STUDIES ON THE DEVELOPMENT OF THE MIXED-FUNCTION OXIDASE OF RAT LIVER ENDOPLASMIC RETICULUM

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ABSTRACT

STUDIES ON THE DEVELOPMENT OF THE
MIXED-FUNCTION OXIDASE OF RAT
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By

Judy Yi-Huei Wu

The postnatal development of the rat liver endoplasmic reticulum has been investigated, especially with respect to the mixed-function oxidases for the metabolism of drugs located therein. The postnatal increase in the activity of this enzyme system occurred most rapidly during the first three weeks of age. The development of cytochrome P-450 and NADPH-cytochrome c reductase did not show the same pattern of development as did aminopyrine demethylase activity, which increased much later. Also, while the treatment of pregnant rats with phenobarbital did increase the aminopyrine demethylase activity in the microsomes of the newborn pups, it did not affect the concentration of cytochrome P-450 equally.

Peroxidation of endogenous lipid in the microsomes of newborn rats also did not occur; however, such activity was obtained when microsomal lipids or methylarachidonate was added to the microsomal preparation, suggesting the

lack of suitable microsomal lipids. However, a comparison of the microsomal lipids and the fatty acid composition of these lipids did not show any significant differences throughout development.

These results suggest that some other factor may be involved in the development of microsomal enzymes for the metabolism of drugs. This factor may be: (1) a lack of the proposed electron carrier "x"; (2) the reduction of cytochrome P-450 as the rate limiting step; (3) the existence of P-450 which is not involved in the demethylation of aminopyrine; or (4) a "maturation" of the mixed-function oxidase into an active configuration. The last possibility could involve lipid.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

NADP	nicotinamide dinucleotide phosphate
NADPH	nicotinamide dinucleotide phosphate, reduced
ADP	adenosine diphosphate
PB	phenobarbital
TLC	thin-layer chromatography
ER	endoplasmic reticulum

INTRODUCTION AND REVIEW OF LITERATURE

A large number of carcinogenic compounds, drugs and steroids are oxidatively metabolized by enzymes in the liver endoplasmic reticulum (1-3). Endoplasmic reticulum is normally prepared by homogenization of the liver to yield membrane fragments which form small enclosed vesicles called microsomes. The hepatic microsomal drug metabolizing enzymes which are isolated in the microsomal fraction are termed mixed-function oxidases (4) because the reactions catalyzed require both NADPH and oxygen (5).

At least two components, cytochrome P-450 and NADPH-cytochrome c reductase are involved in the hepatic metabolism of drugs (6), steroids (7), and fatty acids (8,9). NADPH-cytochrome c reductase is the NADPH specific flavoprotein which has been identified in microsomes. Cytochrome P-450, a CO-binding (10,11) hemoprotein (12), acts as the oxygen activating enzyme (13,14) and the substrate binding protein (15). The reduction-oxidation cycle of cytochrome P-450 during drug metabolism proceeds through several steps. A postulated scheme of microsomal electron transport reactions has been reported by Remmer et al. (16) and Estabrook et al. (17). The first step is

binding of the drug substrate to oxidized cytochrome P-450. This intermediate is then reduced by NADPH-cytochrome c reductase through a proposed electron carrier, "x". The reduced cytochrome P-450 then combines with oxygen to form a transient oxygenated intermediate of reduced cytochrome P-450, which rearranges to form the hydroxylated substrate and to regenerate the original cytochrome P-450.

It had been suggested that the affinity of substrates for cytochrome P-450 is important in determining the rate of hydroxylation by liver microsomes (18-20). Williams and Karler (21) reported that the decrease in drug-metabolizing activity after treating rats with cycloheximide did not parallel the change in NADPH-cytochrome c activity, the cytochrome P-450 content nor the substrate binding spectrum. They suggest the reduction of cytochrome P-450 as the rate-limiting step. Some reports have shown the presence of two forms of P-450 in hepatic microsomes (22,23). These two forms can be distinguished by spectral changes when the substrate interacts with cytochrome P-450. It has been reported that two forms of cytochrome P-450 have different turnover rates (24,25) and can be induced by different chemicals (26). Recently, cytochrome P-450 has been isolated and partially purified by Mitani et al. (27). The isolated P-450 has no cytochrome b_5 and shows little contaminating NADH- or NADPH-cytochrome C reductase.

The metabolism of many drugs is thought to require the association of cytochrome P-450 with lipid components of microsomes (12, 28-31). The loss of microsomal phospholipid following lipase treatment correlates with the loss of drug-metabolizing enzyme activity and the loss of binding between cytochrome P-450 and substrate (29,30). The restoration of activity after addition of lipid to the lipase treated microsomes has also been shown (30). Imai and Sato (28) assume that it is essential for cytochrome P-450 to be in contact with highly hydrophobic protein or buried in the lipids of microsomes. Lu et al. (8,32,33) have resolved three fractions from rabbit liver microsomes which when recombined, will support hydroxylation. These components are cytochrome P-450, NADPH-reductase and a heat stable lipid fraction. All of these were shown to be necessary for maximal drug metabolism.

Cytochrome P-420 is a spectrally altered, biologically inactive form of cytochrome P-450. Imai and Sato (28) suggest that the conversion of P-450 to P-420 is due to alteration of the association of the hemoprotein and microsomal phospholipid. Ichikawa and Yamano (34) have reported the reversibility of the conversion between cytochrome P-450 and P-420 by the addition of polyols and GSH after treatment of microsomes with detergent or sulfhydryl reagent.

Orrenius et al. (35) and Gram and Fouts (36) suggested that hepatic microsomal drug-metabolism and the peroxidation of endogenous lipids compete for reducing equivalents from NADPH. The lipid peroxidation system, which requires iron and ADP (37,38) is thought to involve the microsomal electron transport system and may be involved in the turnover of fatty acids, primarily arachidonate, from the lipids in microsomes (39,40). Lipid peroxidation in microsomes involves the enzymatic formation of fatty acid peroxides by NADPH-cytochrome c reductase and the subsequent nonenzymatic cleavage to form malondialdehyde (41) in the presence of oxygen.

Hepatic microsomal drug metabolizing enzyme activity can be affected by a variety of agents. Phenobarbital is known to increase the smooth endoplasmic reticulum of the liver (42) and to induce microsomal drug-metabolizing enzyme systems (3). Inhibitors of protein synthesis inhibit this increase in enzymatic activity. Many chemicals are known to readily pass across the placenta and are therefore capable of inducing the formation of drug-metabolizing enzymes in the fetus. Pantuck et al. (44) have shown that treatment of pregnant animals with phenobarbital increased the N-demethylation of meperidine and hydroxylation of pentabarbital in the newborn.

Age-dependent changes in drug susceptibility is shown by higher sensitivity in the young. Several different opinions have been suggested for the causes of the greater sensitivity to drugs by young animals. Fouts and Hart (45) suggest that the greater sensitivity is due to low enzyme concentrations, which might be caused by the lack of a stimulus for enzyme synthesis. Feuer and Liscio (46) and Fouts and Adamson (47) reported that the low enzyme activity is caused by the presence of inhibitors of the enzyme systems. Another possibility is that the newly synthesized enzyme is inactive and it becomes activated when the rough membrane converts to smooth endoplasmic reticulum.

Fouts et al. (48) and Henderson and Kersten (49) have reported a decreased level of oxidative enzymes involved in drug metabolism during rat liver regeneration. Liver tumors and livers of newborn animals, all of which show a rapid cellular proliferation, have lower levels of drug metabolism (50,51).

Microsomes from liver of young developing rabbits (47), rats (51), swine (52), and guinea pigs (53) contain very little activity for the transformation of foreign organic compounds.

Howatson and Ham (54) and Peters et al. (55) have reported poorly developed endoplasmic reticulum in fetal hepatic cells. In a study of the distribution of

mixed-function oxidases, it was shown that the drug-metabolizing activity is much higher in the smooth hepatic endoplasmic reticulum than in rough endoplasmic reticulum (56). Electron micrographs suggest that the newborn liver contains a higher proportion of rough ER than smooth ER (43).

Among the possible reasons why the newborn animal cannot metabolize drugs efficiently are the following:

1. a lack of NADPH-linked electron transport system, i.e., NADPH-cytochrome c reductase,
2. the presence of inhibitors or inactive conformation of enzymes,
3. low cytochrome P-450 concentration or the presence of biological inactive cytochrome P-450,
4. a lack of proper lipids to maintain the capacity of cytochrome P-450 to interact with substrate.

The purpose of this study was to describe the development of the hepatic endoplasmic reticulum as a possible step in determining why the newborn rat is deficient in drug metabolism.

The first part of this thesis describes the development of the liver and the drug-metabolizing enzymes located in the endoplasmic reticulum, i.e., aminopyrine demethylase and aryl esterase. These activities are

compared to the development of glucose-6-phosphatase, a microsomal enzyme not related to drug metabolism.

The second part of the thesis describes the development of the components of the hepatic mixed-function oxidase, i.e., NADPH-cytochrome c reductase and cytochrome P-450. Lipid peroxidation, an activity involving NADPH-cytochrome c reductase and endogenous unsaturated fatty acids was also studied.

In the third part of this thesis, an attempt was made to study the microsomal phospholipids and the distribution of fatty acids in total microsomal lipids isolated from hepatic microsomes of different ages.

Efforts were made to correlate the observed alterations of hepatic microsomal drug-metabolizing enzymes, cytochrome P-450 concentration and the development of microsomal phospholipids.

MATERIALS AND METHODS

Chemicals

Aminopyrine (4-dimethylamino-1,5-dimethyl-2-phenyl-3-pyrazolone) and phenobarbital were purchased from K and K Laboratories, Inc., Plainview, New York and Merck and Co., Inc., New Jersey, respectively. D.L.-isocitrate, $NADP^+$, NADPH, HADPH-isocitrate dehydrogenase, ADP, cytochrome c, Na_2 glucose-6-phosphate · H_2O, 1-amino-2-naphthol-4-sulfonic acid, and para-nitrophenyl acetate were all purchased from Sigma Chemical Company, St. Louis, Missouri. Orcinol was purchased from Hartman-Leddon Company, Philadelphia, Pennsylvania, and was purified by dissolving in boiling benzene followed by decoloring with charcoal and crystallizing before use.

Phosphatidyl serine and phosphatidyl ethanolamine were purchased from Applied Science Lab., Inc., State College, Pennsylvania, and used as standards in thin layer chromatography.

Animals

Microsomes were isolated from liver of male rats Spartan strain (Sprague Dawley) of different ages. In experiments involving very young rats it was necessary to

use many rats to obtain sufficient tissue. For example, at one day of age it was necessary to use the liver from 12 to 24 animals in order to obtain sufficient microsomes.

Pregnant rats are pretreated with phenobarbital (PB) 10 days before parturition by including 0.1% PB in their drinking water.

Preparation of Microsomes

All rats were killed by decapitation. The abdominal cavity was opened and the liver of older rats were immediately perfused *in situ* with about 10 ml of cold 1.15% KCl containing 0.2% nicotinamide (pH 7.5) through the portal vein. The perfused liver was rapidly excised, placed in a beaker containing ice cold perfusion fluid, washed, weighed, and chopped with a scissors. Homogenization was accomplished by four strokes with a teflon Potter-Elvehjem homogenizer in four volumes of ice cold 1.15% KCl containing 0.2% nicotinamide. The homogenate was centrifuged for 20 minutes at 10,000 g (8,500 rpm, GSA rotor) in a Sorvall RC 2-B refrigerated centrifuge, and the precipitate containing mitochondria, nuclei, and other organells was discarded. The microsomes were sedimented by centrifuging the supernant at 105,000 xg (30,000 rpm, 30 rotor) for 90 minutes in a Spinco ultracentrifuge. The firmly packed microsomes were resuspended in ice-cold Tris-HCl buffer (0.05*M*, pH 7.5) containing 50% glycerol.

Protein determinations were done by the Lowry method (57). The resultant microsomal suspensions were stored at -20°C under N_2 until they were used.

CO Difference Spectroscopy

Difference spectra were measured on a Perkin-Elmer Coleman 124 spectrophotometer by recording the difference in absorbance between 500 nm and 400 nm (58). Microsomes resuspended in 0.05 M Tris-HCl buffer (pH 7.5) with or without 20% glycerol was placed in reference and sample cuvetts. The solution in sample cell was gassed with deoxygenated CO for at least 30 seconds. Solutions in both cells were then reduced with dithionite by adding few crystals of solid $Na_2S_2O_4$. The extinction coefficient for P-450 was 91 cm^{-1}, mM^{-1} and for P-420 was 111 cm^{-1}, mM^{-1}.

Cytochrome c Reductase

The reported activity refers to the ability of the flavoprotein to use exogenous cytochrome c as the electron acceptor from NADPH in the microsomal NADPH-electron transport chain. Cytochrome c reductase activity was determined on a Beckman DB spectrophotometer with Sargent SRL recorder by following the increase in optical density at 550 nm. The 1.0 ml assays consisted of microsomes, 0.2 ml cytochrome c (4.4 mg/ml in phosphate buffer) and 0.05 M phosphate buffer, pH 7.3. The reaction was started

by the addition of 10 µl of NADPH (11.4 mg/ml in HCO_3^- buffer, pH 10.4). Reduced cytochrome c has an extinction coefficient of 27.7 cm^{-1}, $m\underline{M}^{-1}$.

Lipid Peroxidation Assay

The assay for NADPH-linked peroxidation of endogenous lipids was based on determining the amount of malondialdehyde formed with the thiobarbituric acid reaction (59). The incubation system contained microsomes, 0.05 $\underline{M}$ tris-buffer, pH 7.5, 0.25 ml ADP-Fe^{++} solution (0.03 µmole $Fe(NH_4)_2 \cdot 6H_2O$ and 8 µmole ADP), 7 $m\underline{M}$ $MgCl_2$, and a NADPH generating system consisting of 0.1 $m\underline{M}$ NADPH, 1 $m\underline{M}$ D.L-isocitrate and sufficient isocitrate dehydrogenase to reduce 0.0365 µmoles $NADPH^+$ per minute, in a final volume of 5 ml. The incubation temperature was 37°C in a Dubnoff metabolic shaker. Samples (1.0 ml) were removed at 0, 3, 6, 10 minutes and the reaction was stopped by pipeting into a test tube containing 2.1 ml of TCA-HCl-TBA mixture (15% TCA, 0.375% thiobarbituric acid and 0.25 N HCl). The assay mixture was boiled for 15 minutes, for color development, cooled in ice and centrifuged. The amount of peroxide formed was estimated by measuring the optical density at 535 nm in a Coleman Jr. spectrophotometer. The extinction coefficient is 0.156 cm^{-1}, $\mu\underline{M}^{-1}$ malondialdehyde.

Aminopyrine Demethylase

Microsomal N-demethylation activity was measured by determining formaldehyde generation using the Nash method (60). The composition of the incubation system was the same as for lipid peroxidation, except that ADP-Fe^{++} was omitted and 0.05 M aminopyrine was included as the substrate with a final volume of 5 ml. The reaction was started by adding microsomes to this assay medium. The reaction mixture was incubated at 37°C in a shaking water bath for 0, 2, 5, 10 minutes and the reaction stopped by placing 1 ml aliquots of the incubation mixture into a test tube containing the same volume of 10% trichloroacetic acid. The tubes were allowed to stand for 10 minutes at room temperature for total protein precipitation. Nash reagent (2M $NH_4C_2H_3O_2$, 0.5 M CH_3COOH, 0.02 M 2,4-pentanedione, 2.0 ml) was added to each tube and heated at 60°C for 15 minutes. After centrifugation, optical density was read with a Coleman Jr. spectrophotometer at 412 nm. The extinction coefficient is 7.08 cm^{-1}, mM^{-1} HCHO.

Glucose-6-phosphatase

The rate of hydrolysis of glucose-6-phosphate to glucose and inorganic phosphate was measured by determining the amount of phosphate released after incubation. The incubation mixture consisted of 1 ml of microsome

suspension (less than 1 mg protein) and 1.0 ml glucose-6-phosphate (0.774 gm Na_2 G-6-p $\cdot$ H_2O) in 30 ml H_2O, pH 6.5). The reaction mixture was incubated at 37°C for 20 minutes and terminated by the addition of 0.15 ml of 70% $HClO_4$. After centrifugation, inorganic phosphate was determined in 1 ml aliquots of the supernatant by adding 2.0 ml H_2O and 1.0 ml acid molybdate (6% NH_4 Molybdate in 2N H_2SO_4). After mixing thoroughly, 4.0 ml IBB (Isobutanol:Benzene 1:1) was added and mixed for 30 seconds. The organic layer was separated by centrifugation and the optical density recorded at 410 nm on a Coleman Jr. spectrophotometer. The amount of phosphate in microsomes was determined by comparison with a standard phosphate curve.

Aryl Esterase

Aryl esterase activity was determined by the rate of decomposition of para-nitrophenyl acetate to para-nitrophenol by following the increase in optical density at 420 nm in a Perkin-Elmer Coleman 124 spectrophotometer. Corrections were made for spontaneous hydrolysis of substrate. To 2 ml of 0.05 M tris-HCl buffer, pH 7.5, was added 10 µl of para-nitrophenyl acetate (63 mg/10 ml CH_3OH) and 5 µl of microsomes (2-3 µg of microsomal protein). The extinction coefficient is 0.0528 cm^{-1}, μM^{-1} para-nitrophenol.

RNA Determination

The amount of RNA in microsomes was determined by measuring ribose by the Schneider method (61). Microsomes (10 mg protein) were mixed with 0.5 ml H_2O, 1 ml BSA (1 mg/ml), and 5 ml of ice-cold 10% TCA. Protein was allowed to precipitate at 0-4°C for 20 minutes and the mixture was centrifuged in the Sorvall refrigerated centrifuge for 10 minutes at 15,000 rpm. The sediment was washed with 2.5 ml of cold 10% TCA, 2.5 ml of cold 95% ethanol, and washed three times with 2.5 ml of cold alcohol:ether (3:1). The precipitate was extracted two times with 2.5 ml of 10% TCA, by incubating at 90°C for 30 minutes. After centrifugation, the extracts were combined and saved. The extract was diluted by adding 0.2 ml of extract to 1.3 ml of H_2O. To this was added 1.5 ml of orcinol reagent (0.5 g orcinol in 50 ml conc. HCl containing 0.42 g $FeCl_3 \cdot 6\ H_2O$). This reaction mixture was heated in boiling water bath for 20 minutes and the optical density at 660 nm recorded. From a yeast RNA standard curve, the amount of RNA in microsomes was determinated.

Extraction of Total Microsomal Lipid

Microsomes (20-50 mg protein in 1 ml) were placed in a screw top tube and extracted three times with acidic chloroform:methanol (0.1 ml 0.1 N HCl plus 5 ml of $CHCl_3$: CH_3OH, 60:40). The phases were separated by centrifugation

and the organic phases combined and evaporated to dryness under nitrogen. The lipid was redissolved in 2 ml of chloroform:methanol (60:40) and extracted twice with 2 ml of 0.7% NaCl and 0.1% $MgCl_2$ and 0.5 ml of methanol. This solution was centrifuged to remove the aqueous phase and contaminating protein. The organic phase was transferred to a dried, tared scintillation vial, taken to dryness under nitrogen and dried to constant weight in a vacuum desiccator. After weighing the lipid residue was taken up in 1.0 ml of chloroform for further analysis.

Total Phosphate Analysis

Total phosphate content of microsomes was measured by a modified Fiske and Subbarow method (62). Samples were placed in a 12 ml conical centrifuge tube and 0.5 ml of 10 N H_2SO_4 was added and heated at 150-160°C for at least 3 hours. Two drops of 30% H_2O_2 were added and the sample heated for another 1.5 hours; this step was repeated until the solution was completely clear. This solution was then mixed with 4.6 ml of 0.22% ammonium molybdate and 0.2 ml of Fiske-Subbarow reagent (0.5 g of 1-amino-2-naphthol-4-sulfonic acid was added with mechanical stirring to 200 ml of freshly prepared 15% sodium bisulfite and 1.0 g of anhydrous sodium sulfite. The solution was filtered and stored in a dark bottle). The reaction mixture was heated in a boiling water bath for 7 minutes to allow color development. The optical density was

read with a Coleman Jr. spectrophotometer at 660 nm. The amount of phosphate in the sample was calculated by using a phosphate standard curve.

Thin Layer Chromatography of Total Microsomal Lipid

All TLC separations were performed on 20 x 20 cm Silica Gel F254 thin layer plates. Each plate was spotted with reference samples. The extracted microsomal lipid solution (25 µl, in $CHCl_3$) were spot on the plates. The chromotograms were developed with $CHCl_3$:MeOH:HOAC:H_2O (65:25:8:4) at room temperature. The plates were first visualized in an iodine vapor tank. The spots were circled and the plates were sprayed with ninhydrin and the color developed over a steam bath. The plates were finally sprayed with Dragendorf's reagent.

Fatty Acids Determination

The total microsomal lipid was transferred to a glass stoppered centrifuge tube. Methanol (2 ml) and one drop of concentrate H_2SO_4 were added and the glass stoppers were wired on with copper wire. The mixture was incubated at 40°C for 24 hours. One ml of water was added and the methyl esters were extracted with 2 ml of hexane. The phases were separated by centrifugation and the hexane layer was transferred to a screw topped tube. The hexane extraction was repeated two times. The hexane extracts

were washed with 1 ml of water until the water was neutral to pH paper. The washed extracts were taken to dryness under N_2. After the addition of 0.2 ml of ethanol, the mixture was dried under N_2 again. The fatty acid methyl esters were finally redissolved in 0.1 ml of hexane.

Aliquots of fatty acids esters were applied with a Hamilton syringe to Sargent flame ionization gas chromatography at 170°C; column packing was 3% OV-1 on Chromasorb Q. The peaks observed were compared with the retention time of those of Ivory soap standard. Peaks areas were measured by triangulation.

Preparation of Lipid Riched Microsomes

10 mg of total microsomal lipid was dissolved in chloroform and taken to dryness under nitrogen to form a thin layer on the bottom of a beaker. 37 ml of 1.0 M citrate-K^+, pH 7.6 was added and sonicated with Branson Sonic Power Sonifier Model S 125 until lipid was dispersed. 1 ml microsomes (40 mg/ml) was added to the lipid suspension and stirred in the cold room for at least 30 minutes. The mixture was centrifuged at 105,000 x g (30,000 rpm, 30 rotor) for 90 minutes in a Spinco ultracentrifuge. Microsomes were collected and resuspended in 2 ml tris-HCl buffer (0.05 M, pH 7.5) for assays.

RESULTS AND DISCUSSION

The total liver weight and the liver weight per body weight for male rat from 1 to 61 days of age are shown in Figure 1. From day 15 to 50, the liver weight increased almost linearly. During this time the liver weight per body weight was maximal, indicating a rapid rate of liver growth.

The age-related changes in the content of RNA in microsomes of rat liver are shown in Figure 2. The relatively rapid increase of the RNA content in microsomes of newborn rats is followed by a sharp decrease to the lowest level at 15 days of age. Thereafter, the RNA content tended to show a gradual decrease from 21 days.

The increasing and high RNA content during the immediate postnatal period is expected. Newly formed microsomal membrane first appears as rough endoplasmic reticulum (ER) as shown by Dallner et al. (63). Upon aging, the rough ER converts to smooth ER by ribosome detachment and the microsomal RNA content decreases synchronously.

The development of the enzymes for aminopyrine demethylation is presented in Figure 3. The rate of demethylation of aminopyrine by microsomes from young rats

Figure 1.--Total liver weight (soild line) and the liver weight per body weight (broken line) during postnatal development. Each point represents the mean of two or three measurements on groups of three to twenty-four male rats.

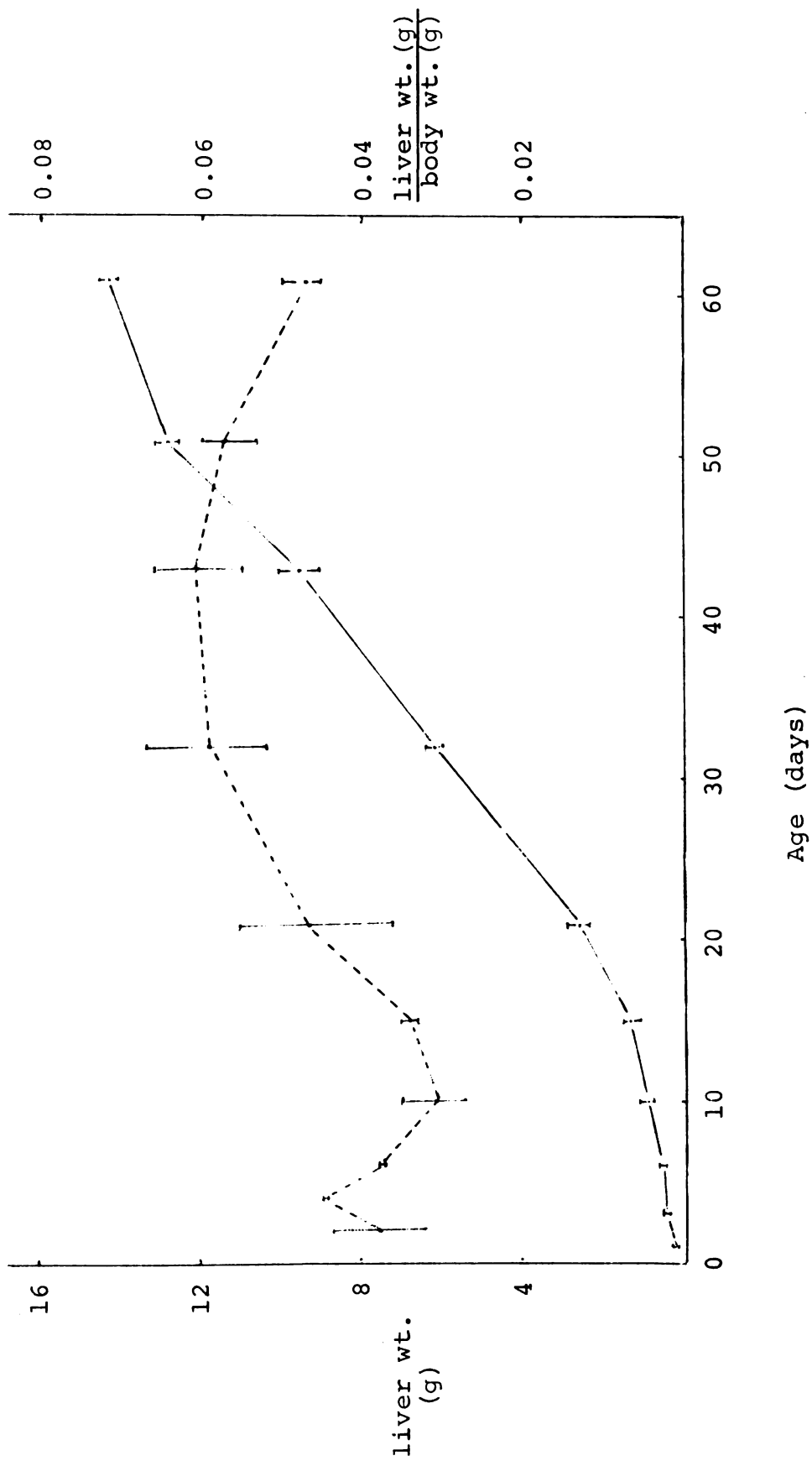

liver wt. (g)
16
12
8
4
0
10
20
30
40
50
60
Age (days)
liver wt. (g) / body wt. (g)
0.08
0.06
0.04
0.02

Figure 2.--Microsomal RNA during postnatal development. The amount of RNA is presented as mg RNA per mg of microsomal protein. Each point represents one assay of hepatic microsomes from three to twenty-four male rats.

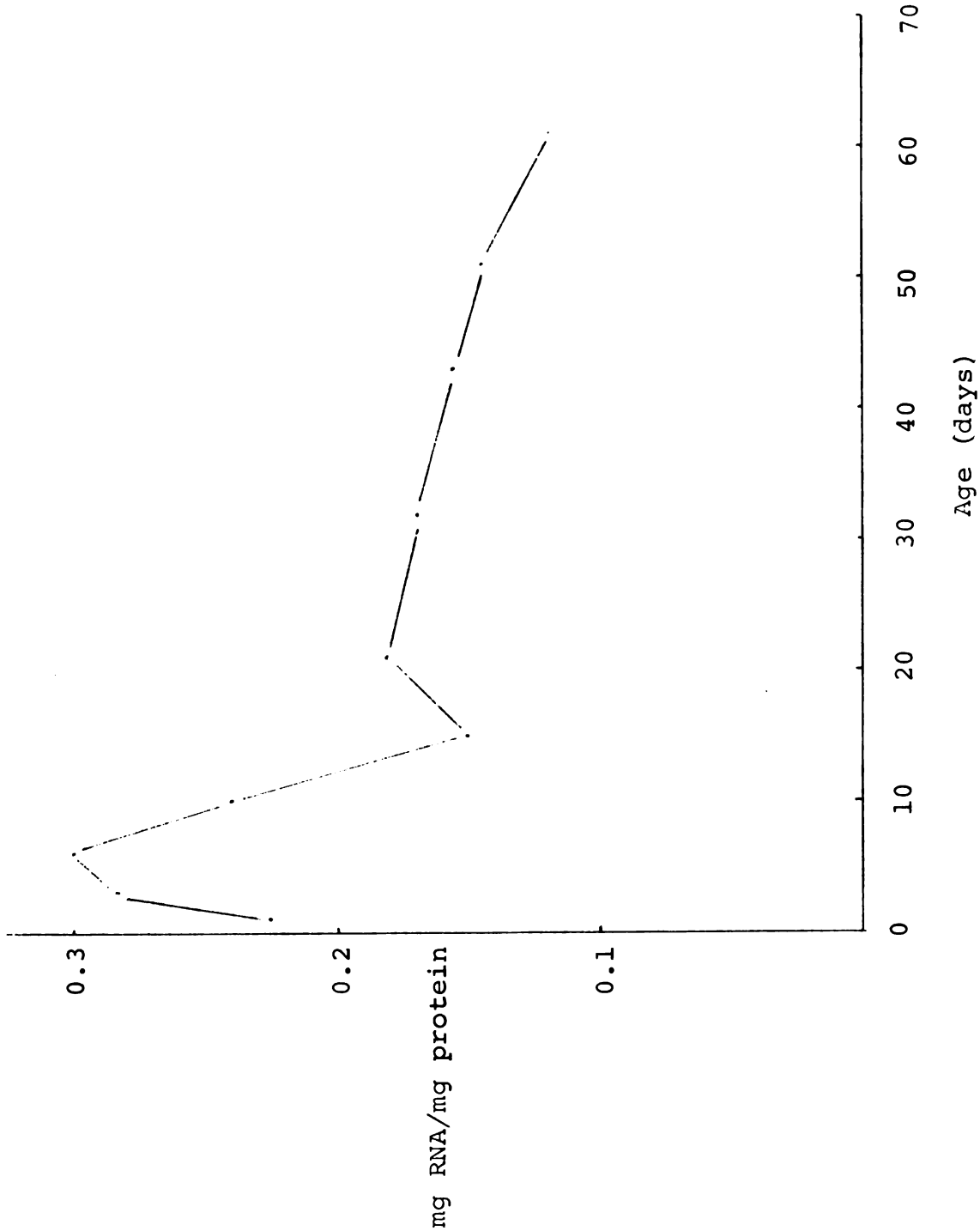

mg RNA/mg protein
0.3
0.2
0.1
0
10
20
30
40
50
60
70
Age (days)

Figure 3.--Aminopyrine demethylase in hepatic microsomes as a function of age. The enzymatic activities are expressed as mμmoles formaldehyde produced per minute per mg of microsomal protein. Each point represents a mean of either three or four assays; individual assays involve hepatic microsomes isolated from groups of from three to twenty-four male rats.

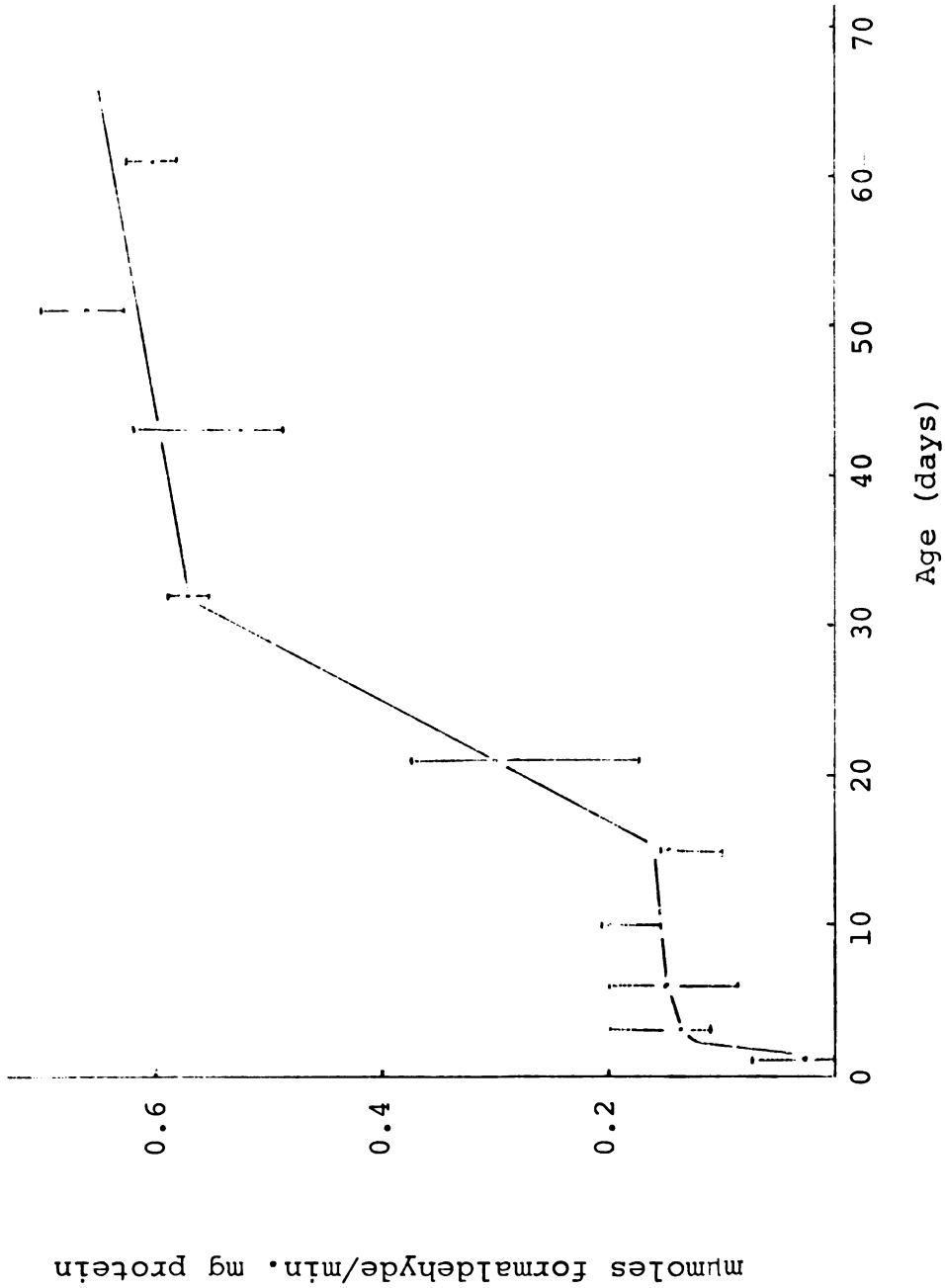
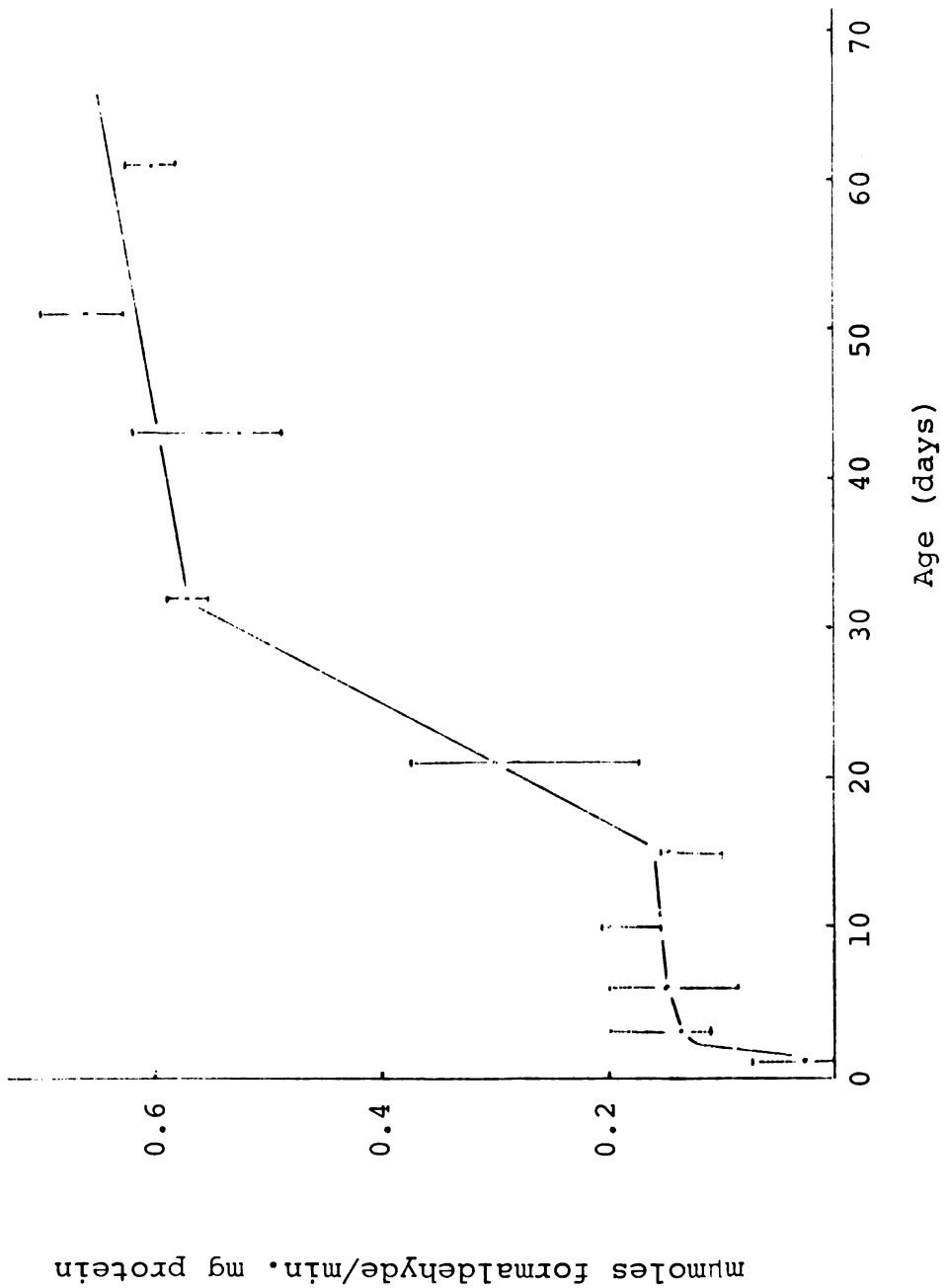

0.6
0.4
0.2
0
10
20
30
40
50
60
70
Age (days)
μmoles formaldehyde/min. mg protein

Figure 3.--Aminopyrine demethylase in hepatic microsomes as a function of age. The enzymatic activities are expressed as mμmoles formaldehyde produced per minute per mg of microsomal protein. Each point represents a mean of either three or four assays; individual assays involve hepatic microsomes isolated from groups of from three to twenty-four male rats.


0.6
0.4
0.2
0
10
20
30
40
50
60
70
Age (days)
μmoles formaldehyde/min. mg protein

was very low and increased during the entire period studied. The curve, representing enzyme specific activity between birth and 61 days of age, appears to have three distinct phases. Before 15 days of age, the activity remains relatively low and constant. Between 15 and 31 days of age the curve depicts a rapid increase in activity by about 6 fold. After that the activity undergoes a constant but slow increase.

The relatively low activity of N-demethylation in rat liver shortly after birth is in accordance with that reported by Dallner et al. (64), Soyka (65) and by Henderson (51). However, as is presented in Figure 3, the present observation reveals a sudden elevation of the enzyme activity at about 15 days of age instead of at 20 days after birth as reported by Henderson. The apparent lack of drug-metabolizing enzyme activity in young rabbits has been demonstrated to be caused by the presence of inhibitors (47). However, no evidence for an inhibitor of aminopyrine metabolism has been found in rats. It has been suggested that differences in enzymatic activity in newborn and adults may result from difference in their pH optima or the activity of inhibitor is pH dependent (65). Gram et al. (66) have demonstrated marked changes in certain kinetic properties of hepatic microsomal enzymes during the first three weeks of postnatal life. They have reported that the Vmax for the N-demethylation of

ethylmorphine increases abruptly between 3 and 4½ weeks of age and during the first 3 weeks of life the apparent Michaelis constant increased markedly. A sex difference for aminopyrine demethylase activity during development has been shown in previous reports (51). The difference appears at puberty, Davies et al. (67), and Castro et al. (68), suggested that androgen may play an important role in regulating the affinity between cytochrome P-450 and substrate. Henderson (51) could show no difference in the activity of drug oxidation in the liver of weanling or preweanling rats of the same age.

Another microsomal enzyme for the metabolism of drugs which is not a mixed-function oxidase is aryl esterase. The development of this enzyme was studied in the hepatic microsomes of rats from birth to adult. The enzymatic activity is low in the newborn but increases almost linearly to 61 days of age (Figure 4).

The development of these activities (aminopyrine demethylase and aryl esterase) were compared to the development of a microsomal enzyme not associated with the metabolism of drugs, namely glucose-6-phosphatase. As shown in Figure 5 the activity of this enzyme decreased throughout development to an adult level of 0.17 μmole min^{-1} mg^{-1} protein.

The lack of drug metabolizing ability in the newborn could most reasonably be attributed to the lack of the

Figure 4.--Postnatal development of hepatic microsomal aryl esterase in rats. Activities are expressed as mμmoles para-nitrophenol formed per minute per mg microsomal protein using para-nitrophenyl acetate as the substrate. Each point represents the mean of three assays; individual assays involve hepatic microsomes isolated from groups of from three to twenty-four male rats.

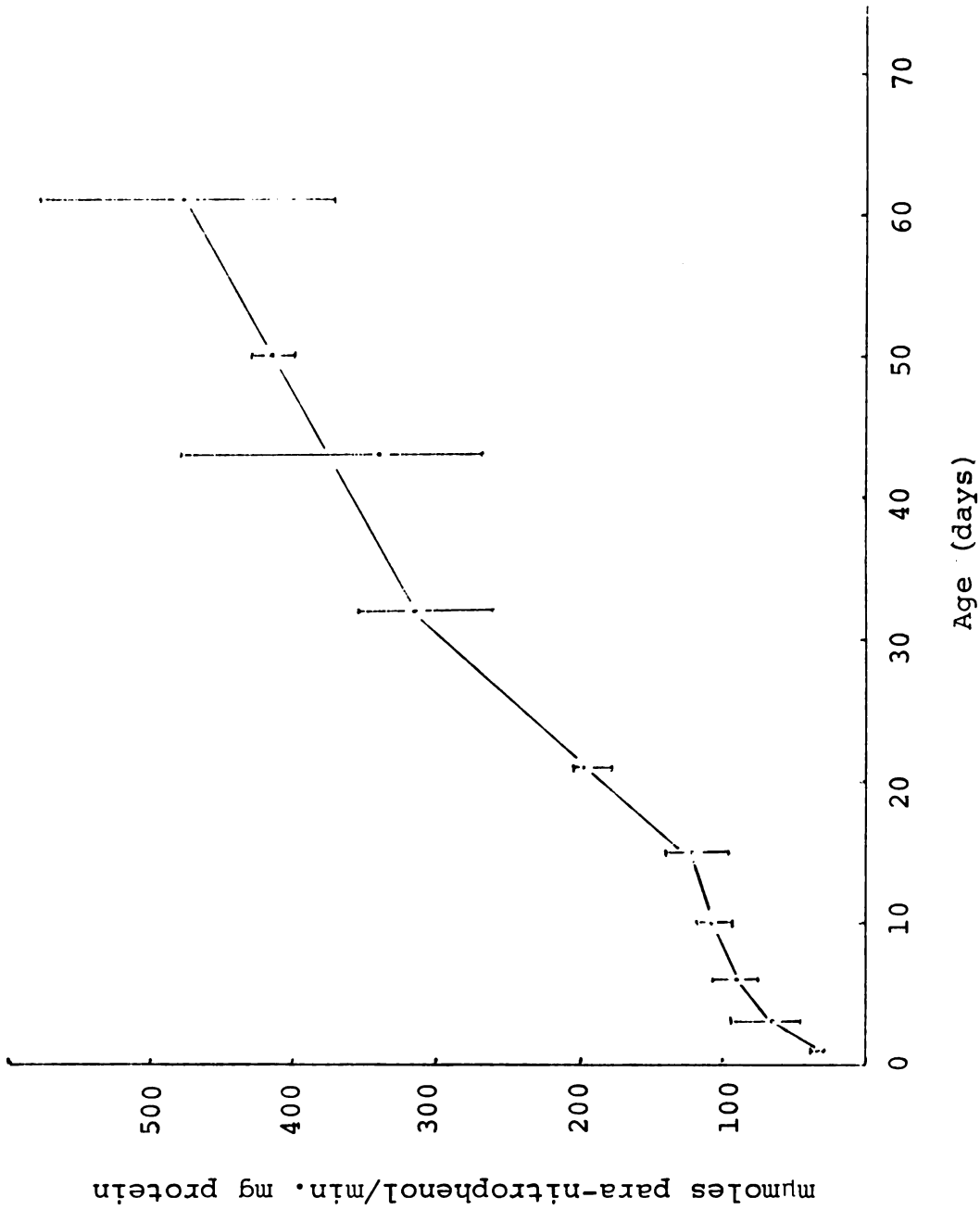

500
400
300
200
100
0
10
20
30
40
50
60
70
Age (days)
μmoles para-nitrophenol/min. mg protein

Figure 4.--Postnatal development of hepatic microsomal aryl esterase in rats. Activities are expressed as mμmoles para-nitrophenol formed per minute per mg microsomal protein using para-nitrophenyl acetate as the substrate. Each point represents the mean of three assays; individual assays involve hepatic microsomes isolated from groups of from three to twenty-four male rats.

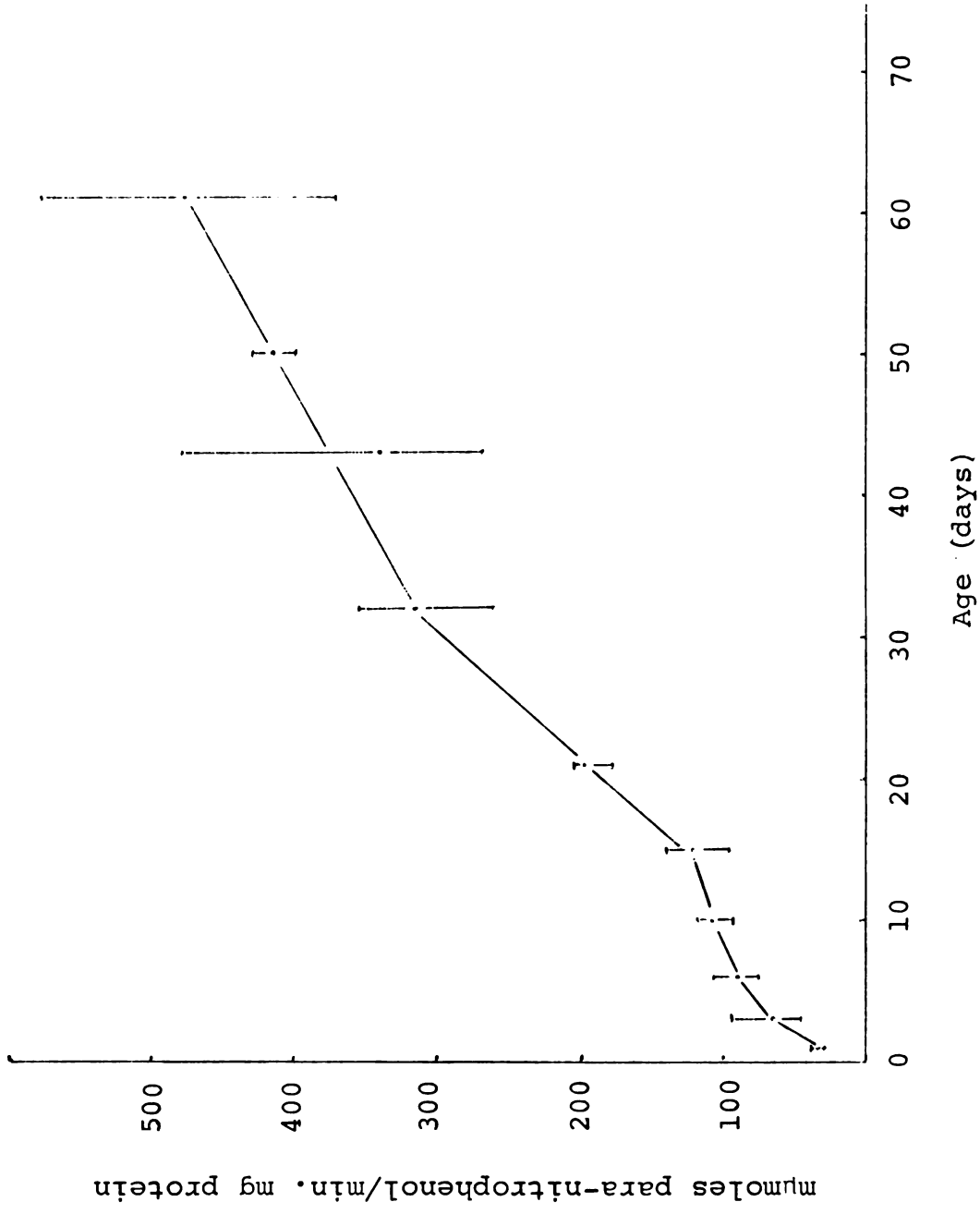

500
400
300
200
100
0
10
20
30
40
50
60
70
Age (days)
μmoles para-nitrophenol/min. mg protein

Figure 4.--Postnatal development of hepatic microsomal aryl esterase in rats. Activities are expressed as mμmoles para-nitrophenol formed per minute per mg microsomal protein using para-nitrophenyl acetate as the substrate. Each point represents the mean of three assays; individual assays involve hepatic microsomes isolated from groups of from three to twenty-four male rats.

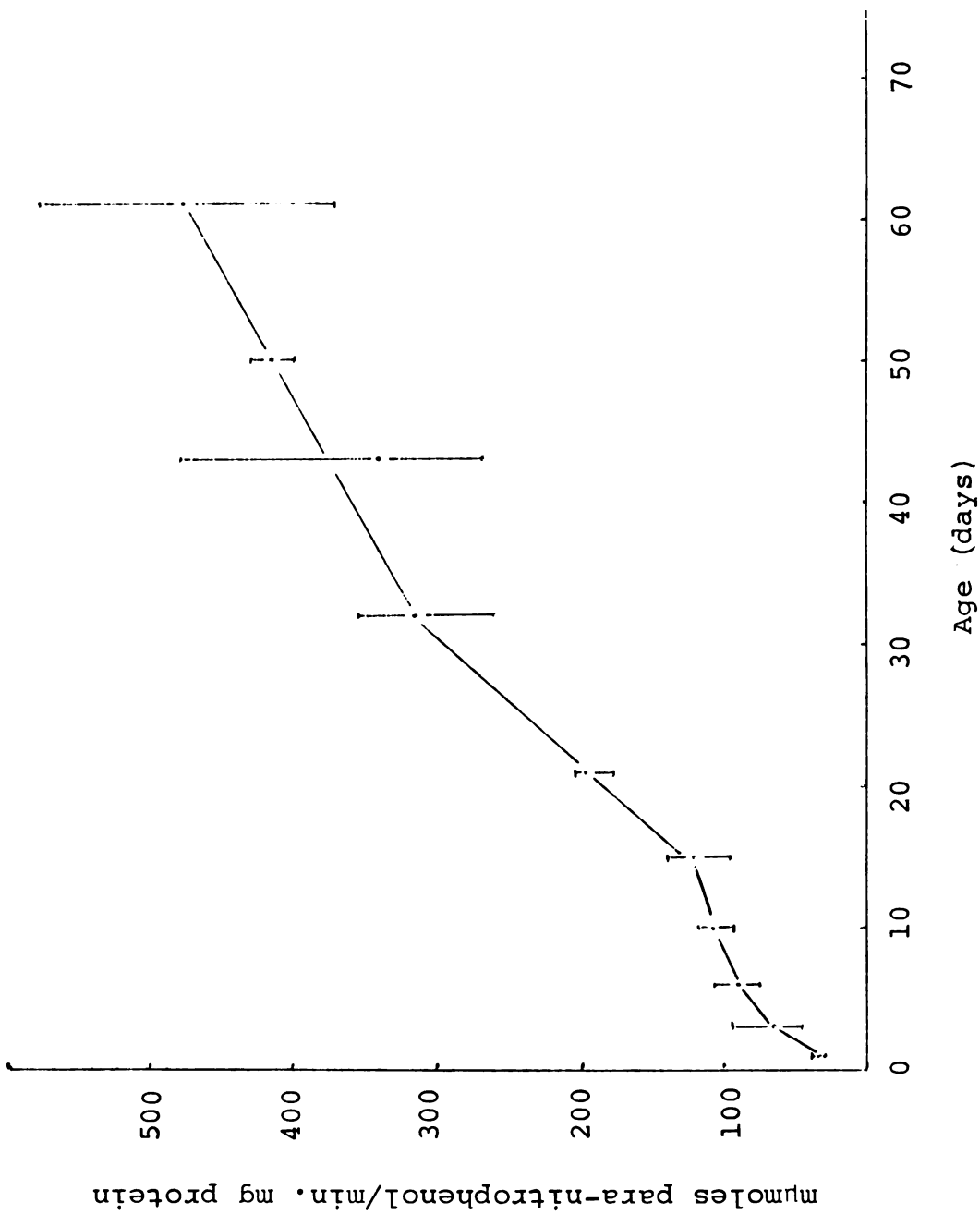
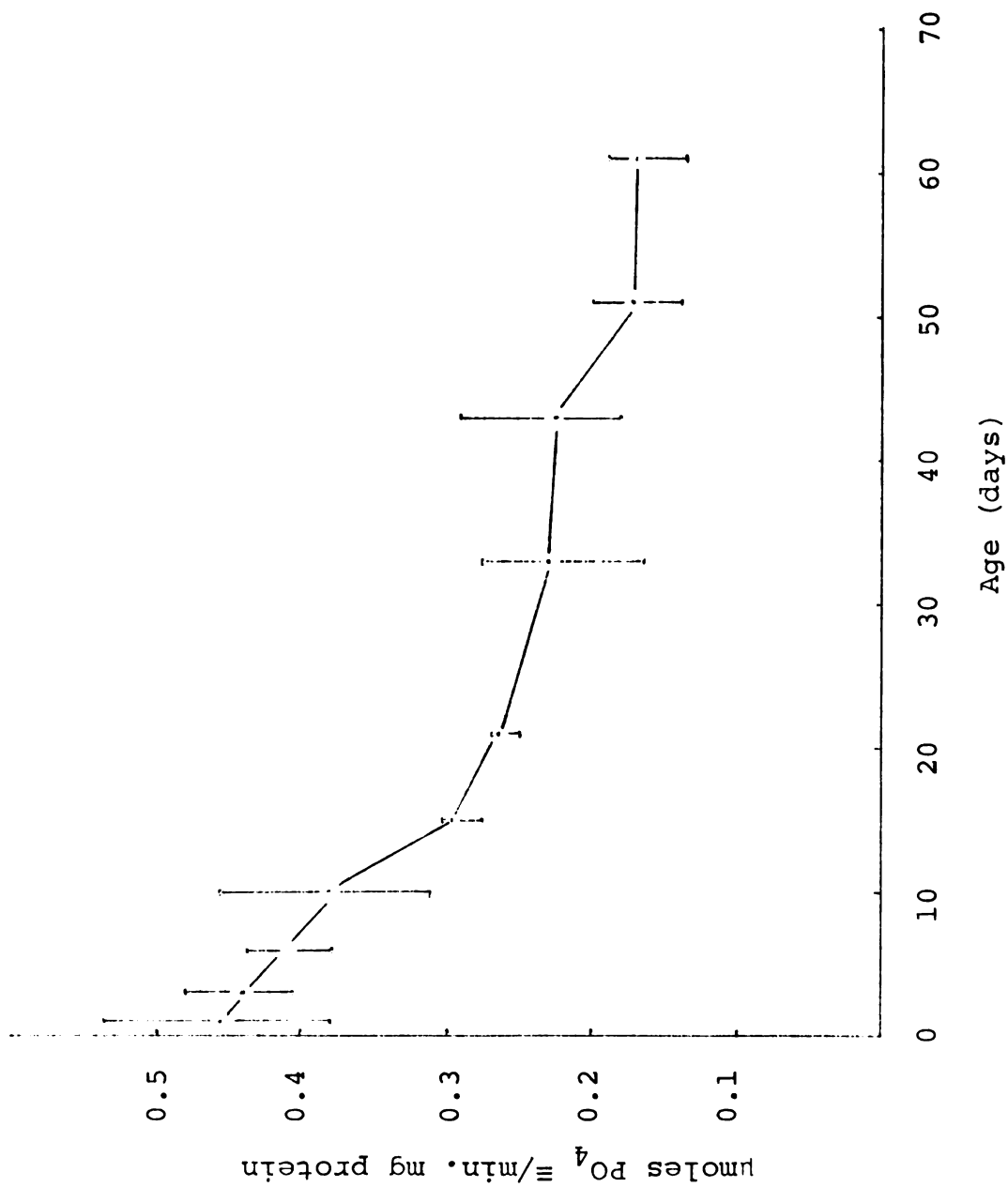

500
400
300
200
100
0
10
20
30
40
50
60
70
Age (days)
μmoles para-nitrophenol/min. mg protein

Figure 5.--Glucose-6-phosphatase activity during postnatal development. Activity is expressed as μmoles of inorganic phosphate released per minute per mg of microsomal protein from glucose-6-phosphate. Each point represents the mean of three assays; individual assays involve hepatic microsomes isolated from groups of from three to twenty-four male rats.


μmoles PO4 ≡/min. mg protein
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
Age (days)

proper enzymes, that is, NADPH-cytochrome c reductase and cytochrome P-450. While these enzymes were not present at adult levels in the newborn (Figures 6 and 7) they increased very rapidly reaching adult levels by the age of 15 days. Therefore while the lack of these components may account for the lack of oxidase activity in the very young they do appear substantially before aminopyrine demethylase activity increases to adult levels.

Dallner et al. (64) have shown that NADPH-cytochrome c reductase activity increased tremendously between the last prenatal and first postnatal day. They also presumed that NADPH-cytochrome c reductase is synthesized in the rough microsomes on the basis that rough microsomes shows higher NADPH-cytochrome c reductase activity than do smooth microsomes. Our observations of the rapidly increased level of NADPH-cytochrome c reductase shortly after birth are not consistent with the data of Dallner et al. (64). They have reported the specific activity of NADPH-cytochrome c reductase reaches adults level at the first day of birth.

Recently Raftell and Orrenius (69) have reported the presence of NADPH-cytochrome c reductase in newborn rat liver by using immunological techniques. They also demonstrated NADPH-cytochrome c reductase could be precipitated from both rough and smooth endoplasmic reticulum

Figure 6.--Cytochrome c reductase activity during post-natal development. The specific activity of the enzyme is expressed as mμmoles of cytochrome c reduced per minute per mg of protein. Each point represents the mean of two assays; individual assays involve hepatic microsomes isolated from groups of from three to twenty-four male rats.

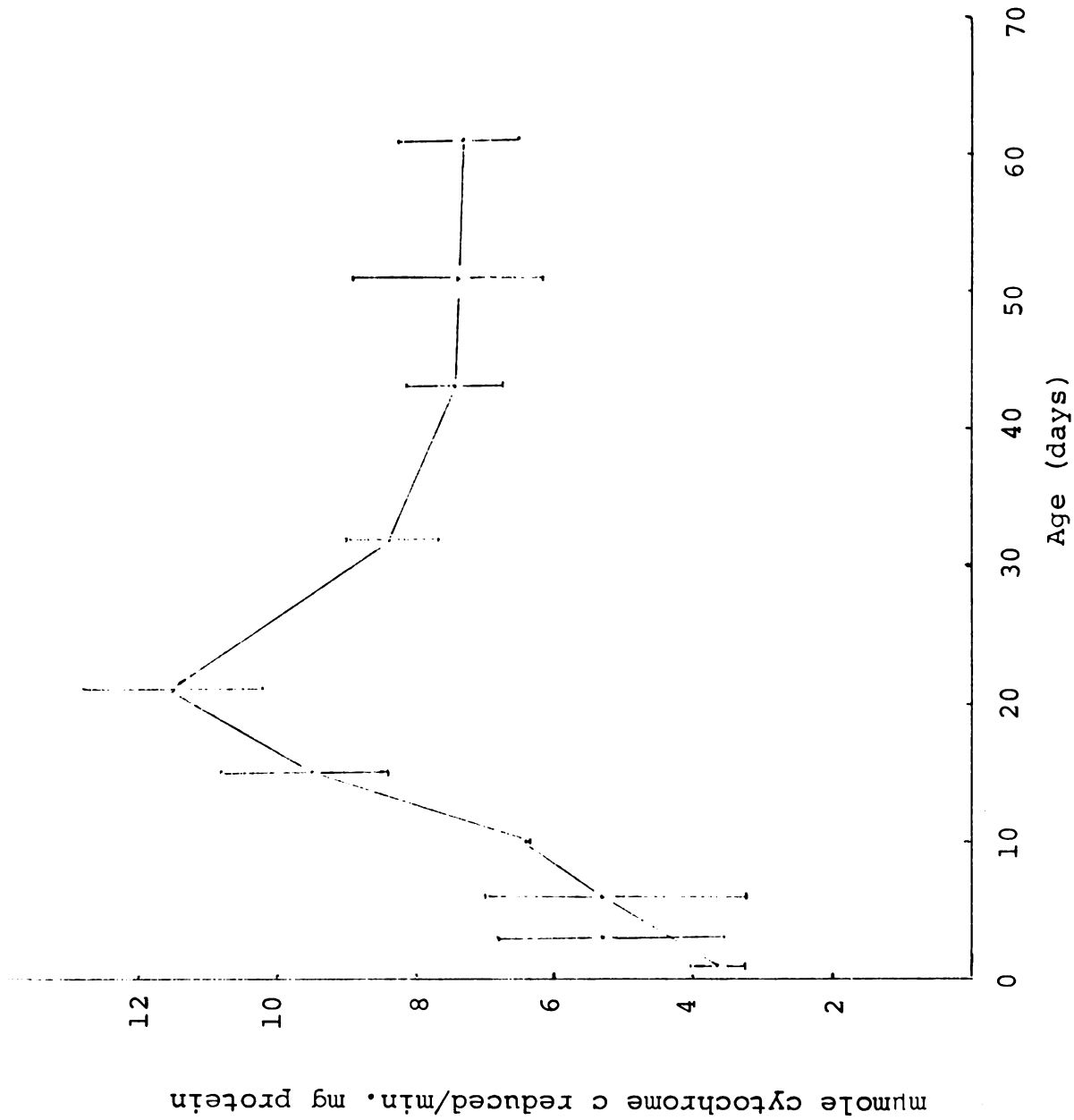

12
10
8
6
4
2
0
10
20
30
40
50
60
70
Age (days)
mμmole cytochrome c reduced/min. mg protein

Figure 6.--Cytochrome c reductase activity during post-natal development. The specific activity of the enzyme is expressed as mμmoles of cytochrome c reduced per minute per mg of protein. Each point represents the mean of two assays; individual assays involve hepatic microsomes isolated from groups of from three to twenty-four male rats.

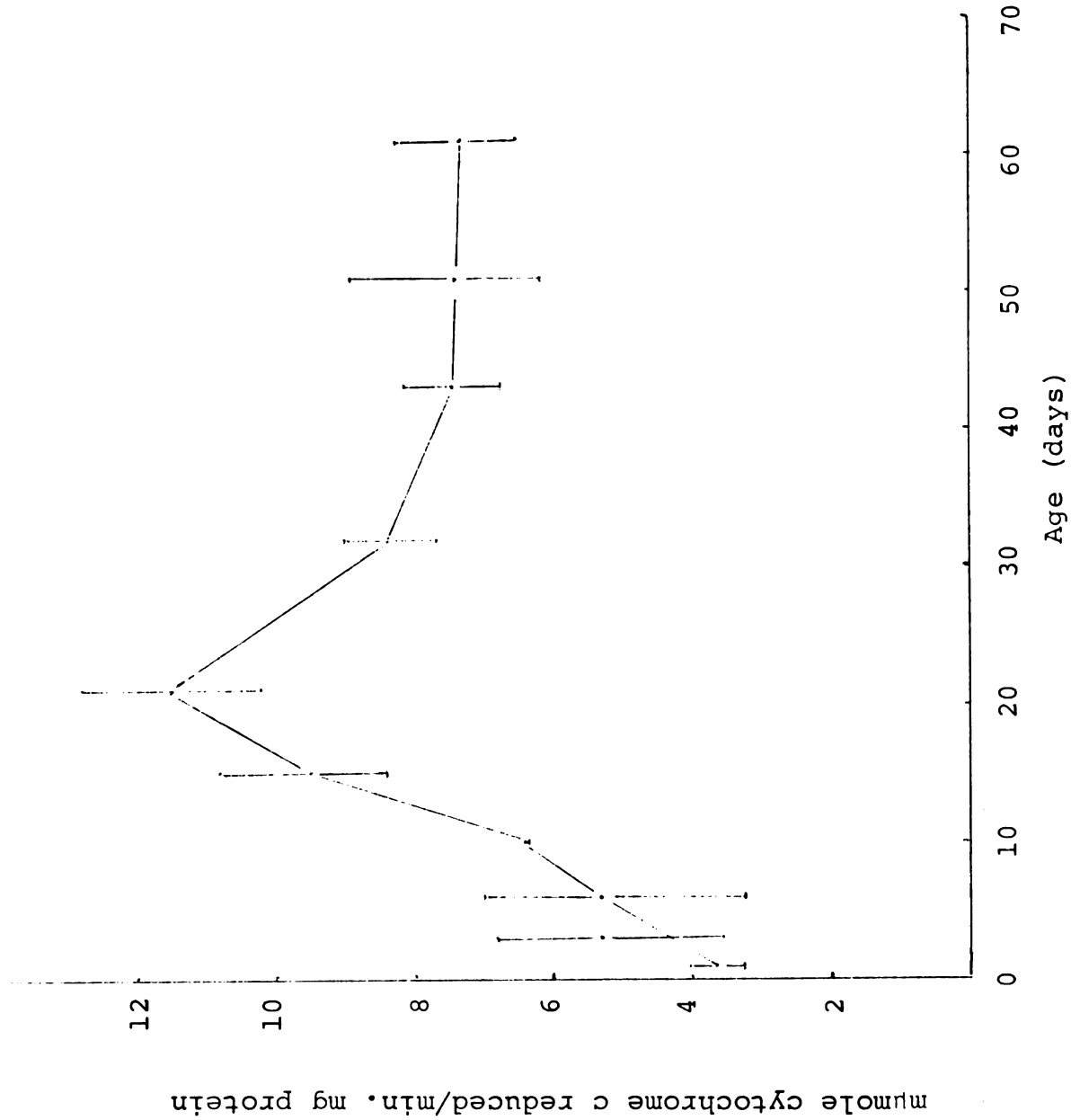

12
10
8
6
4
2
0
10
20
30
40
50
60
70
Age (days)
μmole cytochrome c reduced/min. mg protein

Figure 7.--Cytochrome P-450 (solid line) and P-420 (broken line) levels during postnatal development. The amount of P-450 is expressed as the $\Delta E_{450} - \Delta E_{490}$ per mg of microsomal protein. The amount of P-420 is expressed as E_{420} per mg protein. Values are means of three assays; individual assays involve the hepatic microsomes isolated from groups of from three to twenty-four male rats.

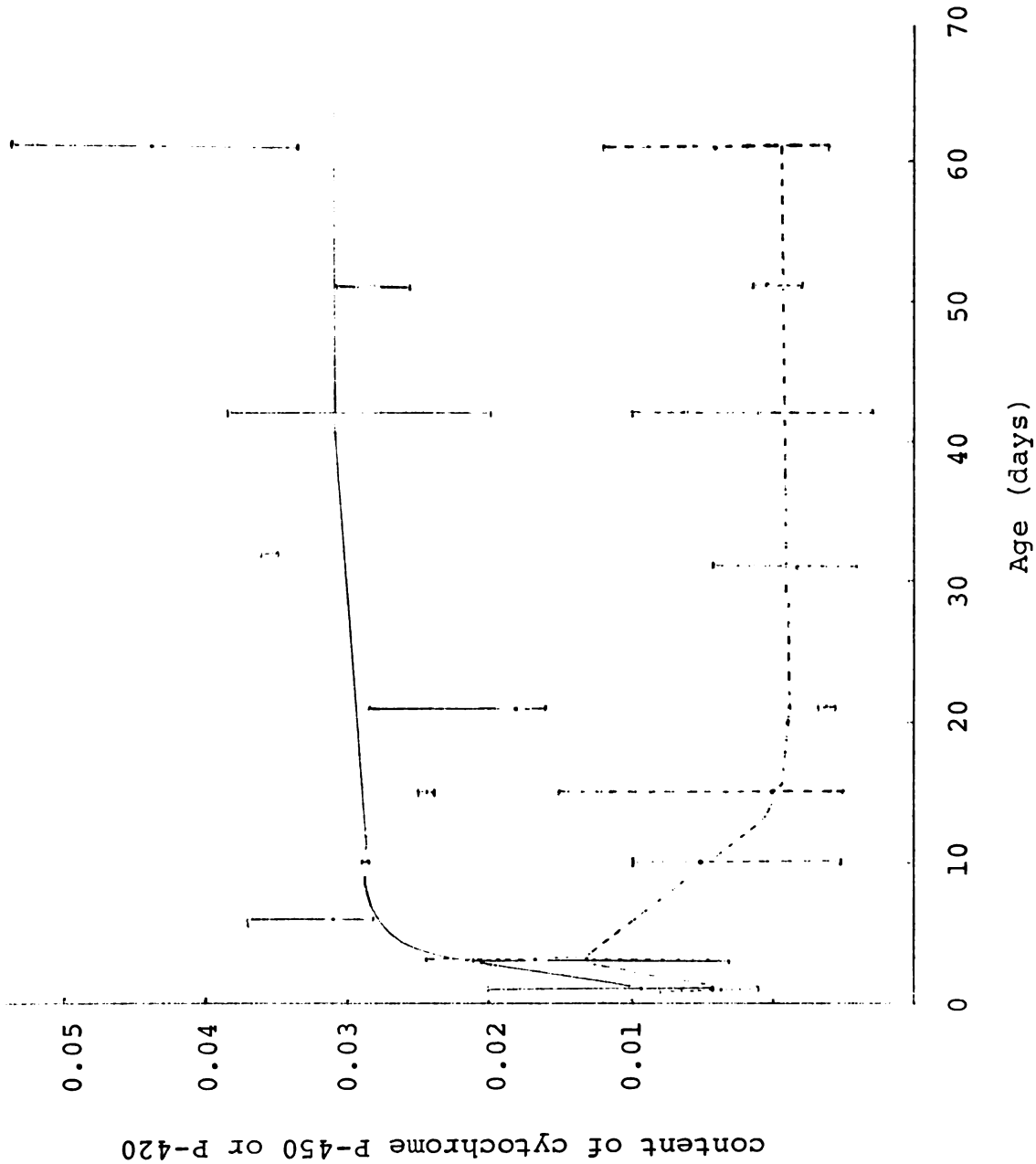

content of cytochrome P-450 or P-420
0.05
0.04
0.03
0.02
0.01
0
10
20
30
40
50
60
70
Age (days)

Figure 7.--Cytochrome P-450 (solid line) and P-420 (broken line) levels during postnatal development. The amount of P-450 is expressed as the $\Delta E_{450} - \Delta E_{490}$ per mg of microsomal protein. The amount of P-420 is expressed as E_{420} per mg protein. Values are means of three assays; individual assays involve the hepatic microsomes isolated from groups of from three to twenty-four male rats.

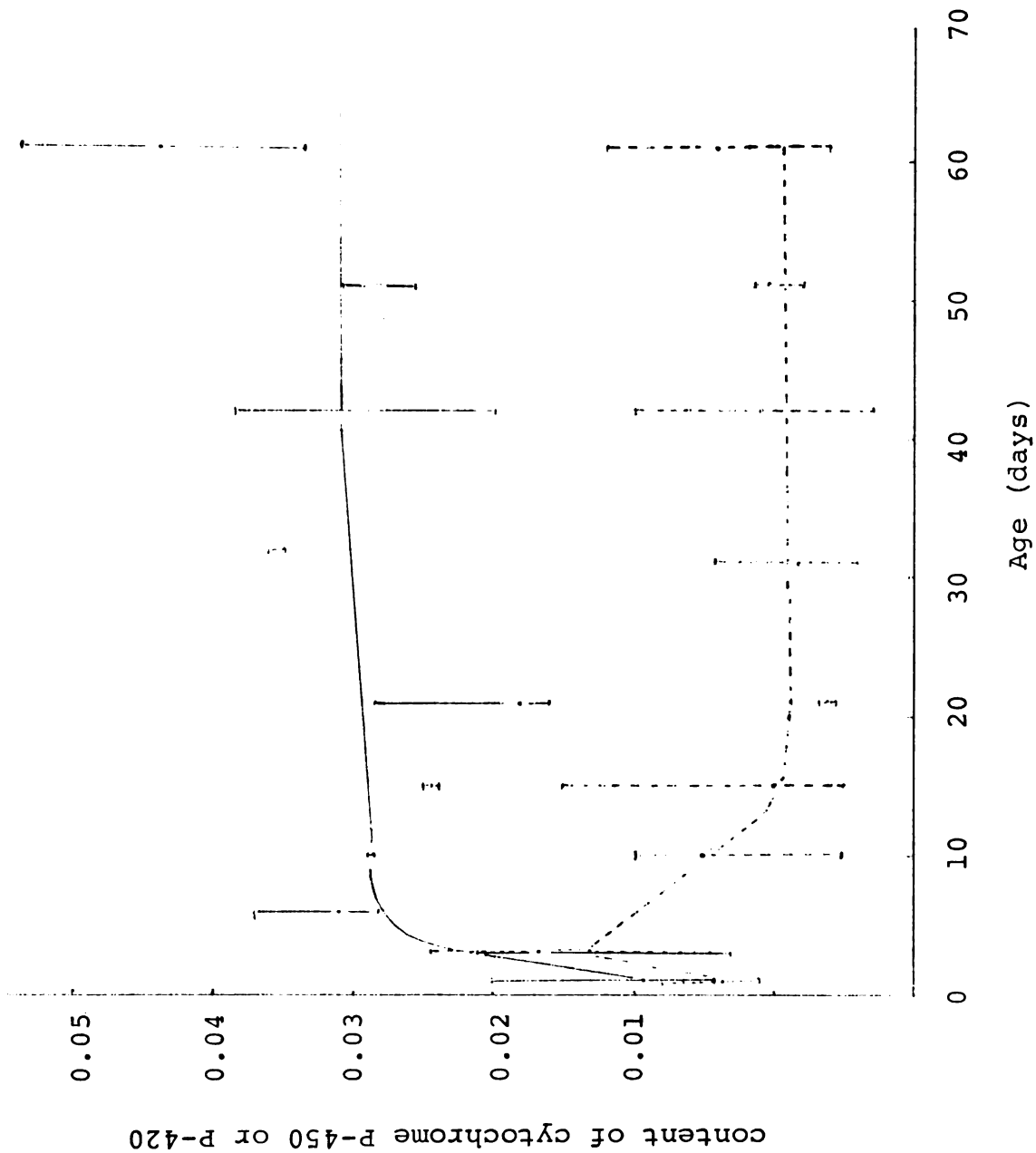
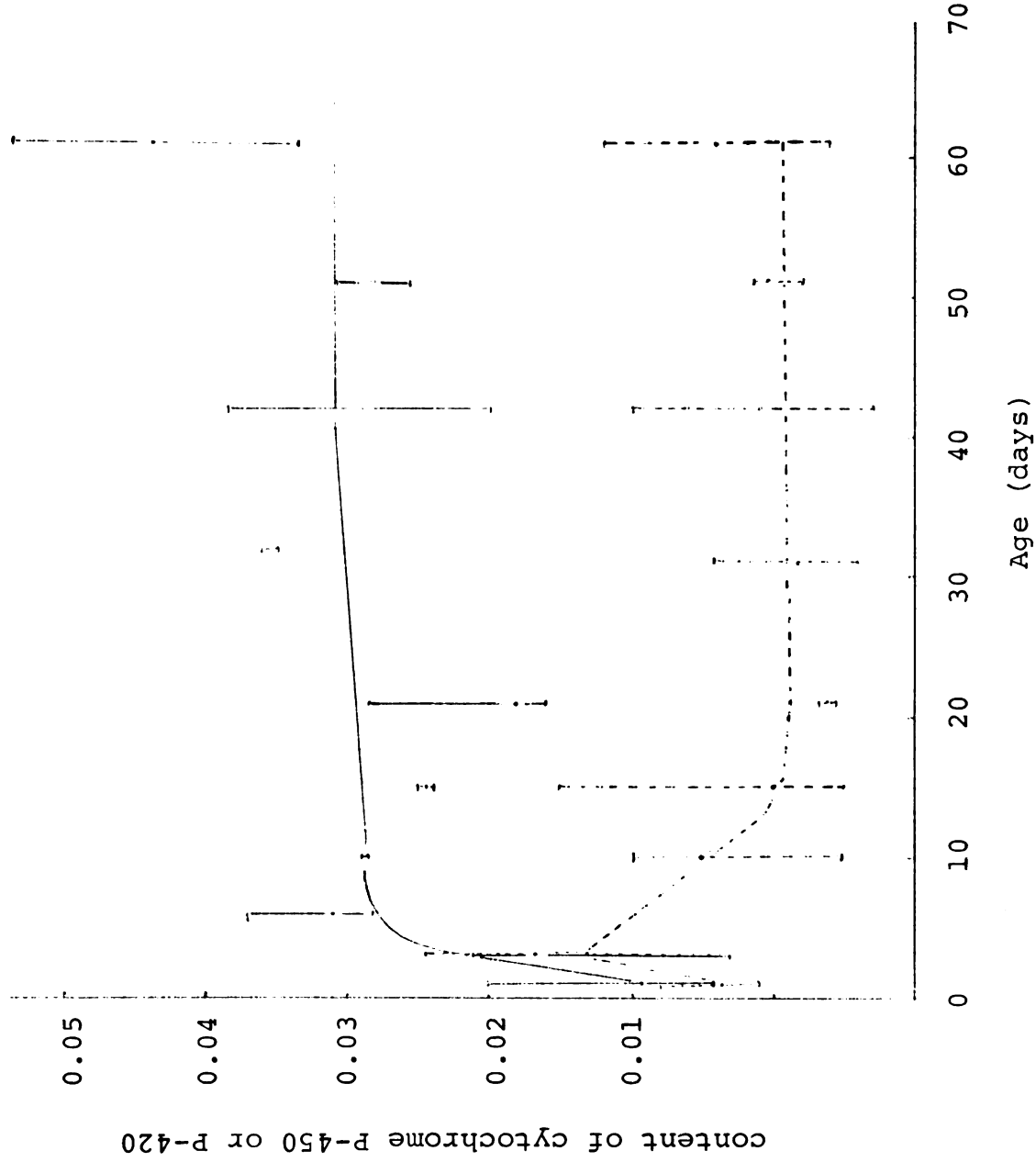

content of cytochrome P-450 or P-420
0.05
0.04
0.03
0.02
0.01
0
10
20
30
40
50
60
70
Age (days)

Figure 7.--Cytochrome P-450 (solid line) and P-420 (broken line) levels during postnatal development. The amount of P-450 is expressed as the $\Delta E_{450} - \Delta E_{490}$ per mg of microsomal protein. The amount of P-420 is expressed as E_{420} per mg protein. Values are means of three assays; individual assays involve the hepatic microsomes isolated from groups of from three to twenty-four male rats.


content of cytochrome P-450 or P-420
0.05
0.04
0.03
0.02
0.01
0
10
20
30
40
50
60
70
Age (days)

and the antigenic properties of NADPH-cytochrome c reductase in both liver and kidney microsome are identical.

The observation that cytochrome P-450 concentrations increase rapidly shortly after birth is consistent with the report by Dallner et al. (64). The amount of cytochrome P-450 in neonatal rats was about 50 per cent that of the adult value in Eling et al. (70) and our studies, while it was only 20 per cent of adult values in those of Dallner et al. However, in the report of Gram et al. (66), microsomal cytochrome P-450 was found to increase linearly and only slightly during the first 12 weeks of life. Elevations in activity of some hepatic drug metabolizing enzyme and in the concentration of cytochrome P-450 have been demonstrated previously by treatment of animals with drugs, such as phenobarbital, 3-methylcholanthrene and diphenylhydratoin (70). It also has been shown that the amount of hepatic microsomal P-450 increased to adult levels by treating newborn rats with diphenylhydratoin (70). Recently, Darby (71) reported increased drug-metabolizing activities in the liver microsomes of nursing rats, after treating the mothers with inducers.

During the studies on the development of cytochrome P-450 several anomalies were noted. In the microsomes isolated from the liver of newborn rats, the assays for cytochrome P-450 showed a considerable amount of the degraded form of cytochrome P-450 (termed cytochrome P-420). The amount of cytochrome P-420 always reached a maximum at

3 days of age (Figure 7). This P-420 could actually be present in the microsomes of newborn rats, possibly as a precursor to P-450, or it could be an artifact of isolation. It could also be contaminating hemoglobin. The latter possibility was investigated by assaying for the hemoglobin-CO complex and indeed a small amount of hemoglobin was present. However, this hemoglobin could be removed by washing the microsomes in 0.25 M NaCl. After washing the cytochrome P-420 was still present.

The possibility that the cytochrome P-420 could be an artifact of the isolation procedure was investigated by isolating microsomes in the presence of 25% glycerol. Glycerol has been shown to protect cytochrome P-450 and convert P-420 to P-450 (34). When this was done, the content of both P-450 and P-420 were decreased, however, the data were not consistent. Frequently, the level of P-450 decreased more than the level of P-420, however, sometimes the results were exactly the reverse. Never, however, did the inclusion of glycerol cause the level of P-420 to decrease and P-450 to increase, as predicted by the literature. This phenomenon is currently being investigated in the laboratory for other polyols were shown to cause a decrease in the amount of cytochrome P-450 present in microsomes.

Pregnant rats were treated with phenobarbital by including 0.1% phenobarbital in their drinking water for 10 days prior to the predicted day of parturition. The liver microsomal fraction was isolated from both mother and newborn pups and assayed for the various components and activities listed in Table 1. The aminopyrine demethylase activity in the microsomes isolated from the liver of the newborn pups was about equal to the activity normally observed in rats from 6 to 15 days of age (Figure 3). Cytochrome P-450 levels remained low while NADPH-cytochrome c reductase levels were increased slightly by the phenobarbital treatment. The only other significant difference was a lower level of glucose-6-phosphatase in the microsomes isolated from the livers of pups from phenobarbital treated mothers.

A comparison between the mother and their pups indicated that enzyme activity associated with drug metabolism was much higher in the mother than the newborn. The activity not associated with drug metabolism (glucose-6-phosphatase) was much lower in the adult.

The number of rats used in the induction studies were low for two reasons. Treatment of the mother with phenobarbital must have induced their steroid metabolism because parturition was delayed, sometimes up to two days. This undoubtedly resulted in some fetal deaths. For example, out of 36 pups only 8 were alive. Secondly, the

TABLE 1.--Effect of phenobarbital on the enzymatic composition of hepatic microsomes from one day old rats by pretreatment of pregnant rats with 0.1% PB in their drinking water.

Specific Activity*	Rats		
	Dam	Baby[a]	Control Baby[b]
aminopyrine demethylase	0.696	0.12	0.042
lipid peroxidation	0.14	0.048	0.042
cytochrome c reductase	7.28	2.70	1.95
P-450 level	0.068	0.014	0.023
aryl esterase	389.4	38.5	34.0
glucose-6-phosphatase	0.054	0.11	0.455
liver weight	13.3	0.31	0.29

*mμmoles formaldehyde formed per min. per mg protein (demethylation).

mμmoles malondialdehyde produced per min. per mg protein.

mμmoles cytochrome c reduced per min. per mg protein.

O.D. 450 - O.D. 490 per mg protein.

O.D. 420 per mg protein.

mμmoles para-nitrophenol formed per min. per mg protein.

μmoles phosphorous formed per minute per mg protein.

g per rat.

[a]One assay of microsomes from 8 newborn rats 3-16 hrs. old from three litters, both sex pooled together.

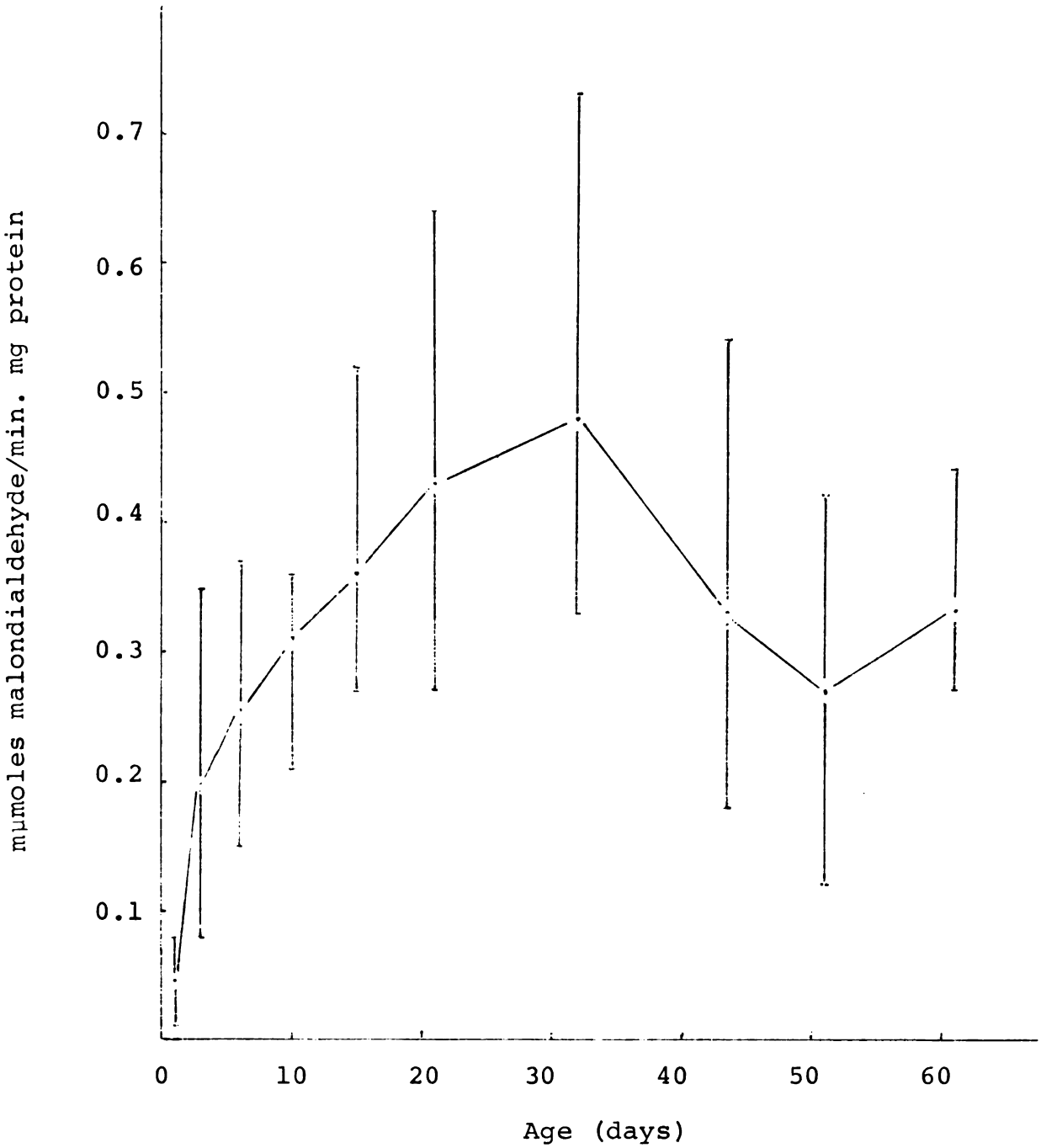
[b]Three assays of microsomes obtained from 12 to 24 rats each, range from 24 to 36 hrs. old, only male rats.

increased metabolism of steroids and presumably low blood levels of steroids, prevented mammary gland development and the newborn pups were unable to obtain milk. It was therefore necessary to sacrifice the pups soon after birth.

The peroxidation of endogenous microsomal lipids is thought to involve NADPH-cytochrome c reductase and the unsaturated fatty acids (primarily arachidonate) of the membrane phospholipids. Therefore, lipid peroxidation could reflect NADPH-cytochrome c reductase activity and/or the presence of arachidonate containing phospholipids. It might also reflect the proper combination of enzyme and lipid. The development of lipid peroxidation activity in the microsomal fraction of male rats is shown in Figure 8. The increase in activity closely parallels the development of NADPH-cytochrome c reductase activity (Figure 6).

Although lipid peroxidation was quite low in the microsomes from newborn rats the activity of NADPH-cytochrome c reductase (Figure 6) was not. It is therefore possible that the lack of lipid peroxidation activity in the microsomes from the newborn rats is due to the absence of suitable substrate. This possibility was investigated by adding total microsomal lipid (isolated from phenobarbital treated adult male rats) to the microsomes from day old rats. Lipid peroxidation activity (measured as malondialdehyde) was increased to the level of activity

Figure 8.--Lipid peroxidation activity in the hepatic microsomes from rats during postnatal development. The activity is presented as mμmoles of malondialdehyde formed per minute per mg microsomal protein. Each point is the mean of three assays; individual assays involve hepatic microsomes isolated from groups of from three to twenty-four male rats.


mμmoles malondialdehyde/min. mg protein
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
Age (days)

found in microsomes from adult rats (Table 2). None of the other activities measured were affected.

TABLE 2.--Activity of enzymes in the lipid enriched, control washed, and no treatment hepatic microsomes from one day old male rats.

Specific Activity*	Lipid Enriched	Control Washed	No Treatment
aminopyrine demethylase	0.138	0.148	0.096
lipid peroxidation	0.22	0.0082	0.008
P-450 level	0.011	0.030	0.009
cytochrome c reductase	4.2	5.76	3.83

*mμmoles formaldehyde formed per min. per mg protein.

mμmoles malondialdehyde produced per min. per mg protein.

O.D. 450 - O.D. 490 per mg protein.

mμmoles cytochrome c reduced per min. per mg protein.

One additional experiment was conducted in which methylarachidonate was added as the lipid. This substrate could also support lipid peroxidation by the microsomes from newborn rats, however, not as well as did total microsomal lipids.

The result presented in Table 2 suggested a lack of suitable unsaturated lipids in the microsomes of newborn rat livers. Therefore, the amount of total lipid and of the individual fatty acids in the hepatic microsomal fraction was assayed throughout development. Total microsomal phosphate did not change greatly (Figure 9) except for some variation during the first 15 days after birth. This could be related to the changes in RNA content (Figure 2). The lowest RNA content of 15 days of age was comparable to the lowest total phosphate content of the same age.

Lipid phosphate was assayed by extracting the total microsomal lipids in 2:1 chloroform:methanol, washing the extracted lipid with a salt solution and assaying for phosphate in the extracted, washed lipid. No large differences were seen (Figure 10) in the lipids extracted from hepatic microsomes of any age except that the newborn did have the lowest amount of lipid phosphate.

The fatty acid composition of total microsomal lipid during rat development is shown in Figure 11. The difference in composition is slight but does vary during the first three weeks of age. Thereafter the fatty acids ratios tended to a constant level. The amount of palmitic and arachidonic acid tended to decrease as a function of age while the concentration of stearic and linoleic acid

Figure 9.--Total phosphate content of rat liver microsomes as a function of age. Phosphate content is expressed as mM phosphate per mg of protein. Each point is the mean of two or three assays; individual assays involve hepatic microsomes isolated from groups of from three to twenty-four male rats.

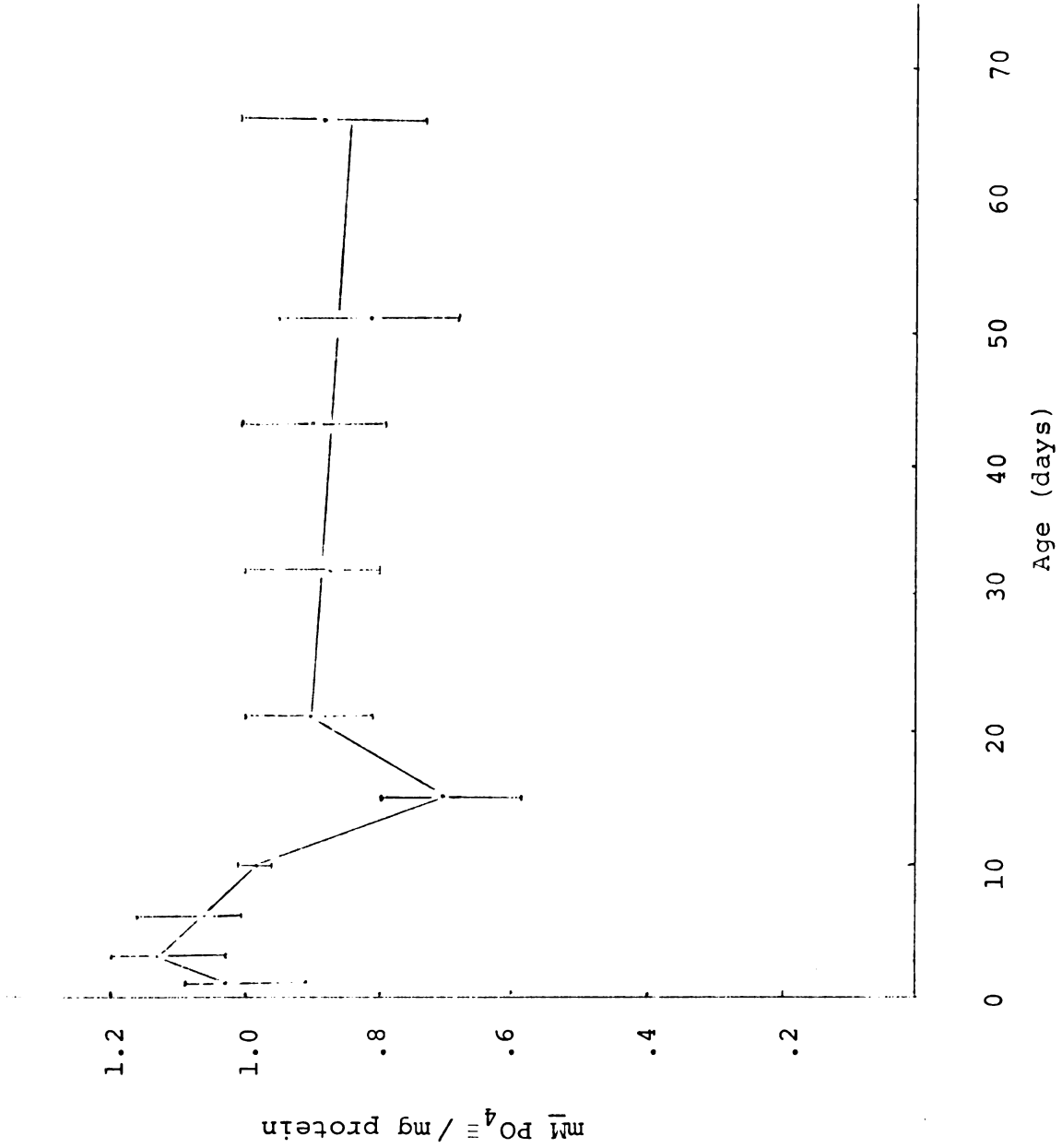
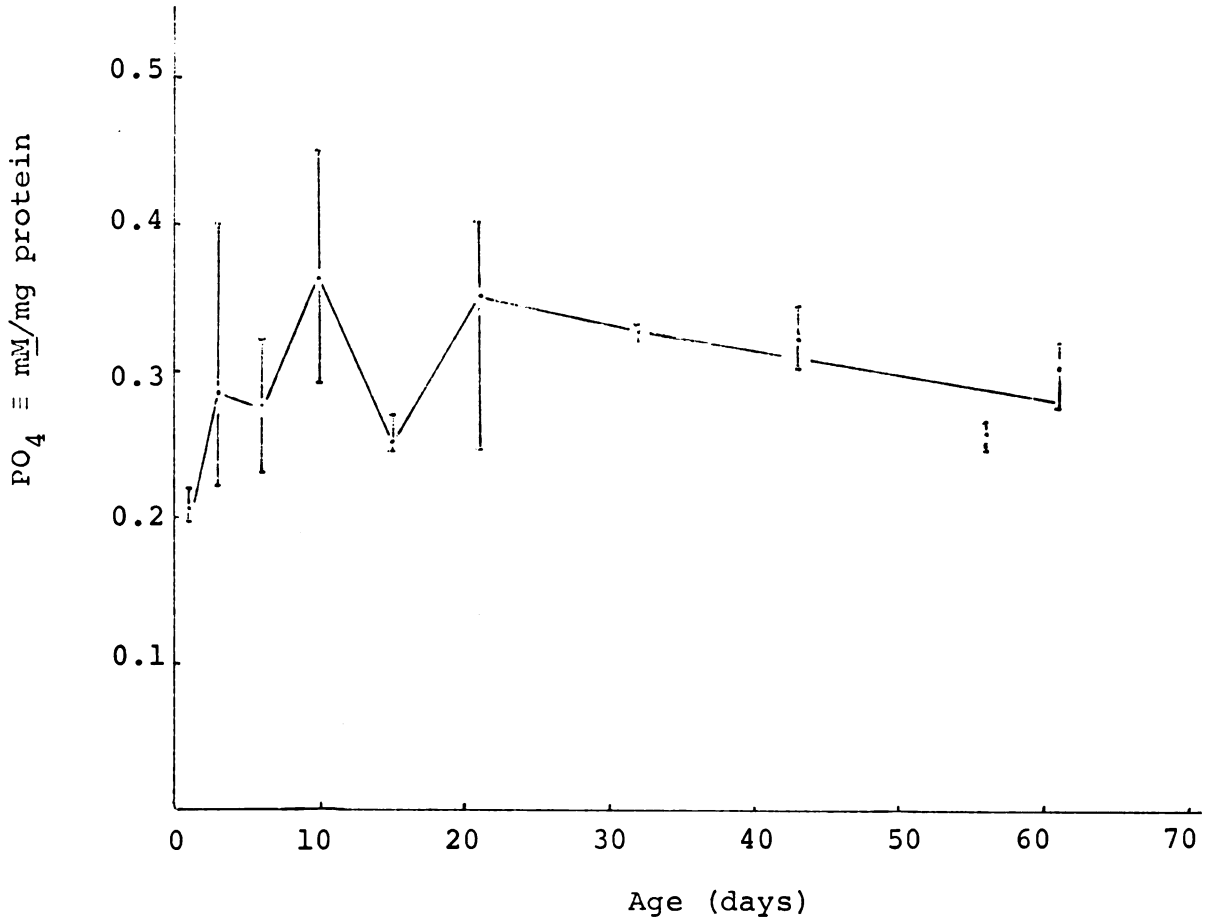

mμ PO4≡ / mg protein
1.2
1.0
.8
.6
.4
.2
0
10
20
30
40
50
60
70
Age (days)

Figure 10.--Postnatal changes in total microsomal phospholipid of rat liver. The amount of phospholipid is represented as mM of phosphate in extracted lipid per mg of microsomal protein. Each point is the mean of two assays; individual assays involve hepatic microsomes isolated from groups of from three to twenty-four male rats.


PO_4 ≡ mM/mg protein
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
Age (days)

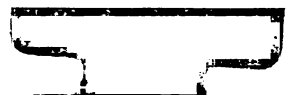


Figure 11. Fatty acid composition of rat liver microsomal phospholipid as a function of age. Each point represents the mean of two assays on lipid extracted from the hepatic microsomes isolated from groups of from three to twenty-four male rats.

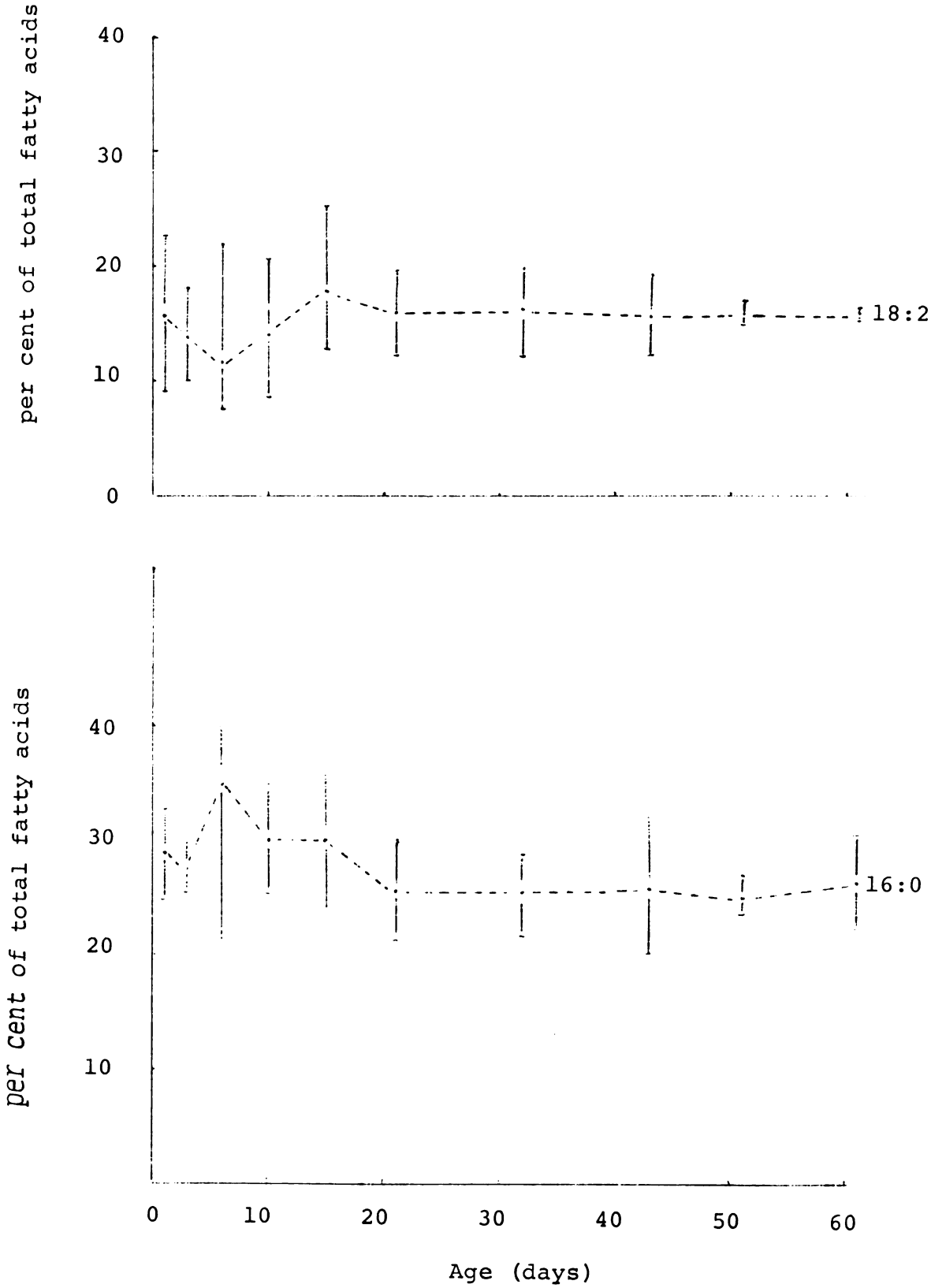
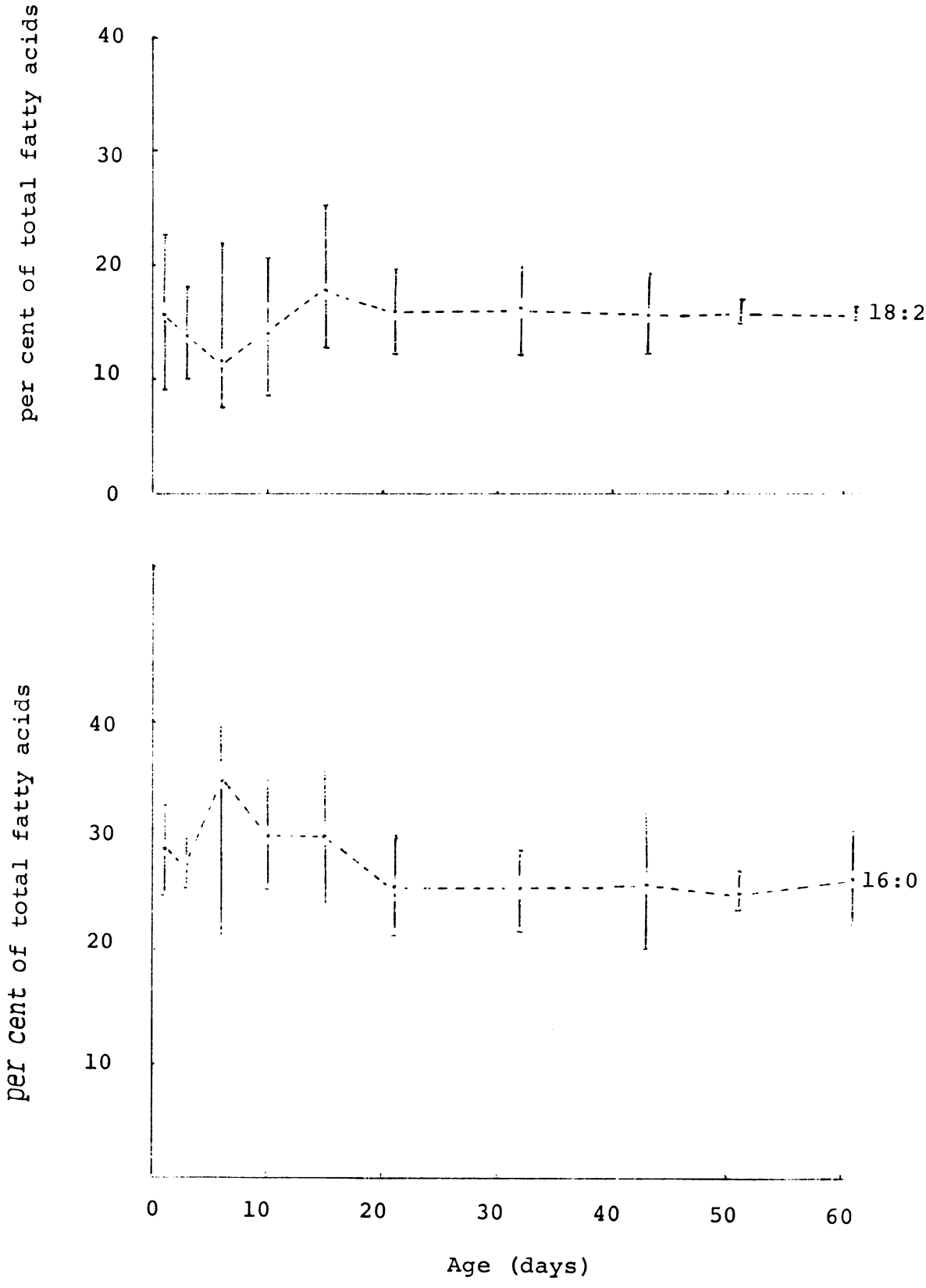
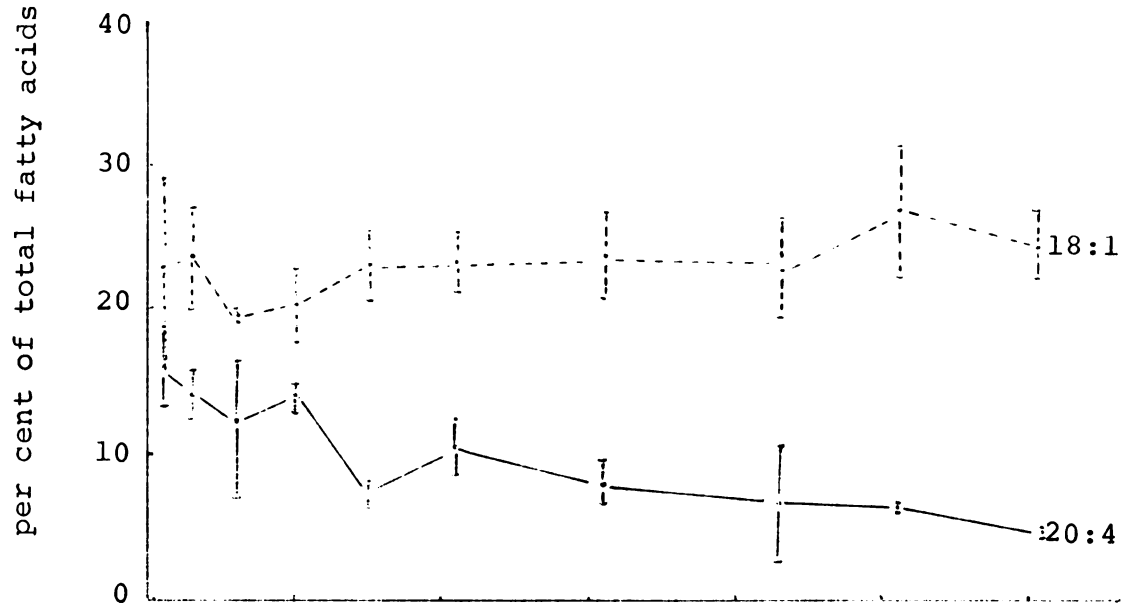
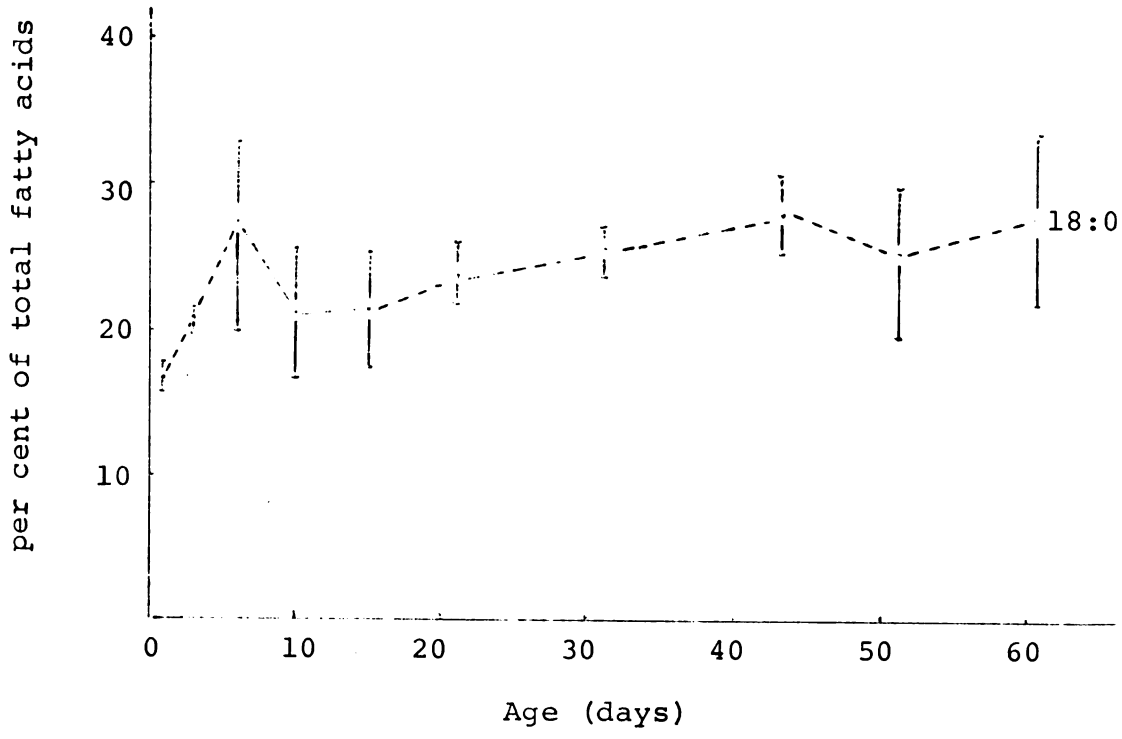

per cent of total fatty acids
40
30
20
10
0
18:2
per cent of total fatty acids
40
30
20
10
16:0
0
10
20
30
40
50
60
Age (days)

Figure 11. Fatty acid composition of rat liver microsomal phospholipid as a function of age. Each point represents the mean of two assays on lipid extracted from the hepatic microsomes isolated from groups of from three to twenty-four male rats.


per cent of total fatty acids
40
30
20
10
0
18:2
per cent of total fatty acids
40
30
20
10
16:0
0
10
20
30
40
50
60
Age (days)


per cent of total fatty acids
40
30
20
10
0
18:1
20:4


per cent of total fatty acids
40
30
20
10
18:0
0
10
20
30
40
50
60
Age (days)

tended to increase. The oleic acid level decreased rapidly to reach the lowest level at 6 days of age, thereafter it increased gradually. The developmental pattern of each fatty acid is fairly consistent with the data from Dallner et al. (63), although they only represented three different ages (0, 5, and 90 days old).

Thin layer chromatography of the total microsomal lipid isolated from the hepatic microsomes from rats of different ages showed no discernable differences. The plates were analyzed visually by exposing to iodine vapors and by spraying with Dragendorf's reagent and ninhydrin. The silica gel was scraped off in 1 cm sections and each section was analyzed for total phosphate. A plot of total phosphate vs cm was almost identical for the chromatograms of the lipid from the microsomes of rats of all ages.

Phosphatidyl choline was the most dominant component in all microsomes. Phosphatidyl ethanolamine is also a major component, while phosphatidyl serine only exist in minor amount. Those observations are consistent with the data of Dallner et al. (63) and Donaldson (72). In the first report (63) the phospholipid composition of hepatic microsomal membrane at three different ages were studied and no major differences were detected. Recently, it has been reported (73) that phosphatidyl choline is required

in the reduction of cytochrome P-450 and phospholipid is required for the activity of several microsomal enzymes, suggesting that phospholipid plays an important role in hepatic microsomes.

SUMMARY

This study presents another example of marked enzymatic changes in developing hepatic microsomes. The activities of postnatal microsomal drug-metabolizing enzymes change independently of each other and do not correlate with the changes in the concentration of microsomal cytochrome P-450 which has been implicated as the terminal oxidase in the metabolism of drugs.

The observation that the rate of development of enzymatic activity appears to be most rapid during the first three weeks of postnatal life is consistent with previous reports (47, 51-53). Enzymes for drug metabolism, aminopyrine demethylase, NADPH-cytochrome c reductase, and cytochrome P-450, an enzyme which may relate to drug metabolism, aryl esterase, and lipid peroxidation activity are low in the newborn animals and slowly increase to adult levels.

The rate of development of aminopyrine demethylase activity is consistent with that reported by Henderson (51), except that in our study the period of rapid increase occurs between 15 and 32 days of age. In the previous report the rapid increase was found to occur between 20 and 30 days of postnatal life.

The pattern of development of NADPH-cytochrome c reductase and lipid peroxidation activity is quite distinct compared to that of other microsomal enzymes. Both of these enzymes are characterized by a rapid and linear rate of development, reaching 130 to 150 per cent of adult activity around the third or fourth weeks postpartum. After this peak, a tendency for the activity to decline was observed. The parallel development of both activities before 21 days of age supports the previous suggestions that lipid peroxidation competes with hepatic microsomal drug-metabolizing enzymes for reducing equivalents from NADPH (35,36).

In the report of Dallner et al. (64), the NADPH-cytochrome c reductase activity was found to reach the adult level on the first day of birth, however, the present study shows that the activity is only about 22 per cent that of the adult level. At 21 days of age, the amount of cytochrome P-450 reaches adult level and the activity of NADPH-cytochrome c reductase is 130 per cent that of the adult level. However, the drug-metabolizing activity, aminopyrine demethylase, reaches only about 50 per cent of the adult level. These unsynchronous phenomena suggest that the mixed-function oxidase activity, when measured by the N-demethylation of aminopyrine, is dependent on some other factor in addition to cytochrome P-450 and NADPH-cytochrome c reductase. This observation

can be variously ascribed to: (1) a deficiency of the proposed electron carrier "x" between NADPH-cytochrome c reductase and cytochrome P-450, as described earlier (64); (2) the reduction of cytochrome P-450 as the rate limiting step, as suggested in the case of rabbit microsomes (56); (3) the existence of an inactive form of aminopyrine demethylase; (4) the indication that only a part of the cytochrome P-450 in microsomes from younger rats is involved in aminopyrine demethylation (74). Recently reported methods for the isolation of cytochrome P-450 (27) may make it possible to answer this question.

A study of the treatment of pregnant animals with drugs to elevate the activity of liver microsomal drug-metabolizing enzymes in the newborn has been reported (44). In the present study, enzymes other than those involved in drug metabolism have been examined in newborns from similarly pretreated mothers. Glucose-6-phosphatase activity is lower in the microsomes from newborn rats whose mothers were given phenobarbital, suggesting that other proteins are being synthesized to decrease the specific activity of non-drug metabolism enzymes.

The specific activity of aminopyrine demethylase is higher in the neonates from pretreated mothers but does not correlate with the amount of cytochrome P-450, in agreement with our observations that the difference in

activities cannot be explained by a difference in the concentration of cytochrome P-450.

The observation of the low hepatic microsomal esterase activity in newborns compared to the adult level is consistent with the data of Schwark et al. (75). Their report also shows a higher esterase activity in immature animals (28 days old), as compared to the adult value. The present study, however, shows an increase in specific activity through the entire period studied. The low esterase activity in newborn rats should somewhat explain the predisposition of the young animals to metabolize drugs with ester linkages (14).

The lipid peroxidation system is thought to be involved in the turnover of fatty acids, primarily arachidonate, from microsomal lipid (39,40). Our studies show that by adding adult total microsomal lipid to the microsomes of one-day old rats, the activity of lipid peroxidation increases about 27 fold. When arachidonate or beef heart lecithin was added to microsomes from one day old animals, lipid peroxidation activity increased 18 fold and 17 fold, respectively. Although analysis of the fatty acid composition of microsomes indicated a higher percentage of arachidonate in the microsomes of newborn rats, the young animals showed relatively low lipid peroxidation activity. These data suggest that, in newborn rats, the membrane is in a conformation such that

arachidonate is masked and cannot act as substrate for the already existent lipid peroxidation system, i.e., the low activity of lipid peroxidation in young animals is caused by a deficiency in available substrate and not by a lack of the lipid peroxidation system.

Previous reports (16,17) have shown a poorly developed endoplasmic reticulum in fetal microsomes. During development, the synthesis of membrane components occurs. Comparison of the total microsomal phosphorous content, the phospholipid content, and the RNA content in hepatic microsomes indicates that the high total microsomal phosphorous content in newborn rats is caused by their high RNA content, which can be expected, since the newborn liver contains a higher proportion of rough endoplasmic reticulum than smooth endoplasmic reticulum.

It has been reported that treatment of microsomes with phospholipase leads to a decrease in the activity of several microsomal enzymes, i.e., glucose-6-phosphatase (76), NADPH-cytochrome c reductase (77), and drug-metabolizing enzymes (29,30). Activity of these enzymes can be restored by adding phospholipids, suggesting that phospholipids are required for microsomal activity. Williamson and O'Donnell (78) have recently reported that phospholipids stabilize hemoprotein P-450 and prevent the conversion of P-450 to P-420.

Administration of phenobarbital to rats preferentially increases phosphatidyl choline (79), which has been reported to be required for the reduction of cytochrome P-450 (78). In this study, no difference in the phospholipid composition of rat hepatic total microsomal lipid could be observed at any age, as was suggested by Dallner et al. (63).

In agreement with previous reports, the fatty acid composition of the total microsomal lipids shows some difference before 21 days of age. Thereafter the ratio of individual fatty acids in microsomal lipid tends to be constant.

While the lack of drug-metabolizing activity in the newborn rat may be initially due to the lack of the proper enzymes, i.e., NADPH-cytochrome c reductase and cytochrome P-450, other factors must be responsible for the observation that the development of oxidase activity does not correlate with the development of these enzymes. The lipid peroxidation studies and all the literature references to the involvement of lipids in microsomal activities leads to the suggestion that maturation of the mixed-function oxidases may involve lipid.

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