microsomes, (3) the high-affinity component of PCA demethylase, which displays age dependency in phenobarbital induction and most likely contributes to the phenobarbital induced, low-affinity activity of aniline hydroxylase. Furthermore, this is probably the form induced by PCA, and (4) the low-affinity components of PCA demethylase and aniline hydroxylase in phenobarbital induced microsomes.

The mechanism(s) by which temporal alterations are manifested in the induction of specific cytochrome P450 subpopulations are presently not known. However, in view of the intimate relationship between endogenous substances, particularly steroid hormones, and the mixed-function monooxygenase system, it is quite possible that these agents may play a role in cellular regulation of P450 subtypes.

CONCLUSIONS

(1) Pentachloroanisole is metabolized in vitro to pentachlorophenol by hepatic microsomes from miniature pigs. The requirement for NADPH, inhibition by carbon monoxide, and production of a substrate binding spectrum indicate that the compound is catalyzed via a cytochrome P450-dependent demethylation reaction.

(2) The relatively large increase in the rate of PCA demethylation following phenobarbital induction strongly suggests that PCA is preferentially catalyzed by a phenobarbital inducible form(s) of cytochrome P450. The very low levels of activity, coupled with the inability to detect a substrate binding spectrum in uninduced microsomes suggests that the substrate-specific form of P450 is absent or present in very low concentration in uninduced microsomes.

(3) The postnatal developmental pattern of hepatic mixed-function monooxygenase activity in miniature pigs is similar to that in other laboratory species. In most parameters measured, an increase between one and four weeks of age is followed by a plateau to eight weeks of age. The additional rise in activity for aniline hydroxylase and PCA demethylase between eight and sixteen weeks of age, observed in kinetic experiments, may be associated with the attainment of sexual maturity in miniature pigs.

(4) Pentachloroanisole treatment produced increases in all MFO parameters measured in one week old minipigs. The qualitative nature of PCA's induction is similar to that of phenobarbital. The greatest effect, a three-fold increase in its own metabolism, suggests that the compound induces a specific form of cytochrome P450, similar to that induced by phenobarbital.

(5) Pentachlorophenol treatment produced increases in some, but not all MFO parameters in one week old piglets. The overall qualitative differences produced by PCA and PCP suggests that the two compounds induce different species of cytochrome P450 which exhibit overlapping substrate specificity in some cases.

(6) The proliferation of smooth endoplasmic reticulum observed following treatment with all three compounds indicates that the magnitude of the biochemical effects in MFO induction does not necessarily correlate with alterations in the cellular content of this organelle.

(7) The age related decrease in catalytic activities observed in phenobarbital and PCA treated pigs is suggestive of changes in the relative contribution of different subpopulations of cytochrome P450 to the observed overall specific activity. The almost identical decreases in PCA demethylase, aniline hydroxylase and nitroanisole demethylase catalytic activities between one and eight weeks of age suggests that the contribution of a particular form of P450, active to varying degrees on all three substrates, decreases with development.

(8) The biphasic kinetic profiles observed for aniline hydroxylase and PCA demethylase activities indicates that multiple forms of cytochrome P450 are active in the metabolism of both substrates. By integrating the effects of age and phenobarbital treatment on the various kinetic species, it is suggested that at least four forms of cytochrome P450 exist in miniature pig microsomes. The inducibility of at least one form by phenobarbital appears to decrease with age and the magnitude of this change correlates well with the effects seen in catalytic activity profiles.

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APPENDIX A

Analysis of Pentachlorophenol and Pentachloroanisole for
Non-Phenolic Contaminants.¹

Source	PCP ² (Aldrich 99%)	PCP	PCA
Dibenzo-p-dioxins			
Hexachloro-	<0.01 ppm	<0.01	<0.01 ppm
Heptachloro-	<0.01	<0.01	<0.01
Octachloro-	≤150.0	≤1.50	<0.01
Dibenzofurans			
Hexachloro-	<0.01	<0.01	<0.01
Heptachloro-	<0.01	<0.01	<0.01
Octachloro-	<0.01	<0.01	<0.01

¹ Samples were analyzed by Gas Chromatography by the Analytical Chemistry section, Office of Pesticides, U.S. EPA, Beltsville, MD. Sensitivity of methods was between 1-10 ppb.

² Aldrich 99% PCP was utilized as source for further purification of PCP and synthesis of PCA for the study.

APPENDIX B

Electron Microscopy Procedures¹

At the time of piglet sacrifice, liver sections were blotted and placed immediately into Karnovsky's fixative containing sucrose.² Tissues were refrigerated for one week and then transferred to Dr. Seibel.

Tissues were removed, cut into smaller slices and returned to fresh fixative for 10 days. At that time, slices were washed with cacodylate buffer (pH 7.4) with 7% sucrose, and allowed to stand overnight in clean buffer. Tissue was next post fixed for 2 hours in OsO_4 and then subjected to graded alcohol dehydration (30% - 100%), followed by 2 changes of propylene oxide and a propylene oxide:Epon mixture (1:1). Tissues were next placed in 100% Epon mixture at room temperature overnight and then embedded for 4 days in Epon mixture at 60°.

Blocks of tissue were thin sectioned with a Porter-Blum MT2 ultramicrotome and double stained with lead citrate and alcoholic uranyl acetate.³ An AEI-6B Transmission Electron Microscope was used for this study.

¹Dr. Werner Seibel, Department of Anatomy, University of Maryland School of Dentistry, Baltimore, MD (personal communication).

²Karnovsky, M.J.: A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27: 137A, 1965.

³Reynolds, E.S.: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17: 208-212, 1963.