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SHORT TITLE:

THYROXIN AND DEVELOPMENT OF DRUG METABOLISM

THYROXIN AND DEVELOPMENT OF HEPATIC MICROSOMAL DRUG OXIDATION AND ELECTRON TRANSPORT IN MAN AND RATS

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ABSTRACT

The neonatal phase of the ontogenetic pattern of hepatic microsomal mixed function oxidases (HMMFO) in man and the influence of thyroid hormone on this ontogenetic pattern in rats were studied. In the neonatal period in man, there is a significant deficiency in the activity of hepatic microsomal aminopyrine N-demethylase, aniline p-hydroxylase, NADPH oxidase, NADPH cytochrome c reductase, and in the amount of cytochrome P-450 and no measurable activity of NADPH cytochrome P-450 reductase. All of these enzymes except aminopyrine N-demethylase significantly correlated with postconceptional age indicating that significant developmental events occur during the newborn period. These developmental events are probably modulated by thyroxin, a hormone closely related to both drug oxidation and organ development as evidenced by changes in the developmental pattern of the hepatic microsomal drug oxidizing enzymes in various altered thyroid states. An acute single dose of thyroxin in immature rats resulted in significant increase in the activity of aniline p-hydroxylase and the microsomal electron transport enzymes. Chronic hyperthyroid state during the first 5 weeks of age resulted in significant acceleration of the development of hepatic microsomal aniline p-hydroxylase, NADPH oxidase and NADPH cytochrome c reductase activity, but with significant deceleration in the development of cytochrome P-450 content. Conversely, hypothyroidism produced by ^{131}I administered at birth resulted in effects opposite to hyperthyroidism (deceleration of NADPH cytochrome c reductase and acceleration of cytochrome P-450), which were reversed by replacement therapy with L-thyroxin. These effects were not demonstrated by the addition of thyroxin

in vitro. Postnatal age is a major determinant in the effect of thyroxin on hepatic microsomal oxidation and electron transport as well as the duration of altered thyroid state. It is proposed that thyroid hormone is an important regulator in the development of hepatic microsomal drug oxidation and electron transport.

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RÉSUMÉ

LA THYROXINE ET LE DEVELOPPEMENT DE L'OXIDATION DES DROGUES ET DU TRANSPORT DES ELECTRONS PAR LES MICROSOMES HEPATIQUES CHEZ L'HOMME ET CHEZ LE RAT

Nous avons étudié l'ontogénèse des oxidases hépatiques à function mixte chèz l'homme, et l'influence des hormones thyroidiennes sur l'ontogénèse de ces énzymes chez le rat.

Durant la période néonatale chez l'homme, on observe une déficience significative de l'aminopyrine-N-démethylase, de l'aniline-p-hydroxylase, de l'NADPH oxidase, NADPH cytochrome c reductase et du cytochrome P-450, tandis que l'NADPH cytochrome P-450 reductase n'est pas mesurable. Il existe une corrélation significative entre l'activité de toutes ces enzymes, exception faite de l'aminopyrine-N-démethylase, et l'âge postnatal, ce qui suggère une évolution significative de leurs activités après la naissance. Ces étapes de développement sont probablement influencées par la thyroxine, hormone intimement associée à la fois à l'oxidation des médicaments et au développement des fonctions organiques comme en témoignent les changements de l'ontogénese des enzymes hépatiques microsomales qui président à l'oxidation des médicaments, au cours d'alterations de la fonction thyroidienne. Une seule dose de thyroxine administrée au rat impubère produit une augmentation significative de l'activité de l'aniline-p-hydroxylase et des enzymes du système microsomal de transport d'électrons. Un état d'hyperthyroidie au cours des 5 premières semaines de vie a pour conséquence une accéleration significative du développement de l'aniline-p-hydroxylase de l'NADPH oxidase et de l'NADPH cytochrome c reductase, associée à un ralentissement significatif du développement du cytochrome P-450. Inversement, l'hypothyroidie produite par l'administration d'iode 131 a la naissance amène des effets contraires (diminution de l'NADPH

cytochrome c reductase, accélération du développement du cytochrome P-450). Ces effets sont réversibles après administration de l-thyroxine. Cette action de la thyroxine n'est toutefois pas démontrable in vitro. L'action de la thyroxine sur le système microsomal d'oxidation hépatique et de transport d'électrons dépend de la durée de l'état d'hyper ou d'hypothyroidie aussi bien que de l'âge postnatal.

Nous proposons que l'hormone thyroidienne est un régulateur important du développement du système microsomal d'oxidation et de transport d'électrons dans le foie.

DEDICATION

to

Dr. Betty I. Sasyniuk

and

Dr. Ted V. Aranda

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PREFACE

FORMAT OF THE THESIS

Pursuant to regulation 4.2.7. (h) of the Faculty of Graduate Studies and Research of McGill University and to the "Thesis Format" adopted by the Department of Pharmacology and Therapeutics of McGill University, the main body of this thesis is presented in manuscript forms suitable for publication in a Learned Journal.

The section entitled "General Introduction and Statement of the Problems" is relatively short, and in order to avoid detraction from the continuity of the contents of this thesis, a review of literature relating to the relationship of hepatic microsomal drug metabolism and thyroid hormone is presented as an appendix (Appendix A). The relevant points which are necessary in the comprehension of the results shown in the individual manuscripts are presented in the appropriate manuscripts. The first manuscript has been published in the Journal of Pediatrics and the other manuscripts have been submitted for publication in the journals indicated in the title page of each of these manuscripts. The first manuscript (MS.I) relates to studies performed in human tissues. Due to ethical and practical considerations and due to the nature of the formulated problems stated in the later part of the General Introduction, subsequent studies were done in experimental animals (MS.II to IV). Some degree of redundancy between the General Introduction, General Discussion and similar sections in the manuscripts was unavoidable. In an attempt to present the relevant points more systematically, a subheading was included in the appropriate sections of the General Discussion. A section entitled "Conclusions and Statement of Original Content" dealing with all the manuscripts is presented between the last manuscript (MS.IV) and the Appendices.

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MANUSCRIPT III: THYROID HORMONE AND POSTNATAL RATE OF REDUCTION OF CYTOCHROME P-450 BY NADPH

GENERAL INTRODUCTION

AND

STATEMENT OF THE PROBLEMS

GENERAL INTRODUCTION AND STATEMENT OF THE PROBLEM

The oxidative biotransformation of a large variety of exogenous and endogenous compounds of physiological and pharmacological interest is known to occur primarily in the liver (Kensler et al., 1947; Mueller and Miller, 1948; Gillette, 1966; Brodie, 1966). The participation in these oxidative reactions, of an enzyme complex associated with the disrupted endoplasmic reticulum (microsomal fraction) of the liver was established in the mid-1950's (Brodie et al., 1955; Cooper and Brodie, 1955). Subsequent studies in the field of drug metabolism during the past two decades have provided evidence that this microsomal enzyme complex catalyzed the biotransformation of drugs (Brodie et al., 1955; Axelrod, 1955a, 1955b, 1956). Evidence was also provided that these oxidative enzymes are NADPH-dependent and are oxygen-requiring (Mueller and Miller, 1953; La Du et al., 1955; Gillette et al., 1957). This requirement for NADPH and molecular oxygen for the formation of an oxidized polar derivative of the parent compound and a molecule of water, led to the classification of this enzyme system as mixed function oxidases (Mason, 1957) or monooxygenases (Hayaishi, 1962) as illustrated in Figure 1 (Manuscript I). Their subcellular localization in the smooth endoplasmic reticulum of the liver (Holtzman, 1968) logically led to the terminology "hepatic microsomal mixed function oxidase system".

In the late 1950's, the activity of the hepatic microsomal enzyme system in the newborn experimental animal was first reported to be

deficient (Fouts and Adamson, 1959; Jondorf et al., 1959). Subsequent biochemical and morphological studies confirmed the observation, and supported the concept that the newborn experimental animal has a negligible activity of the hepatic microsomal drug oxidative enzymes (Kato et al., 1964; Fouts and Devereaux, 1972; MacLeod et al., 1972; Short and Stith, 1973; Basu et al., 1971; Gram et al., 1969; Henderson. 1971; Soyka, 1969; Klinger, 1968; Rane, 1973).

In man, the concept that the newborn infant also has a deficient capability to metabolize drugs was not fully recognized until the advent of the "gray baby syndrome". This was related to the use of chloramphenicol in the premature infants in the late 1950's (Sutherland, 1959; Burns et al., 1959). Chloramphenicol, administered to premature newborn infants in the usual therapeutic doses for older children (100 mg per kg per day) resulted in a syndrome characterized by fatal cardiovascular collapse (Sutherland, 1959) which accounted for a significant neonatal morbidity and mortality (Burns, et al., 1959). Chloramphenicol requires glucuronide conjugation for elimination from the body and because of the deficient glucuronyl transferase activity, chloramphenicol accumulated in toxic concentrations in the blood (Weiss, et al., 1960).

As a deficiency in the hepatic conjugative capacity of the newborn infant was demonstrated (Weiss et al., 1960; Brown and Zuelzer, 1958), suggestive evidence of a deficient hepatic oxidative capacity began to accumulate. Vest and Streiff (1959) gave acetanilid to ten newborn infants and showed that the peak concentration of the oxidative product para-aminophenol, appeared later compared to older children (Vest and Streiff, 1959). Similarly, tolbutamide administered orally or intravenously

to ten normal full term infants had a prolonged plasma retention during the first two days of life (Nitowsky et al., 1969). There was an inverse correlation between the plasma disappearance of the drug and the appearance of the oxidized metabolite, carboxytolbutamide (Nitowsky et al., 1969). Further evidence for a decrease in oxidative capacity was shown when aminopyrine plasma half-lives were measured in the first and eight days of life in fifteen normal full term infants (Reinecke et al., 1970). There was a successive increase with age of aminopyrine elimination rate from the plasma (Reinecke et al., 1970). The lack of significant metabolism of drugs such as diazepam in the human fetus (Idanpaan-Heikkila et al., 1971), and the higher blood level and prolonged serum half-life of diazepam in premature infants relative to older children (Morselli et al., 1973) also supported the concept of a deficient drug oxidative function in the neonatal period. Possible subcellular and biochemical basis for this deficient drug oxidative function in the human aborted fetuses was shown by lower activity of the hepatic microsomal mixed function oxidase system relative to adult liver microsomes (Yaffe et al., 1970; Pelkonen et al., 1973). Presently the data on hepatic microsomal enzymes that are available in the literature are limited to studies on human aborted fetuses with maximum fetal age of 25 weeks (Yaffe et al., 1970; Pelkonen et al., 1973; Rane and Ackermann, 1972); and there are no quantitative determinations of the activity of the hepatic microsomal mixed function system in the later part of gestation, or in the premature newborn infant.

The continuing high degree of exposure of the human fetus and newborn infant to drugs (Tables I and II) warrants the determination of

their capability to handle and metabolize these drugs. Epidemiological studies (Table I) have demonstrated that about 80 to 90% of pregnant mothers have consumed an average number of 3 to 10 drugs (Forfar and Nelson, 1972; Hill, 1972; Peckham and King, 1963). The aggressive diagnostic and therapeutic approaches adopted by the neonatal intensive care units also paralled the increased used of drugs in the newborn infant (Table II). Many of these drugs used in the fetal and neonatal period require biotransformation catalyzed by the hepatic microsomal mixed function oxidase system and the present studies were designed to determine the activity of this system in the premature and full term newborn infant.

The first manuscript in this thesis, "Hepatic Microsomal Drug Oxidation and Electron Transport in the Newborn" , sought to answer three questions:

1. What is the activity of the hepatic drug oxidizing enzymes and the electron transport components in the hepatic microsomes of premature and full term newborn infants? Information obtained from this study may provide quantitative estimates of the functional deficiency in the premature and full term newborn infant.

2. Which electron transport component(s) is/are substrate oxidation closely related to? Correlation of the rate of substrate oxidation with a specific electron transport component has been utilized previously in experimental animals to try to identify the rate limiting step in microsomal drug oxidation (Gigon et al., 1968). The possibility of

identifying the rate limiting step was attempted in the human liver microsomes from premature and full term newborn infants. Identification of the rate limiting step in drug metabolism during the neonatal period might lead to the development of therapeutic regimens which could circumvent a faulty metabolic step or would at least permit the promulgation of rational principles for neonatal drug therapy.

3. Does the activity of the hepatic microsomal drug metabolizing enzymes increase with age during the later part of gestation and in the neonatal period? This problem is a corollary to the major question in human drug metabolism which seeks to determine the time when the deficient neonatal drug oxidative function attains adult level of activity.

Although there is an increasing availability of data relating to the activity of the hepatic microsomal mixed function oxidased system in man (Yaffe et al., 1970; Pelkonen and Karki, 1971; Ackerman et al., 1972; Rane and Ackerman, 1972; Pelkonen et al., 1971; Juchau, 1971; Pomp et al., 1969; Pelkonen, 1973; Pelkonen et al., 1973; Rane and Gustaffson, 1973; Rane, 1974; and Aranda et al., 1974), the developmental pattern of the microsomal drug metabolizing enzymes in humans remains unknown. Furthermore, the available data in the literature are insufficient to predict a possible ontogenetic pattern of these enzymes. Specifically, the question of when the low activity of the microsomal drug metabolizing enzymes in the human fetus and newborn infant undergoes full differentiation or maturation to adult level of activity remains unanswered. In the experimental animals, most microsomal drug-oxidative enzymes approach adult level of activity at approximately 3 to 6 weeks of age (MacLeod et

al., 1972; Fouts and Devereaux, 1972; Short and Davis, 1970; Short and Stith, 1973; Kato et al., 1964; Bresnick and Stevenson, 1968; Basu et al., 1971; Soyka, 1969; Henderson, 1971; Jondorf, et al., 1959). The attainment of adult level of enzyme activity varies according to the substrate oxidative enzyme, electron transport component enzyme and to the sex, strain and species of animal studied. The time periods of the development of various hepatic microsomal drug metabolizing enzymes to adult activity are summarized in Tables III and IV.

The temporal events leading to full maturation of microsomal drug oxidative capability and the factors regulating these events are areas of major interest in perinatal pharmacology. Identification of physiological and pharmacological influences which allow for increased drug-metabolizing enzyme activity in the immature animal may elucidate the normal sequence of biochemical events in enzymic differentiation in drug metabolism. One possible factor that may significantly influence the development of the hepatic microsomal drug oxidative enzymes is thyroid hormone. The basis of this hypothesis is summarized as follows:

1. Thyroid hormone is an important regulator of the development of some organ systems (e.g. brain) (Balazs et al., 1969). Defeciency of thyroid hormone results in retardation of the development of the brain (Balazs et al., 1969). Thyroxin administration to fetal rabbits results in acceleration of the maturation of the lung and increased pulmonary surfactant production (Redding et al., 1972; Wu et al., 1973).

2. Thyroid hormone is intimately related to hepatic microsomal drug metabolism (Kato and Takahashi, 1968; Crooks et al., 1973;

Eichelbaum et al., 1974). This is discussed in Appendix A;

3. The appearance of measurable thyroid hormone seems to coincide with the appearance of measurable drug oxidative enzymes in the human fetus and in experimental animals (Greengard, 1969; Shepard, 1967; Greenberg et al., 1970; Pelkonen, 1973; Fisher et al., 1970).

Since thyroid hormone is related to both organ development and drug metabolism, it seemed possible that it is closely related to the development of hepatic microsomal drug metabolizing enzymes. The possibility was considered that thyroid hormone significantly influences the ontogenesis of the hepatic microsomal mixed function oxidase system.

The second and third manuscripts, "The Effect of Thyroid Hormone on the Development of Hepatic Microsomal Drug Oxidizing Enzymes in Male Rats" and "Thyroid Hormone and Postnatal Rate of Reduction of Cytochrome P-450 by NADPH" respectively, sought to answer the question "What is the influence of thyroid hormone on the development of hepatic microsomal drug oxidation and electron transport?" Delineation of the effect of endogenously occurring growth-promoting substrates such as thyroid hormone may provide an insight into the normal sequence of events involved in microsomal enzymic differentiation and the fundamental mechanisms which regulate these events.

The fourth manuscript entitled "Age Dependent Response of Hepatic Microsomal Mixed Function Oxidase System to Thyroxin", sought to answer the question "What is the influence of age on the inducibility of the hepatic microsomal drug metabolizing enzymes to thyroxin?"

Several factors seem to influence the responsiveness of the

hepatic microsomal drug metabolizing enzymes to induction by exogenous substrate and hormones (Fouts, 1968). One of these factors is age which appears to be a major determinant in the quantitative and perhaps qualitative response of a specific organ or enzyme system such as the drug metabolizing enzymes to a particular agent. The quantitative difference in the response of the microsomal drug metabolizing systems, as a function of age, is evidenced by 50 to 200% greater inducibility of biphenyl-4-hydroxylase, biphenyl-2-hydroxylase, p-nitrobenzoate reductase and cytochrome P-450 by phenobarbital in 12 day old rats compared to 52 day old rats (Basu et al., 1971). Similar age-related responsiveness to phenobarbital has been demonstrated, with a greater induction of aminopyrine N-demethylation, hexobarbital hydroxylation, aniline p-hydroxylation and p-nitrobenzoate reduction in 40 day-old rats compared to older animals (Kato and Takanaka, 1968), thus suggesting that immature rats are intrinsically more responsive to enzyme induction. Furthermore, within the period of development, when several biochemical adaptive changes occur, there is an increased variability in the response of drug metabolizing enzyme system to induction. For instance, oxidative enzymes for hexobarbital and aminopyrine in fetal rabbits studied 4 to 8 days prior to term were refractory to phenobarbital induction, whereas these same enzymes were readily inducible after birth (Hart et al., 1962). These observations indicate that the effectiveness of induction markedly depends upon the age at which the inducing agent is administered.

Clinical applications of age-related inducibility of enzyme systems has been demonstrated in steroid induction of other organ systems

such as the lungs in newborn infants (Liggins et al., 1972; Baden et al., 1972). For instance, induction of pulmonary surfactant to prevent hyaline membrane disease in the newborn infant occurred when steroids were administered before 32 weeks gestation and induction was not successful when steroids were given later than 32 weeks gestation (Liggins et al., 1972) or after birth (Baden et al., 1972). Determination of the degree of inducibility of the hepatic microsomal enzymes by thyroxin as a function of age may allow for optimum and effective induction when such induction is desirable.

TABLE 1: Profile of Maternal Drug Intake during Pregnancy

Percentage of mothers taking drugs (self-medicated and/or prescribed) in pregnancy

Drug	Forfar-1972 Edinburgh n = 911 %	Peckham-1963 Oakland n = 3,072 %	Hill-1972 Houston n = 156 %
Iron	82.0	87	41
Analgesics	63.2	24	64
Vitamins	39.8	78	25
Antacids	34.4	13	35
Barbiturates	28.0	32	24
Diuretics	18.0	32	57
Antiemetics	16.3	15	36
Antibiotics	15.6)	23	41
Sulfonamides	12.5)		6
Cough Medicines	8.3	4	-
Antihistamines	6.8	17	52
Hormones	4.1	8	20
Tranquilizers	3.6	a	-
Bronchodilators	3.1	b	d
Hypnotics	1.4	14	9
Appetite suppressants	1.2	14	10
Narcotic	-	c	5.0
Average number of Drugs consumed/patient	4.2[e] (1-10+)	3.6 (1-10+)	10.3 (3-29)

a, included in barbiturates
b, included in cough medicines
c, included in hypnotics
d, included in antihistamines
e, excluding iron

TABLE II: PROFILE OF DRUG UTILIZATION IN A NEONATAL INTENSIVE CARE UNIT (NICU)[a]

Percentage of newborn infants who received drugs in the Montreal Children's Hospital. NICU (1974-1975) N = 169

DRUG[b]	NUMBER OF PATIENTS (%)
Kanamycin	74 (43.8%)
Penicillin	72 (42.6%)
Calcium Gluconate	50 (29.6%)
Gentamicin	45 (26.6%)
Ampicillin	43 (25.4%)
Sodium Bicarbonate	31 (18.3%)
Infantol[(R)] (Multivitamins)	31 (18.3%)
Fer In Sol[(R)] (Iron)	17 (10.1%)
Chloramphenicol	15 (8.9%)
Furosemide	14 (8.3%)
Cloxacillin	11 (6.5%)
Phenobarbital	10 (5.9%)
Mycostatin	10 (5.9%)
Medium chain triglycerides	7 (4.1%)
Valium[(R)] (Diazepam)	5 (3.0%)
Cyclopentolate	5 (3.0%)
Neosynephrine[(R)] (phenylephrine)	5 (3.0%)
Vitamin C	5 (3.0%)
Hexachlorophene	4 (2.4%)

Table II continued

DRUG[b]	NUMBER OF PATIENTS (%)	
Adrenaline	4	(2.4%)
Morphine	4	(2.4%)
Digoxin	4	(2.4%)
Neosporin	3	(1.7%)
Carbenicillin	3	(1.7%)
Methicillin	3	(1.7%)
Hydrochlorthiazide	3	(1.7%)
Isuprel	3	(1.7%)
Glucose	3	(1.7%)
Polybactrin(R)	2	(1.2%)
Silver Nitrate	2	(1.2%)
Atropine	2	(1.2%)
Caffeine	2	(1.2%)
Vitamine B-12	2	(1.2%)
Magnesium Sulfate	2	(1.2%)
Sodium Sulamyd(R)	2	(1.2%)
Dicloxacillin	1	(0.6%)
Alcohol	1	(0.6%)
Herpes D(R)	1	(0.6%)
Mercuhydrin	1	(0.6%)
Aldactone	1	(0.6%)
Diphenylhydantoin	1	(0.6%)
Propranalol	1	(0.6%)
Acetominophen	1	(0.6%)
Meperidine	1	(0.6%)
Dexamethazone	1	(0.6%)
Insulin	1	(0.6%)
Solu-Cortef(R)	1	(0.6%)
Hydralazine	1	(0.6%)
Diazoxide	1	(0.6%)
Gamma-Globulin	1	(0.6%)
Poly Vi Flor(R)	1	(0.6%)

Table II continued

DRUG[b]	NUMBER OF PATIENTS (%)	
Vipherol[R]	1	(0.6%)
Potassium Chloride	1	(0.6%)
Atarax[R]	1	(0.6%)
Albumin	1	(0.6%)
Cotazyme[R]	1	(0.6%)
Glycerine suppositories	1	(0.6%)
Hypaque[R]	1	(0.6%)
No Drugs	41	(24.3%)

(a) Aranda, J.V., Cohen, S., and Neims, A. H. Epidemiology of drug utilization in the newborn period (Ongoing project).

(b) Excluding prophylactic agents as Vitamin K, and ophthalmic antimicrobial agents and intravenous fluids and alimentation.

(R) Brand (Manufacturer's) name.

TABLE III : THE MATURATION OF THE VARIOUS COMPONENTS OF THE HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE*

Component	Species	Sex	Time when adult or >80% adult activity reached[a]	Reference
NADPH oxidase	Rat (SD)[b]	F	30 days	Kato et al., 1964
	Rat	M & F	18-20 days	Uehleke et al., 1971
	Rat	-	1 day	Dallner et al., 1966
NADPH cytochrome c reductase	Rabbit	M & F	2 weeks	Fouts and Devereux, 1972
	Rat (CRLE)[b]	M	5 weeks	MacLeod et al., 1972
		F	5 weeks	MacLeod et al., 1972
	Pig	M + F	2 weeks	Short and Stith, 1973
NADPH neotetrazolium reductase	Rat	-	1 day	Dallner et al., 1966
Cytochrome b_5	Rabbit	M & F	30 days	Fouts and Devereux, 1972
	Pig	M & F	3 weeks	Short and Stith, 1973
	Rat	-	8 days	Dallner et al., 1966
Cytochrome P-450	Rat (SD)[b]	M	1 week	Gram et al., 1969
	Pig	M & F	4 weeks	Short and Davis, 1970; 1973
	Rat (W)[b]	M & F[d]	3 weeks	Basu et al., 1971
	Rat (CRLE)[b]	M & F	3 weeks	MacLeod et al., 1972
	Rabbit	M & F	30 days	Fouts and Devereux, 1972
	Rat	-	8 days	Dallner et al., 1966
	Rat	M & F	3 weeks	Uehleke et al., 1971
NADPH cytochrome P-450 reductase	Rat (CRLE)[b]	M	5 weeks	MacLeod et al., 1972
		F	4 weeks	MacLeod et al., 1972
	Rabbit	M & F	2 weeks	Fouts and Devereux, 1972
	Pig	M & F	2 weeks	Short and Stith, 1973

* see Footnotes for Table IV

TABLE IV : THE DEVELOPMENT TO ADULT ACTIVITY (>80%) OF VARIOUS SUBSTRATE OXIDATIONS

Substrate oxidized	Species	Sex	Time when adult or >80% adult activity reached	Reference
Aminopyrine	Rat (W)[b]	M	45 days	Henderson, 1971
		F	21 days	Henderson, 1971
	Rat (SD)[b]	M	32 days	Soyka, 1969
		F	12 days	Soyka, 1969
	Rat (CRLE)[b]	M & F	4 weeks	MacLeod et al., 1972
	Rat	M & F	30 days K	Klinger et al., 1968
L-Amphetamine	Pig	M & F	<4 weeks	Short & Davis, 1970
Aniline	Rat (CRLE)[b]	M & F	5 weeks	MacLeod et al., 1972
	Rat (SD)[b]	M	4 weeks	Gram et al., 1969
Benzphetamine	Rabbit	M & F	30 days	Fouts and Devereux, 1972
Benzpyrene	Rabbit	M & F	30 days	Fouts and Devereux, 1972
Biphenyl-2	Rat (W)[b]	M & F[d]	21 days	Basu et al., 1971
Biphenyl-4	Rat (W)[b]	M & F[d]	21 days	Basu et al., 1971
Carisopradol	Rat (SD)[b]	F	20 days	Kato et al., 1964
4-Chloroaniline	Rat (W)	M & F	2-3 days 18-20 days	Uehleke et al., 1971
Chlorpromazine	Rabbit	-	3 weeks	Fouts and Adamson, 1959
Ethylmorphine	Rat (SD)[b]	M	2 weeks	Gram et al., 1969
	Pig	M & F	3 weeks	Short and Stith, 1973

Table IV continued

Substrate oxidized	Species	Sex	Time when adult or >80% adult activity reached	Reference
Hexobarbital	Rat (SD)[b]	F	20 days	Kato et al., 1964
	Guinea pig	F	>57 days	Jondorf et al., 1959
	Pig	M & F	6 weeks	Short and Davis, 1970
Meprobamate	Rat (SD)[b]	F	20 days	Kato et al., 1964
Monomethyl-4-aminoantipyrine	Guinea pig	F	>57 days	Jondorf et al., 1959
Methyl-4-monomethylaminoazobenzene	Rat	M & F	3 days[c]	Bresnick and Stevenson, 1968
N-methyl-aniline	Rat (W)	M & F	2-3 days[e] 18-20 days	Uehleke et al., 1971
N-methyl p-nitriline	Mouse	M & F	20 days	Pomp et al., 1969
Morphine	Rat	M & F	30 days	Klinger et al., 1968
p-Nitroanisole	Pig	M & F	4 weeks	Short and Davis, 1970
	Mouse	M & F	20 days	Pomp et al., 1968
p-Nitrobenzoate	Rat (W)	M & F[d]	31 days	Basu et al., 1971
Pentobarbital	Rat (SD)	F	20 days	Kato et al., 1964
Strychnine	Rat (SD)	F	20 days	Kato et al., 1964
Zoxazolamine	Pig	M & F	4 weeks	Short and Davis, 1970

Table IV continued

a - Adult activity is the level obtained at 60-120 days in rats.

b - Abbreviations:

SD - Sprague Dawley

W - Wistar

CRLE - Charles River Long Evans

M - Male

F - Female

c - Compared to an adult female.

d - Mixed M and F until 21 days, then M were used.

e - Two peaks were noted. First peak reached near adult level; second peak higher than adult level.

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MANUSCRIPT 1

HEPATIC MICROSOMAL DRUG OXIDATION AND ELECTRON TRANSPORT IN THE NEWBORN INFANT

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ABSTRACT

Many drugs require oxidative metabolism for termination of action and/or for elimination from the body. Many oxidative reactions are catalyzed by hepatic microsomal enzymes. The activities of various drug-metabolizing enzymes, namely, NADPH cytochrome c reductase, NADPH oxidase, aminopyrine-N-demethylase and aniline p-hydroxylase and the content of cytochrome P-450, were measured in hepatic microsomes obtained from seven newborn infants and four adult patients. The results in the newborn infant show increasing activities of these enzymes (except aminopyrine-N-demethylase) related to advancing age. Good correlation between three components of the hepatic microsomal mixed function oxidase system and aniline p-hydroxylase was established, whereas only NADPH oxidation correlated with aminopyrine N-demethylation. The rate of substrate or drug oxidation and the activities of the components of the microsomal electron transport pathway were lower than comparable values in the adult. The data demonstrate a possible biochemical basis for the transient deficiency in drug metabolism seen in newborn infants.

INTRODUCTION

The human fetus and newborn infant are continuously at risk of exposure to drugs and environmental pollutants. Epidemiological studies have demonstrated that approximately 90% of pregnant mothers have consumed

an average of 3 to 10 drugs (Forfar and Nelson, 1973; Hill, 1973; Peckham and King, 1963). The aggressive diagnostic and therapeutic approaches adopted by neonatal intensive care units have also paralleled the increased use of drugs in the newborn infant. Thus it is obviously important to determine the functional state of the various factors involved in the pharmacokinetic disposition of drugs, specifically, the hepatic microsomal drug metabolizing enzymes during the neonatal period.

Biochemical and morphological studies in experimental animals have conclusively shown that the fetal and newborn animals have a deficient capability to metabolize drugs (Fouts and Adamson, 1959; MacLeod et al., 1972). Similarly, the lack of significant metabolism of drugs such as diazepam in the human fetus (Idanpaan-Heikkila et al., 1971) and the higher blood level and prolonged serum half life of diazepam in premature infants relative to older children (Morselli et al., 1973) suggested the possibility of deficient drug oxidative function in the neonatal period. Possible subcellular and biochemical basis for this deficient drug oxidative function in the human aborted fetuses was shown by lower activity of the hepatic microsomal mixed function oxidase system (HMMFO, Figure 1) relative to adult liver microsomes (Yaffe et al., 1970; Pelkonen et al., 1973a). The data available in the literature have been limited to studies on human

aborted fetuses with maximum fetal age of 25 weeks (Yaffe et al., 1970; Pelkonen et al., 1973a; Pelkonen, 1973b; Rane and Ackermann, 1972). As yet, there are no quantitative determinations of the activity of the hepatic microsomal mixed function system in the later part of gestation and in the neonatal period, and the present study sought to answer three questions.

First, what is the activity of the hepatic drug oxidizing enzymes and the electron transport components in the hepatic microsomes of premature and full term newborn infants? Such information accrued from this study may provide quantitative estimates of the functional deficiency in the premature and full term newborn infants.

Second, does the activity of the hepatic microsomal drug metabolizing enzymes increase with age during the later part of gestation and in the neonatal period. This problem is a corollary to the major question in human drug metabolism which seeks to determine the time when the deficient neonatal drug oxidative function attains adult level of activity.

Third, which electron transport component(s) is/are substrate oxidation closely related to? Correlation of the rate of substrate oxidation to the electron transport component has been utilized in experimental animals to identify the rate limiting step in microsomal drug oxidation (Gigon et al., 1968). Possibility of identifying the rate limiting step was also attempted in the human liver microsomes from premature and full term newborn infants. Identification of the rate limiting step in drug metabolism and quantitative determinations of the rates of drug metabolism during the neonatal period might lead to the development of therapeutic regimens which could circumvent

a faulty metabolic step or would at least permit the clinician to promulgate rational principles for neonatal drug therapy.

II. MATERIALS AND METHODS

a. Patients and Specimens

Fresh liver tissues obtained either at postmortem or during surgical procedures were taken from four premature and three full term newborn infants and from four adults for comparison. All newborn infants, except one (LaB - Table I-1) were from the Montreal Children's Hospital and the adult patients (transplant donors) were from the Royal Victoria Hospital. Clinical data on these patients are shown in Tables I-1 and I-2. The infants' gestational ages ranged from 28 to 41 weeks. The postnatal ages ranged from 9 hours to 5 weeks and the birth weights ranged from 725 to 3560 grams. Four of the infants were males and three were females. The adult patients' ages varied from 20 to 60 years. Three were males and one was a female.

In the autopsy cases, the time lag between patient death and excision of hepatic tissue sample varied from 15 minutes to 2 hours. Upon excision, the liver specimen was placed on ice for transport to the laboratory and then it was homogenized in ice-cold 0.1 M phosphate buffer, pH 7.4 using 4 volumes of buffer per unit weight for 30 seconds in a high speed Sorvall Omnimixer. All subsequent procedures were carried out between 0 and 4° C.

The homogenate was centrifuged at 10,000 x g for 10 minutes in a refrigerated centrifuge, and the supernatant was decanted and recentrifuged at 100,000 x g for 60 minutes in a Beckman L3-40 refrigerated ultracentrifuge. The microsomal pellet obtained was resuspended in cold buffer using a glass

homogenizer and a volume of buffer sufficient to give a final protein concentration of 20 to 30 mg/ml.

b. Assays

Protein was determined by the biuret method (Kabat and Mayer, 1967). NADPH oxidase was determined by following the absorbance change of NADPH at 340 nm using the method described by Gillette, Brodie and LaDu (1957). NADPH cytochrome c reductase activity was determined by following the absorbance change of cytochrome c at 550 nm after addition of NADPH as described by Phillips and Langdon (1962). Cytochrome P-450 concentration was determined by the method of Omura and Sato (1964) with a microsomal suspension containing 5 mg of protein per ml. NADPH cytochrome P-450 reductase activity was determined by following the absorbance change at 450 nm when carbon monoxide saturated microsomes were reduced by NADPH as described by Gigon et al. (1968). The reaction was started by the anaerobic addition of 2 umoles of NADPH in 50 ul of phosphate buffer. The activity of aminopyrine-N-demethylase was determined by measuring the production of formaldehyde from aminopyrine as described by Cochin and Axelrod (1959) and the activity of aniline p-hydroxylase was determined by measuring the production of p-aminophenol as described by Kato and Gillette (1965). The reaction mixtures used for these assays in our laboratory have been published elsewhere (MacLeod et al., 1972).

Regression analysis by the method of least squares (Campbell, 1969) was used for correlation. The significance of the correlation was tested using the distribution of t (Ferguson, 1971) and significance was accepted at $p < 0.05$. Because of the variability of the gestational age and postnatal

age between newborn infants, postconceptional age was obtained by taking the sum of both ages for use in the correlation. Due to the heterogeneity of patients and to the presence of apparent changes in the activity of enzymes studied as a function of age, range of values rather than mean values were used in this study.

III. RESULTS

Table 1 summarizes the clinical data on the newborn infants studied. Two infants had respiratory distress syndrome, two infants had apnea, two had hyperbilirubenemia, one had purulent meningitis, one had asphyxia neonatorum with convulsions and subarachnoid hemorrhage, and one had biliary atresia. The sample from the patient with biliary atresia (LM) was a biopsy specimen obtained during an exploratory laparotomy. All infants received antibiotics, two received phenobarbital and diazepam, one received diphenylhydantoin, and one received aminophylline. Table 2 shows the clinical data on the adult patients. All were transplant donors with irreversible brain damage. Two had traumatic liver rupture in addition to the irreversible brain damage.

The yield of microsomal protein and the activity of the components of the hepatic microsomal electron transport in newborn infants and adults are shown in Table 3 a, b. In the newborn infants, the microsomal protein yield ranged from 18.8 to 25.5 mg/g liver. The NADPH oxidase activity ranged from 0.97 to 5.6 nmoles x min^{-1} x mg^{-1} protein. NADPH cytochrome c reductase activity in the newborn infants varied between 9.65 to 87.10 nmoles x min^{-1} x mg^{-1} protein; in comparison, the four adults had values ranging from

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45.40 to 207.60 nmoles x min^{-1} x mg^{-1} protein. The cytochrome P-450 content in the newborns ranged from 0 to 0.095 nmoles x mg^{-1} protein whereas in the adults these values ranged from 0.138 to 0.231 nmoles x mg^{-1} protein. The activity of NADPH cytochrome P-450 reductase was not detectable in three newborns, whereas in two adults the activity was 2.6 and 2.7 nmoles x min^{-1} x mg^{-1} protein.

The specific activities of aminopyrine N-demethylase and aniline p-hydroxylase are shown in Table 4 for infants and adults. Aminopyrine N-demethylase activity ranged from 0.035 to 1.880 and 0.980 to 3.760 nmoles x min^{-1} x mg^{-1} protein for newborn infants and adults, respectively. Aniline p-hydroxylase activity ranged from 0 to 0.330 and 0.070 to 0.950 nmoles x min^{-1} x mg^{-1} protein in newborn infants and adults, respectively.

To determine whether the activity of the HMMFO functionally develops during the age period studied, the components of the electron transport chain and the representative substrate oxidation were correlated with post-conceptional age (gestational + postnatal age). Figures 2, 3 and 4 show a positive correlation between postconceptional age and NADPH oxidase activity ($r = 0.853$), NADPH cytochrome c reductase activity ($r = 0.839$) and cytochrome P-450 content ($r = 0.871$), respectively. When postconceptional age was correlated with the activity of aniline p-hydroxylase and aminopyrine N-demethylase, a positive correlation was found with aniline p-hydroxylase activity ($r = 0.945$) and a poor correlation was found between postconceptional age and aminopyrine N-demethylase ($r = 0.655$), as shown in Figure 5.

In an attempt to gain insight into the possible rate limiting step in the two oxidative reactions studied, the activities of aminopyrine N-demethylase and aniline p-hydroxylase were correlated with the activity of

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NADPH oxidase and NADPH cytochrome c reductase, and the amount of cytochrome P-450 (Figure 6 a, b, c). A positive correlation between aminopyrine N-demethylase activity and NADPH oxidase activity ($r = 0.878$) was obtained. No correlation was found between aminopyrine N-demethylase and NADPH cytochrome c reductase activity ($r = 0.631$) and cytochrome P-450 content ($r = 0.592$). The positive correlation between aminopyrine N-demethylase activity and NADPH oxidase activity was noteworthy since there was a correlation between postconceptional age and the former but not with the latter. Further studies with a greater number of subjects would be needed to evaluate this relationship.

A good correlation was found between aniline p-hydroxylase activity and NADPH oxidase activity ($r = 0.963$), NADPH cytochrome c reductase ($r = 0.792$) and cytochrome P-450 ($r = 0.842$) as shown in Figure 7.

IV. DISCUSSION

The hepatic microsomal mixed function oxidase system catalyzes the biotransformation of hormones [e.g., steroids (Conney and Kuntzman, 1971)], bile acids (Conney and Kuntzman, 1971; Voight et al., 1968), exogenous and endogenous substrates such as fatty acids (Lu and Coon, 1968), drugs and environmental pollutants. The newborn infant has a decreased capability to metabolize certain drugs (Done, 1964; Nyhan, 1961; Rane and Sjöqvist, 1972) and the data presented in this study demonstrate a subcellular biochemical basis for this functional deficit. Except for the demonstration by Soyka (1970) of the presence of NADPH cytochrome c reductase activity and of cytochrome b_5 in hepatic microsomes obtained from a full-term and a pre-

mature infant (33 week gestation) respectively, to our knowledge this study represents the first determination of the various components of the hepatic microsomal mixed function oxidase system in the neonatal period.

Cytochrome P-450, the terminal hemooxygenase in microsomal drug oxidation is present in variable quantity in the liver microsomes from newborn infants, an observation in accord with Yaffe's data from aborted fetuses of 14 to 25 week gestation (1970). This hemoprotein increases with age and at term it appears to approach approximately half of the adult values presented in this paper (see Table 3) and of that measured by Alvarez et al. (1969) and Nelson et al. (1971) (Figure 8). However, this proportionality decreased to one-tenth of adult cytochrome P-450 content if compared with the data by Davies et al. (1973) (Figure 8). NADPH cytochrome c reductase activity appears to approach half of adult activity (see Table 3) in the three newborn babies whose postconceptional ages were more than 40 weeks. These values are equal to or much higher than the adult cytochrome c reductase activity reported by Nelson et al. (1971) (Figure 9). However, when compared to the adult activity determined by Davies et al. (1971) (Figure 9), the infant to adult activity is approximately one-third.

The rate of microsomal NADPH oxidation as expressed by NADPH oxidase activity has not been measured in human liver microsomes. Like cytochrome P-450 and NADPH cytochrome c reductase, the activity increases with advancing fetal and postnatal age. In the full term 5-week-old infant (baby L.M.), the activity was 5.6 nmoles NADPH oxidized per minute per mg protein, which approximates 70% of the activity in adult rats (Aranda et al., 1973).

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The tremendous variability of aminopyrine N-demethylase activity is also in accord with Yaffe's observations (Yaffe et al., 1970) in fetal livers. The lack of correlation between aminopyrine N-demethylase activity and age is further presented in this paper. In contrast, aniline p-hydroxylase activity is linearly related to age in the newborn infants studied. Nonetheless, the activity of enzymes in both substrate oxidations in the three term babies is approximately one half of adult.

Neither NADPH cytochrome c reductase nor the amount of cytochrome P-450 seems to be rate limiting in aminopyrine N-demethylase since no correlation between these electron transport components and N-demethylation of aminopyrine was found. Similar lack of correlation between the hemoprotein and substrate oxidation has been noted in adult patients (Nelson et al., 1971). In experimental animals, there is an increasing evidence that the rate limiting step in microsomal drug oxidation is the reduction of cytochrome P-450 (Gigon et al., 1968; Gillette and Gram, 1969). However, the data in this study do not substantiate this concept since substrate oxidation in the newborns proceeded despite relative lack of measurable NADPH cytochrome P-450 reductase. Both aminopyrine N-demethylation and aniline p-hydroxylation were significantly related to NADPH oxidase activity indicating that perhaps the velocity of microsomal NADPH oxidation may be an acceptable indicator of the overall rate of microsomal drug oxidation. Indeed, Stripp et al. (1972) and Sasame et al. (1973) have shown a stoichiometric relationship between drug oxidation and NADPH oxidation in hepatic microsomes. They found that the stoichiometry approaches a 1:1 relationship for a variety of type I substrates when the metabolism of the substrate-dependent NADPH oxidation is corrected

for carbon monoxide sensitive endogenous NADPH oxidation. Further evaluation of this method in human microsomes would be needed to define its applicability as an indicator of the rate of drug oxidation. In contrast to aminopyrine N-demethylation, aniline p-hydroxylation correlated significantly with NADPH cytochrome c reductase activity and cytochrome P-450 content suggesting that the newborn HMMFO is relatively more capable of metabolizing aniline-like compounds. The difference between these two substrate oxidations during the fetal and neonatal period is puzzling. Aminopyrine which gives a typical type I binding spectrum with cytochrome P-450 in adult human liver (Pelkonen, 1973c) and in experimental animals (Schenkman et al., 1967) produces a type II binding spectrum in the human fetal liver microsomes (Yaffe et al., 1970). Animal studies have revealed that type I compounds are better substrates for adult rat liver microsomes because they drive their own metabolism and they stimulate NADPH cytochrome P-450 reductase activity (Gigon et al., 1968; Gillette and Gram, 1969). However, fundamental mechanisms underlying these differences and the significance of these binding spectra in the hepatic microsomes during the newborn period are poorly understood. The presence of substantial ω-oxidation of laurate and hydroxylation of testosterone, both endogenous compounds giving type I binding spectra (Yaffe et al., 1970), lends support to the existence of an intimate relationship between substrate binding and metabolism. This observation and the reversal of aminopyrine binding spectrum to a type II pattern led to the hypothesis (Yaffe et al., 1970; Rane and Sjöqvist, 1972) that there are endogenous substrates with high affinity (low apparent K_m values) for the binding site of the terminal hemo-oxygenase and which can compete more effectively with exogenous substrates.

The intriguing possibility of a qualitatively different cytochrome P-450 between newborns and adults or differences between species cannot be excluded.

The apparent subcellular distribution of the various components of the HMMFO is different in the fetal livers as opposed to adults because of differences in sedimentation properties of subcellular organelles (Ackermann et al., 1972; Rane and Ackermann, 1972; Chatterjee et al., 1965). Electron microscopic studies revealed that the rough endoplasmic reticulum with sparse smooth endoplasmic reticulum sedimented at lower speeds of centrifugation. The smooth endoplasmic reticulum contains the drug metabolizing enzymes (Holtzman et al., 1968; Claude, 1969), and its scarcity in the fetal period provides a morphological explanation for a functional deficit. The time at which the fetal hepatic subcellular localization of the drug metabolizing enzyme approaches adult patterns is not established. Since the measurements of the drug metabolizing enzymes were done on the microsomal fraction and since the smooth endoplasmic reticulum may have not sedimented optimally in the microsomal fraction, it is possible that the levels of the enzyme activity presented in this paper may be lower than the actual value. Further studies are needed to answer this problem.

The great discrepancy and variability of the levels of the various components of the HMMFO and of aminopyrine N-demethylation and aniline p-hydroxylation in the literature and in this study may represent different interindividual genetic expression (LaDu, 1971; Vessell and Page, 1969), variations in tissue sampling and assay techniques, presence of pathophysiologic states, or drug therapy used prior to sampling. The nature of the pre-existent disease states and drug therapy are probably the most important factors.

Darby et al. (1970), who found very low levels of cytochrome P-450 and microsomal drug oxidation in adult patients who succumbed to cardiovascular failure (e.g., myocardial infarction), suggested that anoxia was largely responsible for this observed decrease in drug metabolism. In support of this hypothesis is Cumming's observation that the rate of metabolism of antipyrine was significantly decreased in patients who had severe chronic pulmonary disease with resultant hypoxemia (Cumming, 1972). Hepatic microsomal drug oxidation is an energy- and oxygen-dependent process (Gillette et al., 1957; Mannering, 1971) and a linear relationship between arterial oxygen tension and rate of drug oxidation has been shown (Cumming and Mannering, 1970). Respiratory distress syndrome, where significant ventilation/perfusion abnormality is a major pathophysiologic derrangement (Strang and MacLeish, 1961; Strang, 1966), is characterized by hypoxemia which could alter the determination of the true value of the microsomal enzyme activity in the babies studied. Hyperbilirubinemia (McLuen and Fouts, 1961), starvation (Kato and Gillette, 1965), and high glucose loads (Lamson et al., 1951; Strother et al., 1971) could also decrease drug metabolism and it is possible that the values in this study may not represent the activity of HMMFO in normal infants of similar age. However, the data provide some information on the activities of the various components of the system in the sick newborns who are most apt to receive drug therapy, and for whom such information would be most applicable.

Biochemical and morphologic studies have conclusively shown that the microsomal drug metabolizing enzymes are exquisitely susceptible to the influence of drugs and environmental pollutants (e.g., insecticides, air

pollutants). Adult patients who received phenobarbital, glutethimide, diphenylhydantoin, rifampicin and other drugs showed remarkable induction of cytochrome P-450, NADPH cytochrome c reductase, aminopyrine N-demethylase and p-nitroanisole N-demethylase activity (Remmer et al., 1973; Black et al., 1973). Similarly, phenobarbital therapy in infants and children resulted in 100 to 150 percent increase in the activity of NADPH cytochrome c reductase from hepatic homogenates parallelled with proliferation of the endoplasmic reticulum in two infants with normal hepatic parenchyma (Thaler et al., 1972). However, this inductive effect could be antagonized by the presence of liver disease (Thaler et al., 1972). One of a set of twins in this study (GR 2) who received aminophylline because of apnea, had a 7-fold greater activity of aminopyrine N-demethylase compared to the other twin (GR 1). To our knowledge, there is no available data concerning the effect of aminophylline on hepatic microsomal drug metabolism. If genetic expression of the drug metabolizing enzyme is equal (Vessell et al., 1971; LaDu, 1971; Vessell and Page, 1971; Alexanderson et al., 1969), the differences in enzyme activity may be contingent on environmental or drug effects.

The two infants who received phenobarbital and diphenylhydantoin (Table 1) represent two of the three highest values of microsomal enzyme activities among the infants studied (Tables III and IV). However, these two infants have a more advanced postconceptional age relative to the other subjects and the present study does not allow the delineation between age-related effect and drug-effect on the microsomal enzymes.

The data in this study demonstrate for the first time that the activity of the hepatic microsomal drug oxidative enzymes and the electron

transport components are present in significant amount in the premature and full-term newborn infant. The data also demonstrate that hepatic microsomal enzyme titres determined in this age group are lower compared to the adult enzyme titres. This study also provides the first evidence that there is a progressive increase in the activity of the hepatic microsomal drug metabolizing enzymes during the last trimester of gestation and in the neonatal period. This study further demonstrates that aniline p-hydroxylation is better correlated to the activity of the microsomal electron transport components as against aminopyrine N-demethylation.

This study emphasizes the need for caution in the use of drugs requiring biotransformation (e.g., phenobarbital, diazepam, diphenylhydantoin) in the neonatal period. It further suggests that the capability to metabolize drugs significantly changes during the neonatal period. This implies a need for constant evaluation of the newborn infants' pharmacodynamic responses in order to achieve the desired therapeutic response. This study further suggests that some drugs administered to the newborn infant may be better metabolized as against other drugs. These observations warrant further studies on the rates of metabolism of individual drugs during the newborn period.

The data in this study provide a continuum in the ontogenesis of this system, and supplement the observations on the activity of the hepatic microsomal mixed function oxidase in early fetal life (Yaffe et al., 1970; Pelkonen and Karki, 1971; Ackermann et al., 1972; Rane and Ackermann, 1972; Pelkonen et al., 1971; Juchau, 1971; Pomp et al., 1969; Pelkonen, 1973; Pelkonen et al., 1973; Rane and Gustafsson, 1973; Rane, 1974).

FIGURE 1: The hepatic microsomal electron transport pathway (Scheme modified from Holtzman et al., 1968 and Estabrook, 1971).

The drug binds to oxidized cytochrome P-450 (the terminal hemoprotein oxygenase) forming a drug - oxidized cytochrome P-450 complex. This complex is then reduced by electron flux with NADPH as the source of reducing equivalents, thus forming a drug - reduced cytochrome P-450 complex. Activation of molecular oxygen then occurs and oneatom forms an oxidized drug and the other undergoes two equivalent reductions for the formation of water.

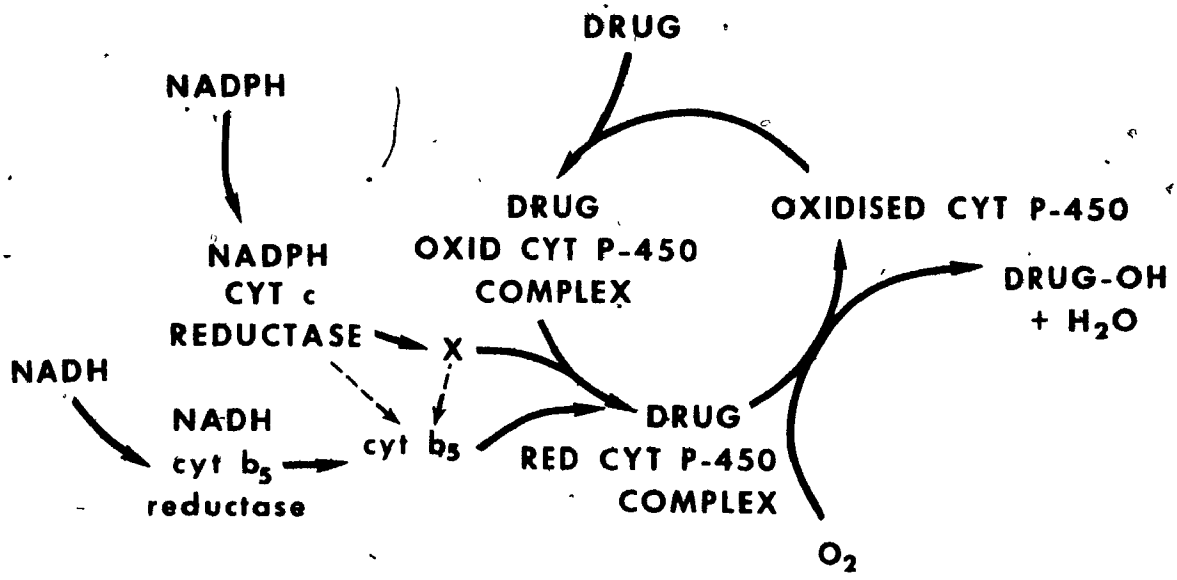

DRUG
NADPH
DRUG
OXIDISED CYT P-450
NADPH
CYT c
REDUCTASE
OXID CYT P-450
COMPLEX
DRUG-OH
+ H_2O
X
NADH
NADH
cyt b_5
reductase
cyt b_5
DRUG
RED CYT P-450
COMPLEX
O_2

FIGURE 2: Correlation between NADPH-oxidase activity and post-conceptional age. Postconceptional age is defined as the sum of gestational age and postnatal age.

SEE* = Standard Error of Estimate.

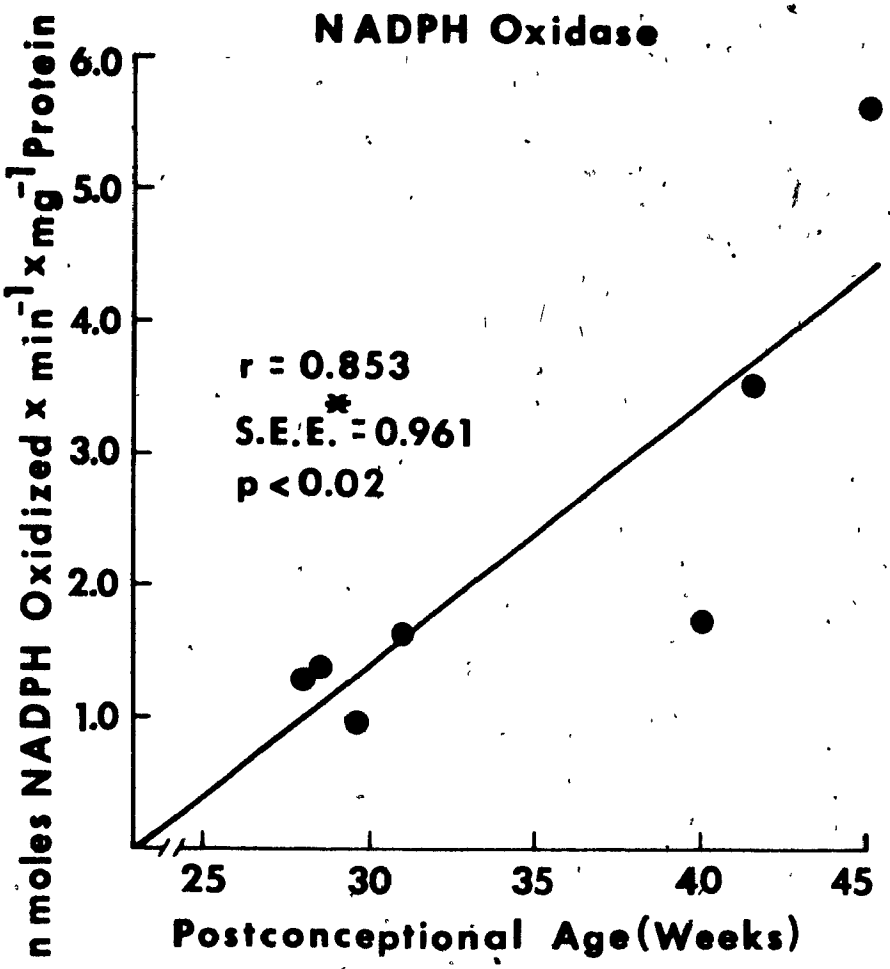

NADPH Oxidase
n moles NADPH Oxidized x min⁻¹ x mg⁻¹ Protein
6.0
5.0
4.0
3.0
2.0
1.0
r = 0.853
S.E.E.* = 0.961
p < 0.02
25
30
35
40
45
Postconceptional Age (Weeks)

FIGURE 3: Correlation between NADPH cytochrome c reductase and post-conceptional age. Post-conceptional age is defined as the sum of gestational age and postnatal age.

SEE* = Standard Error of Estimate.

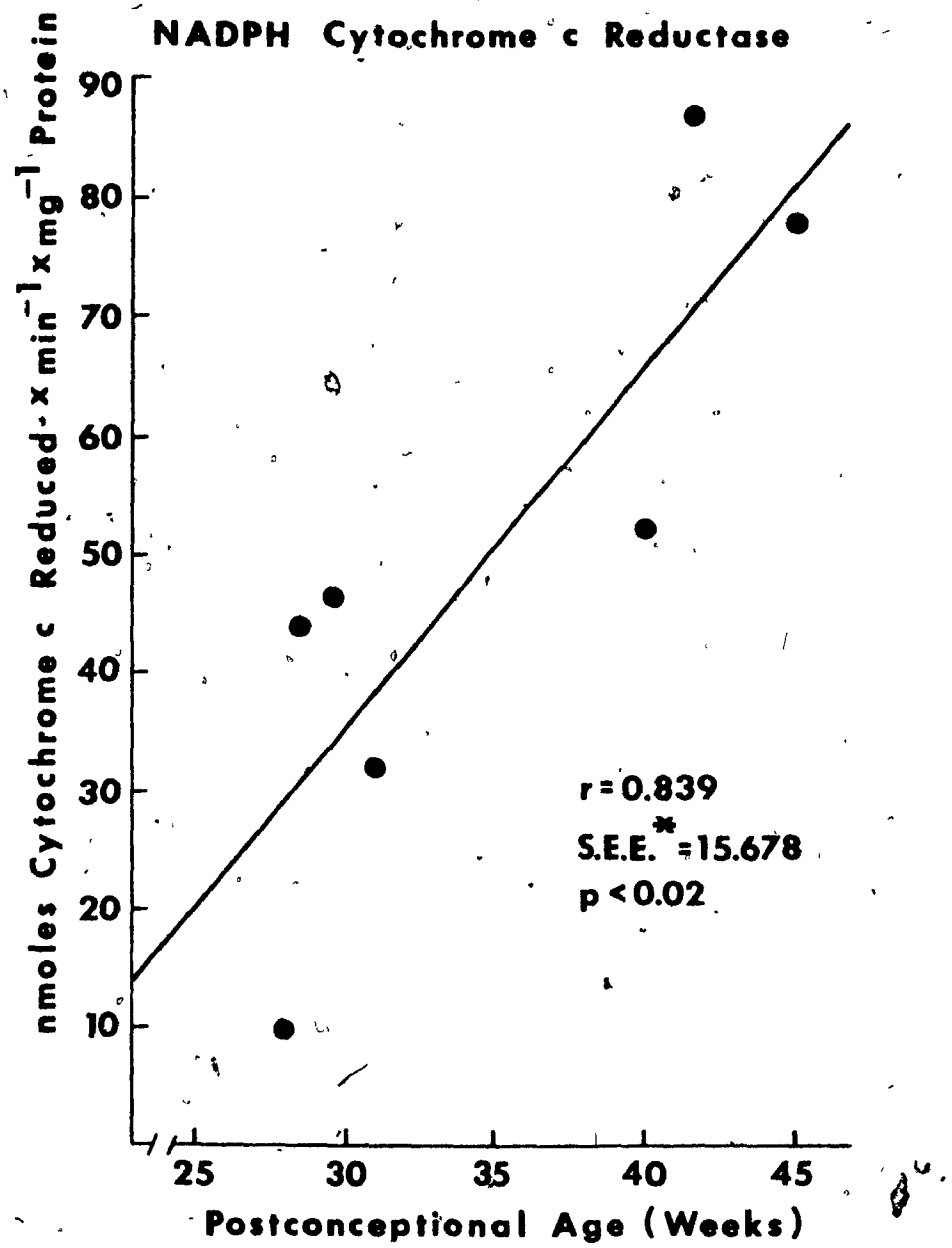

NADPH Cytochrome c Reductase
nmoles Cytochrome c Reduced · x min⁻¹ x mg⁻¹ Protein
90
80
70
60
50
40
30
20
10
25
30
35
40
45
Postconceptional Age (Weeks)
r = 0.839
S.E.E.* = 15.678
p < 0.02

FIGURE 4: Correlation between cytochrome P-450 content and post-conceptional age. Post-conceptional age is defined as the sum of gestational age and postnatal age.

SEE* = Standard Error of Estimate.

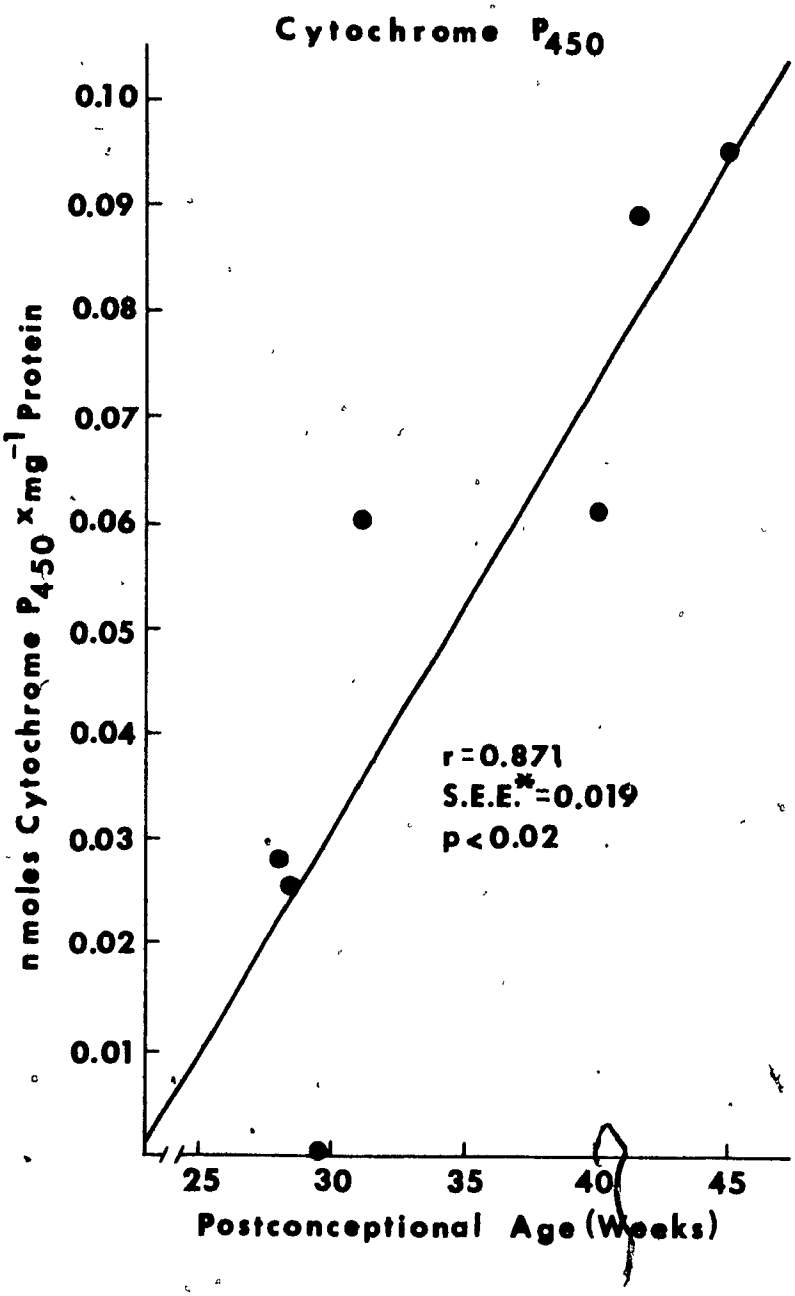

Cytochrome P_{450}
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
n moles Cytochrome P_{450} x mg^{-1} Protein
r=0.871
S.E.*=0.019
p<0.02
25
30
35
40
45
Postconceptional Age (Weeks)

FIGURE 5: Correlation between two substrate oxidations (aminopyrine N-demethylation and aniline p-hydroxylation) and post-conceptional age. Post-conceptional age is defined as the sum of gestational age and postnatal age.

SEE* = Standard Error of Estimate.

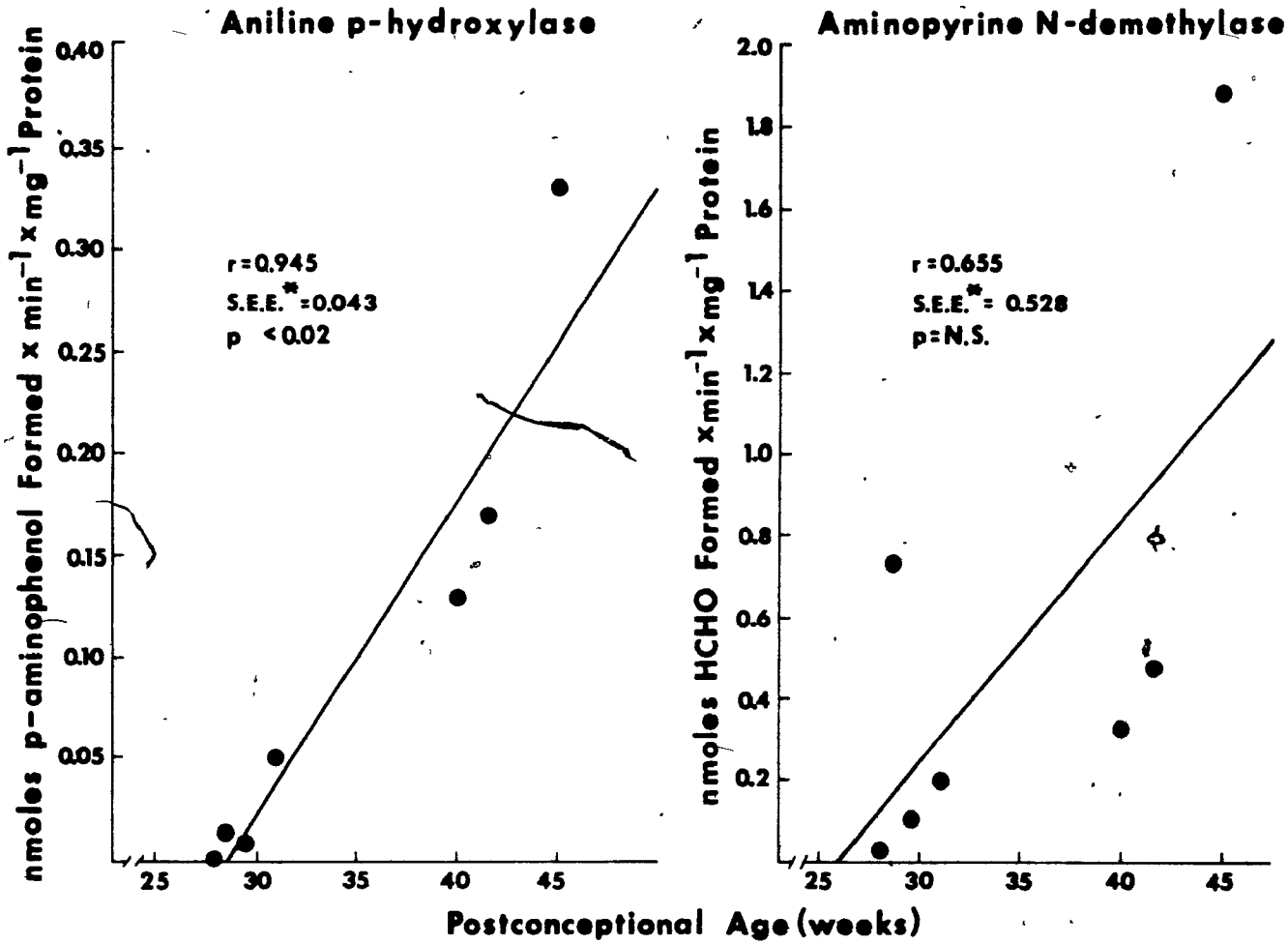
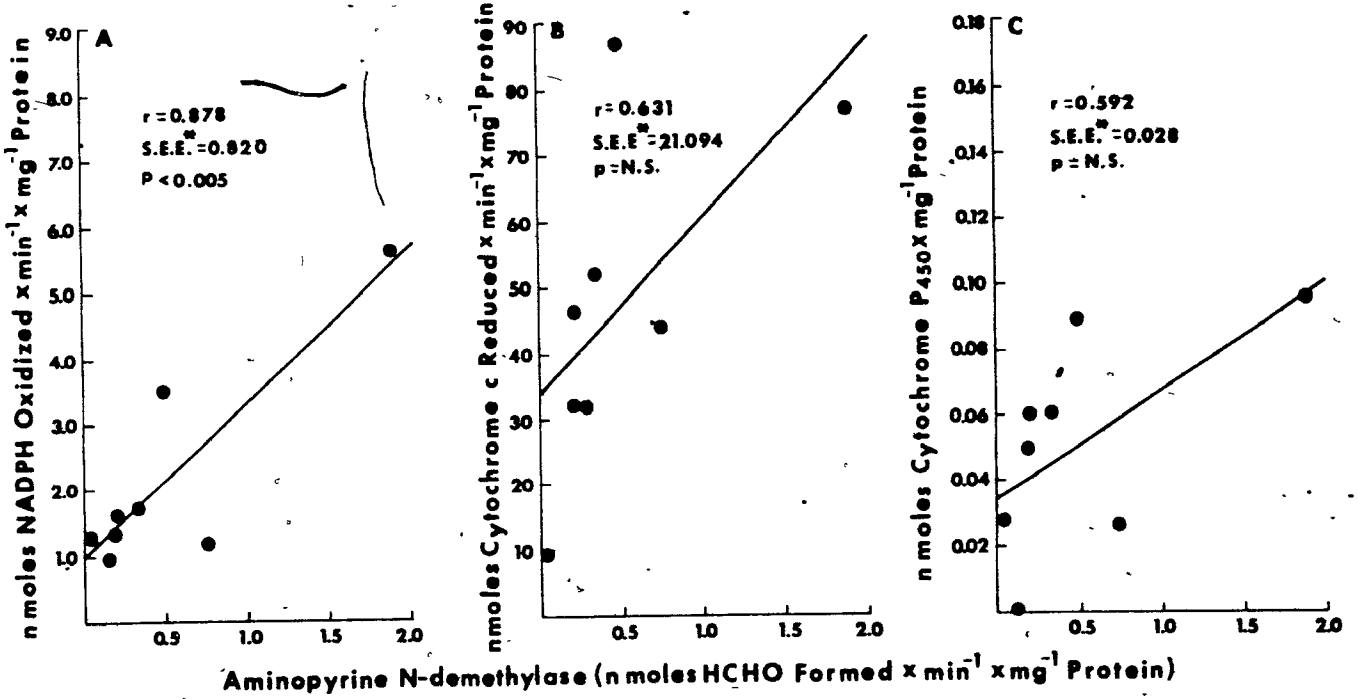

Aniline p-hydroxylase
nmoles p-aminophenol Formed x min⁻¹ x mg⁻¹ Protein
r = 0.945
S.E.E.* = 0.043
p < 0.02
Aminopyrine N-demethylase
nmoles HCHO Formed x min⁻¹ x mg⁻¹ Protein
r = 0.655
S.E.E.* = 0.528
p = N.S.
Postconceptional Age (weeks)

FIGURE 6: Correlation between aminopyrine N-demethylation (abscissa) and NADPH oxidase activity, NADPH cytochrome c reductase activity and cytochrome P-450 content.

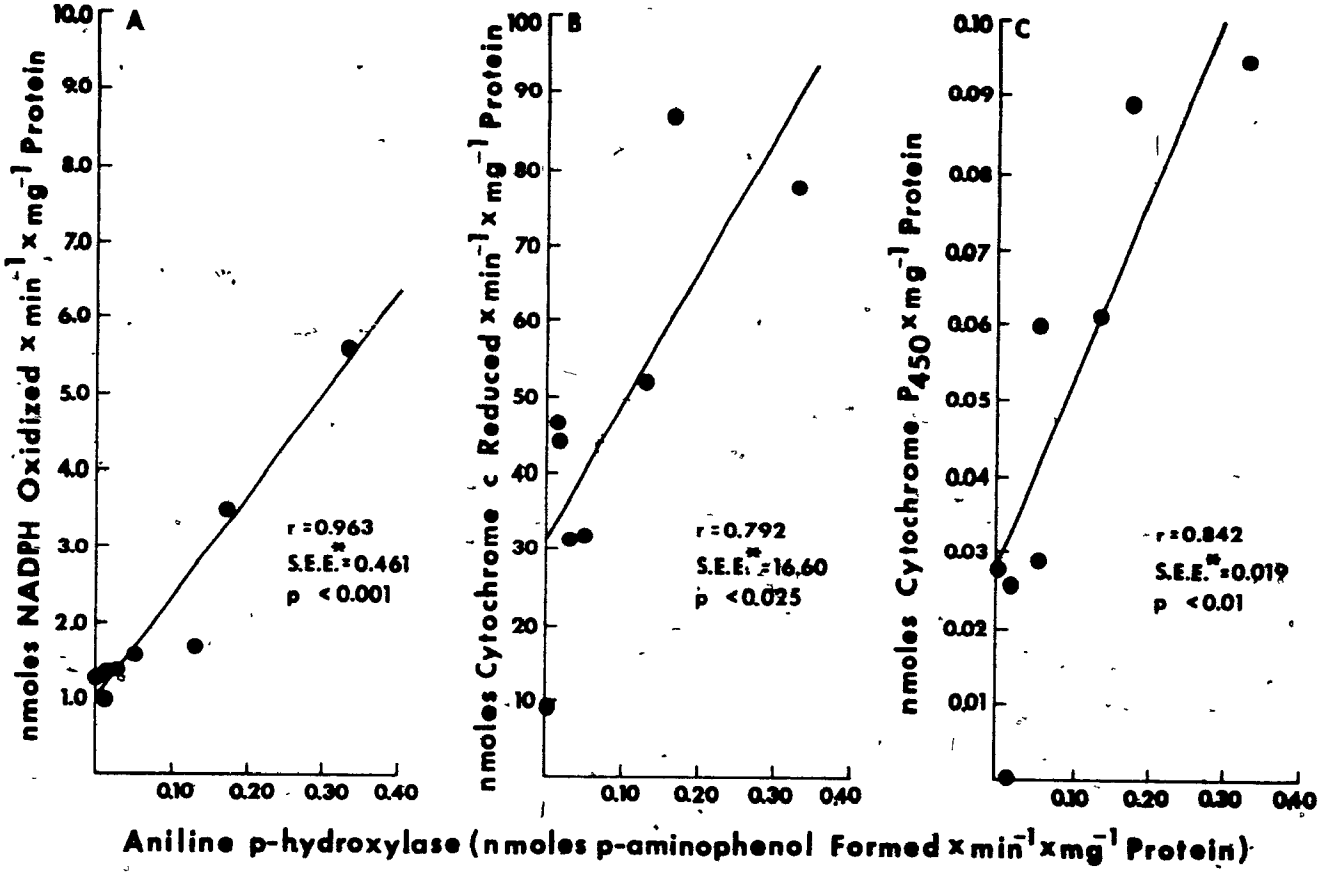
SEE* = Standard Error of Estimate.


A
r = 0.878
S.E.E.* = 0.820
P < 0.005
n moles NADPH Oxidized x min⁻¹ x mg⁻¹ Protein
B
r = 0.631
S.E.E.* = 21.094
p = N.S.
nmoles Cytochrome c Reduced x min⁻¹ x mg⁻¹ Protein
C
r = 0.592
S.E.E.* = 0.028
p = N.S.
n moles Cytochrome P450 x mg⁻¹ Protein
Aminopyrine N-demethylase (n moles HCHO Formed x min⁻¹ x mg⁻¹ Protein)

MS.I

FIGURE 7: Correlation between aniline p-hydroxylation (abscissa) and NADPH oxidase activity, NADPH cytochrome c reductase activity and cytochrome P-450 content.

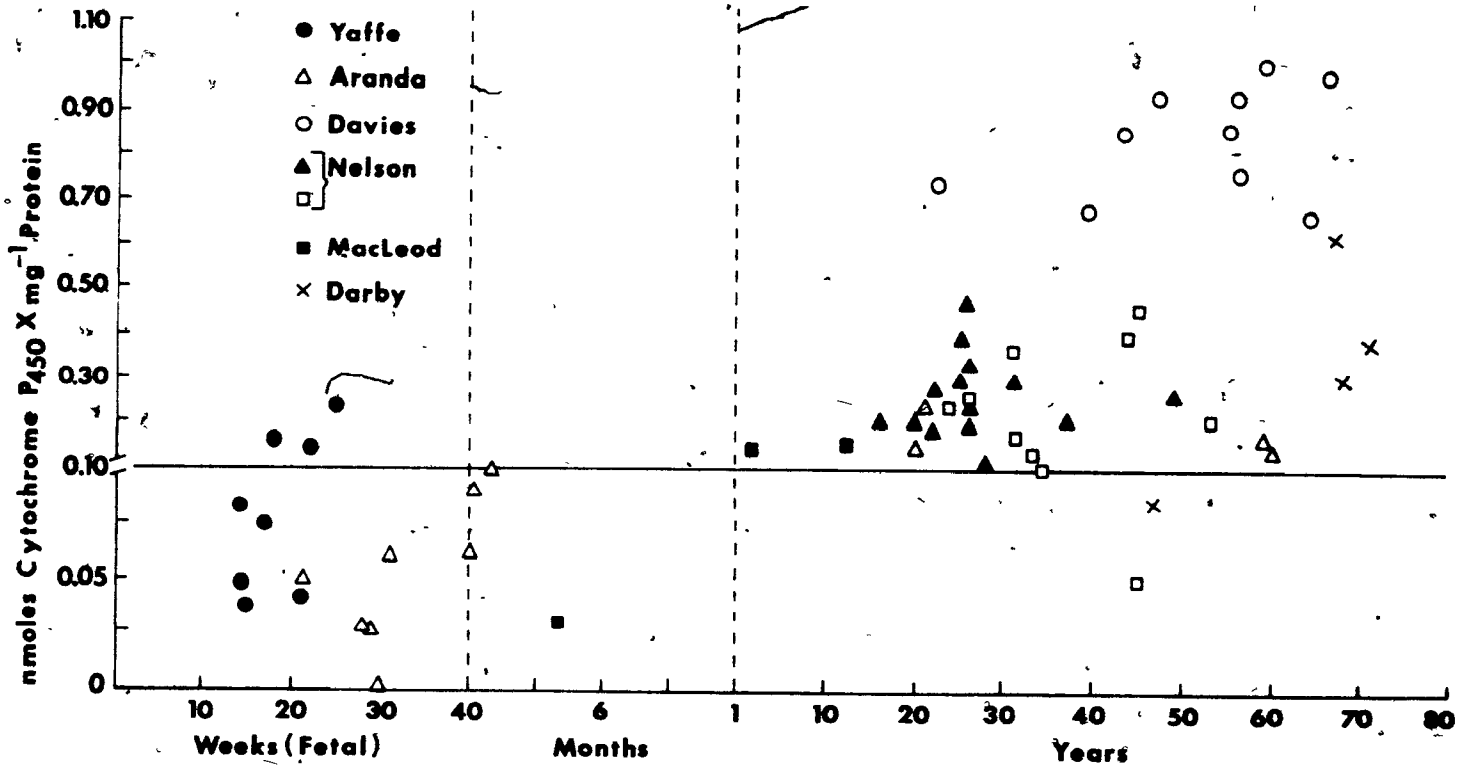
SEE* = Standard Error of Estimate.


A
nmoles NADPH Oxidized x min⁻¹ x mg⁻¹ Protein
r = 0.963
S.E.E.* = 0.461
p < 0.001
B
nmoles Cytochrome c Reduced x min⁻¹ x mg⁻¹ Protein
r = 0.792
S.E.E.* = 16,60
p < 0.025
C
nmoles Cytochrome P450 x mg⁻¹ Protein
r = 0.842
S.E.E.* = 0.019
p < 0.01
Aniline p-hydroxylase (nmoles p-aminophenol Formed x min⁻¹ x mg⁻¹ Protein)

MS.I

FIGURE 8: Comparison of cytochrome P-450 content as determined in various laboratories.

Note the discontinuity in the time scale (abscissa) and in the content of cytochrome P-450 (ordinate). Also note the lack of data during the first decade of life.


Yaffe
Aranda
Davies
Nelson
MacLeod
Darby
nmoles Cytochrome P_{450} X mg^{-1} Protein
1.10
0.90
0.70
0.50
0.30
0.10
0.05
0
10
20
30
40
6
1
10
20
30
40
50
60
70
80
Weeks (Fetal)
Months
Years

MS. I

FIGURE 9: Comparison of the activity of NADPH cytochrome c reductase, a flavoprotein electron acceptor, as determined in various laboratories. Note the discontinuity of the time scale (abscissa) and the lack of data during the first decade of life.

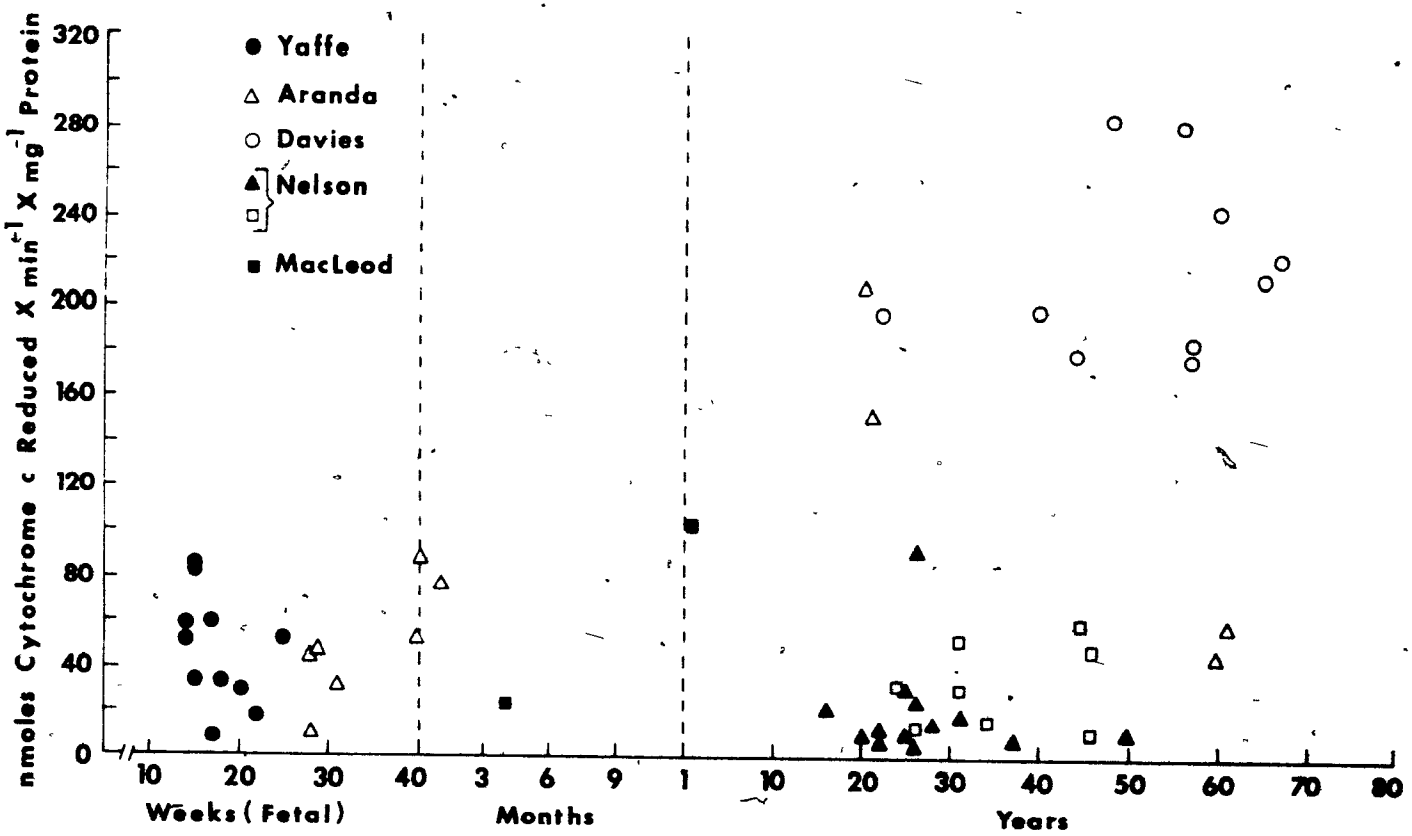

nmoles Cytochrome c Reduced X min⁻¹ X mg⁻¹ Protein
320
280
240
200
160
120
80
40
0
Yaffe
Aranda
Davies
Nelson
MacLeod
10
20
30
40
3
6
9
1
10
20
30
40
50
60
70
80
Weeks (Fetal)
Months
Years

TABLE I: CLINICAL DATA ON NEWBORN INFANTS

Patients	Sex	Birth Weight (grams)	Gestational age (weeks)	Postnatal age	Diagnosis	Drugs Received
POO	Male	936	28	1 day	Respiratory distress syndrome	Pen+, Kana†
GR1	Female	725	28	3 days	Apnea, hyperglycemia	Pen+, Kana†,
GR2	Female	760	28	12 days	Apnea, anemia, hyperbilirubinemia, respiratory failure	Intralipids, i.v. aminoacids, Pen+, Kana† aminophylline
LAV2	Male	1320	31	9 hours	Respiratory distress syndrome, severe pneumothorax	Pen+, Kana†
LaB	Female	3190	39	8 days	Purulent meningitis, cerebral infarct, multicystic kidneys	Pen+, Kana†, Diazepam, Phenobarbital
HUD	Male	3560	41	4 days	Asphyxia, seizures, pneumonia, subarachnoid hemorhage	Phenobarbital, Diphenylhydantoin, Diazepam, Pen+, Kana†
LM	Female	3290	40	5 weeks	Biliary atresia, hyperbilirubinemia (biopsy)	Ampicillin, Kana†, Gentamycin, Cloxacillin, Vit. K., Acetominophen

Pen+ = Penicillin, Kana† = Kanamycin

MS.1

TABLE 2 : CLINICAL DATA ON ADULT PATIENTS (TRANSPLANT DONORS)

Patient	Sex	Age (years)	Diagnosis
JB	Male	20	Brain death (biopsy)
GG	Male	21	Liver rupture
JM	Male	59	Liver rupture (biopsy)
MD	Female	60	Brain death (biopsy)

MS.1

TABLE 3 : HEPATIC MICROSOMAL ELECTRON TRANSPORT COMPONENTS IN NEWBORN, INFANTS (A) AND ADULTS (B)

A. Newborn Infants

Patient	Postconceptional age (wks)	Microsomal protein yield (mg/g liver)	NADPH oxidase nmoles/min/mg protein	NADPH cyt† c reductase nmoles/min/mg protein	Cyt† P-450 nmoles/mg protein	NADPH cyt† P-450 reductase
POO	28	18.8	1.29	9.65	0.028	QND*
GR2	28.4	25.5	1.32	44.16	0.026	QND*
GR1	29.7	22.8	0.99	46.43	0	QND*
LAV	31	19.2	1.61	32.00	0.060	QND*
LAB	40	ND**	1.70	51.90	0.061	ND**
HUD	41.6	ND**	3.50	87.10	0.089	ND**
LM	45	ND**	5.60	77.80	0.095	ND**
Range	28-45	18.8-25.5	0.97-3.5	9.65-87.10	0-0.095	-

B. Adults

Patient	Age (years)	NADPH cyt† c reductase nmoles/min/mg protein	Cyt† P-450 nmoles/mg protein	NADPH cyt P-450 reductase nmoles/min/mg protein
JB	20	207.6	0.143	2.7
GG	21	150.7	0.231	2.6
JM	59	45.4	0.179	ND**
MD	60	58.4	0.138	ND**
Range	20-60	45.4-207.6	0.138-0.231	2.6-2.7

*QND = Activity not detectable by method

**ND = Not determined

†cyt = Cytochrome

MS.1

TABLE 4 : HEPATIC MICROSOMAL SUBSTRATE OXIDATIVE ENZYMES IN NEWBORN INFANTS (A) AND ADULTS (B)

	Patient	Postconceptional age (weeks)	Aminopyrine N-demethylase (nmoles HCHO formed/min/mg protein)	Aniline p-hydroxylase (nmoles p-aminophenol formed/min/mg protein
A. Infants	POO	28	0.035	0
	GR2	28.4	0.734	0.013
	GR1	29.7	0.105	0.010
	LAV	31	0.200	0.050
	LAB	40	0.330	0.130
	HUD	41.6	0.480	0.170
	LM	45	1.880	0.330
	Range	28-45	0.035-1.880	0-0.330
		(years)		
B. Adults	KB	20	3.76	0.95
	GG	21	2.72	0.66
	JM	59	1.16	0.07
	MD	60	0.98	0.22
	Range	20-60	0.98-3.76	0.07-0.95

MS.1

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MANUSCRIPT II

EFFECT OF THYROID HORMONE ON POSTNATAL DEVELOPMENT OF HEPATIC MICROSOMAL DRUG OXIDATION AND ASSOCIATED ELECTRON TRANSPORT

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ABSTRACT

The effect of thyroid hormone on the postnatal development of the hepatic microsomal mixed function oxidase system was studied. A single subcutaneous injection of L-thyroxin, 1 ug/g body weight, given to immature (one week old) male rats, resulted in an increased activity of hepatic microsomal aniline p-hydroxylase, NADPH oxidase and NADPH cytochrome c reductase, and in an increased amount of cytochrome P-450 and microsomal protein, compared to an age-matched non-thyroxin-treated group. Repetitive subcutaneous injections using L-thyroxin, 1 ug/g daily from birth to 5 weeks of age administered to male rats resulted in significant acceleration of the developement of activity of NADPH oxidase, NADPH cytochrome c reductase and aniline p-hydroxylase and in significant repression of the development of cytochrome P-450 and of aminopyrine N-demethylation initially. Male rats thyroidectomized with ^{131}I at birth showed effects opposite to the rats with chronic hyperthyroid state, namely, decreased NADPH cytochrome c reductase activity and increased cytochrome P-450 content at 3 and 5 weeks. These effects were reversed with thyroxin replacement therapy. The effect of a transient neonatal hyperthyroid state on the development of the mixed function oxidase was also determined. Male rats were treated with T_4, 20 ug/g/day (neonatal treatment group) for 5 consecutive days starting at birth. The results suggest that

thyroxin evoked the differentiation of aniline p-hydroxylase and the induction of NADPH cytochrome c reductase. Additional thyroxin injections to the neonatally treated rat at 3 and 5 weeks of age resulted in a decreased aminopyrine N-demethylation at 3 weeks but not at 5 weeks, and a superinduction (i.e. induction of enzyme activity surpassing the normal adult level) of NADPH cytochrome c reductase at 5 weeks but not at 3 weeks suggesting that the quantitative and qualitative response to thyroxin is markedly influenced by age at which thyroxin is administered. The data in this study emphasize the vulnerability of the developmental profile of the microsomal drug metabolizing enzymes to hormonal changes and demonstrate an influence of the thyroid hormone on the ontogenesis of the hepatic microsomal mixed function oxidase system. The data also appear to indicate that the effects of thyroid hormone may be selective to a particular electron transport component, selective to a specific oxidative pathway and may be dependent upon duration and degree of thyroid dysfunction.

INTRODUCTION

An important aspect in the comprehension of the process underlying enzymic differentiation and the ontogenesis of the hepatic microsomal mixed function oxidase system is the delineation of the various effects of hormones on the activity of the mixed function oxidase. Wilson (1970) has shown that growth hormone has a repressive role in the development of ethylmorphine N-demethylation, and aminopyrine N-demethylation. The influence of other growth promoting hormones such as L-thyroxin has not been studied. The possibility that thyroid hormone could act as a physiologic trigger for the differentiation of this enzyme system in rats (Greengard and Dewey, 1968; Greengard, 1969) is suggested by the simultaneous appearance of hepatic NADPH cytochrome c reductase and glucose-6-phosphatase and a functional thyroid gland on the 18th fetal day in rats and the induction of these enzymes by thyroxin. In man, a similar simultaneous appearance of the hepatic microsomal mixed function oxidase activity and measurable thyroxin and thyronines seems to occur during the 10th to 14th week in the human fetus (Pelkonen, 1973; Pelkonen et al., 1973b; Greenberg et al., 1970; Shepard, 1967; Fisher et al., 1970).

The thyroid gland is vital to the normal development of organ systems (Balazs et al., 1969) and to the regulation of several metabolic homeostatic mechanisms. Its inductive role in the development of the lung and pulmonary surfactant has been described (Redding et al., 1972; Wu et al., 1973), and so has its influence on hepatic microsomal drug metabolism in experimental animals (Kato and Gillette, 1965b; Kato and Takahashi, 1968) and in humans (Vessell and Passananitis, 1973; Crooks et al., 1973; Eichelbaum et al., 1974).

Since thyroid hormone influences drug metabolism and organ development, it seemed probable therefore that thyroid hormone could significantly influence the development of the hepatic microsomal mixed function oxidase system. This study was done to test this hypothesis. The experiments described herein examined the influence of chronic hyperthyroid state, transient neonatal hyperthyroid state and hypothyroid state on the postnatal developmental pattern of the components of the hepatic microsomal electron transport pathway and the specific activity of hepatic microsomal drug-oxidative-enzymes catalyzing N-demethylation of aminopyrine (a type I substrate) and p-hydroxylation of aniline (a type II substrate).

MATERIALS AND METHODS

Male Sprague-Dawley rats obtained from Canadian Breeding Farms (St. Constant, Quebec) were used in this study. Adult male rats were at least 10 weeks of age and weighed between 300-340 grams. Female adult rats were bred at the Canadian Breeding Farms and were delivered to the laboratory on the day of parturition at which time the male litters were segregated with their mothers and were used for the experiments. Mothers and litters were maintained at the McGill University McIntyre Animal Centre and in all cases the litters were weaned at 21 days of age. The diet for the nursing mothers and for the weaned litters consisted of Purina Lab rat chow and water ad libitum.

In the experiments on chronic hyperthyroid state, immature rats were given a daily subcutaneous injection of L-thyroxin (Sigma Laboratories, St. Louis, Missouri) at a dose of 1 ug/g body weight starting at birth. L-Thyroxin

was dissolved in 0.01 N NaOH and the concentration was adjusted so that in all experiments the volume injected ranged from 0.05 ml to 0.1 ml to rats from birth to 3 weeks of age, 0.1 ml to 0.3 ml from 3 weeks to 5 weeks of age, and 0.5 ml in adults. Initial experiments in our laboratory showed no effect on the parameters studied using subcutaneously administered 0.01 NaOH at the volumes injected and therefore the control values were obtained from untreated rats of corresponding ages, matched with the thyroxin treated group. These untreated and thyroxin treated rats (1 ug/g body weight daily) were sequentially killed at ages 3 days, 1, 3, and 5 weeks.

Neonatal hyperthyroid state was produced by daily subcutaneous injection (at the nape of the neck) of L-thyroxin (100 ug/day) for the first 5 days of postnatal life. Treatment was started within 18 hours from birth. To test whether thyroxin administration during the neonatal period influences the effect of thyroxin at an older age, additional thyroxin doses (booster dose) of 1 ug/g body weight/day for three days were administered prior to death at age 3 weeks and 5 weeks.

Hypothyroidism was produced by radiothyroidectomy employing radioactive ^{131}I (New England Nuclear, Boston, Mass.) as described by Goldberg (1949). One hundred uC of ^{131}I was subcutaneously administered to newborn rats within 18 hours from birth. These rats were killed at 3 and 5 weeks of age. Replacement therapy using L-thyroxin 1 ug/g body weight daily for 7 days was injected subcutaneously to a group of hypothyroid rats prior to death at age 3 and 5 weeks.

In all experiments on the immature animals, one or more pups were randomly selected from different litters of same treatment group so that at any given age the mean value was derived from a number of individual deter-

minations. Each determination performed in the newborns represents a single value derived from the livers of four littermates pooled prior to homogenization. At one and two weeks of age, livers from three to four littermates were pooled and thereafter single livers were used.

Animals of desired age were sacrificed by decapitation followed by exsanguination. Livers were immediately excised and homogenized for 30 seconds in a high speed Sorvall Omni-Mixer (Sorval Co., Conn.) in ice cold 0.1 M phosphate buffer, pH 7.4 using 4 volumes per unit weight. All subsequent procedures were carried out between 0° C and 4° C.

The homogenate was centrifuged at 10,000 x g for 10 minutes in a refrigerated centrifuge. The 10,000 x g supernatant was carefully removed avoiding contamination by the loosely packed pellet at the bottom and the fatty layer at the top of the centrifuge tube. The 10,000 x g supernatant was centrifuged at 100,000 x g for 60 minutes in a Beckman L3-40 refrigerated ultracentrifuge. The resulting microsomal pellet obtained was separated from its glycogen layer and resuspended in ice-cold buffer using a glass homogenizer with a teflon pestle (Arthur H. Thomas Co., Philadelphia, Pa.), and using a volume of buffer sufficient to provide a final protein concentration of 30 to 40 mg/ml. Protein concentration of the suspension was determined by the biuret method (Kabat and Mayer, 1967).

NADPH oxidase activity was determined by following the absorbance change of NADPH at 340 nm by the method described by Gillette, Brodie and LaDu (1957). NADPH cytochrome c reductase activity was determined by following the absorbance change at 550 nm reflecting the appearance of reduced cytochrome c after addition of NADPH and cytochrome c to the microsomal preparation, as described by Phillips and Langdon (1962). Cyto-

chrome P-450 concentration was determined by the method of Omura and Sato (1964) with a microsomal suspension containing 5 mg of protein per ml.

The activity of aminopyrine N-demethylase was determined by measuring the production of formaldehyde from aminopyrine as described by Cochin and Axelrod (1959). The reaction mixture contained 0.1 ml of microsomal suspension, 36 umol of $MgCl_2$, 24 umol of neutralized semicarbazide HCl, 40 umol of nicotinamide, 0.66 umol of $NADP^+$, 16 umol of sodium isocitrate, 0.5 unit of isocitric dehydrogenase and 10 umol of aminopyrine in a final volume of 2.0 ml of 0.1 M phosphate buffer (pH 7.4). Nicotinamide has been reported to inhibit the microsomal N-demethylation of aminopyrine (Schenkman et al., 1967) but in our experiments addition of amounts up to 100 umol had no such effect.

The activity of aniline p-hydroxylase was determined using the above reaction mixture minus semicarbazide and with 10 umol of aniline substituted for aminopyrine. The production of p-aminophenol was measured as described by Kato and Gillette (1965a). In all assays of aniline p-hydroxylase and aminopyrine N-demethylase activity, blank tubes were prepared simultaneously with each assay.

Serum thyroxin levels were measured by competitive protein binding as described by Murphy and Pattee (1966).

Student's t test for unpaired data was used for statistical analysis. A p value of < 0.05 was considered significant.

RESULTS

Effect of acute thyroxin treatment in immature rats

To determine the effect of acute thyroxin treatment, groups of 6-day-old male suckling rats were given a single dose of thyroxin at a dose of 1 ug/g. Twenty-four hours later, determination of the electron transport components of the hepatic microsomal mixed function oxidase system and two representative substrate oxidations were performed. The results, expressed as percentage of adult activity, are shown in Fig. 1. Compared to the non-L-thyroxin treated control group, there was a significant increase in the activity of NADPH oxidase, NADPH cytochrome c reductase, aniline p-hydroxylase and in the content of cytochrome P-450 and yield of microsomal protein per gram of liver. A remarkable increase to adult levels in NADPH oxidase activity and aniline p-hydroxylation was noted. In contrast, no effect on aminopyrine N-demethylation was observed.

Influence of chronic thyroxin treatment on the ontogenesis of hepatic microsomal drug oxidation

To determine the effect of a sustained hyperthyroid state on the postnatal development of the hepatic mixed function oxidase system, thyroxin, 1.0 ug/g body weight was administered subcutaneously to male rats daily starting from the first day of life. Groups of these treated rats were sacrificed at age 3 days, 1 week, 3 weeks and 5 weeks, and the activity of the hepatic mixed function oxidase system was measured. The results with respect to liver weight, microsomal protein yield, and total microsomal

protein per liver are shown in Table 1. There was a decrease in liver weight at age 3 weeks which returned to control value at age 5 weeks. There was a significant increase in the microsomal protein yield per gram of liver which again returned to comparable control value at age 5 weeks. However, when the total microsomal protein per liver was calculated by taking the product of the microsomal protein yield per gram of liver and the liver wet weight, a decrease in the total microsomal protein was noted at three and five weeks. No effect was seen at age 1 week.

The effect of a continuous hyperthyroid state of NADPH oxidase is shown in Fig. 2. Compared to the normal developmental pattern of NADPH oxidase activity, the pattern in the thyroxin treated group showed a significant augmentation which was observed starting from 3 days postnatally. The activity surpassed adult levels at age 3 weeks and returned to adult levels by age 5 weeks.

The effect of sustained hyperthyroidism on NADPH cytochrome c reductase is shown in Fig. 3. In the normal non-thyroxin treated group, adult level of activity was attained at 5 weeks. In contrast, the thyroxin treated group reached adult level of NADPH cytochrome c reductase within one week. The inductive effect of thyroxin on this flavoprotein was noted at age 3 days, followed by a plateau at adult level of activity from age 1 week to 5 weeks, thereafter.

The effect of continuous thyroxin treatment on the developmental pattern of cytochrome P-450 is shown in Fig. 4. In the normal rat, a biphasic development was noted, with an initial rapid rise during the first week followed by a plateau until 3 weeks. A second rapid rise was noted after 3 weeks and approached adult level of activity at 4 to 5 weeks. In

contrast, a considerable lag in the development of cytochrome P-450 was noted in the thyroxin treated rats starting at age 3 days. In one week, a significantly lower cytochrome P-450 content was observed relative to the age matched control group. However, some degree of compensation must have occurred since the hemoprotein content increased to an equivalent value compared to that of the control group at age 3 weeks. But whereas the normal rats showed an increase to near adult levels of cytochrome P-450 after 3 weeks, the thyroxin treated rats showed an arrest in the development at this level so that by age 5 weeks the cytochrome P-450 content was equivalent to that at age 3 weeks.

The influence of sustained hyperthyroidism on the development of aminopyrine N-demethylase and aniline p-hydroxylase is shown in Fig. 5 and Fig. 6, respectively. A significantly lower activity of aminopyrine N-demethylase was noted at first week which rose to control value at 3 and 5 weeks. In contrast to aminopyrine N-demethylase activity, aniline p-hydroxylase activity was significantly increased in the thyroxin treated group compared to the untreated control group. This increase was noted as early as 3 days postnatal age and at 1 week the activity of aniline p-hydroxylase was higher than normal adult activity, and appeared to reach plateau up to age 5 weeks.

Influence of neonatal thyroxin therapy with or without booster thyroxin dose on the development of the microsomal mixed function oxidase system

To determine the influence of a transient hyperthyroid state shortly after birth, rats were treated subcutaneously with thyroxin 20 ug/g/day for

the first 5 days of life. This was the maximum thyroxin dose that was tolerated by the animals and which produced significant hyperthyroidism during the immediate newborn period. To determine whether neonatal treatment would alter the magnitude of response to thyroxin during the 3rd and 5th week of life prior to full maturation of the system, the rats treated with thyroxin during the neonatal period were given an additional or booster dose of thyroxin 1 ug/g/day for 3 days prior to sacrifice. The booster dose was given to determine whether an earlier episode of hyperthyroid state would alter the response to a dose of thyroxin at a later age. The results of this treatment are summarized in Tables 2 and 3. The liver weights of the neonatally treated rats were significantly lower at 5 weeks. This decrease in the liver weight was slightly potentiated by the booster thyroxin dose. An increase in the microsomal protein yield per gram liver was observed during the first three weeks and in the group given a booster dose at 5 weeks of age. A decrease in the activity of aminopyrine N-demethylase was noted at age 1 week, which rose to control values at age 3 and 5 weeks (Table 2). Booster dose of thyroxin at 3 weeks reduced the activity of aminopyrine N-demethylase at 3 weeks but did not do so at 5 weeks of age indicating some possible age related influence of thyroxin effect. In contrast to aminopyrine N-demethylase, aniline p-hydroxylase was induced to levels equal to or above adult levels during the first 3 weeks of life in the neonatally treated froup (Table 2). The activity fell below age matched control at 5 weeks but this was restored to the age matched control value with the thyroxin booster dose. No significant effect was noted with NADPH oxidase activity. On the other hand, a significant induction of NADPH cytochrome c reductase was obtained with the neonatally treated

group at age one week which interestingly fell below age matched control level at 3 and 5 weeks of age. Similar to aniline p-hydroxylase this fall in the activity of NADPH cytochrome c reductase was reversed by thyroxin booster dose (Table 3). In fact, an activity greater than the age matched control was obtained at 5 weeks, a finding not in accord with that observed at 3 weeks. This again suggests an age related influence of thyroxin effect on hepatic microsomal drug oxidation. The hemoprotein monooxygenase cytochrome P-450 was significantly decreased at age one week in the neonatally treated rats (Table 3). However, an increase was noted at age 3 weeks followed by a return to age matched control value at age 5 weeks.

Influence of hypothyroidism on the development of the hepatic mixed function oxidase activity

Rats were thyroidectomized with a single dose of 100 uC of ^{131}I at birth. One group received replacement therapy using daily administered L-thyroxin 1 ug/g body weight subcutaneously for seven days prior to death at 3 and 5 weeks of age. These procedures resulted in effects as shown in Table 4. There was a significant decrease in the liver wet weight of hypothyroid rats at 3 and 5 weeks of age. Replacement therapy with thyroxin produced a slight increase in liver weight over the non-treated hypothyroid group; however, this did not result in a complete recovery to control values. The hypothyroid rats were myxedematous and their livers were pale and edematous. Replacement therapy with thyroxin resulted in weight loss secondary to correction of edema (data not shown) and the livers of this thyroxin treated group became reddish and firm. The microsomal yield per gram of liver

was significantly increased in the hypothyroid group with or without thyroxin replacement in both 3 and 5 week old rats. However, the total liver microsomal protein per rat, obtained as the product of the microsomal protein yield per mg protein and the liver wet weight, was much lower compared to the control group. The activity of aminopyrine N-demethylase was significantly lower in the 3 week old hypothyroid rat which was not corrected by one week replacement therapy with thyroxin (Table 4). This may be due to the fact that thyroxin per se can decrease aminopyrine N-demethylase activity in the male rat. Some compensatory mechanisms may have occurred on prolonged hypothyroidism so that at 5 weeks of age the hypothyroid rat with or without thyroxin replacement therapy showed equiactivity with the age matched control group. In contrast to aminopyrine N-demethylase, aniline p-hydroxylase activity was significantly increased in the 3 week old hypothyroid rat which was further increased by replacement therapy with thyroxin (Table 4). Again, compensation must have occurred, hence an equiactivity in aniline p-hydroxylase between the control group and the 5 week old hypothyroid group was obtained. Replacement therapy with thyroxin significantly increased the activity of aniline p-hydroxylase in the 5 week old hypothyroid rat. NADPH oxidase activity was unchanged in the 3 week old hypothyroid rat, and with replacement therapy with thyroxin, a significant increase in activity was noted (Table 4). The 5 week old rat, however, showed no significant change in NADPH oxidase activity. NADPH cytochrome c reductase showed a significant decrease in the 3 and 5 week old hypothyroid group (Table 4). This decrease was not only corrected by replacement thyroxin treatment but was significantly enhanced above age matched control values. Cytochrome P-450 content was significantly increased in the 3

week old hypothyroid rat (Table 4). Replacement therapy with thyroxin resulted in an over-reversal of this finding and a significant decrease in cytochrome P-450 content was obtained. With prolongation of the hypothyroid state, a compensation may have occurred since only a slight insignificant increase of cytochrome P-450 content was noted in the 5 week old hypothyroid which was reversed by thyroxin therapy. The increase in cytochrome P-450 content in hypothyroidism has also been noted in adult surgically thyroidectomized rats (Suzuki, 1967).

Effect of thyroxin in vitro

To evaluate the possibility of a direct thyroxin-substrate oxidative enzyme effect, thyroxin was added to the reaction mixtures for the assays of NADPH cytochrome c reductase and aniline p-hydroxylase activity. Thyroxin concentrations varying from 5×10^{-9} to 5×10^{-4} M had no effect on NADPH cytochrome c reductase activity as shown in Table 5. Similarly, thyroxin concentrations varying from 1×10^{-7} to 1×10^{-3} M had no effect on aniline p-hydroxylase activity. The lack of effect of thyroxin added *in vitro* on NADPH oxidase activity has already been demonstrated (Suzuki *et al.*, 1967).

Effect of subacute (3 days) and chronic (3 - 5 weeks) thyroxin therapy on the activity of the hepatic mixed function oxidase in immature rats

To determine the magnitude of changes in hepatic microsomal oxidation due to chronic hyperthyroidism and partly to determine the influence of subacute and chronic hyperthyroid state, the change in drug oxidation in rats that were treated with thyroxin 1 µg/g body weight for 3 and 5 weeks were

compared to those of corresponding age which received thyroxin 1 ug/g body weight daily for 3 days. The results are shown in Tables 6 and 7. At 3 weeks of age, there was a significant increase in the microsomal protein yield per gram liver in both sub-acutely and chronically thyroxin treated rats. No significant change was noted with NADPH oxidase and NADPH cytochrome c reductase activity in the sub-acute treatment group whereas a significant induction was observed in the chronically thyroxin treated group. Conversely, a significant decrease in cytochrome P-450 content and aminopyrine N-demethylase activity was only observed in the subacutely treated group. However, a significant increase was observed in aniline p-hydroxylase activity in both sub-acute and chronic thyroxin treatment.

In the 5 week old rat, the pattern of effects is different. No change was noted in the microsomal protein yield per gram of liver. A significant induction of NADPH oxidase was only noted in the chronically treated group whereas an induction of NADPH cytochrome c reductase occurred only in the subacutely treated group. A significant decrease in cytochrome P-450 content occurred in both the sub-acute and chronic treatment. An increase in aminopyrine N-demethylase activity occurred only in the sub-acute treatment groups. An interesting finding is the decreased aniline p-hydroxylation in the sub-acutely treated group whereas an increase was noted in the chronically treated group. These observations suggest that the quantitative (magnitude) and qualitative (direction of change) effects of thyroxin are greatly modified by the duration of thyroid dysfunction and the age at which thyroxin is administered. The data (Table 7) further suggest that compensatory mechanisms that bring the enzyme activity to comparable

control levels occur with prolonged hyperthyroidism.

Serum thyroxin levels and evidence for thyroid dysfunction

To determine the degree of deviation from euthyroid state, the serum thyroxin levels were measured in the radiothyroidectomized rats with and without thyroxin replacement therapy and in the rats treated with thyroxin since birth. The results are shown in Table 8. The control serum thyroxin levels of the 3 and 5 week old rat are within the lower human euthyroid values (4 - 11 ug%). The radiothyroidectomized rats had low serum thyroxin levels at 3 weeks of age (2.08 $\pm$ 0.15 ug%) which decreased further at 5 weeks of age (1.70 $\pm$ 0.24 ug%). Clinical manifestations of hypothyroidism (myxedema, hypoactivity, weight loss, decreased heart rate, decreased sensitivity of isolated heart to noradrenaline (Kunos et al., 1974) were noted. Replacement therapy with T_4 corrected some of these findings. Serum T_4 levels in these rats were 16.46 $\pm$ 1.51 ug% at 3 weeks and 50.00 $\pm$ 0 ug% at 5 weeks. These high T_4 values represent in part, the accumulation of the exogenous hormone due to decreased T_4 turnover seen in hypothyroidism. The rats treated daily with T_4 since birth had high T_4 values at 3 weeks (36.28 $\pm$ 4.37 ug%) which decreased at 5 weeks (6.63 $\pm$ 1.22 ug%). The latter value represents, in part, an increased capacity to excrete exogenous thyroxin as T_4 turnover in hyperthyroidism is increased and T_4 half-life is shortened remarkably (Sterling, 1964). Clinically, these rats were hyperthyroid as evidenced by low weight gain, hyperactivity and increased heart rate.

DISCUSSION

The optimal capacity for oxidative function represents the culmination of the processes of differentiation that have progressively occurred in the hepatic microsomes. The temporal events involved in these processes of differentiation may be modified by pathologic derrangements in the various mechanisms regulating growth and development. The data in this study demonstrate that postnatal aberrations in thyroid function significantly alter the developmental pattern of hepatic microsomal drug oxidation and the electron transport components of the mixed function oxidases, namely acceleration of the maturation of NADPH oxidase, NADPH cytochrome c reductase and aniline p-hydroxylase activity and deceleration of the maturation of cytochrome P-450 and aminopyrine N-demethylation under the influence of a sustained hyperthyroid state. Furthermore, the data demonstrate that the effects on maturational pattern appear to be specific to a particular electron transport component and to a substrate oxidative enzyme, and are related to age and duration of thyroid dysfunction. The differential effects on microsomal electron components is illustrated by the sustained age-matched supranormal values of NADPH cytochrome c reductase (Fig. 3) and NADPH oxidase activity (Fig. 2) and conversely by the infranormal cytochrome P-450 content (Fig. 4) during continuous thyroxin treatment. Also, converse effects on Types I and II substrate oxidations have been noted. Whereas aminopyrine N-demethylation (Fig. 5) was decreased at one week, aniline p-hydroxylation was accelerated significantly and attained adult levels at the same stated age (Fig. 6) under the influence of a sustained hyperthyroid state. These observations are in accord with

the findings in thyroxin treated or thyroidectomized adult male rats (Kato et al., 1968; Suzuki, 1967).

The age-relatedness to thyroxin response is dramatically illustrated by the effect of a "booster" dose of thyroxin on aminopyrine N-demethylation (Table 2). Whereas a significant inhibition of this substrate oxidation is observed at age 3 weeks, a slight but insignificant increase is noted at age 5 weeks (Table 2). Similar age related responsiveness to thyroxin is further shown by the effect of a booster dose of thyroxin on NADPH cytochrome c reductase activity on the neonatal thyroxin treated rat where a superinduction was exhibited at age 5 weeks but not at age 3 weeks (Table 3). The duration of postnatal thyroid dysfunction also influences the alteration in the maturational pattern of microsomal drug oxidation. This is exemplified by a decrease in aminopyrine N-demethylation in the three week old hypothyroid rat but not in the 5 week old hypothyroid rat. Similarly, but in a converse fashion, an increase in aniline p-hydroxylation is noted in the three week old but not in the 5 week old hypothyroid rat. These findings suggest some degree of compensatory mechanism inherent in prolonged hormonal imbalance. Indeed, a major difficulty in studying cell or enzymic differentiation in vivo, using endogenous substrates, is the presence of feedback control mechanisms that intervene to maintain the steady state (Thrasher, 1971). For instance, in hypothyroidism, the thyroid stimulating hormone (TSH) of the anterior pituitary gland is greatly elevated (Catt, 1970). It is not inconceivable that TSH per se may directly or indirectly alter the development of microsomal drug oxidation. To our knowledge, this hormonal interplay has not been studied as it pertains to the ontogenesis of the hepatic mixed function oxidase system.

The most sensitive indicator of the effect of thyroid hormone or of the deficiency thereof (as in hypothyroidism) on the developing hepatic mixed function oxidase system is NADPH cytochrome c reductase activity. This is supported by a marked increase in the activity 24 hours after thyroxin administration (Fig. 1); sustained high activity with continuous thyroxin treatment (Fig. 3); decreased activity to preinduction levels upon withdrawal of thyroxin (Table 3); a significant fall below control levels in hypothyroidism and a rise to above control levels with thyroxin replacement therapy in hypothyroidism (Table 4). These findings are in accord with the observations obtained in hypothyroid and hyperthyroid adult rats (Phillips and Langdon, 1956; Kato and Takahashi, 1968; Suzuki et al., 1967). Evidence has been presented that the metabolism of the flavoproteins is under the control of the thyroid gland through the regulation of flavokinase (Rivlin and Langdon, 1966; Rivlin et al., 1968; Rivlin, 1970), and since NADPH cytochrome c reductase is a flavoprotein (Williams and Kamin, 1962) therefore, the regulation of the enzyme titre is most probably mediated by the thyroid gland (Phillips and Langdon, 1956). Biochemical similarities between hypothyroidism and riboflavin deficiency have been demonstrated (Rivlin et al., 1968) and similarities between microsomal drug metabolism in hypothyroid immature and adult rat and the riboflavin deficient post-weaning mice (Catz et al., 1970), namely, increased cytochrome b_5 and cytochrome P-450 content, and increased aniline p-hydroxylase have also been shown (Table 4; Suzuki et al., 1967).

Thyroxin appears to exert a repressive role to the development of cytochrome P-450 and aminopyrine N-demethylase. This repressive role was more defined in the case of cytochrome P-450 as evidenced by a decrease

of this hemoprotein in chronic thyroxin treatment (Fig. 4); an initial decrease in neonatal thyroxin treated rat followed by an increase, above age matched control levels at three weeks with the withdrawal of thyroid effect (Table 3) and an increase in the three week old radiothyroidectomized rat (Table 4). The reason for the repressive effect of thyroxin on the development of cytochrome P-450 is not clear. The hypothesis that the low drug metabolizing capacity in the newborn may be due to the presence of inhibitory substances (Fouts and Adamson, 1959) that may have been derived from the mother (Feuer and Liscio, 1970) has been advanced. In support of this hypothesis is the inhibition of hepatic microsomal drug metabolism by endogenous substrates such as somatotropin (Wilson, 1969), pregnenolone and progesterone metabolites (Soyka and Lang, 1972); Soyka and Deckert, 1973) and the repression of the postnatal development of ethyl morphine N-demethylation and aminopyrine N-demethylation by growth hormone (Wilson, 1970). The newborn infant at term has a higher free serum thyroxin level with a transient 8- to 9-fold rise in serum thyroxin stimulating hormone immediately after birth (Fisher et al., 1970; Greenberg et al., 1970) added to a total pool of circulating endogenous substrates. Increased circulating thyroid hormone may produce altered substrate interaction with cytochrome P-450 as evidenced by decreased $\Delta O.D._{max}$ value and increased V_{max} for aniline; and increased K_s value, increased K_m value and decreased V_{max} for hexobarbital in thyroxin treated adult male rats (Kato et al., 1970). The possibility that relatively high thyroxin levels may indirectly act as a repressor of the development of aminopyrine N-demethylation and cytochrome P-450 through a permissive effect on growth hormone secretion (Catt, 1970) cannot be excluded.

Greengard (1969) also raised the concept that the thyroid gland is the probable physiologic trigger in NADPH generating system in fetal rat on the basis of simultaneous appearance of fetal thyroid function and NADPH cytochrome c reductase and glucose-6-phosphatase, and a precocious differentiation of these enzymes with exogenously administered thyroxin. However, the teleologic significance of a transient neonatal thyroid gland hyperfunction in relation to the differentiation of NADPH and oxygen requiring drug oxidative pathways and their associated electron transport components is as yet speculative. Moreover, the fundamental mechanism underlying the selective but variable effect of thyroxin on the various components of the microsomal electron transport chain and various oxidative pathways is still undefined.

In the human neonate, p-hydroxylation of aniline correlates with advancing postnatal age whereas aminopyrine N-demethylation does not correlate (Aranda et al., 1974). Furthermore, aniline p-hydroxylation is directly related to the activities of NADPH oxidase and NADPH cytochrome c reductase and cytochrome P-450 content (Aranda et al., 1974). The accelerated developmental pattern of aniline p-hydroxylase and NADPH cytochrome c reductase activity induced by thyroid hormone are relatively parallel. These observations suggest that aniline p-hydroxylation may be a better indicator of the activity of microsomal drug oxidative capacity as against aminopyrine N-demethylation in the immature organism. Marked variability in aminopyrine N-demethylation has also been observed in the human fetal liver (Yaffe et al., 1970).

Whether the effect of thyroxin on the development of the hepatic mixed function oxidase system is one of permanent enzyme differentiation

or merely a transient induction is not resolved in these experiments. The concept of enzyme differentiation entails maintenance of enzyme titres and the physiological variations of these titres, which are normally exhibited in a fully differentiated enzyme system as that seen in the adult organism. On the other hand, transient induction entails a rise in enzyme titres followed by a fall to pre-induction levels upon withdrawal of the inducing agent. Suggestive evidence for enzyme differentiation is provided by the effect of transient neonatal hyperthyroid state on the developmental pattern of aniline p-hydroxylase activity, an oxidative enzyme which exhibits the "overshoot phenomenon" (Moog, 1971). This phenomenon, whereby enzymes that undergo a large increase in activity during a short period of time, rise above their adult level and subsequently fall back to a relatively stable level, has been described in various substrate oxidations (Kato et al., 1964; Gram et al., 1969; Uehleke, 1971). Gram et al. (1969) had shown that the V_{max} for aniline p-hydroxylase peaked at 1 to 2 weeks followed by a decline thereafter. In contrast, MacLeod et al. (1972) and the present studies show that the peak activity of aniline p-hydroxylase above adult level was observed later at age 5 weeks. This peak activity was shifted to age one week by thyroxin treatment followed by a slow decline to lower adult value attained at 5 weeks of age (Fig. 8). This represents an advanced shift in the maturational pattern of approximately four weeks.

Suggestive evidence for a transient induction is that observed with the effect of neonatal thyroxin therapy which resulted in an increase in the activity of NADPH cytochrome c reductase at one week followed by a fall to subcontrol levels (Table 3), and an infranormal shift of the developmental

pattern at three to five weeks of age. This finding also raises a possible consequence of induction, that is, a fall in the enzyme activity to subnormal levels upon withdrawal of the influence of the inducing agent. Blackburn et al. (1972) and Carson et al. (1973) have demonstrated in the lung that steroids which are inducers of pulmonary surfactant production enhance cell differentiation at the expense of cell division, resulting in fewer but more mature cells that are functioning at an optimal capacity. With growth the metabolic demands of the organism increase commensurately with its increased body size, and it is possible that the decreased number of cells could no longer meet this increased demand leading to an infranormal shift in the developmental pattern, as shown by NADPH cytochrome c reductase activity.

The fundamental mechanism underlying all of the known effects of thyroid hormone is still unsettled (Catt, 1970; Sokoloff, 1972). It is highly probable that the primary action of thyroid hormone involves a process so basic to cellular function that its regulation eventually ramifies into all the metabolic processes, such as microsomal drug oxidation, which have been shown to be modified in thyroid dysfunction. A basic process of cellular activity is protein synthesis, which has been shown to be stimulated by thyroid hormone both in vitro and in vivo (Tata, 1963; Sokoloff, 1972; Sokoloff et al., 1963; Tata et al., 1968). The postnatal increase in the drug-oxidative enzymes probably represents de novo protein synthesis (Dallner et al., 1965) and it is likely that an increase in thyroid hormone would increase the rate of postnatal synthesis of enzyme proteins as reflected by increased NADPH oxidase, NADPH cyto-

chrome c reductase and aniline p-hydroxylase activity (Figs. 2, 3, and 6). However, this mechanism does not explain the repression of the development of cytochrome P-450 and aminopyrine N-demethylation (Figs. 4 and 5). Nonetheless, a unified mechanism involving stimulation of protein synthesis is likely if one invokes the possibility of an imbalance between rate of synthesis and rate of degradation resulting in a net increased or net decreased protein synthesis. Such an imbalance between synthesis and degradation has been demonstrated by the effect of methylmercury hydroxide on hepatic cytochrome P-450 (Lucier et al., 1973). The rate of incorporation of radioactivity from labelled δ-aminolevulinic acid into cytochrome P-450 did not appear to be affected by methylmercury, but the degradation of the labeled cytochrome P-450 occurred twice as fast in the methylmercury treated rats compared to the controls (Lucier et al., 1973). Increased protein turnover in hyperthyroidism and decreased protein turnover in hypothyroidism in the mitochondria have been demonstrated (Gross, 1971) and the possibility of increased microsomal protein turnover under hyperthyroid state is highly probable. The coherent sequence in integrating all of these effects of thyroid hormone on the ontogenesis of the mixed function oxidase system needs further investigation.

It has been suggested that thyroxin may increase drug oxidation by facilitating the availability of oxygen to the substrate (Kato, 1970). This intriguing hypothesis gains support with the observation that hyperthyroid patients have a left to right shift in the hemoglobin oxygen dissociation curve with a resultant increase in P_{50} (Miller et al., 1970; Snyder and Reddy, 1970), the oxygen tension at which 50% of the hemoglobin is saturated with oxygen. This decreased hemoglobin oxygen affinity

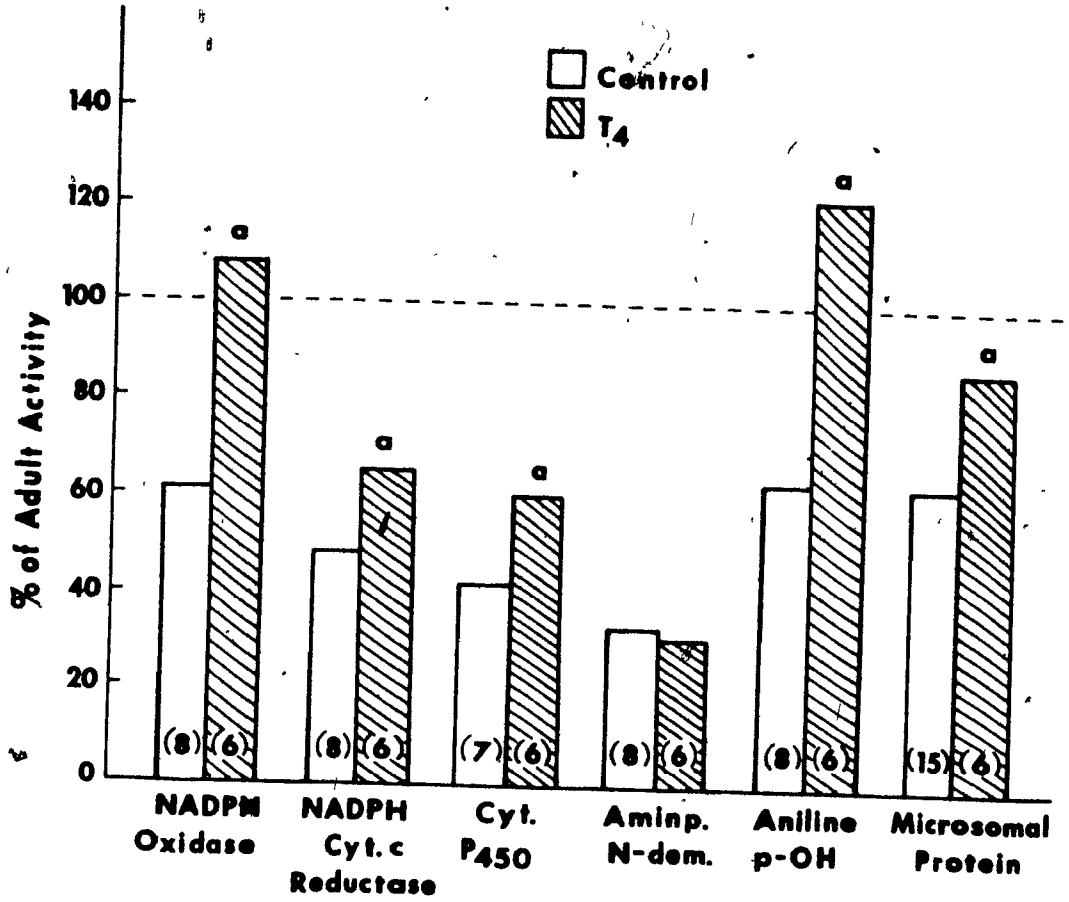
facilitates oxygen transfer to the tissues (Miller et al., 1970; Schussler et al., 1971) and a similar facilitation of oxygen transfer from the hemoprotein to the substrate undergoing oxidation may occur. However, the exact biochemical mechanism for oxygen transfer from the cytochrome P-450 to the substrate is not completely understood yet (Ullrich, 1971). Nonetheless, the possibility remains that thyroxin may exert an effect at this step of drug oxidation.

The events leading to full maturation of microsomal drug oxidative capability and the factors regulating these events are areas of major interest in perinatal pharmacology. Identification of physiological influences which allow for increased drug-metabolizing enzyme activity in the immature animal may elucidate the normal sequence of biochemical events in drug metabolism. The data in this study emphasize the vulnerability of the developmental profile of the microsomal drug metabolizing enzymes to alteration in thyroid states and suggest a possible regulatory effect of the thyroid gland in the ontogenesis of the hepatic mixed function oxidase system.

FIGURE 1: The effect of a single thyroxin dose on the hepatic microsomal electron transport and substrate oxidation in immature male rats.

Thyroxin 1 ug/g subcutaneously was administered 24 hours prior to death. Thyroxin was dissolved in 0.01 N NaOH at a concentration sufficient to deliver the 1 ug/g dose in 0.05 ml. The control rats received 0.05 ml of 0.01 N NaOH. The bars indicate percentage of the adult activity. The adult enzyme activities are: NADPH oxidase = 7.98 $\pm$ 0.57 nmoles x min^{-1} x mg^{-1} protein; NADPH cytochrome c reductase = 95.02 $\pm$ 8.55 nmoles x min^{-1} x mg^{-1} protein; cytochrome P-450 = 0.452 $\pm$ 0.028 nmoles x mg^{-1} protein, aminopyrine N-demethylation = 2.52 $\pm$ 0.15 nmoles x min^{-1} x mg^{-1} protein, aniline p-hydroxylation = 0.392 $\pm$ 0.020 nmoles x min^{-1} x mg^{-1} protein and microsomal protein = 49.77 $\pm$ 3.08 mg x g^{-1} liver. Numbers in parenthesis indicate numbers of individual determinations. Each individual determination represents a pool of liver from 4 immature rats.

a = $p < 0.01$.


Control
T_4
% of Adult Activity
140
120
100
80
60
40
20
0
a
(8) (6)
(7) (6)
(15) (6)
NADPH Oxidase
NADPH Cyt.c Reductase
Cyt. P_{450}
Aminp. N-dem.
Aniline p-OH
Microsomal Protein

MS: II

FIGURE 2: Effect of chronic thyroxin therapy on the development of NADPH oxidase activity in male rats.

Rats were given L-thyroxin 1 ug/g/d subcutaneously daily after birth. Values indicate mean ± SEM of 6 to 20 individual determinations.

a = $p < 0.05$.

b = $p < 0.01$.

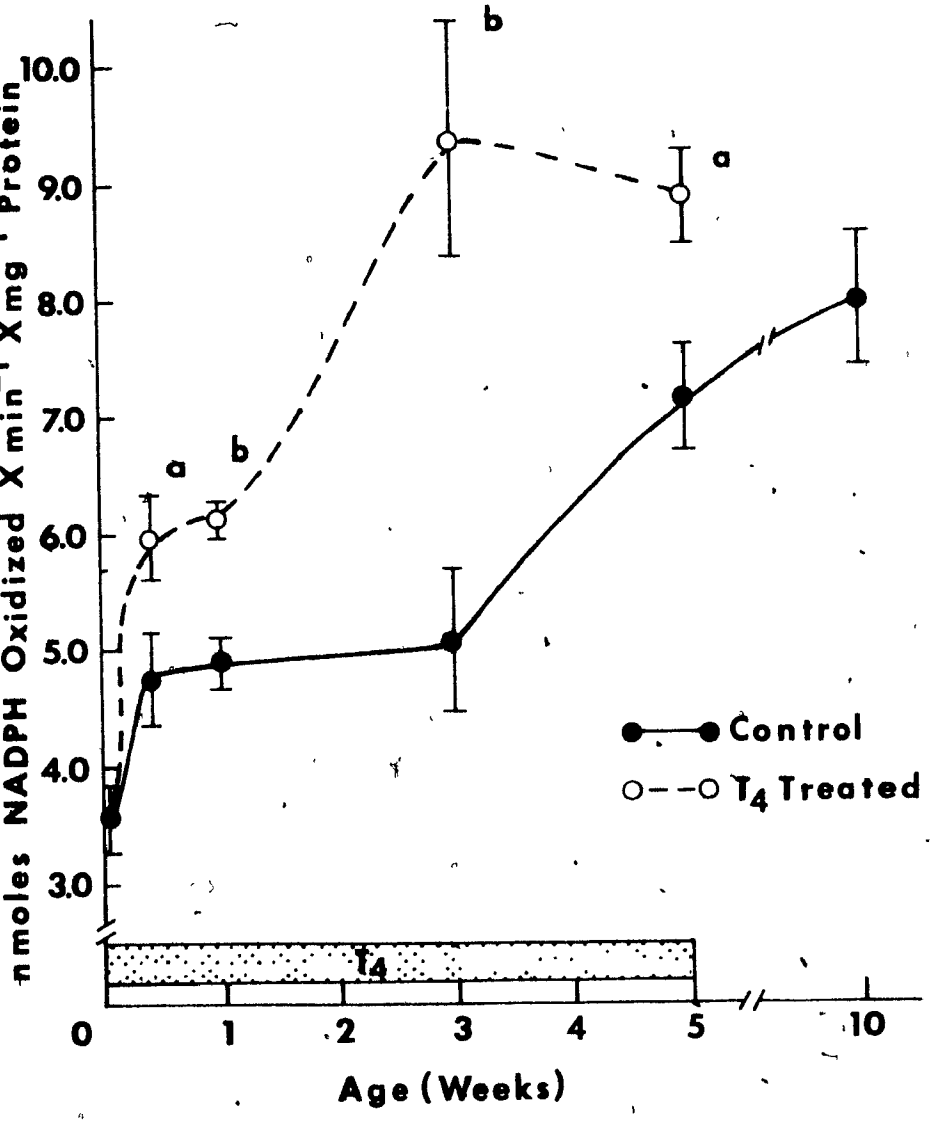

nmoles NADPH Oxidized X min⁻¹ X mg⁻¹ Protein
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
a
b
b
a
Control
T4 Treated
T4
0
1
2
3
4
5
10
Age (Weeks)

FIGURE 3: The effect of continuous thyroxin therapy on the development of cytochrome c reductase activity. Rats were given L-thyroxin 1 ug/g/d subcutaneously since birth. Values indicate mean ± SEM of 6 to 14 determinations obtained from individual pools in the newborn rat or from a single animal in the older rats.

a = $p < 0.01$.

b = $p < 0.001$.

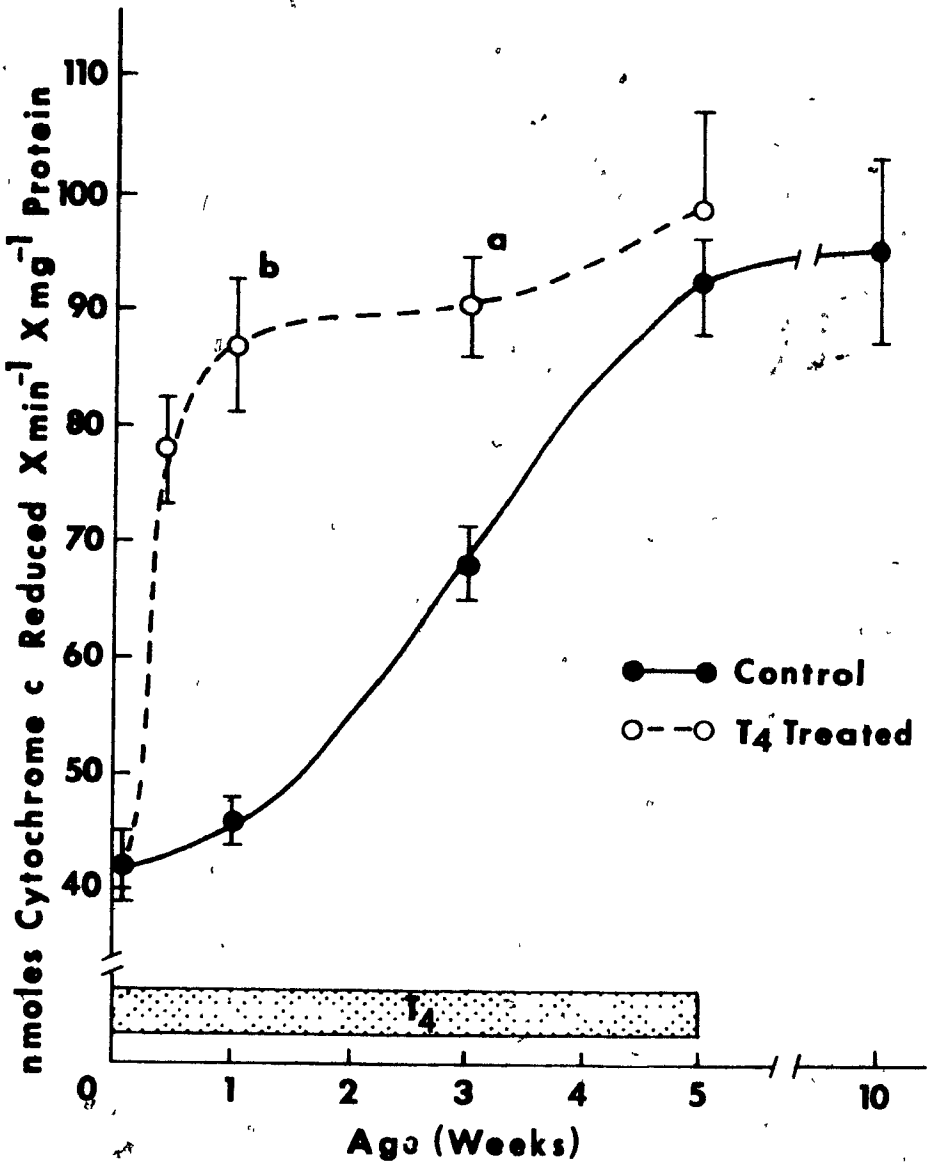
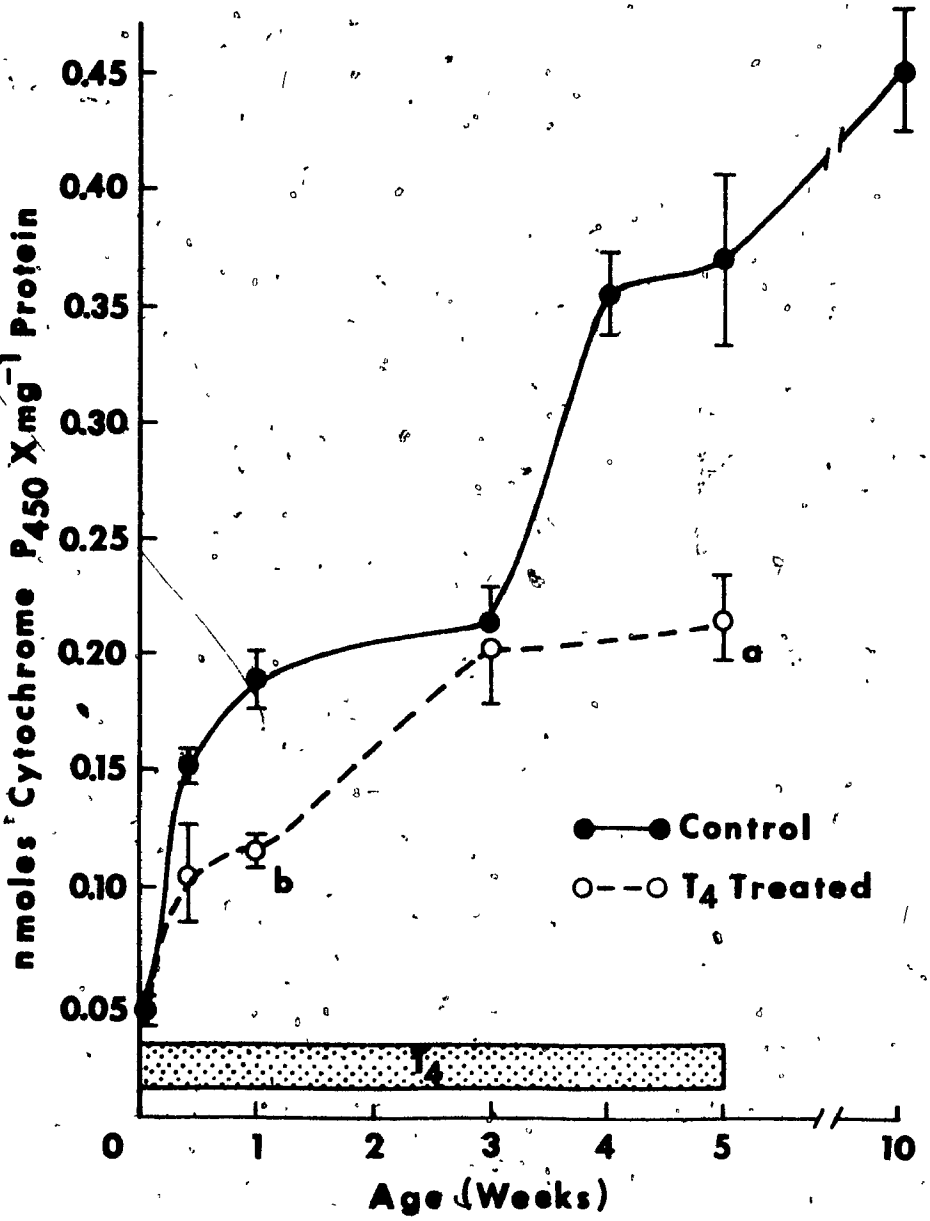

nmoles Cytochrome c Reduced X min⁻¹ X mg⁻¹ Protein
110
100
90
80
70
60
50
40
0
1
2
3
4
5
10
Age (Weeks)
a
b
Control
T4 Treated
T4

FIGURE 4: Effect of continuous thyroxin therapy on the development of cytochrome P-450. Rats were given a daily subcutaneous injection of 1 ug/g/d of L-thyroxin. Each value represents mean ± SEM of 5 to 14 determinations.

a = $p < 0.05$.

b = $p < 0.01$.


0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
nmoles Cytochrome P_{450} X mg^{-1} Protein
a
b
Control
T_4 Treated
T_4
0
1
2
3
4
5
10
Age (Weeks)

MS. II

FIGURE 5: Effect of chronic hyperthyroid state on development of aminopyrine N-demethylase activity in male rats. Rats were given daily injection of L-thyroxin 1 ug/g/d subcutaneously since birth. Values indicate mean ± SEM of 6 to 10 determinations.

a = $p < 0.01$ compared to the control group.

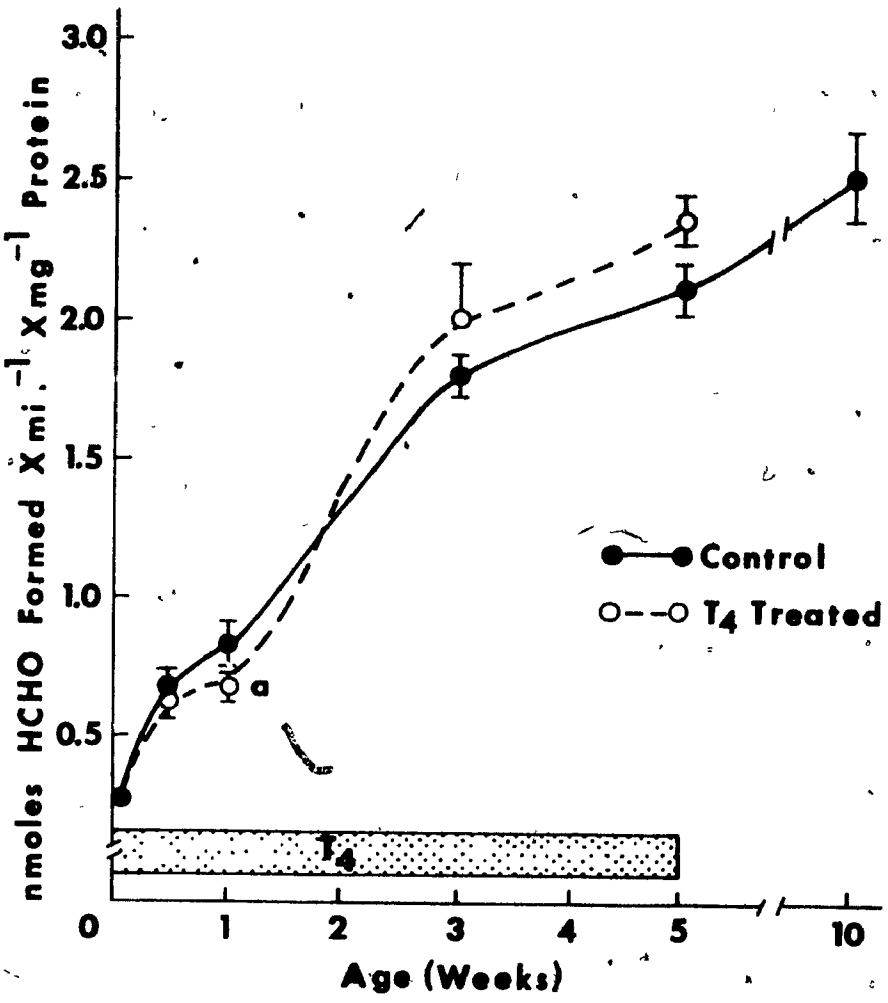
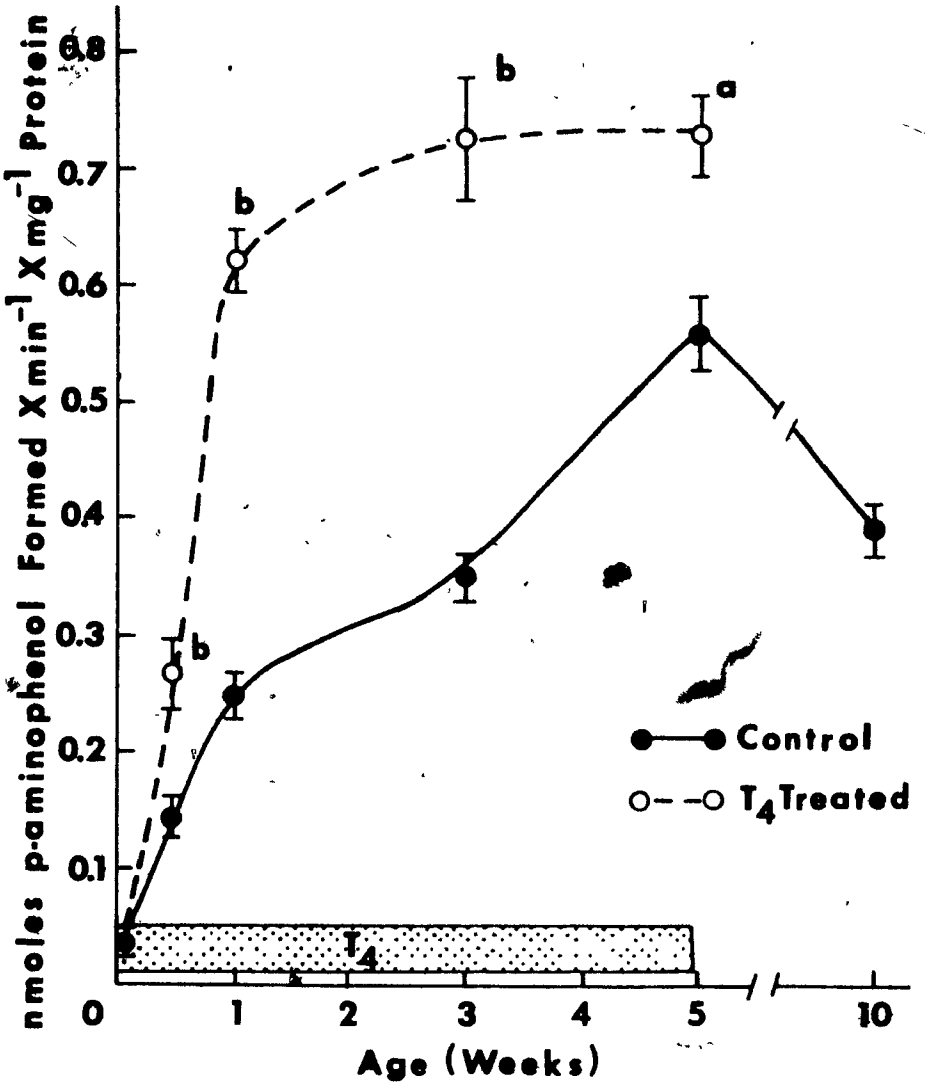

3.0
2.5
2.0
1.5
1.0
0.5
0
1
2
3
4
5
10
nmoles HCHO Formed X mi.$^{-1}$ X mg^{-1} Protein
Age (Weeks)
Control
T_4 Treated
a
T_4

FIGURE 6: Effect of chronic hyperthyroid state on the development of aniline p-hydroxylase activity in male rats. Rats were given daily injections of L-thyroxin 1 ug/g/d subcutaneously since birth. Values indicates mean ± SEM of 6 to 14 individual determinations.

a = $p < 0.01$.

b = $p < 0.001$.


nmoles p-aminophenol Formed X min^{-1} X mg^{-1} Protein
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
10
Age (Weeks)
a
b
b
b
Control
T_4 Treated
T_4

MS. II

TABLE 1

Effect of chronic thyroxin administration on liver weight, microsomal protein yield and total microsomal protein per liver in immature male rats.

Rats were treated daily with L-thyroxin (1 ug/g body weight). Total microsomal protein per liver was obtained from the product of liver weight and microsomal protein yield. Values indicate mean ± SEM. Numbers in parentheses indicate number of individual determinations.

Age (wks)	Liver wet weight (g)		Microsomal protein (mg/g liver)		Total microsomal protein (mg/liver)	
	Control	T4 treated	Control	T4 treated	Control	T4 Treated
1	0.52±0.01 (24)	0.52±0.01 (30)	31.71±1.24 (15)	34.92±1.84 (6)	16.49	18.16
3	2.38±0.10 (20)	1.34±0.14 (8)[b]	30.51±0.71 (10)	36.47±1.98 (8)[a]	72.61	48.89
5	6.93±0.16 (10)	5.13±0.24 (6)	39.11±1.04 (5)	39.56±2.83 (7)	271.03	202.94

a = $p < 0.01$ compared to control value.

b = $p < 0.001$ compared to control value.

MS. II

TABLE 2

Effect of neonatal thyroxin administration on liver weight, microsomal protein and substrate oxidation in immature male rats.

Rats were treated daily with 100 ug of thyroxin for the first 5 days of life. Thyroxin was also administered to neonatally treated rats at a dose of 1 ug/g body weight daily ("booster"dose) for three days prior to death at age 3 and 5 weeks. Values indicate mean ± SEM. Numbers in parentheses indicate number of individual determinations.

Age (wks)	Group	Liver wet weight (g)	Microsomal protein (mg/g liver)	Aminopyrine N-demethylase, nmoles $x\ min^{-1} x\ mg^{-1}$ protein	Aniline p-hydroxylase, nmoles $x\ min^{-1} x\ mg^{-1}$ protein
1	control	0.52±0.01 (24)	31.71±1.24 (15)	0.83±0.08 (8)	0.250±0.016 (8)
	neonatal T4	0.51±0.01 (24)	43.70±3.50 (6)[c]	0.63±0.05 (6)[b]	0.604±0.076 (6)[c]
3	control	2.39±0.10 (20)	30.51±0.71 (10)	1.80±0.07 (10)	0.349±0.018 (10)
	neonatal T4	1.94±0.11 (6)	43.16±2.48 (6)[c]	1.91±0.13 (6)	0.548±0.015 (6)
	neonatal T4 + booster T4	1.15±0.06 (6)[c]	52.05±3.99 (6)[c]	0.83±0.08 (6)[c]	0.581±0.027 (6)[c]
5	control	6.93±0.16 (10)	39.11±1.04 (5)	2.11±0.10 (10)	0.562±0.029 (14)
	neonatal T4	5.85±0.35 (6)b	34.01±2.70 (6)	2.11±0.09 (6)	0.311±0.013 (6)
	neonatal T4 + booster T4	4.15±0.15 (6)[c]	48.13±2.51 (6)[a]	2.43±0.09 (6)	0.570±0.016 (6)

(a) $p < 0.05$ (b) $p < 0.01$ (c) $p < 0.001$ compared to the age matched control.

MS. II

TABLE 3

The effect of neonatal thyroxin treatment with and without "booster" thyroxin dose on the development of microsomal electron transport components in male rats.

Rats were injected with 100 ug of thyroxin during the first 5 days of life. Booster dose of thyroxin was administered to a group of these neonatally treated rats at a dose of 1 ug/g body weight daily for three days prior to death at age 3 and 5 weeks. Values indicate mean ± SEM. Numbers in parentheses indicate number of individual determinations.

Age (wks)	Group	NADPH oxidase nmoles x min^{-1} x mg^{-1} protein	NADPH cytochrome c reductase nmoles x min^{-1} x mg^{-1} protein	Cytochrome P-450 nmoles x mg^{-1} protein
1	control	4.89±0.23 (8)	45.97±1.77 (8)	0.190±0.013 (7)
	neonatal T4	5.62±0.55 (6)	76.68±6.82 (6)[c]	0.120±0.003 (6)[c]
3	control	5.07±0.64 (18)	68.22±3.12 (20)	0.214±0.014 (10)
	neonatal T4	6.08±0.27 (6)	41.63±4.29 (6)[c]	0.367±0.034 (5)[c]
	neonatal T4 + booster T4	5.05±0.29 (6)	54.50±4.19 (6)[a]	---
5	control	7.12±0.48 (15)	96.16±4.16 (10)	0.371±0.037 (14)
	neonatal T4	6.56±0.52 (6)	60.43±4.95 (6)[c]	0.377±0.025 (5)
	neonatal T4 + booster T4	8.43±0.64 (6)	125.78±8.06 (6)[b]	---

(a) $p < 0.05$ (b) $p < 0.001$ (c) $p < 0.001$

MS. II

TABLE 4

Effect of hypothyroidism with and without thyroxin replacement therapy on liver weight, microsomal protein yield, substrate oxidations and electron transport components in immature male rats.

Rats were thyroidectomized with ^{131}I (100 ug) at birth. Replacement therapy with L-thyroxin (1 ug/g/day) was started 7 days prior to the experiments. Values represent mean ± SEM. Numbers in parentheses indicate number of individual determinations.

	3 weeks			5 weeks		
Parameters	Control	Hypothyroid	Hypothyroid + T4 x 7 d	Control	Hypothyroid	Hypothyroid + T4 x 7dd
Liver wet wt. (g)	2.38±0.11 (20)	1.60±0.05[c] (18)	1.66±0.04[c] (18)	6.93±0.16 (10)	1.78±0.09[c] (14)	2.48±0.10[c] (13)
Microsomal protein yield mg/g liver	30.51±0.71 (10)	38.12±2.63[b] (6)	40.90±1.53[c] (6)	39.11±1.04 (5)	49.31±3.69[a] (6)	47.62±2.05[b] (6)
Aminopyrine N-demethylase[d]	1.80±0.07 (10)	1.02±0.03[c] (6)	1.06±0.06[c] (6)	2.11±0.10 (10)	1.93±0.20 (6)	2.17±0.08 (6)
Aniline p-hydroxylase[d]	0.349±0.018 (10)	0.526±0.017[c] (6)	0.805±0.041[c] (6)	0.562±0.029 (6)	0.534±0.033 (6)	0.805±0.041[c] (6)
NADPH oxidase[d]	5.13±0.62 (18)	4.85±0.16 (6)	8.64±0.040[b] (6)	7.12±0.48 (15)	5.48±0.48 (6)	8.64±0.47 (6)
NADPH cytochrome c reductase[d]	68.22±3.12 (20)	37.71±1.37[c] (6)	119.07±10.06[c] (6)	92.16±4.16 (10)	60.48±4.69[c] (6)	120.57±8.55[b] (6)
Cytochrome P-450[e]	0.213±0.014 (10)	0.383±0.012[c] (6)	0.087±0.024[c] (6)	0.371±0.037 (14)	0.485±0.025 (6)	0.272±0.023 (6)

(a) $p < 0.05$ (b) $p < 0.01$ (c) $p < 0.001$ compared to control.
(d) values expressed as nmoles x min^{-1} x mg^{-1} protein. (e) values expressed as nmoles x mg^{-1} protein.

MS.II

TABLE 5

Effect of thyroxin *in vitro* on NADPH cytochrome c reductase and aniline p-hydroxylase activity.

L-thyroxin was dissolved in 0.01 M NaOH and added directly to the reaction mixtures for the assay of NADPH cytochrome c reductase and aniline p-hydroxylase. Values are expressed as means ± SEM of 8 individual values for NADPH cytochrome c reductase activity and 6 individual values for aniline p-hydroxylase activity. Each individual value represents a pool of microsomes obtained from three one-week old rats. No significant differences in the activity of NADPH cytochrome c reductase and aniline p-hydroxylase were obtained between the solvent (0.01 M NaOH) containing reaction mixture and those containing variable concentrations of thyroxin. The hormone precipitated out at concentration of 1×10^{-4}.

T4 M Concentration	NADPH cytochrome c reductase (nmoles cyt. c reduced $\times$ min^{-1} $\times$ mg^{-1} protein	T4 M Concentration	Aniline p-hydroxylase (nmoles p-aminophenol formed $\times$ min^{-1} $\times$ mg^{-1} protein
0.00	52.19 ± 2.22	0.00	0.250 ± 0.016
0.00 + 0.01 M NaOH	45.86 ± 3.03	0.00 + 0.01 NaOH	0.250 ± 0.016
5×10^{-9}	46.83 ± 2.79	1×10^{-7}	0.267 ± 0.011
5×10^{-8}	46.43 ± 2.61		
5×10^{-7}	46.35 ± 3.93	1×10^{-5}	0.274 ± 0.011
5×10^{-6}	44.40 ± 2.60		
5×10^{-5}	46.02 ± 2.79	1×10^{-3}	0.279 ± 0.004
5×10^{-4}	46.80 ± 4.83		

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TABLE 6

Comparison between subacute thyroxin therapy and chronic therapy in 3 week old rats.

Male rats were treated with L-thyroxin (1 ug/g) daily for 3 days in the subacute treatment group and daily for 3 weeks in the chronic treatment group. Values are expressed as mean ± SEM. Numbers in parentheses represent number of rats individually studied. Δ% indicates percent change from the control group.

		Subacute		Chronic	
	Control	T4 1 mg/kg/ d x 3d	Δ%	T4 1 mg/kg/ d x 3 wks	Δ%
Microsomal protein yield mg/g liver	30.51±0.71 (10)	49.69±2.36 (7)	+62.9[b]	36.47±1.98 (8)	+19.53[a]
NADPH oxidase nmoles x min^{-1} x mg^{-1} protein	5.33±0.67 (20)	6.87±0.35 (7)	+31.6	9.36±1.04 (8)	+75.6[a]
NADPH cytochrome c reductase min^{-1} x mg^{-1} protein	68.22±3.11 (20)	74.55±5.94 (7)	+ 9.28	90.11±4.68 (7)	+30.1[a]
Cytochrome P-450 nmoles x mg^{-1} protein	0.214±0.014 (10)	0.120±0.006 (7)	-43.9[b]	0.203±0.026 (5)	-5.1
Aminopyrine N-demethylase nmoles x min^{-1} x mg^{-1} protein	1.80±0.06 (10)	1.22±0.05 (7)	-32.2	2.07±0.13 (8)	+15.0
Aniline p-hydroxylase nmoles x min^{-1} x mg^{-1} protein	0.349±0.019 (10)	0.594±0.016 (7)	+70.2[b]	0.727±0.053 (8)	+108.3[b]

(a) $p < 0.01$

(b) $p < 0.001$

MS. II

TABLE 7

Comparison between subacute and chronic thyroxin therapy in 5 week old rats.

Male rats were treated with L-thyroxin (1 ug/g) for 3 days in the subacute treatment group and daily for 5 weeks in the chronic treatment group. Values are expressed as mean ± SEM. Numbers in parentheses indicate number of rats individually studied. Δ% indicates percent change from the control group.

		Subacute		Chronic	
	Control	T4 1 mg/kg/ d x 3d	Δ%	T4 1 mg/kg/ d x 5 wks	Δ%
Microsomal protein yield mg/g liver	39.11±1.04 (5)	37.18±2.90 (6)	-4.9	39.56±2.83 (7)	+1.1
NADPH oxidase nmoles x min^{-1} x mg^{-1} protein	7.11±0.48 (15)	7.79±0.55 (6)	-9.6	8.84±0.40 (7)	+24.1[a]
NADPH cytochrome c reductase nmoles x min^{-1} x mg^{-1} protein	92.16±4.16 (10)	154.22±15.02 (6)	+67.31[c]	98.44±8.69 (7)	+5.7
Cytochrome P-450 nmoles x mg^{-1} protein	0.371±0.036 (14)	0.244±0.007 (6)	-34.2[a]	0.216±0.019 (5)	-41.8[a]
Aminopyrine N-demethylase nmoles x min^{-1} x mg^{-1} protein	2.11±0.11 (10)	2.57±0.17 (6)	+21.8[a]	2.36±0.09 (7)	+11.8
Aniline p-hydroxylase nmoles x min^{-1} x mg^{-1} protein	0.562±0.029	0.363±0.032	-35.4[c]	0.728±0.036	+29.5[b]

(a) $p < 0.05$ (b) $p < 0.01$ (c) $p < 0.001$

MS. II

TABLE 8

Serum thyroxin levels in euthyroid, radiothyroidectomized and hyperthyroid immature rats.

Groups	Serum Thyroxin (ug %)[a]	
	3 weeks	5 weeks
Control	4.31 ± 0.28 (8)[b]	4.17 ± 0.33 (9)
Chronic T4[c]	36.28 ± 4.37 (7)	6.63 ± 1.22 (11)
Hypothyroid[d]	2.08 ± 0.15 (6)	1.70 ± 0.24 (10)
Hypothyroid + T4[e]	16.46 ± 1.51 (12)	50.00 ± 0.00 (6)

(a) Values are expressed as Mean ± SEM.

(b) Numbers in parentheses indicate number of individual determinations.

(c) Chronic T4 indicates the group of rats treated with L-thyroxin (1 ug/g) daily for 3 or 5 weeks.

(d) Hypothyroid group indicates rats treated with ^{131}I at birth.

(e) As in (d), with replacement L-thyroxin therapy at a dose of 1 ug/g body weight daily for 1 week prior to T4 measurements.

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MANUSCRIPT III

THYROID HORMONE AND POSTNATAL RATE OF REDUCTION OF CYTOCHROME P-450 BY NADPH

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ABSTRACT

The influence of thyroid hormone on the development of NADPH cytochrome P-450 reductase activity was studied. Hepatic microsomes obtained from normal male rats of varying ages from birth to 10 weeks of age showed an increasing rate of reduction of cytochrome P-450 by NADPH, as a function of postnatal age with attainment of 50% adult activity by 5 weeks of age. Addition of 1 mM aminopyrine in vitro into the reaction mixture enhanced the rate of reduction of cytochrome P-450 by NADPH at all ages except at 3 weeks of age.

Male rats were made hypothyroid with ^{131}I at birth. Hypothyroidism increased the non-aminopyrine enhanced NADPH cytochrome P-450 reductase activity and was greater than the control rats at age 3 and 5 weeks. Hypothyroidism also increased the aminopyrine enhanced NADPH cytochrome P-450 reductase at age 3 weeks but decreased it at age 5 weeks. Therapy with L-thyroxin (1 ug/gram body weight/day for 7 days prior to sacrifice) partly reversed the observed changes in non-substrate enhanced activity of NADPH cytochrome P-450 reduction in hypothyroidism at age 3 weeks and was completely reversed at age 5 weeks.

Male rats made hyperthyroid during the neonatal period (100 ug/day from birth to 5 days) showed a significant increase in the rate of reduction of cytochrome P-450 and was greater than the control rats at ages 3 and 5 weeks. Chronic hyperthyroidism produced by daily subcutaneous injections of L-thyroxin (1 ug/gram body weight/day for 3 and 5 weeks) resulted in increased rate of reduction of cytochrome P-450 at age 3 weeks and had no observed effects at age 5 weeks indicating some degree of adaptation.

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Aminopyrine added *in vitro* at 1×10^{-3} M concentration significantly enhanced the rate of reduction of cytochrome P-450 by NADPH in the control rats, decreased the rate of reduction of cytochrome P-450 by NADPH in the 3 week old hypothyroid rat and had no effect in the chronic hyperthyroid rat. These observations indicate that a euthyroid state may be necessary for substrate enhancement of NADPH cytochrome P-450 reductase activity.

The data demonstrate that the thyroid hormone significantly influences the development of NADPH cytochrome P-450 reductase. Thyroid hormone may be an important regulator in the development of hepatic microsomal electron transport and substrate oxidative enzymes.

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INTRODUCTION

The reduction of cytochrome P-450 by NADPH is one of the most important steps in microsomal hydroxylation reactions (Diehl, et al., 1970). Evidence has been presented that the rate of reduction of cytochrome P-450 by NADPH often expressed as NADPH cytochrome P-450 reductase activity is rate limiting in the reactions catalyzed by the hepatic microsomal mixed function oxidase system in the adult rat (Gigon et al., 1968; Gigon et al., 1969; Gram et al., 1968). The N-demethylation of ethyl morphine by liver microsomes is more closely related to NADPH cytochrome P-450 reductase activity than to the amount of cytochrome P-450, the binding of substrate to cytochrome P-450 as measured by the magnitude of the spectral change, or the activity of NADPH cytochrome c reductase (Voltzman et al., 1968).

The activity of hepatic microsomal substrate oxidative enzymes is deficient in the newborn animal (Fouts and Adamson, 1959; Jondorf et al., 1959), relative to the adult animal of the same species. The activity increases postnatally, approaching adult level of activity by approximately 3 to 5 weeks (MacLeod et al., 1972; Fouts and Devereux, 1972). The increase in substrate oxidative capacity is parallelled by an increase in the activity of NADPH cytochrome P-450 reductase (MacLeod et al., 1972). Since the NADPH cytochrome P-450 reductase is the possible rate limiting step in microsomal hydroxylation reactions (Diehl et al., 1970), it is important to study possible factors that may influence the postnatal development of this reductase activity.

Studies in our laboratory (Aranda and Fade, 1974) demonstrated that thyroid hormone or lack thereof significantly influenced the rate of NADPH and substrate oxidation, the rate of NADPH cytochrome c reduction and the

content of cytochrome P-450 in the immature male rats. The possibility that thyroid hormone might also influence the activity of NADPH cytochrome P-450 reductase was therefore tested in the present study, in which the rate of reduction of cytochrome P-450 by NADPH was measured in hepatic microsomes from thyroxin treated and in radiothyroidectomized immature male rats. The rate of substrate enhanced reduction of cytochrome P-450 by NADPH was also measured.

MATERIALS AND METHODS

Adult and immature male Sprague-Dawley rats obtained from Canadian Breeding Farms (St. Constant, Que.) were used in this study. The adult rats were at least 10 weeks of age and weighed between 300-340 grams. Pregnant female rats, bred at the Canadian Breeding Farms, were brought to the laboratory on the day of parturition at which time the male litters were segregated with their mothers and were used for the experiments. Mothers and litters were maintained at the McGill University McIntyre Animal Centre and the litters were weaned at 21 days of age. The diet for the nursing mothers and for the weaned litters consisted of Purina Lab Rat chow and water ad libitum. For the developmental pattern of NADPH cytochrome P-450 reductase activity, normal male rats were sacrificed at birth, 1, 3, 5 and 10 weeks of age. The developmental pattern in female rats was not studied.

The effect of thyroid hormone on the rate of reduction of cytochrome P-450 by NADPH was studied and the rats were divided into four major groups:

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Group	Thyroid Treatment	Aminopyrine in vitro
I. Control	0	0
	0	1mM
II. Hypothyroid	0	0
	0	1mM
	+	0
	+	1mM
III. Chronic hyperthyroid	+	0
	+	1mM
IV. Neonatal hyperthyroid	+	0
	+	1mM

Hypothyroidism was produced by thyroidectomy employing radioactive ^{131}I (New England Nuclear, Boston, Mass.) as described by Goldberg (1949). One hundred uC of ^{131}I was subcutaneously administered to newborn rats within 18 hours from birth. These rats were later killed at 3 and 5 weeks of age. Replacement therapy using L-thyroxin 1 ug/gram body weight daily for 7 days was injected subcutaneously to a group of hypothyroid rats prior to sacrifice at age 3 and 5 weeks.

To determine the effect of surgical thyroidectomy as compared to radiothyroidectomy by ^{131}I, surgical thyroidectomy was performed on a group of weanling rats (ages 3 to 4 weeks) under ether anesthesia. These rats were allowed to survive until 6 weeks post-thyroidectomy.

In the experiments on chronic hyperthyroid state, immature rats were given a daily subcutaneous injection of L-thyroxin (Sigma Laboratories, St. Louis, Missouri) at a dose of 1 ug/gram body weight starting at birth. L-

thyroxin was dissolved in 0.01 N NaOH and the concentration was adjusted so that in all experiments the volume injected ranged from 0.05 ml to 0.1 ml to rats from birth to 3 weeks of age, 0.1 ml to 0.3 ml from 3 weeks to 5 weeks of age and 0.5 ml in adults. Initial experiments in our laboratory showed no effect on the parameters studied when 0.01 N was subcutaneously administered at the volumes equivalent to the thyroxin solutions used, and therefore the control values were obtained from untreated rats of corresponding ages matched with the thyroxin treated group. Groups of immature rats from these untreated and thyroxin treated rats (1 ug/g body weight daily) were sequentially killed at ages 3 and 5 weeks.

To determine the effect of hyperthyroidism during the early neonatal period (first five days of age), neonatal hyperthyroid state was produced by subcutaneous injection (at the nape of the neck) of L-thyroxin 100 ug/day for the first 5 days of postnatal life. Treatment was started within 18 hours from birth. To determine whether an early neonatal hyperthyroid state would alter the response of the rat to thyroxin at a later age, additional thyroxin doses (booster dose) of 1 ug/g body weight/day for three days were administered to these neonatally hyperthyroid rats prior to sacrifice at age 3 weeks and 5 weeks.

In all experiments on the immature animals, one or more pups were randomly selected from different litters of same treatment group so that at any given age the mean value was derived from a number of individual determinations. Each determination performed in the newborns represents a single value derived from the livers of four littermates pooled prior to homogenization. At one and two weeks of age, livers from three to four littermates were pooled and thereafter individual livers were used.

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Animals of desired age were sacrificed by decapitation followed by exsanguination. Livers were immediately excised and homogenized on ice-cold 0.1 M phosphate buffer, pH 7.4 using 4 volumes per unit weight for 30 seconds in a high speed Sorvall Omni-Mixer (Sorval Co., Conn.). All subsequent procedures were carried out between 0^{o} C and 4^{o} C.

The homogenate was centrifuged at 10,000 x g for 10 minutes in a refrigerated centrifuge. The 10,000 x g supernatant was carefully removed avoiding any contamination by the loosely packed pellet at the bottom or the fatty layer at the top of the centrifuge tube. The 10,000 x g supernatant was centrifuged at 100,000 x g for 60 minutes in a Beckman L3-40 refrigerated ultracentrifuge. The microsomal pellet obtained was separated from its glycogen layer and resuspended in ice-cold buffer using a glass homogenizer with a Teflon pestle (Arthur H. Thomas Co., Philadelphia, Pa.) and using a volume of buffer sufficient to provide a final protein concentration of 30 to 40 mg/ml. Protein concentration of the suspension was determined by the biuret method (Kabat and Mayer, 1964).

NADPH cytochrome P-450 reductase activity was determined by following the absorbance change at 450 nm (in a Beckman DBGT recording spectrophotometer) when carbon monoxide saturated microsomes were reduced by NADPH in a modification of the method described by Gigon et al. (1968). Carbon monoxide which was deoxygenated by passage through an alkaline dithionite solution (0.05% sodium anthraquinones beta sulfonate and 0.5% sodium dithionite in 0.1 N sodium hydroxide) was bubbled for 5 minutes through a microsomal suspension containing 5 mg of protein per ml of 0.1 M phosphate buffer (pH 7.4). Three ml of the suspension was then transferred to an anaerobic Aminco cell (A1-65085) and a plunger assembly containing 2 umoles of NADPH in 50 ul of buffer

was fitted to the cuvette. Carbon monoxide was passed through the air space within the cell for an additional 3 minutes (diffusion of CO in the suspension was enhanced by use of a small magnetic stirring bar) and placed in a spectrophotometer where it was allowed to equilibrate for 2 minutes at 37° C. The reference cell contained an untreated microsomal suspension of identical protein concentration. The plunger was depressed and the change in absorbance at 450 nm with the rapid appearance of a CO-reduced cytochrome P-450 complex was recorded on chart paper moving at 10 inches per minute. The velocity of the reaction was determined from the slope of the initial linear phase after addition of NADPH. In our experiments, this phase was complete in 6 seconds.

The aminopyrine-enhanced rate of cytochrome P-450 reduction by NADPH was measured, in a manner identical to that just described, except that aminopyrine was added to the microsomal suspension to a final concentration of 1 mM. The protein concentration of the microsomal suspension was again 5 mg per ml.

Serum thyroxin levels were measured to document the degree of thyroid dysfunction and these have been described elsewhere (Aranda and Eade, 1974a).

Student's t test for unpaired data was used for statistical analysis. A level of significance was accepted at $p<0.05$.

RESULTS

Development of substrate and non-substrate enhanced NADPH cytochrome P-450 reductase activity

The rate of reduction of cytochrome P-450 by NADPH with or without aminopyrine added to the microsomes *in vitro* was measured from liver microsomes

obtained from untreated rats from birth to 5 weeks of age and compared to adult rat (10 weeks old). The results are shown in Fig. 1. At birth, negligible activity of non-substrate enhanced NADPH cytochrome P-450 reductase was noted (mean ± SEM = 0.180 ± 0.030 nmoles x min^{-1} x mg^{-1} protein). The activity increased five-fold at five weeks (mean ± SEM = 1.020 ± 0.166 nmoles x min^{-1} x mg^{-1} protein), however, this increased activity was only half of that observed in adult rats (mean ± SEM = 1.954 ± 0.166 nmoles x min^{-1} x mg^{-1} protein).

The addition of 1 mM aminopyrine enhanced the rate of reduction of cytochrome P-450 by NADPH in the 1 and 5 week old but not in the 3 week old immature rat (Fig. 1). Similar enhancement was also noted in the adult rat. The aminopyrine-enhanced NADPH cytochrome P-450 reductase activity at 5 weeks was equivalent to the non-substrate enhanced cytochrome P-450 reductase activity in the adult rat.

Effect of hypothyroidism, chronic hyperthyroidism and neonatal thyroxin therapy on the development of non-substrate enhanced NADPH cytochrome P-450 reductase activity

The effects of these state of thyroid dysfunction on NADPH cytochrome P-450 reductase activity are shown in Table 1. At 3 weeks of age a two-fold and a 3.3 fold increase in NADPH cytochrome P-450 reductase activity was observed in the hypothyroid animals with and without thyroid hormone replacement therapy respectively. A 2.8 fold increase was observed in the group that was chronically treated with thyroxin, and a 3.7 fold increase was observed in the neonatally treated rats. This increased activity of NADPH cytochrome P-450

reductase among the rats with thyroid dysfunction was significantly different ($p < 0.005$) compared to the control group.

At 5 weeks of age, a significant increase was noted only in the hypothyroid rats and in the thyroxin, neonatally treated group as compared with the age-matched control group ($p < 0.05$).

Effect of hypothyroidism, chronic hyperthyroidism and neonatal thyroxin therapy on the development of aminopyrine-enhanced NADPH cytochrome P-450 reductase activity

The effects of these states of thyroid dysfunction on aminopyrine enhanced NADPH cytochrome P-450 reductase activity are shown in Table 2. At 3 weeks, a 1.5 fold and 1.6 fold increase was observed in the hypothyroid rat with and without thyroxin replacement therapy respectively. A 2.3 fold increase was observed in the group that was chronically treated with thyroxin daily. A 2.5 fold increase and the greatest increase was noted in the neonatally treated group. All of these increases in activity of aminopyrine enhanced NADPH cytochrome P-450 reductase activity were significantly different from control group ($p < 0.05$).

At 5 weeks of age, there was a significant decrease in the aminopyrine enhanced NADPH cytochrome P-450 reductase activity in the hypothyroid rat which was reversed by replacement therapy with thyroxin for one week. The increase in activity of aminopyrine enhanced NADPH cytochrome P-450 reductase noted at 3 weeks in the neonatally treated rat was sustained at the same level in the 5 week old neonatally treated rat.

MS. III

Effect of surgical thyroidectomy on NADPH cytochrome P-450 reductase activity

Liver microsomes from adult male rats that were surgically thyroidectomized during the weaning period showed no significant difference from age matched control group as shown in Table 3. However, it is possible that significant changes may have occurred in the interval between surgical thyroidectomy and the time of the assay as noted in adult rats (Suzuki et al., 1967).

Effect of substrate added in vitro on the reduction of cytochrome P-450 by NADPH, in hypothyroid, hyperthyroid and thyroxin-neonatally treated young rats

The effect of 1×10^{-3} M aminopyrine added in vitro on the activity of NADPH cytochrome P450 reductase in euthyroid state and in altered thyroid state is shown in Fig. 2. In the normal rat, a significant increase in NADPH cytochrome P-450 reductase activity was noted at 5 weeks when aminopyrine was added in vitro. Conversely, the hypothyroid rat showed a decrease in NADPH cytochrome P-450 reductase activity when aminopyrine was added in vitro. This decreased noted in the hypothyroid rat was reversed with the administration of replacement doses of thyroxin given for a period of one week.

The rats treated with thyroxin during the neonatal period showed a significant increase in NADPH cytochrome P-450 reductase activity when aminopyrine was added in vitro. In contrast, rats treated with thyroxin chronically (1 ug daily) showed no significant change with the in vitro addition of aminopyrine.

DISCUSSION

The capacity for NADPH reduction of the terminal hemooxygenase increases progressively with advancing post-natal age. The data in this study demonstrate that in the male Sprague-Dawley rat, a full adult complement of NADPH cytochrome P-450 reductase activity is not attained by 5 weeks. This observation contrasts significantly with the data obtained from pigs (Short and Stith, 1973) and rabbits (Fouts and Devereux, 1972) where NADPH cytochrome P-450 reductase approached adult level of activity by approximately 2 weeks of age. Studies in our laboratory using Charles River Long Evans rats showed that adult activity of NADPH cytochrome P-450 reductase is attained at 5 weeks in the male rat and at 4 weeks in the female rat (MacLeod et al., 1972). The discrepancy between the maturational pattern of NADPH cytochrome P-450 reduction previously reported (MacLeod et al., 1972; Fouts and Devereux, 1972; Short and Stith, 1973) and in the present study may be due to species and strain variation.

The in vitro addition of aminopyrine 1 mM concentration to the hepatic microsomes obtained from adult rats significantly enhanced the rate of reduction of cytochrome P-450 by NADPH. These observations are in accord with the findings of Gigon et al. (1969). Similar aminopyrine-enhancement in NADPH cytochrome P-450 reduction was observed in the one week and 5 week old rat but not in the 3 week old rat. The latter observation suggests an age related responsiveness of NADPH cytochrome P-450 reductase to the effect of aminopyrine.

A marked variability in the response of the hepatic microsomal mixed function oxidase system to induction or inhibition by various exogenous and endogenous substrates as a function of age have been reported (Hart, 1962;

Basu, 1971; Aranda, 1974b). Thus, the intrinsic sensitivity of the hepatic microsomal enzymes to the effects of drugs is determined to a large extent by the age at which the drug is administered.

The possible role of the thyroid hormone in the ontogenesis of the hepatic microsomal drug oxidative enzymes and the microsomal electron transport components have been demonstrated by previous studies in our laboratory (Aranda and Eade, 1974a). The effect of thyroid hormone on the development of NADPH cytochrome P-450 reductase is examined in the present study. The data demonstrate that deviation from euthyroid state results in altered activity of NADPH cytochrome P-450 reductase. At three weeks of age the activity of the reductase was increased in the hypothyroid rats. This increase was partly reversed by thyroxin replacement therapy (Table 1). Chronic thyroid treatment and neonatal thyroxin therapy both resulted in significant increase in the reduction of P-450 (Table 1). The observed increases of P-450 reductase in both hypothyroid and hyperthyroid rats contrast to the findings observed in other electron transport components in similar states of thyroid dysfunction (Aranda and Eade, 1974a). Whereas cytochrome P-450 was increased and NADPH cytochrome c reductase was decreased in hypothyroidism, cytochrome P-450 was decreased and NADPH cytochrome c reductase was increased in hyperthyroidism (Aranda and Eade, 1974a). It is possible that the increased reduction of cytochrome P-450 by NADPH in hypothyroidism may reflect the availability of more cytochrome P-450 for reduction. In contrast, the possibility also exists that the increased reduction of cytochrome P-450 by NADPH in hyperthyroidism may reflect the greater effectiveness in the utilization of decreased cytochrome P-450 content for reduction. Thus, although there is a markedly decreased cytochrome P-450 content in chronic thyroid treatment, the

percentage of the total amount of cytochrome P-450 that is effectively reduced for ultimate drug oxidation may be much higher. This possibility may be resolved by the determination of the percentage of P-450 reducible by NADPH for a given time.

Biochemical adaptation to thyroid dysfunction as a function of the duration of the latter may occur. In accordance with observations by Suzuki et al. (1967) wherein some microsomal electron transport components increased after an initial decrease following surgical thyroidectomy, NADPH cytochrome P-450 reductase from liver microsomes of rats treated chronically with thyroxin was equiactive to the reductase of euthyroid control rats at 5 weeks of age. This adaptation to thyroid dysfunction may partly explain the lack of change of NADPH cytochrome c reductase activity in surgically thyroidectomized adult rats (Table 3) compared to euthyroid rats.

Thyroid dysfunction influences the effect of aminopyrine added in vitro to NADPH cytochrome P-450 reductase (Fig. 2). In the normal 5 week old rat, aminopyrine significantly enhanced the reduction of cytochrome P-450 by NADPH. Similar observations were noted in the 5 week old neonatally thyroxin treated rat. In contrast aminopyrine decreased the reduction of cytochrome P-450 by NADPH in the 3 week old hypothyroid rat. These observations suggest that a euthyroid state may be necessary for substrate enhancement to occur. Furthermore, the substrate enhanced cytochrome P-450 reduction is significantly lower in the 5 week old hypothyroid and hyperthyroid rat compared to the euthyroid control rat (Table 2).

The data in this study demonstrate that thyroid hormone significantly influences the development of NADPH cytochrome P-450 reductase. To our knowledge, the study also presents the first determination of the normal age related changes in substrate enhanced P-450 reduction by NADPH.

FIGURE 1. The development of substrate and non-substrate enhanced NADPH cytochrome P-450 reductase activity in male rats.

Liver microsomes were obtained from normal rats and determinations (shown by numbers in parentheses) of NADPH cytochrome P-450 reductase with or without the addition of 1 mM aminopyrine *in vitro* were performed. Values are mean ± SEM.

a: indicates significant difference ($p < 0.05$) between the determinations performed with and without aminopyrine within the same age group.

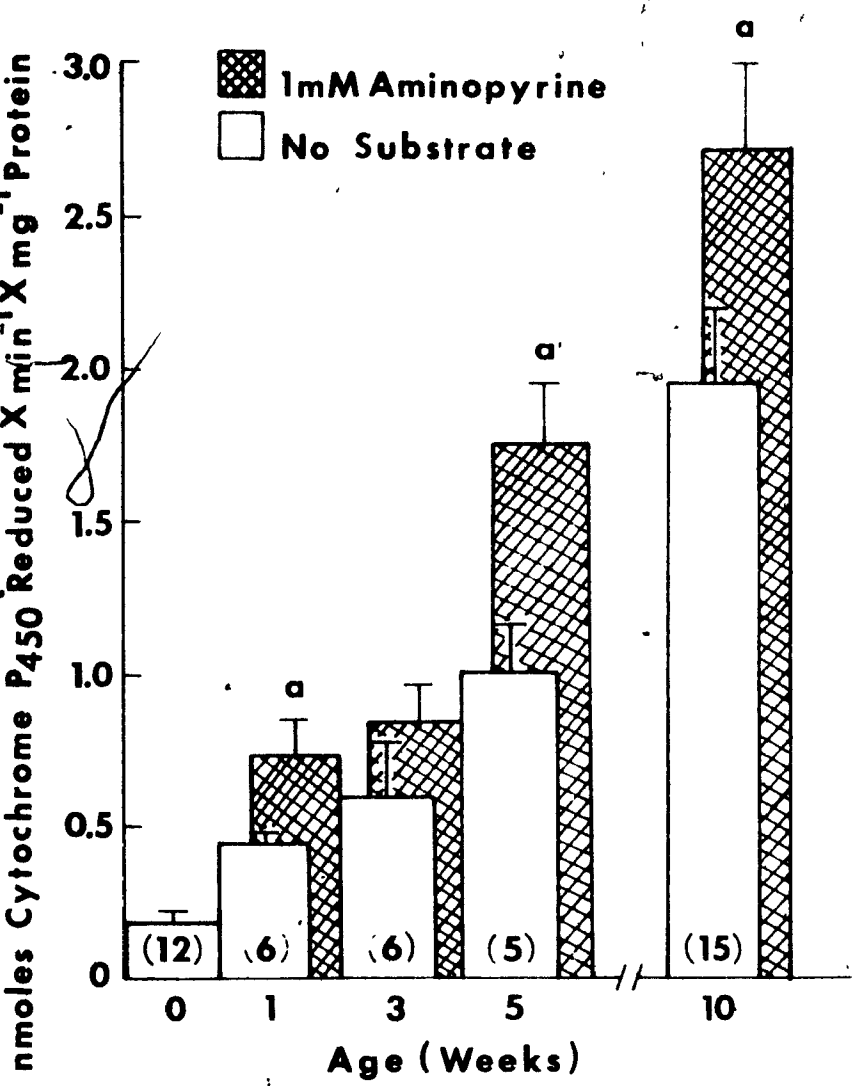

1mM Aminopyrine
No Substrate
nmoles Cytochrome P450 Reduced X min-1 X mg-1 Protein
3.0
2.5
2.0
1.5
1.0
0.5
0
a
a'
a
(12)
(6)
(6)
(5)
(15)
0
1
3
5
10
Age (Weeks)

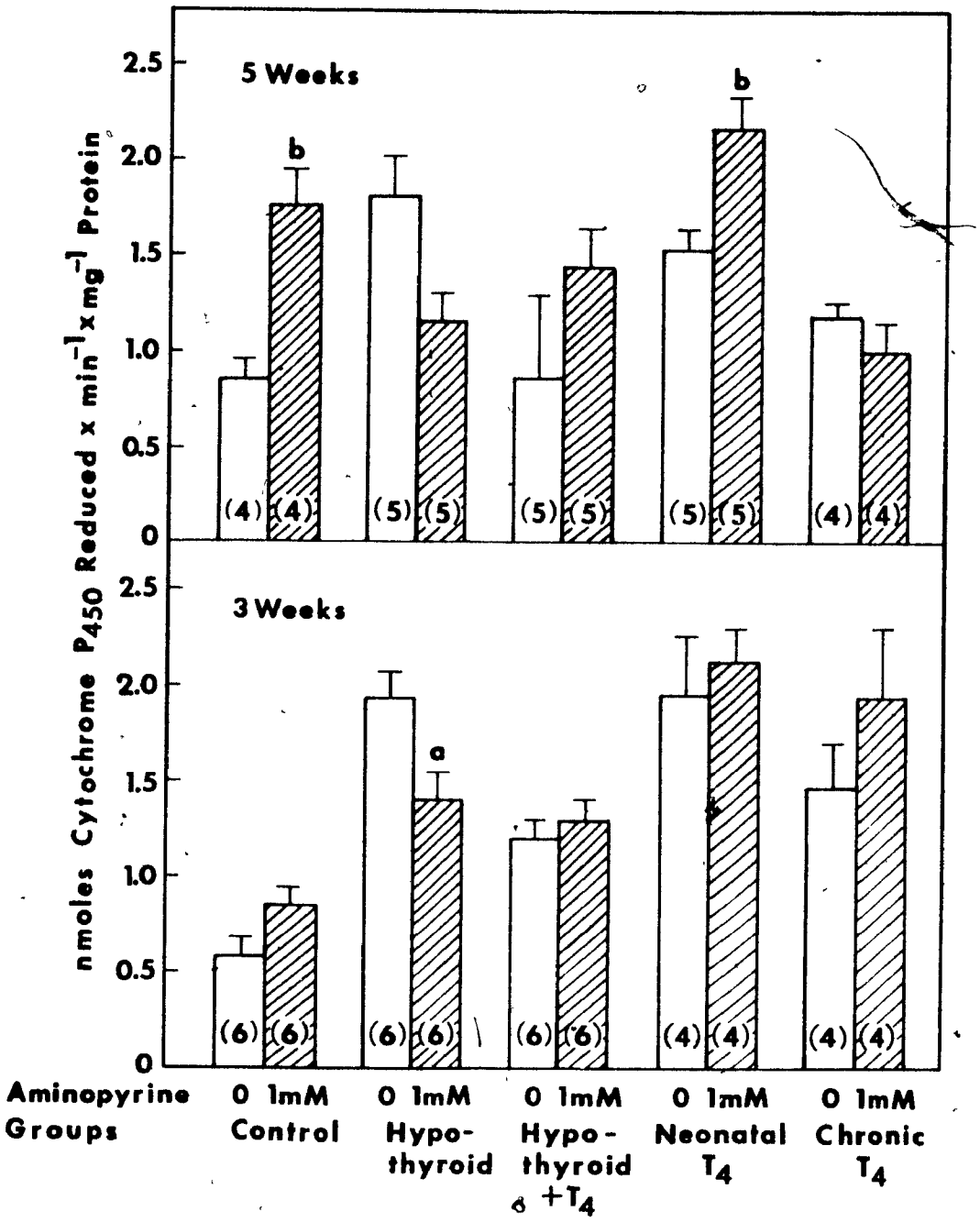
FIGURE 2: The effect of aminopyrine added *in vitro* on NADPH cytochrome P-450 reductase activity in immature male rats with and without thyroid dysfunction.

Hypothyroidism was produced by radiothyroidectomy with ^{131}I at birth. Replacement therapy with L-thyroxin(1ug/g body weight per day for one week) was given to the hypothyroid + T4 group. Treatment with L-thyroxin (100 ug/ day from birth to 5 days of age) was given to the neonatal T4 group. Chronic hyperthyroidism (chronic T4 group) was produced by daily subcutaneous injection of L-thyroxin 1 ug/g body weight per day.

Values are mean ± SEM and numbers in parentheses indicate individual determinations.

a: indicates $p < 0.05$ and

b: indicates $p < 0.01$ between the determinations with and without aminopyrine addition *in vitro* within the same group.


5 Weeks
3 Weeks
nmoles Cytochrome P_{450} Reduced x min^{-1} x mg^{-1} Protein
2.5
2.0
1.5
1.0
0.5
0
b
a
(4) (4) (5) (5) (5) (5) (5) (5) (4) (4)
(6) (6) (6) (6) (6) (6) (4) (4) (4) (4)
Aminopyrine
0 1mM
0 1mM
0 1mM
0 1mM
0 1mM
Groups
Control
Hypo-thyroid
Hypo-thyroid +T_4
Neonatal T_4
Chronic T_4

MS.III

TABLE 1: The effect of hypothyroidism, chronic hyperthyroidism and neonatal thyroxin therapy on non-substrate enhanced NADPH cytochrome P-450 reductase activity in young rats.

AGE	GROUP	CYTOCHROME P-450 REDUCED x min^{-1} x mg^{-1} protein
1 week	Control	0.439 ± 0.044 (6)
3 weeks	Control	0.585 ± 0.110 (6)
	Hypothyroid	1.927 ± 0.159 (6)[b]
	Hypothyroid T4	1.202 ± 0.099 (6)[b]
	Neonatal T4	2.145 ± 0.308 (5)[b]
	Chronic T4	1.627 ± 0.230 (5)[b]
5 weeks	Control	1.020 ± 0.166 (5)
	Hypothyroid	1.677 ± 0.220 (6)[a]
	Hypothyroid T4	0.885 ± 0.466 (6)
	Neonatal T4	1.538 ± 0.086 (5)[a]
	Chronic T4	1.168 ± 0.056 (5)
10 weeks	Control	1.954 ± 0.166 (15)

a: $p < 0.05$; b: $p < 0.01$, compared to age-matched control.

TABLE 2: The effect of hypothyroidism, chronic hyperthyroidism and neonatal thyroxin therapy, on substrate enhanced reduction of cytochrome P-450 in young rats.

AGE	GROUP	CYTOCHROME P-450 REDUCED x min^{-1} x mg^{-1} protein
1 week	Control	0.733 ± 0.119 (6)
3 weeks	Control	0.850 ± 0.102 (6)
	Hypothyroid	1.399 ± 0.144 (6)[a]
	Hypothyroid T4	1.297 ± 0.110 (6)[a]
	Neonatal T4	2.121 ± 0.341 (4)[b]
	Chronic T4	1.945 ± 0.348 (4)[a]
5 weeks	Control	1.757 ± 0.200 (4)
	Hypothyroid	1.151 ± 0.135 (5)[a]
	Hypothyroid T4	1.448 ± 0.191 (5)
	Neonatal T4	2.178 ± 0.206 (5)
	Chronic T4	1.011 ± 0.155 (4)[a]
10 Weeks	Control	2.720 ± 0.313 (9)

a: $p < 0.05$; b: $p < 0.01$, compared to control group of similar age.

MS.III

TABLE 3: The reduction of cytochrome P-450 in surgically thyroidectomized adult rats.

Rats were thyroidectomized at age 4 weeks (post-weaning) and allowed to survive until age 10 weeks. Values represent mean ± SEM. Numbers in parentheses indicate number of rats individually studied.

Group	Cytochrome P-450 reduced x min^{-1} x mg^{-1} protein
Control	1.954 ± 0.166 (15)
Thyroidectomized	2.103 ± 0.369 (6)

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MANUSCRIPT IV

AGE DEPENDENT RESPONSE OF HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE SYSTEM TO THYROXIN

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MS. IV

ABSTRACT

To determine whether postnatal age exerts quantitative and qualitative (induction or inhibition) effects in the response of hepatic microsomal mixed function oxidase system (HMMFO) to drugs, thyroxin (T4), known to induce enzyme maturation, was given in equivalent doses of 1 ug/g/day for 3 days to male rats of varying ages (birth to 10 weeks) followed by measurements of the component enzymes of HMMFO and substrate oxidations. Untreated rats of corresponding ages served as controls. The results show varying responses of HMMFO to T4 as a function of age. T4 decreased aminopyrine N-demethylase activity at 1-3 weeks, had no effect at 4 weeks, and induced the same enzyme at 5-10 weeks. Opposite effects were noted with aniline p-hydroxylase, where induction was noted from 3 days to 4 weeks followed by inhibition at 5-10 weeks. Induction of NADPH oxidase was noted only at ages 3 days and 4 weeks. NADPH cytochrome c reductase was highly inducible at all ages except at age 3 weeks. Cytochrome P-450 was inhibited at all ages. The data show that the enzyme titres achieved in response to T4 is dependent on age. Where induction is desirable the effectiveness of induction may depend on the age at which the inducer is administered.

MS.IV

INTRODUCTION

Age is a major determinant in the response of an organism to drugs and other substrates as evidenced by increased sensitivity of the developing fetus and newborn infant to the effects of many pharmacologic agents (Nyhan, 1961, Done, 1964, Goldenthal, 1971) compared to adults. Furthermore, age also appears to be a major determinant in the quantitative and perhaps qualitative response to an inducing agent (Hospador and Manthei, 1968, Hospador et al., 1971). The quantitative difference in the response of the microsomal drug metabolizing systems as a function of age is evidenced by 50 to 200% greater inducibility of biphenyl-4-hydroxylase, biphenyl-2-hydroxylase, p-nitrobenzoate reductase and cytochrome P-450 by phenobarbital in 12 day old rat compared to 52 day old rats (Basu et al., 1971). Similar age-related responsiveness to phenobarbital has been demonstrated with a greater induction of aminopyrine N-demethylation, hexobarbital hydroxylation, aniline p-hydroxylation and p-nitrobenzoate reduction in 40 day old rats compared to older animals (Kato and Takanaka, 1968), thus suggesting that immature rats are intrinsically more responsive to enzyme induction.

Within the period of development, wherein several biochemical adaptive changes occur, there is a probable augmented variability in the response of drug metabolizing enzyme system to induction. Oxidative enzymes for hexobarbital and aminopyrine in fetal rabbits

studied 4 to 8 days prior to term were refractory to phenobarbital induction, whereas these same enzymes were readily inducible after birth (Hart et al., 1962). These observations imply that the effectiveness of induction markedly depends upon the age at which the inducing agent is administered.

Previous studies involving the ontogenesis of the hepatic microsomal drug metabolizing enzymes in our laboratory (Aranda and Eade, 1974) have provided evidence that thyroid hormone induces the maturation of NADPH cytochrome c reductase and the differentiation of aniline p-hydroxylase in the male rats. Suggestive evidence of an age-related effect of thyroxin on the hepatic microsomal drug oxidative enzymes was also demonstrated. Male rats treated with thyroxin during the neonatal period showed no effect on NADPH cytochrome c reductase by an additional dose of thyroxin at 3 weeks whereas a superinduction was noted by an equivalent dose of thyroxin at 5 weeks indicating that the magnitude of the response to thyroxin is dependent upon the age at which thyroxin was administered. The decrease in aminopyrine N-demethylase activity by thyroxin administered at 3 weeks and the slight increased activity of the same enzyme at 5 weeks (Aranda and Eade, 1974) suggested that thyroxin may exert converse effects (induction or inhibition) depending upon the age at which it was administered. This study was performed to define the quantitative and qualitative (inhibition or induction) effects of thyroxin on substrate oxidation and the various components of the hepatic microsomal drug oxidative enzymes, as a function of postnatal age.

MS. IV

MATERIALS AND METHODS

Male Sprague-Dawley rats obtained from Canadian Breeding Farms (St. Constant, Que.) were used in this study. Adult male rats were at least 10 weeks of age and weighed between 300-340 grams. Female adult rats were bred at the Canadian Breeding Farms and brought to the laboratory on the day of parturition at which time the male litters were segregated with their mothers and were used for the experiments. Mothers and litters were maintained at the McGill University McIntyre Animal Centre and in all cases the litters were weaned at 21 days of age. The diet for the nursing mothers and for the weaned litters consisted of Purina Lab Rat chow and water ad libitum.

To determine the influence of age on the effect of thyroxin on substrate oxidations and on the components of the microsomal electron transport chain, male rats of various ages were given the same subcutaneous dose of L-thyroxin (Sigma, St. Louis, Mo.) 1 ug/g body weight per day for three days prior to sacrifice. L-thyroxin was dissolved in 0.01 N NaOH and the concentration was adjusted so that in all experiments the volume injected ranged from 0.05 ml to 0.1 ml to rats from birth to 3 weeks of age, 0.1 ml to 0.3 ml from 3 weeks to 5 weeks of age and 0.5 ml in adults. These rats were sacrificed at ages 3 days, 1, 2, 3, 4, 5 and 10 weeks. Untreated rats matched according to age served as controls. Since

initial experiments in our laboratory showed no effect on the parameters studied using subcutaneously administered 0.01 NaOH at the volumes injected, all of the control values were obtained from untreated rats of corresponding ages, matched with the thyroxin treated group.

To determine a possible difference in the sensitivity of the drug metabolizing enzymes to thyroxin between the young and the adult animal, a dose response relationship was done using doses varying from 0.1 ug/g body weight to 10 ug/g body weight/day administered for three days prior to sacrifice to one week old and 10 week old rats.

To determine further the difference in the response of the drug metabolizing enzymes to thyroxin between the young and the adult animal as a function of the duration of thyroid hormone treatment, L-thyroxin at a dose of 1 ug/g body weight/day was given to one week old and 10 week old rats for a period varying from 1 to 7 days prior to sacrifice.

In all experiments on the immature animals one or more pups were randomly selected from different litters of the same treatment group so that at any given age the mean value was derived from a number of individual determinations. Each determination performed in the newborn rats represents a single value derived from the livers of four littermates pooled prior to homogenization. At one and two weeks of age, livers from two to three littermates were pooled and

thereafter individual livers were used.

Animals of desired age were sacrificed by decapitation followed by exsanguination. Livers were immediately excised and homogenized on ice cold 0.1 M phosphate buffer, pH 7.4 using 4 volumes per unit weight for 30 seconds in a high speed Sorvall Omni-Mixer (Sorvall Co., Conn). All subsequent procedures were carried out between $0^{o}C$ and $4^{o}C$.

The homogenate was centrifuged at 10,000 x g for 10 minutes in a refrigerated centrifuge. The 10,000 x g supernatant was carefully removed avoiding any contamination by the loosely packed pellet at the bottom or the fatty layer at the top of the centrifuge tube. The 10,000 x g supernatant was centrifuged at 100,000 x g for 60 minutes in a Beckman L3-40 refrigerated ultracentrifuge. The microsomal pellet obtained was separated from its glycogen layer and resuspended in ice cold buffer using a glass homogenizer with a Teflon pestle (Arthur H. Thomas Co., Philadelphia, Pa.) and a volume of buffer sufficient to provide a final protein concentration of 30 to 40 mg per ml. Protein concentration of the suspension was determined by the biuret method (Kabat and Mayer, 1967).

NADPH oxidase activity was determined by following the absorbance change of NADPH at 340 nm by the method described by Gillette, Brodie and LaDu (1957). NADPH cytochrome c reductase activity was determined by following the absorbance change at 550 nm reflecting the appearance of reduced cytochrome c after addition of NADPH as described by Phillips and Langdon (1962). Cytochrome P-450

concentration was determined by the method of Omura and Sato (1964) with a microsomal suspension containing 5 mg of protein per ml.

The activity of aminopyrine N-demethylase was determined by measuring the production of formaldehyde from aminopyrine as described by Cochin and Axelrod (1959). The reaction mixture contained 0.1 ml of microsomal suspension, 36 µmol of $MgCl_2$, 24 µmol of neutralized semicarbazide HCl, 40 µmol of nicotinamide, 0.66 µmol of $NADP^+$, 16 µmol of sodium isocitrate, 0.5 unit of isocitric dehydrogenase and 10 µmol of aminopyrine in a final volume of 2.0 ml of 0.1 M phosphate buffer (pH 7.4). Nicotinamide has been reported to inhibit the microsomal N-demethylation of aminopyrine (Schenkman et al., 1967) but in our experiments addition of amounts up to 100 µmol had no such effect.

The activity of aniline p-hydroxylase was determined using the above reaction mixture minus semicarbazide and with 10 µmol of aniline substituted for aminopyrine. The production of p-aminophenol was measured as described by Kato and Gillette (1965).

Difference spectra for aniline were determined using the method of Schenkman et al., 1967.

Serum thyroxin levels were measured by competitive protein binding as described by Murphy and Pattee (1966).

Students' test for unpaired data was used for statistical analysis. A significant difference was accepted at $p < 0.05$.

RESULTS

Influence of age on the effect of thyroxin on type I and type II substrate oxidation

L-thyroxin administered at the same dose (1 ug/g body weight/day for 3 days) to rats of various ages produced opposite effects on substrate oxidations as a function of age (Fig. 1). Thyroxin significantly decreased the activity of aminopyrine N-demethylation at 1 to 3 weeks, did not affect the same oxidative enzyme at 4 weeks, and significantly increased it at 5 and 10 weeks. Conversely, thyroxin significantly increased the activity of aniline p-hydroxylase from birth up to 4 weeks and then decreased the same enzyme at 5 and 10 weeks. When plotted as percentage difference from age-matched controls as a function of age (Fig. 1) the opposite effects of thyroxin in aminopyrine N-demethylation and aniline p-hydroxylation was demonstrated.

Influence of age on the effect of thyroxin on liver weight and microsomal protein yield

A significant decrease in the liver weights of thyroxin treated rats was observed as shown in Table 1. This decrease in the liver weight was observed at all ages except at 3 days. This decrease in the liver weight within a 3 day period and an almost negligible

glycogen deposit in the 100,000 x g pellet suggest that most of the decrease in liver weight is accounted for by the increased breakdown of glycogen by thyroxin. Indeed, it has been shown that there is a complete absence of glycogen in thyrotoxicosis (Kimberg 1971, Coggeshall and Greene, 1933, Pipher and Paulsen, 1947). The depletion of glycogen may also explain why thyroxin did not change the liver weight at age 3 days. At birth most of the glycogen is utilized by the newborn to maintain normoglycemia upon withdrawal of maternal glucose supply (Adams, 1971, Senior, 1973). Thyroxin could not deplete what is not available. The effect of thyroid hormone on the microsomal protein yield as a function of age was not as remarkable as its effect on the liver weight. There was a significant increase in the microsomal protein yield per gram liver at 2 and 3 weeks only (Table 1). This variation may reflect the differences in the rate of protein synthesis as a function of age (Winick and Noble, 1965, Winick and Noble, 1966, Yousef and Johnson, 1970, Waterlow and Stephen, 1968, Young et al., 1974). This may also account, in part, for the alteration in liver weight seen in these experiments.

Influence of age on the effect of thyroxin on microsomal electron transport

Thyroxin administered for 3 days to rats of various ages resulted in changes as shown in Table 2. NADPH oxidase activity was significantly increased at 3 days and 4 weeks. NADPH cytochrome c reductase activity was significantly increased at all ages except at

3 days and at 3 weeks where it appeared to be refractory to the effect of thyroxin. The terminal hemoxygenase cytochrome P-450 was significantly decreased at all ages.

The effect of various doses of thyroxin on cytochrome P-450 content, NADPH cytochrome c reductase activity and aminopyrine N-demethylase activity

Table 3 shows the comparison in the response of cytochrome P-450, NADPH cytochrome c reductase and aminopyrine N-demethylase activity to varying doses of thyroxin in immature and adult rats. In the one week old rats a significant decrease in the cytochrome P-450 content was only demonstrated with the 1.0 mg/kg thyroxin dose whereas the adult rat showed a significant decrease in the hemoprotein with thyroxin doses of 0.1 mg/kg to 10.0 mg/kg. This finding indicates that the adult rat cytochrome P-450 is relatively more sensitive to thyroxin effect. NADPH cytochrome c reductase was significantly increased in all doses of thyroxin used indicating equisensitivity of NADPH cytochrome c reductase of both ages to thyroxin effect. The immature rats showed a decreased aminopyrine N-demethylation with 0.1 mg/kg and 1.0 mg/kg dose of thyroxin but no significant change at a high dose (10.0 mg/kg). In contrast, the adult rat showed an increase in aminopyrine N-demethylation with 0.1 mg/kg and 1.0 mg/kg but no significant change at a high dose (10.0 mg/kg). This dose related effect of thyroxin has been shown to occur with

the erythrocyte 2,3-diphosphoglyceric acid synthesis (Snyder et al., 1970).

Effect of age and thyroxin on aniline difference spectra

The opposite effect of aminopyrine N-demethylation (a type I substrate oxidation) and aniline p-hydroxylation (a type II substrate oxidation) suggested the possibility of substrate-cytochrome P-450 binding interaction. Type II substrates such as aniline exhibit a difference spectrum which is a "mirror-image" of a type I substrate such as aminopyrine (Schenkman et al., 1967). The former inhibits mixed function oxidase activity whereas the latter stimulates its own metabolism and enhances the reduction of cytochrome P-450 by NADPH (Gigon et al., 1968). Furthermore, aminopyrine, a type I substrate in the adult human and rat microsome (Pelkonen, 1973, Schenkman, 1967) exhibits a type II difference spectrum in the fetal human microsome (Yaffe, 1970). It seemed possible that aniline acts as a type I compound enhancing its own oxidation in the neonatal period and then reverting to a classic type II spectrum later in the adult period. To test this possibility, aniline difference spectra were performed on the hepatic microsomes of immature (one week old) and adult (10 week old) rat with and without pretreatment with thyroxin. The difference spectra shown in Fig. 2, show that aniline exhibits a type II spectrum in both the immature and adult rat with an apparent decrease in the absorbance change in the immature rat, a finding in

accord with Eling et al., 1970. Thyroxin pretreatment resulted in an obliteration of the observed aniline difference spectrum in the immature animal and in a decreased absorbance change in the adult animal. The latter observation is in accord with the decreased Δ-OD max of aniline in thyroxin treated adult rats shown by Kato (1968).

Influence of the duration of thyroxin therapy on NADPH cytochrome c reductase, cytochrome P-450 and aminopyrine N-demethylation in immature and adult male rats

One week and ten week old rats were pretreated with thyroxin 1 ug/g body weight per day one to seven days prior to sacrifice. The percentage difference in NADPH cytochrome c reductase activity, cytochrome P-450 content and aminopyrine N-demethylation over age matched nontreated control group was plotted as a function of the duration of thyroxin treatment as shown in Figs. 3, 4 and 5 respectively. In the one week old rat, NADPH cytochrome c reductase activity (Fig. 3) rose significantly after one day of therapy but appeared to reach lower levels compared to the adult after one week of thyroxin treatment. In contrast, the adult rat showed an initial slow rise with a significant increase over the non-thyroxin control group at 3 days followed by a rapid increase thereafter to levels greater than the young rat. In the immature one week old rat cytochrome P-450 content was significantly increased after one day therapy followed by a significant decrease in this hemoprotein concentration after

3 days which persisted up to seven days. In contrast, the adult rat showed an immediate 50% fall occurring during the first day of thyroxin treatment and remained at that decreased level throughout the seven days of thyroxin treatment (Fig. 4). In the young one week old rat, aminopyrine N-demethylation was decreased after 2 days of thyroxin therapy reaching a nadir at 3 days (-50% of control value) then appeared to increase to near control values after 7 days of treatment. In contrast, the adult animal showed an initial increase in aminopyrine N-demethylase activity with a peak at three days followed by a fall below control values after 5 days of thyroxin therapy and a significant decrease after 7 days.

DISCUSSION

The well known altered sensitivity of the newborn infant and immature animals to drugs has been attributed to a great extent to their altered capability to handle drugs as exemplified by differences in protein binding (Krasner, 1973), drug distribution (Kufferberg and Way, 1965), decreased hepatic oxidative and conjugative biotransforming capacity (Aranda et al., 1974, Fouts and Adamson, 1959, Yaffe et al., 1970, Pelkonen et al., 1974, Weiss et al., 1960) and decreased renal excretion of drugs (Barnett et al., 1949, McCracken et al., 1973). It has also been shown that the immature rat has a greater tolerance to the effect of thyroid hormone (Bodansky and Duff, 1936). The data in this study support this observation and also show that there is a marked difference between the response of an immature and an adult

animal to the effect of thyroxin on the hepatic microsomal drug oxidizing enzymes. The data further demonstrate that within the 5 week postnatal period, dynamic variations on the responsiveness of the hepatic microsomal enzymes to thyroxin occur. This is best illustrated by the opposite effect of thyroxin on hepatic microsomal aminopyrine N-demethylase and aniline p-hydroxylase activity as a function of age (Fig. 1). This observation appears to be independent of substrate - cytochrome P-450 interaction as suggested by the same type II difference spectra obtained with aniline added to the liver microsomes from immature and adult rat with or without thyroxin therapy (Fig. 2). This difference in response to thyroxin could not be accounted for by changes in the electron transport components due to thyroxin treatment, since there was no observed parallel changes between electron transport component and substrate oxidation. For instance, there was a decrease in cytochrome P-450 varying from 30% to 60% throughout the first 5 weeks of age and in the adult period after thyroxin treatment. On the other hand, aniline p-hydroxylation was significantly increased during the first 4 weeks of age followed by a decrease during 5 and 10 weeks of age. This change did not parallel the observed effect of thyroxin on the electron transport components.

NADPH cytochrome c reductase is a flavoprotein (Williams and Kamin, 1962) and its activity is dependent upon the state of thyroid function since its metabolism is regulated by the thyroid hormone (Rivlin and Langdon, 1966, Rivlin et al., 1968). However, even the influence of thyroid hormone appears to be dependent upon age since

the 3 week old male rat was relatively insensitive to the influence of thyroid hormone (Table 2). A significant induction of NADPH cytochrome c reductase by thyroxin was noted at all ages studied, except at age 3 weeks. It appears that marked differences in response to thyroxin may occur at this age which is coincident with the weaning period. The reason for this differences in the response to thyroxin is not yet established. However the weaning period seems to be a transition from the neonatal pattern to the adult pattern of response to thyroxin of both aniline p-hydroxylation and aminopyrine N-demethylation (Fig. 1).

In accord with the observation of Phillips and Langdon (1956) this study demonstrates that in the adult rat, there is an initial slow increase followed by a rapid increase in NADPH cytochrome c reductase activity during the first week as a function of the duration of thyroxin treatment. In contrast to the adult rat, the immature animal responds much earlier and appears to plateau at a lower level (Fig. 3). The difference between the young and the adult animal's response to thyroxin as a function of the duration of treatment is further illustrated by the effect of thyroxin treatment on cytochrome P-450 and aminopyrine N-demethylase in the young and adult animal (Fig. 4 and 5). In the young rat, thyroxin treatment produced an initial increase in the cytochrome P-450 content followed by a decrease with continued thyroxin treatment. In contrast, thyroxin treatment produced an immediate decrease in cytochrome P-450 content in the adult rat (Fig. 4). This implies that in comparing the age-related response

of the hepatic microsomal mixed function oxidase system, the duration of treatment may be a determining factor to the quantitative or qualitative changes observed. Although the magnitude or degree of quantitative change may be partly dose-related (Table 3), the qualitative change (inhibition or induction) as a function of age appears to be not dose-related (Table 3).

Although age as a major determinant in the host response to drug is generally accepted and is underscored in several reviews (Done, 1964, Sereni and Principi, 1968, Yaffe, 1974, Nyhan, 1964, Weinstein, 1971) the fundamental mechanism underlying the age dependent responsiveness to an inducing agent remains unsettled. The failure to induce hepatic microsomal drug-metabolizing enzymes by phenobarbital few days prior to birth (Hart et al., 1962) and the ready inducibility of these same enzymes after birth suggest that the machinery for enzyme synthesis is a prerequisite for the activation of enzyme synthesis (Greengard, 1971). Postnatally, however, it appears that the required co-factors for hepatic microsomal drug oxidation are present (Fouts et al., 1959) and the progressive increase in the synthesis of these enzymes (Dallner et al., 1966) occurring after birth indicates that the machinery for the synthesis of microsomal enzymes are developed. This argument does not explain the decrease responsiveness of the microsomal enzymes at a certain period postnatally such as that observed in the lack of effect of thyroxin on NADPH cytochrome c reductase at age 3 weeks. One fundamental cellular function that may change with age is the rate of protein synthesis (Young et al., 1975,

Winick and Noble, 1965, Waterlow and Stephen, 1968, Yousef and Johnson, 1970). The rate of protein synthesis changes as a function of age and it is possible that a specific enzyme may have varying rates of synthesis and degradation that changes with age. This would result in quantitative variations in the response of an enzyme system to a specific inducing agent, that is, induction when protein synthesis is maximal may result in maximal response to the inducing agent. To our knowledge, enzyme turnover studies of hepatic microsomal enzymes as a function of age and in the presence of inducing agents have not yet been done.

Clinical applications of age-related inducibility of enzyme systems have been demonstrated in steroid induction of other organ systems such as the lungs (Liggins and Howie, 1972, Baden et al., 1972). Induction of pulmonary surfactant to prevent hyaline membrane disease in newborn infant occurred when steroids were administered before 32 weeks gestation and induction was not successful when steroids were given later than 32 weeks gestation (Liggins et al., 1972) or after birth (Baden et al., 1972).

The data in this study demonstrate that the microsomal enzyme titres achieved in response to thyroxin is markedly dependent on the age at which thyroxin was administered.. Furthermore the response of the hepatic microsomal enzymes as a function of the duration of treatment differs between the immature and the adult rats. This quantitative difference in response is partly dose-dependent, and appears to be unrelated to substrate-cytochrome P-450 interaction.

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FIGURE 1: Influence of age on the effect of thyroxin on microsomal substrate oxidation.

Rats were subcutaneously injected with L-thyroxin 1 ug/g body weight/day for three days prior to sacrifice. The points represent percentage change from the control value derived from the mean of 6 to 16 individual determinations. The significance of the difference from the age-matched control group is indicated by (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$.

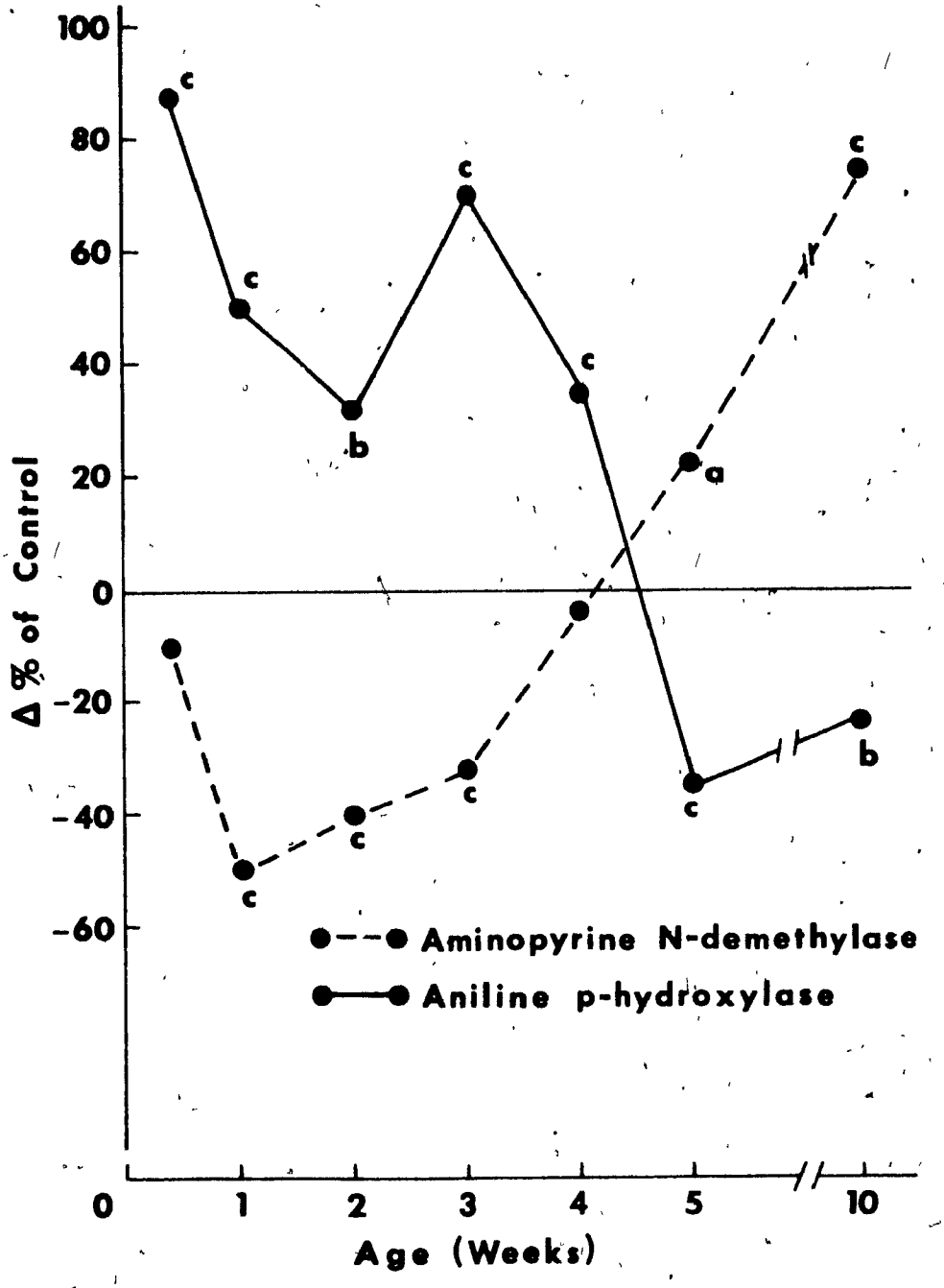

100
80
60
40
20
0
-20
-40
-60
Δ % of Control
0
1
2
3
4
5
10
Age (Weeks)
Aminopyrine N-demethylase
Aniline p-hydroxylase
a
b
c

FIGURE 2: The difference spectra of aniline and cytochrome P-450 in the liver microsome from immature and adult rats with and without treatment with thyroxin.

Liver microsomes were obtained from one week old and ten week old rats treated and not treated with thyroxin. Thyroxin was administered subcutaneously at a dose of 1 ug/g body weight/day for three days prior to sacrifice. Control non-thyroxin treated group received the diluent equal to the amount given in the thyroxin treated group. Aniline 3 mM concentration was added to the cuvette containing 2 mg/ml of microsomal protein.

BINDING SPECTRA - ANILINE 3.0mM

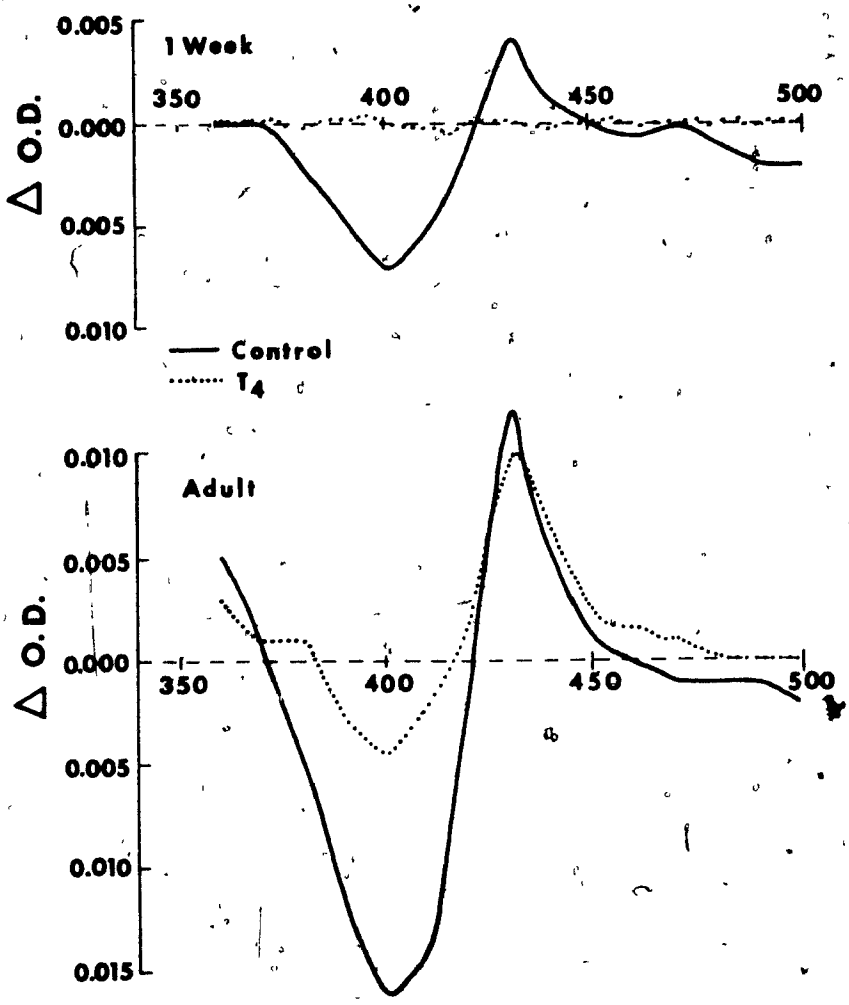


FIGURE 3: Influence of age and duration of thyroxin therapy on the activity of NADPH cytochrome c reductase.

One week old and ten week old rats were given L-thyroxin 1 ug/g body weight/day for one to seven days prior to sacrifice. Untreated rats served as controls. The points represent the percentage change from the control value derived from the mean of 6 to 8 individual determinations. Control values are: one week old rat = 46.05±1.75 nmoles cytochrome c reduced x min^{-1} x mg^{-1} protein; ten week old rat = 95.08±8.55 nmoles x min^{-1} x mg^{-1} protein. The significance of the change from the age-matched control is indicated by (a) $p < 0.01$, (b) $p < 0.001$.

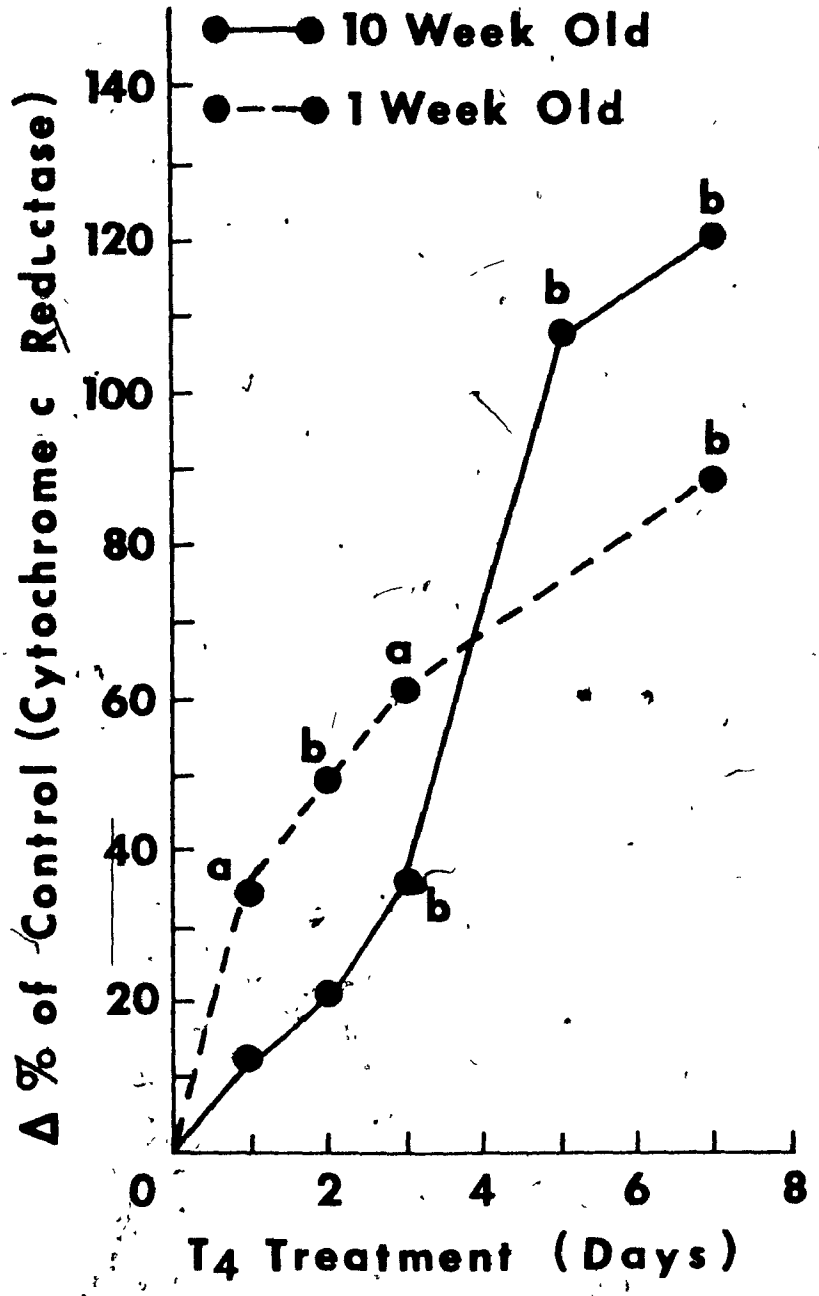

10 Week Old
1 Week Old
Δ % of Control (Cytochrome c Reductase)
140
120
100
80
60
40
20
0
2
4
6
8
T4 Treatment (Days)
a
b
b
b
a
b
b

FIGURE 4: The influence of age and duration of thyroxin therapy on the amount of hepatic microsomal cytochrome P-450.

One week old and ten week old rats were given L-thyroxin 1 ug/g body weight/day for one to seven days prior to sacrifice. Untreated rats served as controls. The points represent the percentage change from the control value derived from the mean of 5 to 8 individual determinations. Control values are: one week old rat = 0.190±0.013 nmoles x mg^{-1} protein, ten week old rat = 0.452±0.028 x mg^{-1} protein. The significance of the change from the age-matched control is indicated by (a) $p < 0.01$, (b) $p < 0.001$.

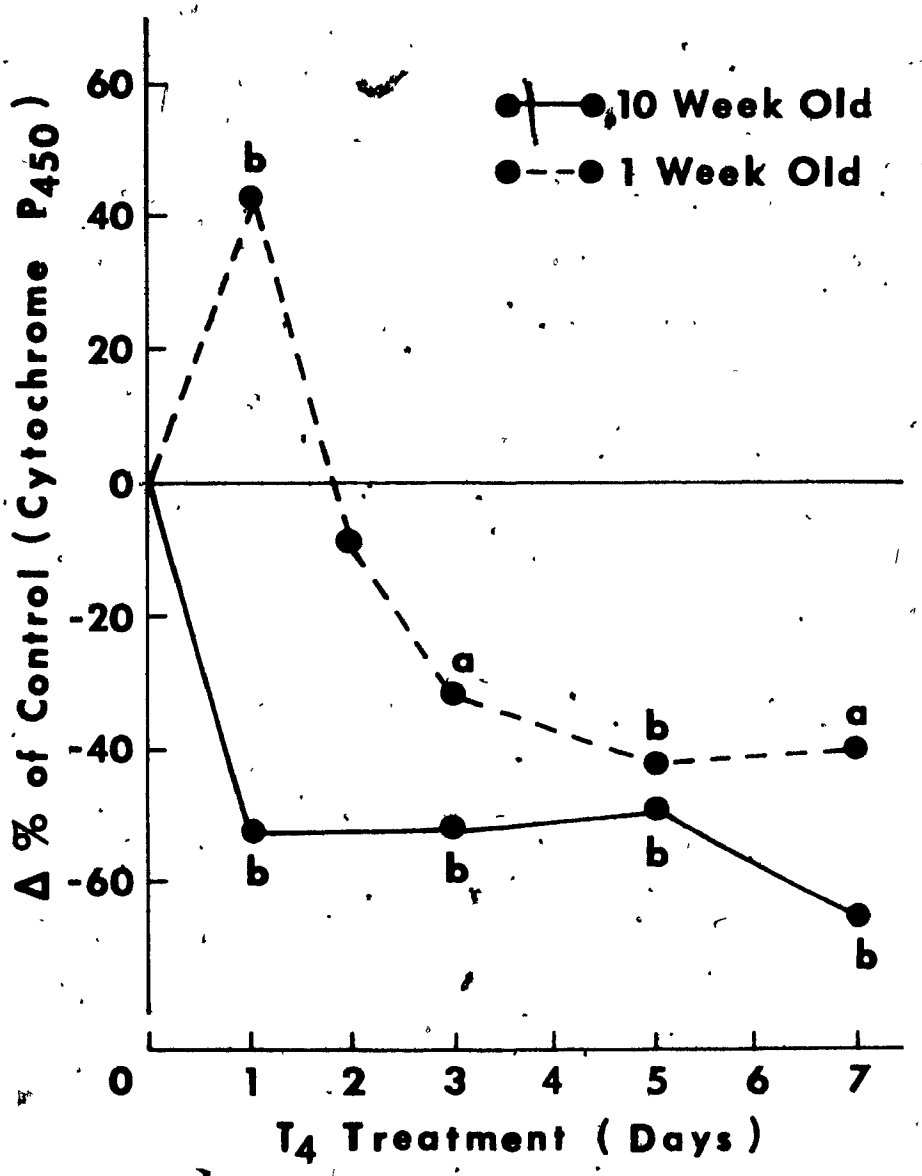
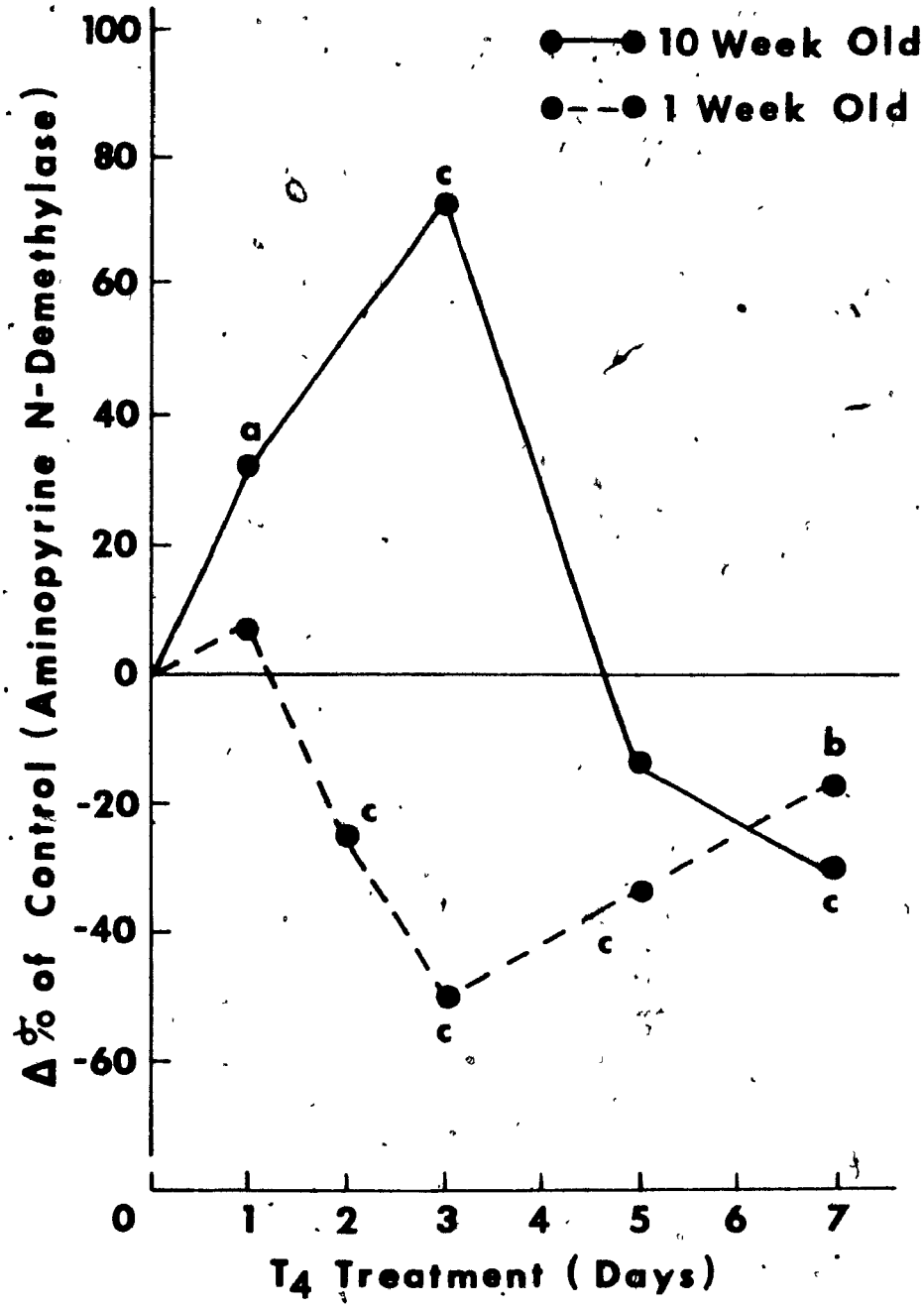

10 Week Old
1 Week Old
Δ % of Control (Cytochrome P450)
60
40
20
0
-20
-40
-60
b
a
b
a
b
b
b
b
0
1
2
3
4
5
6
7
T4 Treatment (Days)

FIGURE 5: The influence of age and duration of thyroxin therapy on the activity of hepatic microsomal aminophyrine N-demethylase.

One week old and ten week old rats were given L-thyroxin 1 ug/g body weight/day for one to seven days prior to sacrifice. Untreated rats served as controls. The points represent the percentage change from the control value derived from the mean of 6 to 8 individual determinations. Control values are: one week old rats = 0.83±0.08 nmoles HCHO formed x min^{-1} x mg^{-1} protein. Ten week old rats = 2.52 ± 0.15 nmoles HCHO formed x min^{-1} x mg^{-1} protein. The significance of the change from the age-matched control group is indicated by (a) $p < 0.05$, (b) $p < 0.01$, (c) $p < 0.001$.


10 Week Old
1 Week Old
Δ% of Control (Aminopyrine N-Demethylase)
100
80
60
40
20
0
-20
-40
-60
0
1
2
3
4
5
6
7
T4 Treatment (Days)
a
c
c
c
c
c
b
c

MS. IV

TABLE 1

Influence of age on the effect of L-thyroxin on liver weight and microsomal protein yield.

Rats were given L-thyroxin 1 ug/g body weight daily for 3 days prior to the experiments. Values are mean ± SEM of individual determinations. Numbers in parentheses indicate number of individual determinations.

Age	Groups	Liver wet weight (gram)	Microsomal protein mg/g wet weight liver
3 days	Control	0.36±0.01 (20)	33.81±2.96 (6)
	L-thyroxin	0.37±0.01 (25)	22.62±1.65 (6)[a]
1 week	Control	0.52±0.01 (24)	31.71±1.25 (15)
	L-thyroxin	0.39±0.01 (18)[b]	30.72±1.62 (6)
2 weeks	Control	0.96±0.01 (30)	30.43±1.59 (16)
	L-thyroxin	0.63±0.01 (18)[b]	36.55±2.07 (6)[a]
3 weeks	Control	2.38±0.10 (20)	30.51±0.71 (10)
	L-thyroxin	1.33±0.08 (12)[b]	49.69±2.36 (7)[b]
4 weeks	Control	4.38±0.15 (6)	37.18±1.48 (6)
	L-thyroxin	3.13±0.07 (7)[b]	41.04±2.43 (6)
5 weeks	Control	6.93±0.16 (10)	39.11±1.04 (5)
	L-thyroxin	5.70±0.15 (6)[b]	37.18±2.90 (6)
10 weeks (Adult)	Control	14.65±0.32 (6)	49.77±3.08 (6)
	L-thyroxin	12.47±0.39 (6)[a]	43.05±1.75 (7)

(a) $p < 0.05$, (b) $p < 0.001$ compared to control group

TABLE 2

Influence of age on the response of the microsomal electron transport components to thyroxin.

Male rats were treated with subcutaneous injection of L-thyroxin 1 ug/g body weight/day for 3 days prior to death. Values are expressed as mean ± SEM. Number in parentheses indicate number of individual determinations.

Age	NADPH oxidase, nmoles NADPH oxidized x min^{-1} x mg^{-1} protein			NADPH cytochrome c reductase (nmoles cyt. c reduced x min^{-1} x mg^{-1} protein)			Cytochrome P-450 nmoles x mg^{-1} protein		
	Control	Thyroxin treated	% change	Control	Thyroxin treated	% change	Control	Thyroxin treated	% change
3 days	4.76±0.39 (6)	5.98±0.36 (6)	+25.6[a]	59.40±3.90 (6)	77.79±4.78 (6)	+31.0	0.152±0.007 (6)	0.106±0.021 (6)	-30.2
1 week	4.89±0.22 (8)	4.05±0.34 (6)	-17.2	46.05±1.75 (8)	74.15±10.45 (6)	+61.1[b]	0.190±0.013 (7)	0.130±0.013 (6)	-31.6[b]
2 weeks	5.00±0.40 (6)	5.79±0.63 (6)	+15.8	38.89±2.96 (6)	66.87±7.05 (6)	+71.2[a]	0.266±0.010 (16)	0.103±0.021 (6)	-61.3[c]
3 weeks	5.22±0.67 (20)	6.87±0.35 (7)	+31.6	68.22±3.11 (20)	74.55±5.94 (7)	+ 9.3	0.214±0.014 (10)	0.120±0.006 (7)	-43.9[c]
4 weeks	7.09±0.55 (6)	9.01±0.62 (6)	+27.1[a]	91.34±3.87 (6)	157.1±12.73 (6)	+72.0[c]	0.357±0.017 (6)	0.170±0.023 (6)	-52.4[c]
5 weeks	7.11±0.48 (15)	7.79±0.55 (6)	+ 9.6	92.16±4.16 (10)	154.22±15.02 (6)	+67.3[c]	0.371±0.036 (14)	0.244±0.007 (6)	-34.2[a]
10 weeks	7.98±0.57 (6)	6.53±0.31 (6)	-18.2	95.02±8.55 (10)	129.15±4.31 (6)	+35.9[c]	0.452±0.028 (6)	0.218±0.024 (6)	-51.7[a]

(a) $p < 0.05$, (b) $p < 0.01$, (c) $p \leq 0.001$.

TABLE 3

Thyroxin dose-response comparison between immature (1 week old) and adult (10 week old).

Rats were given L-thyroxin subcutaneously daily for 3 days prior to the experiments. Values are mean ± SEM of individual determinations which are indicated by numbers in parentheses. Each determination in the one week old rat represents a pool of 3 rat livers. Percentage changes from control group are shown below the mean value.

Age (Wks)	L-thyroxin (dose per Kg body weight)	Cytochrome P-450 (nmoles x mg^{-1} protein)	NADPH cytochrome c reductase (n-moles cytochrome c reduced x min^{-1} x mg^{-1} protein)	Aminopyrine N-demethylase (n-moles HCHO formed x min^{-1} x mg^{-1} protein)
1	0.00 (control)	0.190±0.013 (7)	46.05±1.75 (8)	0.83±0.08 (8)
	0.1 mg	0.218±0.013 (6)	81.65±5.76 (6)	0.73±0.03 (6)
		+14.7%[a]	+77.3%[c]	-12.1%[b]
	1.0 mg	0.130±0.013 (6)	74.15±10.45(5)	0.42±0.03 (6)
		-31.6%[b]	+61.0%[b]	-49.4%[c]
	10.0 mg	0.155±0.016 (6)	83.88±2.54 (6)	0.78±0.03 (6)
		-18.4%[a]	+82.1%[c]	- 6.0%[a]
10	0.00 (control)	0.452±0.028 (6)	95.02±8.55 (6)	2.52±0.15 (6)
	0.1 mg	0.360±0.019 (6)	156.48±10.76(6)	2.89±0.05 (6)
		-20.3%[b]	+64.7%[b]	+14.7%[b]
	1.0 mg	0.218±0.024 (6)	129.15±4.31 (6)	4.38±0.14 (6)
		-51.8%[c]	+35.9%[c]	+73.8%[c]
	10.0 mg	0.215±0.024 (6)	158.31±6.79 (6)	2.25±0.15 (6)
		-52.4%[c]	+66.6%[c]	-10.7%[a]

(a) $p < 0.05$)
(b) $p < 0.01$) compared to control group
(c) $p < 0.001$)

TABLE 4

Serum thyroxin (T4) levels of immature (1 week old) and adult rat (10 week old) with or without treatment with thyroxin (1 ug/g body weight/day for 3 days). Values are expressed as mean ± SEM of individual determinations indicated by numbers in parentheses.

Age	Serum thyroxin (ug %)	
	Control	T4 treated
1 week	2.37±0.23 (8)	37.50±1.36 (8)
10 weeks	5.44±0.63 (8)	34.37±2.52 (8)

GENERAL DISCUSSION

GENERAL DISCUSSION

The data presented in the foregoing manuscripts in this thesis demonstrate that the activity of the hepatic microsomal mixed function oxidase system in the premature and full term newborn infant is present in significant amount. However, the activity of this system in the newborn infant is much lower compared to that of the adult man. The data provide a biochemical basis for a transient deficiency in drug oxidation during the newborn period and support the well-recognized concept that the rate of drug metabolism in the newborn period is relatively slower compared to the adult man.

The data also provide evidence that the activity of the hepatic microsomal mixed function oxidase system increases with age during the newborn period. A factor that may modulate the development of this system is thyroxin and the data presented in this thesis support the concept that thyroxin significantly influences the ontogenesis of this system.

I. HEPATIC MICROSOMAL DRUG METABOLISM AND ELECTRON TRANSPORT IN THE PREMATURE AND FULL TERM NEWBORN INFANT

1. Evidence for the presence of significant hepatic microsomal drug oxidation in the premature and full term newborn infant.

The hepatic microsomal fixed function oxidase system catalyzes

the oxidation of drugs (Gillette et al., 1972, Mannering, 1971), environmental pollutants and various endogenous substrates (Conney and Kuntzman, 1971). The optimal capacity for this oxidative function represents the culmination of the processes of differentiation that have gone forward in the hepatic microsomes. In the fetus and newborn animals, this drug oxidative capability is negligible (Fouts and Adamson, 1959, Jondorf et al., 1959, Kato et al., 1964, Gram et al., 1969, Fouts, 1968, Fouts and Hart, 1965, Rane et al., 1973, MacLeod et al., 1972). However, the studies presented in this thesis show that unlike the fetus and newborn of experimental animals, the premature and full term newborn infant shows a significant drug oxidative capability (Aranda et al., 1974). The microsomes from livers obtained during post-mortem and surgical procedures in premature and full term infants have a significantly measurable activity of hepatic microsomal aminopyrine N-demethylase activity and aniline p-hydroxylase and the activity of these oxidative enzymes is approximately one-fourth to one-third of the observed values obtained from adults (Aranda et al., 1974). These observations provide a continuum in the ontogenesis of hepatic microsomal drug metabolizing enzymes. Previous studies in aborted human fetuses limited to 25 weeks gestation indicated that the human fetus as early as

eighth week of gestation can metabolize a variety of exogenous substrates such as chlorpromazine, methylaniline, ethylmorphine, aniline, and some endogenous substrates such as testosterone and fatty acid laurate (Yaffe et al., 1970, Pelkonen et al., 1971, Pelkonen, 1973a, Rane, 1972, Rane, 1972, Pelkonen et al., 1973).

The difference in the drug metabolizing capability between the human and animal fetus and newborn may reflect species differences. However, the possibility that the presence of these drug metabolizing enzymes in humans could be due to non-specific induction by smoking, drugs and environmental pollutants cannot be excluded. The experimental animals are usually kept under relatively controlled environmental conditions. In contrast, the pregnant woman is constantly exposed to environmental pollutants and drugs. For instance phenobarbital a known enzyme inducer (Ernster et al., 1965) is one of the major drugs consumed by the pregnant woman (Forfar, 1972). Estimates have indicated that about 80 to 90% of mothers have received some drugs during the period of pregnancy and the average number of drugs consumed per pregnant mother varies from 3 to 10 drugs (Forfar, 1972, Hill, 1972, Peckham, 1963).

2. Evidence for the presence of hepatic microsomal electron transport components in the premature and full term newborn infant.

Cytochrome P-450, the terminal hemooxygenase in microsomal drug oxidation is present in variable but significant quantity in the newborn liver microsomes as shown in the present study (Aranda et al., 1974). This is in accord with Yaffe's data from abortuses of 14 to 25 week gestation (Yaffe et al, 1970). This hemoprotein increases with age and at term it appears to approach almost half of the adult values

(Aranda et al., 1974, Alvarez et al., 1969, Nelson et al., 1971). However, this proportionality decreases to one-tenth of adult cytochrome P-450 content if compared with the data by Davies et al (1973). NADPH cytochrome c reductase activity appears to approach half of adult activity in the three newborn babies whose postconceptional ages were more than 40 weeks (Aranda et al., 1974). These values are equal to or much higher than the adult cytochrome c reductase activity reported by Nelson et al (1971). However, when compared to the adult activity determined by Davies et al (1973), the infant to adult activity is approximately one third. The rate of microsomal NADPH oxidation as expressed by NADPH oxidase activity has not been measured in human liver microsomes. In a full term 5 week old infant (Aranda et al.,1974), the activity was 5.6 nmoles NADPH oxidized per minute per mg protein, which approximates 70% of the activity in adult rats (Aranda et al., 1973). Like cytochrome P-450 and NADPH cytochrome c reductase, the activity of NADPH oxidase increases with advancing fetal and postnatal age.

3. Factors influencing the activity of the hepatic microsomal drug metabolizing enzymes.

The great discrepancy and variability of the levels of the various components of the HMMFO and of aminopyrine N-demethylation and aniline p-hydroxylation may represent different interindividual genetic expression (LaDu, 1971, Vessell and Page, 1969) variations in tissue sampling and assay techniques, presence of pathophysiologic states, and drug therapy used prior to sampling. The nature of the preexistent disease states and drug therapy are probably the most important factors. Darby (1970) who found very low levels of cytochrome P-450 and microsomal drug oxidation

in adult patients who succumbed to cardio-vascular failure (e.g., myocardial infarction) suggested that anoxia was largely responsible for the observed decrease in drug metabolism. In support of this hypothesis is Cumming's observation that the rate of metabolism of antipyrine was significantly decreased in patients who had severe chronic pulmonary disease with resultant hypoxemia (Cumming, 1972). Hepatic microsomal drug oxidation is an energy- and oxygen-dependent process (Gillette et al., 1957, Mannering, 1971) and a linear relationship between arterial oxygen tension and rate of drug oxidation has been shown (Cumming and Mannering, 1970). Respiratory distress syndrome, where significant ventilation/perfusion abnormality is a major pathophysiologic derrangement (Strang and MacLeish, 1961, Strang, 1966), is characterized by hypoxemia which could alter the true value of the microsomal enzymes studied. Hyperbilirubinemia (McLuen and Fouts, 1961), starvation (Kato and Gillette, 1965), and high glucose loads (Lamson et al., 1951, Strother et al., 1971) could also decrease drug metabolism and it is possible that the values presented in this study might not represent the actual activity of HMMFO in normal infants of similar age (Aranda et al., 1974). However, the data provide some information on the activities of the various components of the system in the sick newborns who are most apt to receive drug therapy, and for whom such information would be of greater relevance.

Exogenous substrates significantly alter the activity of the hepatic microsomal drug metabolizing enzymes. Biochemical and morphologic studies have conclusively shown that the microsomal drug metabolizing enzymes are exquisitely susceptible to the influence of drugs and environmental pollutants. Adult patients who received phenobarbital,

glutethamide, diphenylhydantoin, rifampicin and other drugs showed remarkable induction of cytochrome P-450, NADPH cytochrome c reductase, aminopyrine N-demethylase, p-nitroanisole and p-demethylase activity (Remmer et al., 1973, Black et al., 1973). Similar phenobarbital therapy in infants and children resulted in 100 to 150 per cent increase in the activity of NADPH cytochrome c reductase from hepatic homogenates and a parallel proliferation of the endoplasmic reticulum in two infants with normal hepatic parenchyma (Thaler et al, 1972). However, this inductive effect could be antagonized by the presence of liver disease (Thaler et al., 1972). One of a set of twins (Aranda et al., 1974) who received aminophylline because of apnea, had a 7 fold greater activity of aminopyrine N-demethylase compared to the other twin. The possibility that aminophylline might account for this difference cannot be excluded, however, to our knowledge there are no data available concerning the influence of aminophylline on drug metabolism. If the genetic expression of the drug metabolizing enzyme is equal in twins (Vessell et al, 1971, LaDu, 1971, Vessell and Page, 1971, Alexanderson et al., 1969), the difference in enzyme activity may be contingent on environmental or drug effect. Two infants who received phenobarbital and diphenylhydantoin constituted two of the three infants who have the highest activity of hepatic microsomal enzymes determined (Aranda et al., 1974). However, these infants were of advanced post conceptional age relative to the other infants studied, and the data did not permit the delineation of age-related effect from drug effect on the hepatic microsomal drug metabolizing enzymes.

The apparent subcellular distribution of the various components of the HMMFO is different in the fetal livers as against adults because

of differences in sedimentation properties of subcellular organelles (Ackerman et al., 1972, Rane and Ackerman, 1972, Chatterjee et al., 1965). Electron microscopic studies revealed that the rough endoplasmic reticulum with sparse smooth endoplasmic reticulum sedimented at lower speeds of centrifugation. The smooth endoplasmic reticulum contains the drug metabolizing enzymes (Holtzman et al., 1968, Claude, 1969) and its scarcity in the fetal period provides a morphological explanation for a functional deficit. The time at which the fetal hepatic subcellular localization of the drug metabolizing enzyme approaches adult patterns is not established. Since the hepatic microsomal drug metabolizing enzymes are localized in the smooth endoplasmic reticulum (Holtzman et al., 1968) and since the smooth endoplasmic reticulum may not have precipitated in the microsomal pellet in the neonatal period, determinations of the hepatic microsomal drug metabolizing enzymes may represent lower than the actual value. Further studies are needed to evaluate this possibility.

4. Relationship of hepatic microsomal substrate oxidations to microsomal electron transport in the neonatal period.

Identification of possible rate determining steps in hepatic microsomal drug metabolism in the newborn infant was attempted in this study (Aranda et al., 1974), by evaluating the correlation between the rate of substrate oxidation, and the activity or amount of microsomal electron transport component. Neither NADPH cytochrome c reductase nor the amount of cytochrome P-450 seems to be rate limiting in aminopyrine N-demethylation since no correlation between these electron transport components and N-demethylation of aminopyrine was found. Similar lack of correlation between the hemoprotein and substrate oxidation has been noted in adult

patients (Nelson et al., 1971). In experimental animals there is an increasing evidence that the rate limiting step in microsomal drug oxidation is the reduction of cytochrome P-450 (Gigon et al., 1968, Gillette and Gram, 1969, Diehl et al., 1970). However, this concept was not substantiated in our laboratory since substrate oxidation in the newborns proceeded despite lack of measurable NADPH cytochrome P-450 reductase (Aranda et al., 1974).

Both aminopyrine N-demethylation and aniline p-hydroxylation were significantly related to NADPH oxidase activity indicating that perhaps the velocity of microsomal NADPH oxidation may be an acceptable indicator of the overall rate of microsomal drug oxidation. Indeed, Gillette's laboratory (Stripp et al., 1972, Sasame et al, 1973) have shown a stoichiometric relationship between drug oxidation and NADPH oxidation in hepatic microsomes. They found that the stoichiometry approaches a 1:1 relationship for a variety of type I substrates when the metabolism of the substrate-dependent NADPH oxidation is corrected for carbon monoxide sensitive, endogenous NADPH oxidation. Further evaluation of this method in human microsomes would be needed to define its applicability as an indicator of the rate of drug oxidation. In contrast to aminopyrine N-demethylation, aniline p-hydroxylation correlated significantly with NADPH cytochrome c reductase activity and cytochrome P-450 content, suggesting that the newborn HMMFO is relatively more capable of metabolizing aniline-like compounds. The difference between these two substrate oxidations during the fetal and neonatal period is puzzling. Aminopyrine which gives a typical type I binding spectrum with cytochrome P-450 in adult human liver (Pelkonen, 1973b) and in experimental

animals (Schenkman et al., 1967) produces a type II binding spectrum in the human fetal liver microsomes (Yaffe et al., 1970). Animal studies revealed that type I compounds are better substrates because they drive their own metabolism and they stimulated NADPH cytochrome P-450 reductase activity (Gigon et al., 1968, Gillette and Gram, 1969). However, fundamental mechanisms underlying these differences and the significance of these binding spectra in the hepatic microsomes during the newborn period are poorly understood. The presence of substantial ω-oxidation of laurate and hydroxylation of testosterone, both endogenous compounds giving a type I binding spectrum (Yaffe et al., 1970), lends support to the existence of an intimate relationship between substrate binding and metabolism. This observation and the reversal of aminopyrine binding spectrum to a type II pattern led to the hypothesis (Yaffe et al, 1970, Rane and Sjoqvist, 1972) that there are endogenous substrates with high affinity (low apparent K_m values) for the binding site of the terminal hemooxygenase and which can compete more effectively with exogenous substrates. The intriguing possibility of a qualitatively different cytochrome P-450 between the newborn and adult human being or between species could not be excluded.

5. Evidence for age-related increases in the activity of drug metabolizing enzymes in the premature and full term newborn infant.

A very important but unanswered issue in human drug metabolism is the time at which the deficient neonatal drug metabolic function attains adult level of metabolic capability. Corollary to this problem is the possibility of a demonstratable increase in the activity of these microsomal enzymes during the later part of gestation and in the newborn

period. Studies in our laboratory indicate that there is an increase in the activity of these enzymes as a function of age in the perinatal period (Aranda et al., 1974). Postconceptional age defined as the sum of gestational age and postnatal age, positively correlated with NADPH oxidase activity, NADPH cytochrome c reductase activity, and cytochrome P-450 content. Postconceptional age was also positively correlated with aniline p-hydroxylase activity but not with aminopyrine N-demethylase activity suggesting that the developmental pattern of one substrate oxidative enzyme may be different from another (Aranda et al., 1974). The tremendous variability in the developmental pattern of various enzymes systems regulating protein and carbohydrate metabolism and the difference of the enzyme titres as a function of age are well established phenomena (Kretchmer, 1969, Greengard, 1971). In the early fetal period dynamic changes as a function of age, similar to the events observed in the later part of gestation and in the neonatal period seem to occur. An intriguing observation has been put forth by Pelkonen (1973), who demonstrated in human aborted fetuses from 8 to 20 weeks gestation, a phase of rapid exponential development of the oxidative metabolism of chlorpromazine, p-nitrobenzene, benzpyrene and methylaniline during 8 to 13 weeks of gestation which tended to plateau after 13 to 14 weeks. This suggest that the development of hepatic microsomal mixed function oxidases begins in the early phase of gestation.

Although there is an increased availability of data relating to the activity of the hepatic microsomal mixed function oxidase system in humans (Yaffe et al., 1970, Pelkonen and Karki, 1971, Ackermann et al., 1972, Rane and Ackermann, 1972, Pelkonen et al., 1971, Juchau, 1971, Pomp et al., 1969, Pelkonen, 1973a, Pelkonen et al., 1973, Rane and Gustaffson,

1973, Rane, 1974 and Aranda et al., 1974), the developmental pattern of the microsomal drug metabolizing enzymes in human remains unknown. Furthermore, the available data in the literature are insufficient to predict a possible ontogenetic pattern of these enzymes. Specifically, the question that relates to the time when the low activity microsomal drug metabolizing enzymes in the human fetus and newborn infant undergoes full differentiation or maturation to adult activity remains unanswered. In the experimental animals, most microsomal drug-oxidative enzymes approach adult level of activity at approximately 3 to 6 weeks of age (MacLeod et al., 1972, Fouts and Devereaux, 1972, Short and Davis, 1970, Short and Stith, 1973, Kato et al., 1964, Bresnick and Stevenson, 1968, Basu et al., 1971, Soyka, 1969, Henderson, 1971, Jonsdorf et al., 1959). Some aspects of drug metabolism that are relatively unexplored are the underlying mechanism(s) which regulate(s) the ontogenesis of the hepatic microsomal mixed function oxidase system and the various factors which may modify the pattern of development of this system. One such factor is thyroid hormone.

II. EFFECT OF THYROID HORMONE ON THE DEVELOPMENT OF THE HEPATIC MICROSOMAL DRUG METABOLIZING ENZYMES

1. Possible role of the thyroid hormone on the postnatal development of hepatic microsomal drug metabolizing enzymes.

Vital to the comprehension of the process underlying enzymic differentiation and the ontogenesis of the hepatic microsomal mixed function oxidase system is the delineation of the various effects of endogenous substrates on the activity of the mixed function oxidase. Wilson (1970)

has shown that growth hormone has a repressive role in the development of ethylmorphine N-demethylation, and aminopyrine N-demethylation. The influence of other growth promoting hormones such as thyroxin has been determined in our laboratory, and the basis for these investigations is as follows: First, the simultaneous appearance of hepatic TPNH cytochrome c reductase, glucose-6-phosphatase and a functional thyroid gland on the 18th fetal day in rats and the induction of this enzyme by thyroxin in the fetal rat suggest that the thyroid hormone might act as a physiologic trigger for the differentiation of this enzyme system (Greengard and Dewey, 1968, Greengard, 1969). Similarly, a simultaneous appearance of the hepatic microsomal mixed function oxidase activity and measurable thyroxin and thyronines seems to occur during the 10th to 14th week in the human fetus (Pelkonen, 1973a, Greenberg, 1970, Shepard, 1967, Fisher, 1970).

Second, the thyroid gland, through obscure mechanisms, regulates the development of organ systems. The thyroid gland is vital to the normal development of the central nervous system (Balazs et al., 1969) and to the regulation of several metabolic homeostatic mechanisms. Its inductive role in the development of the lung and pulmonary surfactant has been described (Redding et al., 1972, Wu et al., 1973). Third, thyroid hormone has a major influence on hepatic microsomal drug metabolism in experimental animals (Kato and Gillette, 1965, Kato et al., 1968) and in man (Vessell and Passanintis, 1973, Crooks et al., 1973). Since thyroid hormone is related to both organ development and drug metabolism, the hypothesis that thyroid hormone significantly influences the development of hepatic microsomal mixed function oxidase system was tested and subsequently confirmed in the present study (Aranda and Eade; 1974a).

2. Evidence for the influence of thyroid hormone on the maturation of hepatic microsomal drug metabolizing enzymes:

(a) Acceleration of the development of drug metabolizing enzymes by thyroid hormone.

(i) Effect of acute thyroid hormone therapy:

Postnatal aberrations in thyroid function significantly altered the developmental pattern of hepatic microsomal mixed function oxidase system (Aranda and Eade, 1974, Aranda and Eade, 1975a). A single dose of thyroxin administered to immature rats resulted in significant increase in the activity of NADPH oxidase, NADPH cytochrome c reductase, aniline p-hydroxylase and in the content of cytochrome P-450 and microsomal protein yield. Adult levels of activity were attained with NADPH oxidase activity and aniline p-hydroxylase activity twenty-four hours after thyroid hormone injection (Aranda and Eade, 1975a). Significant increases in protein, DNA and RNA have been demonstrated few hours after a single injection of thyroxin (Sokoloff, 1971, Tata et al., 1963, Tata, 1968). The increase in the microsomal drug metabolizing enzymes may represent de novo enzyme synthesis however the possibility of shifts in the protein pools cannot be excluded. Studies on the effect of thyroxin on the subcellular distribution of the hepatic microsomal drug metabolizing enzymes would probably resolve this possibility.

(ii) Effect of repetitive administration of thyroxin on the developmental pattern of hepatic drug metabolizing enzymes:

Repetitive treatment of male rats with thyroid hormone during the first five days after birth or throughout the first

five weeks of life resulted in significant acceleration in the maturation of the activity of NADPH oxidase, NADPH cytochrome c reductase and aniline p-hydroxylase. Whereas the adult activity of these enzymes are attained in five weeks in the normal male rat, the adult enzyme titres are achieved and are surpassed in one week in the thyroxin treated rats.

Whether the effect of thyroxin on the development of the hepatic mixed function oxidase system is one of permanent enzyme differentiation or merely a transient induction is not resolved in these experiments. The concept of enzyme differentiation entails maintenance of enzyme titres and the physiological variations of these titres, which are normally exhibited in a fully differentiated enzyme system as that seen in the adult organism. On the other hand, transient induction entails a rise in enzyme titres followed by a fall to pre-induction levels upon withdrawal of the inducing agent. Suggestive evidence for enzyme differentiation is provided by the effect of transient neonatal hyperthyroid state on the developmental pattern of aniline p-hydroxylase activity, an oxidative enzyme which exhibits the "overshoot phenomenon" (Moog, 1971). This phenomenon, whereby enzymes that undergo a large increase in activity during a short period of time, rise above their adult level and subsequently fall back to a relatively stable level, has been described in various substrate oxidations (Kato et al., 1964, Gram et al., 1969, Uehleke, 1971). Gram et al (1969) had shown that the Vmax for aniline p-hydroxylase peaked at 1 to 2 weeks followed by a decline thereafter. In contrast, MacLeod et al (1972) and the present studies show that the peak activity of aniline p-hydroxylase

above adult level was observed later at age 5 weeks. This peak activity was shifted to age one week, followed by a slow decline to lower adult value attained at 5 weeks of age (Aranda and Eade, 1974a). This represents an advanced shift in the maturational pattern of approximately four weeks, suggesting that enzyme differentiation have occurred.

Suggestive evidence for a transient induction is that observed with the effect of neonatal thyroxin therapy which resulted in an increase in the activity of NADPH cytochrome c reductase at one week followed by a fall to subcontrol levels (Aranda and Eade, 1975a), and an infranormal shift of the developmental pattern at three to five weeks of age. This finding also raises a possible consequence of induction, that is, a fall in the enzyme activity to subnormal levels upon withdrawal of the influence of the inducing agent. Blackburn et al (1972) and Carson et al (1973) have demonstrated in the lung, that steroids which are inducers of pulmonary surfactant production enhance cell differentiation at the expense of cell division, resulting in fewer but more mature cells that are functioning at an optimal capacity. With growth the metabolic demands of the organism increase commensurately with its increased body size, and it is possible that the decreased number of cells could no longer meet this increased demand leading to an infranormal shift in the developmental pattern, as shown by NADPH cytochrome c reductase activity.

(b) Repression of the development of cytochrome P-450 by thyroid hormone.

Thyroxin appears to exert a repressive role on the development of cytochrome P-450 and aminopyrine N-demethylase. This

repressive role was more defined in the case of cytochrome P-450 as evidenced by a decrease of this hemoprotein in chronic thyroxin treatment; an initial decrease in neonatal thyroxin treated rat followed by an increase above age-matched control levels at three weeks with the withdrawal of thyroid effect and an increase in the three week old radiothyroidectomized rat (Aranda and Eade, 1974a). The basis for the repressive effect is unclear. The hypothesis that the low drug metabolizing capacity in the newborn may be due to the presence of inhibitory substances (Fouts and Adamson, 1959) that may have been derived from the mother (Feuer and Liscio, 1970) has been advanced. In support to this hypothesis is the inhibition of hepatic microsomal drug metabolism by endogenous substrates such as somatotropin (Wilson, 1969), pregnenolone and progesterone metabolites (Soyka, 1972, Soyka, 1973) and the repression of the postnatal development of ethyl morphine N-demethylation and aminopyrine N-demethylation by growth hormone (Wilson, 1970). The newborn infant at term has a higher free serum thyroxin level with a transient 8 to 9 fold rise in serum thyroid stimulating hormone immediately after birth (Fisher, 1970, Greenberg, 1970) and the increased thyroid hormone and thyroid stimulating hormone may augment the total pool of circulating endogenous substrates. Increased circulating thyroid hormone may produce altered substrate interaction with cytochrome P-450 as evidenced by decreased $O.D._{max}$ value and increased Vmax for aniline; and increased K_s value, increased K_m value and decreased Vmax value for hexobarbital in thyroxin treated adult male rats (Kato et al., 1970). The other possibility

that relatively high thyroxin levels may indirectly act as a repressor of the development of aminopyrine N-demethylation and cytochrome P-450 through a permissive effect on growth hormone secretion (Catt, 1970) cannot be excluded.

The increased thyroid hormone during the immediate postnatal period is of dubious significance. Greengard (1969) raised the concept that the thyroid gland is the probable physiologic trigger in NADPH generating system in fetal rat on the basis of simultaneous appearance of fetal thyroid function and NADPH cytochrome c reductase and glucose-6 phosphatase, and a precocious differentiation of these enzymes with exogenously administered thyroxin. However, the teleologic significance of a transient neonatal thyroid gland hyperfunction in relation to the differentiation of NADPH and oxygen requiring drug oxidative pathways and their associated electron transport components is as yet speculative. Moreover, the fundamental mechanism underlying the selective but variable effect of thyroxin on the various components of the microsomal electron transport chain and various oxidative pathways is still undefined.

(c) Effect of hypothyroidism on hepatic microsomal drug metabolizing enzymes, converse to effect of repetitive thyroid hormone therapy. Reversibility of hypothyroid effect by thyroid hormone replacement therapy.

Hypothyroidism achieved by administration of ^{131}I at birth resulted in some effects opposite to those obtained by repetitive thyroid hormone treatment as shown in the present study (Aranda and Eade, 1975a). There was a significant decrease in the activity of the NADPH cytochrome c reductase in the immature rats which was not only corrected by replacement therapy with thyroxin but which was significantly enhanced above age matched control values. Conversely, cytochrome

P-450 content was significantly increased in the three week old hypothyroid rat and this finding was reversed with the administration of thyroxin (Aranda and Eade, 1974).

The most sensitive indicator of the effect of thyroid hormone or of the deficiency thereof on the developing hepatic mixed function oxidase system is NADPH cytochrome c reductase activity. This is supported by a marked increase in the activity 24 hours after thyroxin administration, sustained high activity with continuous thyroxin treatment, decreased activity to preinduction levels upon withdrawal of thyroxin; a significant fall below control levels in hypothyroidism and a rise to above control levels with thyroxin replacement therapy in hypothyroidism (Aranda and Eade, 1975a). These findings are in accord with the observations obtained in hypothyroid and hyperthyroid adult rats (Phillips and Langdon, 1956, Kato and Takahashi, 1968, Suzuki, et al., 1967). Evidence has been presented that the metabolism of the flavoproteins is under the control of the thyroid gland through the regulation of flavokinase (Rivlin and Langdon, 1966, Rivlin et al., 1968, Rivlin, 1970) and since NADPH cytochrome c reductase is a flavoprotein (Williams and Kamin, 1962) therefore, the regulation of the enzyme titre is most probably mediated by the thyroid gland (Phillips and Langdon, 1956). Biochemical similarities between hypothyroidism and riboflavin deficiency have been demonstrated (Rivlin et al., 1968) and similarities between microsomal drug metabolism in hypothyroid immature and adult rat and the riboflavin deficient postweaning mice (Catz et al., 1970), namely, increased cytochrome b5 and cytochrome P-450 content and increased aniline p-hydroxylation, have also been shown (Aranda and Eade, 1975a, Suzuki et al., 1967).

3. Effect of thyroid hormone on the postnatal changes in the reduction of cytochrome P-450 by NADPH.

Thyroid hormone significantly influences the development of the hepatic microsomal NADPH cytochrome P-450 reductase activity. Experimentally produced deviation from euthyroid state resulted in altered activity of NADPH cytochrome P-450 reductase. At three weeks of age the activity of the reductase was increased in the hypothyroid rats which was partly reversed by thyroxin replacement therapy (Aranda and Eade, 1975b). These observed increases of P-450 reductase in both hypothyroid and hyperthyroid rats are not similar to the findings observed in other electron transport components in similar states of thyroid dysfunction (Aranda and Eade, 1975a). Cytochrome P-450 was increased and NADPH cytochrome c reductase was decreased in hypothyroidism. In contrast cytochrome P-450 was decreased and NADPH cytochrome c reductase was increased in hyperthyroidism (Aranda and Eade, 1975a). The increased reduction of cytochrome P-450 by NADPH in hypothyroidism may reflect a greater effectiveness in the utilization of decreased cytochrome P-450 content for reduction. Thus, although there is a markedly decreased cytochrome P-450 content in chronic thyroid treatment, the percentage of the total amount of cytochrome P-450 which may be effectively reduced for ultimate drug oxidation is probably much higher. This possibility may be resolved by the determination of the percentage of P-450 reducible by NADPH for a given time.

Biochemical adaptation to thyroid dysfunction as a function of the duration of the latter may occur. In accord to observations by Suzuki et al., (1967), wherein some microsomal electron transport

components increased after an initial decrease following surgical thyroidectomy, NADPH cytochrome P-450 reductase from liver microsomes of chronically thyroxin treated rats shown in this study was equiactive to the euthyroid control rats at 5 weeks age. This adaptation to thyroid dysfunction may partly explain the lack of change of NADPH cytochrome c reductase activity in surgically thyroidectomized adult rats (Aranda and Eade, 1975b) compared to euthyroid rats.

Thyroid dysfunction influences the effect of aminopyrine added in vitro to NADPH cytochrome P-450 reductase (Aranda and Eade, 1975b). In the normal 5 week old rat, aminopyrine significantly accelerates the reduction of cytochrome P-450 by NADPH. Similar observations are noted in the 5 week old neonatally thyroxin treated rat. In contrast, aminopyrine decelerates the reduction of cytochrome P-450 by NADPH in the 3 week old hypothyroid rat. These observations suggest that a euthyroid state may be necessary for substrate enhancement to occur. Furthermore the substrate enhanced cytochrome P-450 reduction is significantly lower in the 5 week old hypothyroid and hyperthyroid rat compared to the euthyroid control rat (Aranda and Eade, 1975b). The mechanism underlying the influence of thyroid hormone on the in vitro effect of aminopyrine on the reduction of cytochrome P-450 is not known. The possibility of direct substrate-thyroid hormone interaction is negated by the lack of effect of thyroxin added in vitro (Aranda and Eade, 1975a).

4. Characteristics of the effect of thyroid hormone on the hepatic microsomal mixed function oxidase system.

The effect of thyroid hormone on the hepatic microsomal mixed function oxidase system appears to be selective to a specific substrate oxidative pathway; selective to a specific electron transport component;

dependent upon the duration of the altered thyroid state; and markedly influenced by the age at which thyroid hormone is administered.

The selectivity of the effect of thyroid hormone on substrate oxidation is evidenced by the significant induction on the activity of aniline p-hydroxylase after a single dose of thyroxin in the immature animal and by significant acceleration of the maturation of this enzyme by repetitive administration of thyroid hormone. In contrast, aminopyrine N-demethylase activity is not changed by acute thyroxin treatment. Furthermore, repetitive treatment of immature animals with thyroid hormone result in initial repression of the activity of aminopyrine demethylation. Similar selectivity of the effect of thyroid hormone on specific substrate oxidations have been demonstrated in adult rats (Kato and Gillette, 1965, Kato and Takahashi, 1968, Suzuki et al., 1967). The basis for the selective effect of thyroid hormone on a specific substrate oxidation is not clear. The possibility that the substrate specificity of the effect of thyroid hormone is probably due to androgenic effect has been proposed (Kato and Gillette, 1965, Kato and Takahashi, 1968). This hypothesis has not been adequately tested in the immature animals.

The selectivity of the effect of thyroid hormone on specific microsomal electron transport components is illustrated by significant acceleration of the maturation of NADPH oxidase activity and NADPH cytochrome c reductase activity in immature rats treated with repetitive doses of thyroxin. In contrast, a significant repression of the development of the terminal hemooxygenase, cytochrome P-450 is observed following similar treatment with thyroid hormone (Aranda and Eade, 1975a). Similar observations in adult rats of the selectivity of thyroid hormone

effect on the microsomal electron transport component has been noted (Kato and Takahashi, 1968, Suzuki et al., 1967). Again the basis for this selectivity is unclear. The influence of thyroid hormone on the metabolism of flavoprotein NADPH cytochrome c reductase has been discussed (supra vide) which may provide partial explanation for this selectivity. The repressive effect of thyroid hormone on cytochrome P-450 has also been discussed (supra vide).

The effect of thyroid hormone on the hepatic microsomal mixed function oxidase system is also dependent upon the duration of thyroid dysfunction. A five week old hypothyroid rat is significantly more responsive to the effect of thyroid hormone replacement therapy as against a 3 week old hypothyroid rat (Aranda and Eade, 1975a). This observation is probably due to age-related changes in sensitivity to thyroid hormone, however, the changes in the activity of the drug metabolizing enzymes as a function of the duration of hypothyroidism in adult rats suggest that the duration of altered thyroid state may be largely responsible for these changes (Suzuki et al., 1967).

The duration of postnatal thyroid dysfunction also influences the maturational pattern of microsomal drug oxidation. This is exemplified by a decrease in aminopyrine N-demethylation in the three week old hypothyroid rat but not in the 5 week old hypothyroid rat (Aranda and Eade, 1975a). Similarly but in a converse fashion, an increase in aniline p-hydroxylation is noted in the three week old but not in the 5 week old hypothyroid rats (Aranda and Eade, 1975a). These findings suggest some degree of compensatory mechanisms inherent in prolonged hormonal imbalance. Indeed, a major difficulty in studying cell or enzymic differentiation in vivo using endogenous substrates is the

presence of feedback control mechanisms that intervene to maintain the steady state (Thrasher, 1971). For instance, in hypothyroidism, the thyroid stimulating hormone (TSH) of the anterior pituitary gland is greatly elevated (Catt, 1970). It is not inconceivable that TSH per se may directly or indirectly alter the development of microsomal drug oxidation. To our knowledge, this hormonal interplay has not been studied as it pertains to the ontogenesis of the hepatic mixed function oxidase system.

The effect of thyroid hormone is determined to a great extent by the age at which the hormone is administered. The age-relatedness to thyroxin response is dramatically illustrated by the effect of a "booster" dose of thyroxin on aminopyrine N-demethylation. Whereas a significant inhibition of this substrate oxidation is observed at age 3 weeks, a slight but insignificant increase is noted at age 5 weeks (Aranda and Eade, 1975a). Similar age-related responsiveness to thyroxin is further shown by the effect of a booster dose of thyroxin on NADPH cytochrome c reductase activity on the neonatal thyroxin treated rat where a super induction was exhibited at age 5 weeks but not at age 3 weeks (Aranda, 1974).

These findings indicate that a major determinant in the effect of thyroid hormone on the hepatic microsomal mixed function oxidase system is the age at which the hormone is administered. Evidence for the validity of this assumption is provided below.

III. EVIDENCE THAT AGE IS A MAJOR DETERMINANT IN THE EFFECT OF THYROID HORMONE ON THE HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE SYSTEM

The well known altered sensitivity of the newborn infant and

immature animals to drugs has been attributed to a great extent to their altered capability to handle drugs as exemplified by differences in protein binding (Krasner, 1973), drug distribution (Kupferberg and Way, 1965), decreased hepatic oxidative and conjugative biotransforming capacity (Fouts and Adamson, 1959, Yaffe et al., 1970, Pelkonen et al., 1973, Weiss et al., 1960) and decreased renal excretion of drugs (Barnett et al., 1949, Nelson and McCracken, 1973, Simon and Axline, 1966). Evidence is now provided that there is variability in the intrinsic sensitivity of hepatic microsomal enzymes to inducing agents such as thyroxin. There is a marked difference between the response of an immature and an adult animal to the effect of hormones (thyroxin) on the hepatic microsomal drug oxidizing enzymes (Aranda and Eade, 1975c). Within the 5 week postnatal period, dynamic variations on the responsiveness of the hepatic microsomal enzymes to thyroxin occur, and this is best illustrated by the opposite effects of thyroxin on the hepatic microsomal aminopyrine N-demethylase and aniline p-hydroxylase activity as a function of age (Aranda and Eade, 1975c). This observations appears to be independent of differences in substrate-cytochrome P-450 interaction as evidence by the type II difference spectra obtained with aniline added to the liver microsomes from immature and adult rat with or without thyroxin therapy (Aranda and Eade, 1975c). This difference in response to thyroxin could not be accounted for by changes in the electron transport components due to thyroxin treatment since there was no observed parallel change between electron transport component and substrate oxidation. For instance, there was a decrease in cytochrome P-450 varying from 30% to 60% throughout the first 5 weeks of age and in the adult period

after thyroxin treatment. On the other hand, aniline p-hydroxylation was significantly increased during the first 4 weeks of age followed by a decrease during 5 and 10 weeks of age. This change did not parallel the observed effect of thyroxin on the electron transport components.

As discussed earlier (supra vide) NADPH cytochrome c reductase is a flavoprotein (Williams and Kamin, 1962) and its activity is dependent upon the state of thyroid function since its metabolism is regulated by the thyroid hormone (Rivlin and Langdon, 1966, Rivlin et al., 1968). However, even the influence of thyroid hormone appears to be dependent upon age since the 3 week old male rat was relatively insensitive to the influence of thyroid hormone (Aranda and Eade, 1975c). A significant induction of NADPH cytochrome c reductase by thyroxin was noted at all ages studied, except at age 3 weeks. It appears that marked differences in response to thyroxin may occur at this age which coincides with the weaning period. The weaning period seems to be a transition from the neonatal pattern to the adult pattern of response to thyroxin of both aniline p-hydroxylation and aminopyrine N-demethylation (Aranda and Eade, 1975c). The mechanism underlying this transition is still poorly understood.

In accord with the observation of Phillips and Langdon (1956), studies in our laboratory demonstrate that in the adult animal, an almost linear increase in NADPH cytochrome c reductase activity as a function of the duration of thyroxin treatment occurs during the first week (Aranda and Eade, 1975c). In contrast the immature animal responds much earlier and appears to plateau at a lower level. This difference

between the young and the adult animal's response to thyroxin as a function of the duration of treatment is dramatically illustrated by the effect of thyroxin treatment on cytochrome P-450 and aminopyrine N-demethylase in the young and adult animal (Aranda and Eade, 1975c). Thyroxin treatment produced an initial increase in the cytochrome P-450 content followed by a decrease with prolongation of thyroxin treatment. In contrast, thyroxin treatment produced an immediate decrease in cytochrome P-450 content. The obvious implication of this observation rests on the fact that in comparing the age-related responses of the hepatic microsomal mixed function oxidase system, the duration of treatment may be a determining factor for the quantitative or qualitative changes observed. Although the magnitude or degree of quantitative change may be partly dose-related, the qualitative change (inhibition or induction) as a function of age appears to be independent of the dose (Aranda and Eade, 1975c).

Although age as a major determinant in the host response to drug is generally accepted and is underscored in several reviews (Done, 1964, Sereni and Principi, 1968, Yaffe, 1974, Nyhan, 1961, Weinstein, 1968) the fundamental reason(s) underlying the variability of response of an organ system to a drug as a function of age remains unsettled. The failure to induce hepatic microsomal drug-metabolizing enzymes by phenobarbital few days prior to birth (Hart et al., 1962) and the ready inducibility of these same enzymes after birth suggest that the machinery for enzyme synthesis is an obvious prerequisite for the activation of enzyme synthesis (Greengard, 1971). Postnatally, however, it appears that the required co-factors for hepatic microsomal drug oxidation are

present (Fouts et al., 1959) and the progressive increase in the synthesis of these enzymes (Dallner et al., 1966) occurring after birth indicates that the machinery for the synthesis of microsomal enzymes are developed. This argument does not explain the decreased responsiveness of the microsomal enzymes at a certain period postnatally such as the lack of effect of thyroxin on NADPH cytochrome c reductase at age 3 weeks. One fundamental cellular function that may change with age is the rate of protein synthesis (Waterlow, 1968, Winick and Noble, 1965, Winick and Noble, 1966). The rate of protein synthesis changes as a function of age and it is possible that a specific enzyme may have varying rates of synthesis and degradation that changes with age. This would result in quantitative variations in the response of an enzyme system to a specific inducing agent, that is, induction when protein synthesis is maximal may result in maximal response to the inducing agent. To our knowledge, turnover studies of hepatic microsomal enzymes as a function of age and in the presence of inducing agents have not yet been done.

Clinical applications of age-related inducibility of enzyme systems has been demonstrated in steroid induction of other organ systems such as the lungs (Liggins et al., 1972, Baden et al., 1972). For instance, induction of pulmonary surfactant to prevent hyaline membrane disease in newborn infant occurred when steroids were administered before 32 weeks of gestation and induction was not successful when steroids were given later than 32 weeks gestation (Liggins et al., 1972) or after birth (Baden et al., 1972).

IV. POSSIBLE MECHANISMS WHEREBY THE EFFECT OF THYROID HORMONE ON THE HEPATIC MICROSOMAL DRUG OXIDIZING ENZYMES IS MEDIATED

The fundamental mechanism underlying all of the known effects of thyroid hormone is still unsettled (Catt, 1970, Sokoloff, 1971). It is highly probable that the primary action of thyroid hormone involves a process so basic to cellular function that its regulation eventually ramifies into all the metabolic processes, such as microsomal drug oxidation, which have been shown to be modified in thyroid dysfunction. As mentioned earlier, a basic process of cellular activity is protein synthesis, which has been shown to be stimulated by thyroid hormone both in vitro and in vivo (Tata, 1967, Sokoloff et al., 1963, Tata et al., 1963). The postnatal increase in the drug-oxidative enzymes probably represents de novo protein synthesis (Dallner et al., 1966) and it is likely that an increase in thyroid hormone would increase the rate of postnatal synthesis of enzyme proteins as reflected by increased NADPH oxidase, NADPH cytochrome c reductase and aniline p-hydroxylase activity (Aranda and Eade, 1975a). However, this mechanism does not explain the repression of the development of cytochrome P-450 and aminopyrine N-demethylation (Aranda and Eade, 1975a). Nonetheless, a unified mechanism involving stimulation of protein synthesis is likely if one invokes the possibility of an imbalance between rate of synthesis and rate of degradation resulting in a net increased or net decreased protein synthesis. Such an imbalance between synthesis and degradation has been demonstrated by the effect of methylmercury hydroxide on hepatic cytochrome P-450 (Lucier et al., 1973). The rate of incorporation of radio-activity from labelled δ-aminolevulinic acid into cytochrome P-450 did not appear to be affected by methylmercury but the degradation

of the labelled cytochrome P-450 occurred twice as fast in the methylmercury treated rats compared to controls (Lucier et al., 1973). Increased protein turnover in hyperthyroidism and decreased protein turnover in hypothyroidism in the mitochondria have been demonstrated (Gross, 1971) and the possibility of altered microsomal protein turnover under states of thyroid dysfunction is highly probable. The coherent sequence in integrating all of these effects of thyroid hormone on the ontogenesis of the mixed function oxidase system needs further investigation.

It has been suggested that thyroxin may increase drug oxidation by facilitating the availability of oxygen to the substrate (Kato, 1970). This intriguing hypothesis gains support with the observation that hyperthyroid patients have a left to right shift in the hemoglobin oxygen dissociation curve with a resultant increase in P-50, the oxygen tension at which 50% of the hemoglobin is oxygen-saturated (Miller et al., 1970, Synder and Reddy, 1970). This decreased hemoglobin oxygen affinity facilitates oxygen transfer to the tissues (Miller et al., 1970, Schussler et al., 1971) and a similar facilitation of oxygen transfer from the hemoprotein to the substrate undergoing oxidation may occur. However, the exact biochemical mechanism for oxygen transfer from the cytochrome P-450 to the substrate is not completely understood yet (Ullrich, 1971). Nonetheless, the possibility remains that thyroxin may exert an effect at this step of drug oxidation.

Other possible mechanisms whereby thyroid hormone may exert its effect on the hepatic microsomal drug metabolizing enzymes are through the regulation of the metabolism of flavoprotein electron

acceptors such as NADPH cytochrome c reductase, and through the permissive effect of thyroid hormone on growth hormone. Both of these possibilities have been discussed previously.

The temporal events leading to full maturation of microsomal drug oxidative capability and the factors regulating these events are areas of major interest in perinatal pharmacology. Identification of physiological and pharmacological influences which allow for increased drug-metabolizing enzyme activity in the immature animal may elucidate the normal sequence of the maturation of biochemical events in drug metabolism. This study emphasizes the vulnerability of the developmental profile of the microsomal drug metabolizing enzymes to changes in thyroid state and provides a basis for the possible role of the thyroid gland in the ontogenesis of the hepatic mixed function oxidase system.

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CONCLUSIONS AND STATEMENT OF ORIGINAL CONTENT

CONCLUSIONS and STATEMENT OF ORIGINAL CONTENT

The following points established in the foregoing thesis, represent original descriptions and contributions to knowledge.

1. The human newborn infant, unlike the newborn experimental animal, has significant, although quantitatively deficient activity and amount, of hepatic microsomal drug metabolizing enzymes as compared to the adults. In the premature infant, there is a significant deficiency in the rate of hepatic microsomal drug oxidation (aniline p-hydroxylation and aminopyrine N-demethylation) and in the activity of the hepatic microsomal electron transport enzymes.

2. In the premature and full term infant, aniline is a better substrate for the hepatic microsomal mixed function oxidase compared to aminopyrine, thus providing a suggestive evidence for preferential subcellular substrate oxidation (i.e., some drugs are more efficiently metabolized in the neonatal period).

3. In the premature and full term infants, the p-hydroxylation of aniline correlated with postconceptional age whereas the N-demethylation of aminopyrine did not, suggesting that there is a differential development of various drug oxidative pathways during the neonatal period.

4. In the premature and full term infant, there is a progressive increase in the activity of the hepatic microsomal drug-metabolizing enzymes as a function of postconceptional age. This implies that within the neonatal period there is a significant change towards an increasing capability to

metabolize drugs. This suggests a need for constant evaluation of the newborn infant's pharmacodynamic response in order to achieve the desired therapeutic response and obviate toxic reactions or undertreatment.

5. In the premature and full term infant, there is no measurable activity of NADPH cytochrome P-450 reductase, the possible rate limiting step in drug oxidation.

6. In immature male rats, an acute dose of thyroxin produced significant induction of hepatic microsomal aniline p-hydroxylase, NADPH oxidase, NADPH cytochrome c reductase cytochrome P-450 and microsomal protein content. No effect was noted with aminopyrine N-demethylase suggesting that the effect of thyroid hormone in the immature animal may be selective to certain substrate oxidative enzymes.

7. In the male immature rats, a chronic hyperthyroid state produced acceleration of the development of hepatic microsomal aniline p-hydroxylase, NADPH oxidase and NADPH cytochrome c reductase. This observation suggests that thyroxin may act as "inducer" for these microsomal enzymes.

8. In the male immature rat, chronic hyperthyroid state resulted in deceleration of the development of cytochrome P-450 suggesting selectivity of thyroid hormone effect on the development of the hepatic microsomal enzymes. Whereas acceleration of the development is noted in most enzymes, deceleration of cytochrome P-450 development occurs.

9. In the male immature rats, hypothyroidism produced by ^{131}I injection at birth resulted in effects opposite to hyperthyroidism (i.e., deceleration of the development of NADPH cytochrome c reductase and acceleration of the

development of cytochrome P-450). These effects were reversed by treatment with L-thyroxin in the hypothyroid rats.

10. In the male immature rat, the peak activity of aniline p-hydroxylase is shifted from five weeks to one week by short term administration of thyroxin immediately after birth. This suggests enzymic differentiation of aniline p-hydroxylase by thyroxin.

11. In the male immature rat, there is a transient acceleration in the development of NADPH cytochrome c reductase induced by short term treatment with thyroxin immediately after birth.

12. The most sensitive enzyme to thyroxin effect in the hepatic microsomal drug oxidizing enzymes in the male immature rat appears to be NADPH cytochrome c reductase.

13. In the male rat, subacute treatment (3 days) using equivalent doses of thyroxin (1 ug/g/day) administered at varying postnatal ages, resulted in induction of aniline p-hydroxylase at ages 3 days to 4 weeks and in inhibition at ages 5 and 10 weeks. Conversely, the same treatment with thyroxin resulted in inhibition of aminopyrine N-demethylase at ages 1 to 3 weeks, no effect at 4 weeks and induction at 5 and 10 weeks. When expressed as percentage change of control value as a function of postnatal age, the opposite effects of thyroxin on aniline p-hydroxylase and aminopyrine N-demethylase becomes obvious. These results indicate that the enzyme titres achieved in response to thyroxin is greatly determined by the age at which thyroxin is administered. This suggests that the effectiveness of induction may depend upon the timing of administration of the

inducing agent.

14. The changes in activity or content of hepatic microsomal drug oxidative enzymes as a function of the duration of altered thyroid state is different in the immature rat as compared to the adult rat. Whereas a 50 to 60% decrease in cytochrome P-450 content is obtained within 24 hours in the adult rat, an initial increase followed by a decrease in cytochrome P-450 content is obtained in the immature rat.

15. The foregoing observations demonstrate for the first time that thyroxin significantly alters the ontogenetic pattern of the hepatic microsomal drug oxidizing enzymes. This suggests that thyroxin may be an important factor in the regulation of the development of the hepatic microsomal mixed function oxidase system.

APPENDICES

APPENDIX A

THYROID HORMONE AND HEPATIC DRUG METABOLISM

A. HISTORICAL BACKGROUND:

The concept that thyroid hormone is a significant determinant in hepatic drug metabolism has its roots in the early 1900's, however its potential significance remained unrecognised until recently. The probable basis of the acetonitrile test for hyperthyroidism described by Hunt (1905, 1907) and Hunt and Seidel (1909) rested on the observation that chemically hyperthyroid mice resisted toxic doses of acetonitrile, a methyl cyanide compound. Carlson and Woelfel (1910) fed mice with dessicated thyroid daily and confirmed Hunt's observation (Hunt, 1905, 1907) by demonstrating that a 2.5 fold increase in the acetonitrile dose that killed the normal mice failed to kill the thyroid-fed ones. These findings were not adequately explained. Acetonitrile a cyanide, is biotransformed into thiocyanate by mitochrondrial thiosulphate sulfurtransferase (rhodanase) resulting into a 200-fold reduction in toxicity (Williams, 1971) and it is possible that thyroid hormone enhanced this detoxication process. Olds (1910) provided further evidence that a euthyroid state is necessary for a normal drug response. Surgically thyroidectomized rats that were given morphine had a 95.6% mortality compared to 47.6% mortality in the control rats which were given the same dose of morphine, a drug which undergoes hepatic glucuronide conjugation and microsomal N-demethylation for termination of drug action and elimination (Jaffe, 1970). The increased sensitivity to morphine in hypothyroidism was later confirmed in rats (Busco, 1925) and rabbits (Newton, 1924) and the converse demonstration of increased

tolerance to morphine administration in chronic thyroid feeding (Scarborough, 1926) led to an increased suspicion that the thyroid gland influenced the duration of drug action.

The possible clinical significance of these animal observations was demonstrated by Lund and Benedict (1929) who reported a 36 year old hypothyroid female, with a markedly prolonged morphine effect post-operatively, characterized by a profound respiratory depression attributed to a pre-operative medication of 10 mg morphine sulphate. This observed idiosyncratic reaction to morphine, and the increase activity of Δ4-3-ketosteroid hydrogenases in hepatic microsomes of rats pretreated with thyroxin (McGuire and Tomkins, 1959) led to the demonstration (Conney and Garren, 1961) that thyroxin pretreatment of rats resulted in an altered hepatic drug biotransformation as evidenced by increased zoxazolamine metabolism in vivo paralleled with shortened paralysis time and increased activity of NADPH generating enzymes.

B. THYROID HORMONE AND HEPATIC MICROSOMAL ELECTRON TRANSPORT AND SUBSTRATE OXIDATION:

1. Animal Studies

The increased turnover of steroids in thyrotoxicosis (Levin and Daughadays, 1955, Petersen, 1958) led to the elucidation of the role of thyroxin in NADPH dependent biotransformation of steroids by the liver (McGuire and Tompkins, 1959). These hepatic enzymes involved with steroid biotransformation have a remarkable similarity with those involved in drug oxidation. For instance, enzymes for the biotrans-

formation of both steroids and exogenous substrates are localized in the liver microsomes, require NADPH and are oxygen dependent (Mannering, 1971, Conney, 1967, Conney, 1971, Gillette et al., 1957). Because of these observed similarities between the hydroxylases of both steroids and exogenous substrates, and because thyroid hormone increased the activity of the steroid hydroxylating enzymes, it was postulated and subsequently shown that thyroid hormone has a significant influence on the hepatic microsomal drug oxidative enzymes (Conney and Garren, 1961, Prange et al., 1966, Kato and Gillette, 1965).

Tables I to IV summarize the effect of thyroidectomy and of thyroid hormone treatment, on various substrate oxidations and the components of the hepatic microsomal electron transport pathway. Notwithstanding a few inconsistencies, the literature allows the following generalizations: in male rats, thyroxin administered for at least 10 days decreases Type I substrate oxidations (aminopyrine N-demethylation, hexobarbital hydroxylation, morphine N-demethylation, testosterone hydroxylation and progesterone hydroxylation), increases Type II substrate oxidation (aniline p-hydroxylation), increases NADPH oxidase activity and NADPH cytochrome c reductase activity but decreases cytochrome P-450 content.

In female rats, thyroxin augments or does not affect Type I (supra vide) oxidation and enhances Type II substrate oxidations. However, there appears to be no sex difference in the effect of thyroxin on the hepatic microsomal electron flux, for thyroxin increases the NADPH oxidase activity and NADPH cytochrome c reductase but

decreases cytochrome P-450 in both sexes.

Thyroidectomy results in a decrease in electron flux as evidenced by decreased activity of NADPH oxidase and NADPH cytochrome c reductase in both sexes. No effect was observable in cytochrome P-450 in either sex. The decrease in the carrier flavoprotein was reversible by tri-iodothyronine administration. Cytochrome P-450 showed the typical response to thyroxin, i.e. a decrease in the hemoprotein content, with the replacement therapy using tri-iodothyronine.

The activity of the substrate oxidative enzymes (aminopyrine N-demethylase, hexobarbital hydroxylase, aniline p-hydroxylase) and p-nitrobenzoic acid reduction were uniformly decreased by surgical thyroidectomy. These were all reversed by replacement therapy with tri-iodothyronine in the female rat but not in the male rat. These obvious differences in response due to sex which have been observed also in other pathophysiologic states, e.g., starvation (Kato and Gillette, 1965) raised the concept of androgen dependent microsomal oxidative enzymes (Kato and Takahashi, 1968) as exemplified by aminopyrine N-demethylase and hexobarbital hydroxylase. This hypothesis advanced the possibility of bipartite composition of the enzymes, one part, the lesser portion, representing the basal activity which is increased by thyroxin and the greater portion, the androgen-dependent activity which is decreased by thyroxin. This concept gained support from the observations that male castrated rats treated with thyroxin showed the female pattern in aminopyrine N-demethylation, hexobarbital hydroxylation and aniline p-hydroxylation and that methyltestosterone therapy reverted this pattern back into the male response to thyroxin (Kato and Gillette,

1965). The male-female variation has been attributed to differences in sex hormones (Quinn et al., 1958, Kato and Onada, 1970) to quantitative differences in substrate binding to cytochrome P-450 (Schenkman et al., 1967a, b) and to differences in the substrate-enhanced rate of NADPH cytochrome P-450 reductase (Gillette and Gram, 1969).

Gillette (1963) found that thyroidectomy of immature female rats or treatment of mature male rats with ^{131}I produced no significant effect on the microsomal enzymes that catalyze the metabolism of hexobarbital, acetanilid and monomethyl-4-aminopyrine. Orrenius et al. (1965) found similar observations that surgical thyroidectomy produced no effect on aminopyrine N-demethylation. These observations which are inconsistent with the work of other investigators (Kato and Takahashi, 1968) could probably be explained by the duration of the thyroidectomized state relative to the measurements of the microsomal oxidative process. Adaptive response occurs in thyroidectomy as evidenced by an abrupt increase in cytochrome P-450 and cytochrome b5 in thyroidectomized rats 15 days post-thyroidectomy (Suzuki et al., 1967). Similarly, an initial decrease in aminopyrine N-demethylation followed by a rise to near normal level occurs in prolonged (5 weeks) thyroxin therapy in immature male rats (Aranda et al., 1974). The isolated observation of an increase cytochrome P-450 and cytochrome b5 (Raw and Anes de Silva, 1965) in hyperthyroidism however remains unexplained.

2. Human Studies

The prolonged morphine effect in a hypothyroid female patient reported by Lund and Benedict in 1929 probably represents the earliest published observations of the potential clinical significance of the

influence of the thyroid hormone on hepatic drug biotransformation. Presently, there is very little information regarding the relationship of thyroid function to hepatic microsomal drug metabolism in man. Brunk et al (1969, 1974) showed a prolonged antipyrine plasma half-life in myxedematons hypothyroid patients. Similarly Vessel and Passananti (1973) demonstrated a prolonged antipyrine half-life in 2 hypothyroid patients ($T\frac{1}{2}$ = 24 and 36 hours) and shortened antipyrine half-life in 2 hyperthyroid patients ($T\frac{1}{2}$ = 6.9 and 7.2 hours) relative to the half-life in normal healthy volunteers ($T\frac{1}{2}$ = 10.9±0.5 hours). More recently, Crooks et al. (1973) confirmed Vessel's observation by the demonstration of an increased antipyrine half-life in 20 hypothyroid female patients ($T\frac{1}{2}$ = 16.2±2.3 hours) and shortened half-life in 13 hyperthyroid patients ($T\frac{1}{2}$ = 7.9±0.6 hours) relative to 36 normal adults ($T\frac{1}{2}$ = 10.8±0.4 hours). Evidence has been provided that the level of circulating thyroid hormone may be important in controlling the rate of drug metabolism. A close inverse correlation between the serum thyroxin levels and the corresponding serum half-life of antipyrine in four patients with hyperthyroidism before and after sub-total thyroidectomy and in four patients with hypothyroidism before and after treatment with thyroid hormone has been demonstrated (Eichelbaum et al., 1974). In thyrotoxic patients treated with the antithyroid drug carbimozole, the plasma half-life of antipyrine increased from 8.9±1.0 hours after one week of therapy to 11.4±1.1 hours after nine weeks. (Crooks et al., 1973). If tri-iodothyronine was administered concomitantly with carbimozole, the normalization of antipyrine half-life occurred much less rapidly (Crooks, 1973).

Since a variety of endogenous substrates such as fatty acids, bile acids and hormones (Lu et al., 1968, Conney and Kuntzman, 1971,

Voight et al., 1968) undergoes biotransformation catalyzed by the microsomal mixed function oxidases, it is not unexpected that the turnover of some endogenous substrates is altered in thyroid dysfunction. Indeed, increased steroid metabolism has been demonstrated in thyrotoxicosis (Levin and Daughaday, 1955, Petersen, 1958).

The available data in the literature do not allow definitive generalization regarding the relationship of thyroid status and hepatic biotransformation of drugs in man. It appears that a hyperthyroid state may accelerate the rate of hepatic microsomal biotransformation of drugs, and conversely, a hypothyroid state may decrease the same biotransformation process. Further studies are needed to evaluate this relationship in man.

TABLE I : EFFECT OF THYROID HORMONE ON HEPATIC MICROSOMAL ELECTRON TRANSPORT

Enzyme	Species, sex, weight	Thyroid hormone dose/duration	Other drugs dose, duration	Result	Reference
NADPH oxidase	Rat, wistar M 190 g F 160 g	T4 1 mg/kg i.p./ day x 10 days	--	Increased[1] in M & F[4]	A
	M 190 g	as above	Phenobarbital 80 mg/kg 48 & 72 hrs	Increased[2]	A
NADPH cytochrome c reductase	Rat, wistar M 190 g F 160 g	as above	--	Increased[1] in M & F	A
	M 190 g	as above	Phenobarbital 80 mg/kg 48 & 72 hrs	Increased[2]	A
NADPH neotetrazolium reductase	Rat, wistar M 190 g F 160 g	T4 1 ug/kg/day o.p. x 10 days	--	Increased[1] in M & F	A
	M 190 g	as above	Phenobarbital 80 mg/kg i.p. (2 doses)	Increased[2]	A

TABLE 1 continued

Enzyme	Species sex, weight	Thyroid hormone treatment	Other drugs Dose/duration	Results	Reference
NADH oxidase	Rat, wistar M 190 g	T4 1 mg/kg/day i.p. x 10 days	--	Increased[1] in M & F	A
	M 190 g	as above	Phenobarbital 80 mg/kg i.p. (2 doses)	Decreased[2]	A
NADH cytochrome c reductase	Rat, wistar M 190 g F 160 g	T4 1 mg/kg/day i.p. x 10 days	--	Decreased[1] in M & F	A
	M 190 g	as above	Phenobarbital 80 mg/kg i.p. (2 doses)	No change[2]	A
Cytochrome b_5	Rat, wistar M 190 g F 160 g	T4 1 mg/kg/day i.p. x 10 days	--	Increased[1] in M	A
	M 190 g	as above	Phenobarbital 80 mg/kg i.p. (2 doses)	No change[1,2]	A

TABLE 1 continued

Enzyme + other parameters	Species, sex, weight	Thyroid hormone	Other drugs	Results	Reference
Cytochrome P-450	Rat, wistar M	as above	--	Decreased[1] in M & F	B
	M 190 g	as above	Phenobarbital 80 mg/kg i.p. (2 doses)	Increased[2]	A
>	M) F) 160-80 g	T4 1.5 mg/kg/ day x 10 days	--	Decreased[1] in M & F	B
Microsomal protein	as above	as above	--	No change[1]	B

A - Kato and Takahashi, 1968
B - Kato et al., 1970

1 - Compared to control (untreated) groups
2 - Compared to thyroxin treated groups
3 - Dose prior to sacrifice

TABLE II : EFFECT OF THYROID HORMONE THERAPY ON HEPATIC MICROSOMAL SUBSTRATE OXIDATION

Substrate oxidized	Animal, sex, weight	Thyroid hormone treatment	Surgical procedures	Other drug pretreatment	Results	Reference
Aminopyrine	Rat, wistar M & F 160-180 g	T4 1.5 mg/kg/ day x 10 days	-	-	Decreased[1] in M	B
	Rat, Sprague Dawley M 180 g F 160 g	T4 1.5 mg/kg/ day x 14 days	-	-	Decreased[1] in M Increased[1] in F	A A
	Rat, wistar M 190 g F 160 g	T4 1 mg/kg/ day x 10 days	-	-	Decreased[1] in M Increased[1] in F	C
	M 190 g	as above	-	Phenobarbital 80 mg/kg i.p. (2 doses)	Increased[2]	C
	Rat, SD, M	T4 1.5 mg/kg/ day x 14 days	Castration	-	No change[1]	A
	Rat, SD, M	as above	Castration	Methyltestos-terone	Decreased[1]	A
	Rat, SD, F (100-110 g)	as above	castration	-	No change[1]	A
		as above	castration	Methyltestos-terone	Decreased[1]	A

TABLE II continued

Substrate oxidized	Animal, sex, weight	Thyroid hormone treatment	Surgical procedures	Other drug pretreatment	Results	Reference
Aniline	Rat, wistar M & F 160-180 g	T4 1.5 mg/kg/ day x 10 days	-	-	Increased in M & F[1]	B
	M 190 g	T4 1 mg/kg/ day x 10 days	-	-	Increased in both M & F[1]	C
	Rat, wistar M	T4 1 mg/kg/ day x 10 days	-	Phenobarbital 80 mg/kg (2 doses)	Increased[2]	C
	Rat, SD M 180 g F 160 g	T4 1.5 mg/kg/ day x 14 days	-	-	Increased in M & F[1]	A
	M	as above	Castration	-	Increased[1]	A
	M	as above	Castration	Methyltestos-terone	Increased[1]	A
	F	as above	Castration	-	Increased[1]	A
	F	as above	Castration	Methyltestos-terone	Increased[1]	A
Hexobarbi-tal (sleep-ing time)	Rat, SD M 70-80 g	T4 0.2 mg/day x 18-20 days	-	-	Prolonged[1]	D

TABLE II continued

Substrate oxidized	Animal sex, weight	Thyroid hormone treatment	Surgical procedures	Other drug pretreatment	Results	Reference
Hexobarbital	Rat, SD M 70-80 g	T4 0.2 g daily x 18-20 days	-	-	Decreased[1]	D
	Rat, wistar M & F 160-180 g	T4 1.5 mg/kg/ day x 10 days	-	-	Decreased in M[1] Increased in F[1]	B
	Rat, wistar M 190 g F 160 g	T4 1 mg/kg/ day x 10 days	-	-	Decreased in M[1] Increased in F[1]	C
	M	as above	-	Phenobarbital 80 mg/kg (2 doses)	Increased[2]	C
	Rat, SD M 180 g F 160 g	T4 1.5 mg/kg/ day x 14 days	-	-	Decreased in M[1]	A
	M	as above	Castration	-	No change[1]	A
	M	as above	Castration	Methyltestos-terone	Decreased[1]	A
	F	as above	Castration	-	No change[1]	A
		as above	Castration	Methyltestos-terone	Decreased[1]	A

TABLE II continued

Substrate oxidized	Animal, sex, weight	Thyroid hormone treatment	Surgical procedures	Other drug pretreatment	Results	Reference
Morphine	Rat, NIH M 200-300 g	T4 90 ug/day x 7 days	-	-	No change[1]	E
		T4 90 ug/day x 10 days	-	-	Decreased[1]	E
		T4 90 ug/day 14-19 days	-	-	Decreased[1]	E
p-Nitro-benzoic acid	Rat, wistar M 190 g F 160 g	T4 1 ug/kg/day x 10 days	-	-	Increased in M[1]	C
	M	as above	-	Phenobarbital 80 mg/kg (2 doses)	Increased[2]	C
Proges-terone	Rat, wistar M & F 180-200 g	T4 1 mg/kg/day x 10 days	-	-	Decreased in M[1]	F
Testos-terone	as above	as above	-	-	Decreased in M[1]	G
Zoxazol-amine (paraly-sis time)	Rat, SD	T4 0.2 ngm/day x 18-20 days	-	-	Shortened[1]	D

TABLE II continued

Substrate oxidized	Animal, sex, weight	Thyroid hormone treatment	Surgical procedures	Other drug pretreatment	Results	Reference
Zoxazol-amine (in vivo metab)	as above	as above	-	-	Increased[1]	D
Zoxazol-amine (in vitro)	Rat, SD M 180 g F 160 g	1.5 ngm/kg/day x 14 days	-	-	Increased in M[1]	A
Zoxazol-amine	Rat, wistar M & F 160-180 g	T4 1.5 mg/kg/day x 10 days	-	-	No change	B

A - Kato and Gillette, 1965
B - Kato et al., 1970a
C - Kato and Takahashi, 1968
D - Conney and Garren, 1961
E - Cochin and Sokoloff, 1960
F - Kato et al, 1970b
G - Kato et al., 1970c
1 - Compared to control untreated group
2 - Compared to thyroxin treated group

TABLE III : EFFECT OF THYROIDECTOMY WITH AND WITHOUT THYROID HORMONE THERAPY ON HEPATIC MICROSOMAL ELECTRON TRANSPORT

Enzymes	Species, sex, weight	Thyroidectomy (duration)	Thyroid hormone therapy	Other drugs	Results	Ref.
NADPH oxidase nmoles x min^{-1} x mg^{-1} protein	Rat, wistar M 190 g F 160 g	23 days	T3 (100 ug/g, i.p.) 2 doses, 24 hrs	-	No significant change[1]	A
		23 days	-	-	Decreased in M & F[1]	A
	Rat, wistar M 110-140 g	10-17 days	-	-	Decreased[1]	B
		10-17 days	T3 30 ug/100 g single dose[4]	-	Increased[2]	B
	Rat, wistar F 160 g	23 days	-	Phenobarbital 60 mg/kg i.p. (2 doses)	Increased[2]	A
NADPH cytochrome c reductase activity	Rat, wistar M 190 g. F 160 g	23 days	T3 (100 ug/kg i.p.) 2 doses in 48 hrs	-	Increased in F only[1]	A
	Rat, wistar M 190 g F 160 g	23 days	-	-	Decreased in M & F[1]	A
	Rat, wistar M 110-140 g	10-17 days	-	-	Decreased[1]	B

TABLE III continued

Enzymes	Species sex, weight	Thyroidectomy (duration)	Thyroid hormone therapy	Other drugs	Results	Ref.
NADPH cytochrome c reductase activity	Rat, wistar M 110-140 g	10-17 days	T3 30 ug/100 g body weight (single dose)	-	Increased[2]	B
	Rat, wistar F 160 g	23 days	-	Phenobarbital 60 mg/kg i.p. (2 doses)	Increased[2]	A
NADPH neotetrazolium reductase	Rat, wistar M 190 g F 160 g	23 days	T3 (100 ug/kg i.p.) 2 doses 48 hrs[4]	-	Decreased in M[1] Increased in F[1]	A
	Rat, wistar M 190 g F 160 g	23 days	-	-	Decreased in M & F[1]	A
	F 160 g	23 days	-	Phenobarbital 60 mg/kg i.p. 48, 72 hrs[4]	Increased[1]	A
NADPH diaphorase	Rat, wistar M 110-140 g	10-17 days	-	-	Decreased[1]	B
		10-17 days	T3 (30 ug/100 g) single dose	-	Increased[2]	B

TABLE III continued

Enzyme	Species, sex, weight	Thyroidectomy (duration)	Thyroid hormone Therapy	Other drugs	Results	Ref.
NADPH oxidase	Rat, wistar M 190 g F 160 g	23 days	T3 (100 ug/kg i.p.) 2 doses in 48 hrs[4]	-	Increased in M & F	A
	M 190 g F 160 g	23 days	-	-	Decreased in M & F[1]	A
NADPH cytochrome c reductase	Rat, wistar M 190 g F 160 g	23 days	T3 (100 ug/kg i.p.) 2 doses in 48 hrs[4]	-	No change	A
Cytochrome b_5	Rat, wistar M 190 g F 160 g	23 days	-	-	No change[1]	A
	Rat, wistar M 190 g F 160 g	23 days	T3 (100 ug/kg i.p.) 2 doses in 48 hrs	-	No change[1]	A
	M 110-140 g	10-17 days	-	-	No change[1]	B
		>17 days	-	-	Increased[1]	B
		10-17 days	T3 30 ug/100 g single dose	-	Decreased[2]	B

TABLE III continued

Enzyme	Species, sex, weight	Thyroidectomy (duration)	Thyroid hormone therapy	Other drugs	Results	Ref.
Cytochrome P-450	Rat, wistar M 190 g F 160 g	23 days	-	-	No change[1]	A
	Rat, wistar M 190 g F 160 g	23 days	T3 (100 ug/kg i.p.) 2 doses in 48 hrs	-	Decreased in M only[1]	A
	F 160 g	23 days	-	Phenobarbital 60 mg/kg 48 & 72 hrs	Increased[1]	A
	M 180-200 g	23 days	-	-	Decreased in M[1]	C
	M 110-140 g	10-17 days	-	-	No change[1]	B
	M 110-140 g	>17 days	-	-	Increased[1]	B
	M 110-140 g	10-17 days	T3 (30 ug/100 g) single dose	-	Decreased[2]	B

A - Kato and Takahashi, 1968
B - Suzuki et al., 1967
C - Kato et al., 1970

1 - Compared to control untreated group
2 - Compared to thyroxin treated group

TABLE IV : EFFECT OF THYROIDECTOMY WITH AND WITHOUT THYROID HORMONE REPLACEMENT AND HEPATIC MICROSOMAL SUBSTRATE OXIDATION .

Substrate oxidized	Species, sex, weight	Thyroidectomy (duration)	Thyroid hormone (therapy)	Other drugs	Results	Ref.
Aminopyrine	Rat, wistar M 190 g F 160 g	23 days	-	-	Decreased in M & F[1]	A
		23 days	T3 (100 ug/g x 2 days[4]	-	Decreased in M only[1]	A
	F 160 g	23 days	-	Phenobarbital 60 mg/kg 48 and 72 hrs	Increased[2]	A
Aniline	Rat, wistar M 190 g F 160 g	23 days	-	-	Decreased in F & M[1]	A
		23 days	T3 100 ug/kg i.p. 48 and 72 hrs	-	Increased in F only[1]	A
	F 160 g	23 days	-	Phenobarbital 60 mg/kg i.p. 48 and 72 hrs	Increased[2]	A

TABLE IV continued

Substrate oxidized	Species sex, weight	Thyroidectomy (duration)	Thyroid hormone therapy	Other drugs	Results	Ref.
Hex barbital	Rat, wistar M 190 g	23 days	-	-	Decreased in M & F[1]	A
	F 160 g	23 days	T3 100 ug i.p. x 2 days	-	Decreased in M[1] Increased in F[1]	A
	F 160 g	23 days	-	Phenobarbital 60 mg/kg i.p. 48 and 72 hrs	Increased[2]	A
p-Nitro-benzoic acid	Rat, wistar M 190 g	23 days	-	-	Decreased in M & F[1]	A
	F 160 g	23 days	T3 100 ug i.p. x 2 days	-	Increased in F only[1]	A
	F 160 g	23 days	-	Phenobarbital 60 mg/kg i.p. 48 and 72 hrs	Increased[2]	A
Proges-terone	Rat, wistar M & F 180-200 g	23 days	-	-	Decreased in M[1]	B
Testos-terone	Rat, wistar M & F 180-200 g	23 days	-	-	Decreased in M[1]	C

A - Kato and Takahashi, 1968
B - Kato et al., 1970
C - Kato et al., 1970c

1 - Compared to control untreated group
2 - Compared to thyroxin treated group

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APPENDIX B

MATERIALS AND METHODS

1. MATERIALS:

(a) Tissue Samples and experimental animals:

The tissue samples and experimental animals in these studies have been presented in the foregoing individual manuscripts. Therefore, these will not be discussed in this section. Reference is made to the respective manuscripts in this thesis.

(b) Chemicals and Reagents:

Acetyl Acetone, reagent grade: Fisher Scientific Co., Fair Lawn, New Jersey.

Albumin, purified: J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Aminopyrine: CIBA Co. Ltd., Dorval, Quebec.

Ammonium Acetate: J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Aniline, certified grade: Fisher Scientific Co., Fair Lawn, New Jersey.

Barium hydroxide: J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Biuret Reagent: Gornall-Bardawill-David formula: Harleco Chemicals, Philadelphia, Pennsylvania.

Cytochrome c (from horse heart): Sigma Chemical Co., St. Louis, Missouri.

Disodium monohydrogen phosphate: J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Ethyl ether (anhydrous), analytic grade: Mallinckrodt Chemical Works, St. Louis, Missouri.

Formaldehyde, analytic grade: British Drug Houses (Canada) Ltd., Montreal,

Iodine 131 (as Sodium Iodide) New England Nuclear Co., Boston.

Isocitric Dehydrogenase (from pig heart) in 50% glycerol solution: Sigma Chemical Co., St. Louis, Missouri.

Magnesium Chloride ($6H_20$): J.T. Baker Chemical Co., Phillipsburg, New Jersey.

NADP (Triphosphopyridine nucleotide): Sigma Chemical Co., St. Louis, Missouri.

NADPH, (Triphosphopyridine nucleotide reduced): Sigma Chemical Co., St. Louis, Missouri.

Nicotinamide: Sigma Chemical Co., St. Louis, Missouri.

Para-aminophenol, practical grade: Eastman Kodak Co., Rochester, New York.

Phenol, U.S.P.: J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Potassium dihydrogen phosphate: J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Semicarbazide hydrochloride: Sigma Chemical Co., St. Louis, Missouri.

Sodium anthraquinone beta sulfonate: Fisher Scientific Co., Fair Lawn, New Jersey.

Sodium Chloride: J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Sodium dithionite (sodium hydrosulfite): Fisher Scientific Co., Fair Lawn, New Jersey.

Sodium hydroxide: J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Sodium isocitrate, (Trisodium salt of DL-isocitric acid): Sigma Chemical Co., St. Louis, Missouri.

L-Thyroxine (Sodium salt): Sigma Chemical Co., St. Louis, Missouri.

Zinc Sulfate ($7H_2O$): J.T. Baker Chemical Co., Phillipsburg, New Jersey.

2. METHODS:

(a) Experimental designs and statistical analyses:

These sections have been presented in the foregoing manuscripts.

(b) Assays:

(i) Protein determination

The biuret method (Kabat & Mayer, 1967) was used to determine the protein concentration of the microsomal suspension. A standard curve was prepared using purified albumin (J.T. Baker) at concentrations from 1 to 7 mg per ml.

(ii) Assay of hepatic microsomal NADPH oxidase

The activity of hepatic microsomal NADPH oxidase was determined spectrophotometrically by measuring the rate of oxidation of NADPH as described by Gillette et al (1957). The reaction mixture contained 1.8 ml of 0.1 M phosphate buffer, pH 7.4, 36 µmoles of magnesium chloride, 0.1 ml of the microsomal preparation and 1 µmole of NADPH in 0.1 ml of phosphate buffer to a final volume of 2.1 ml. NADPH was added last. The control cuvette contained all the components except the reduced coenzyme. A decrease in absorbance at 340 nm was recorded in a Beckman DBGT recording sepctrophotometer and the specific activity of NADPH oxidase was determined from the change in the absorbance during the initial 5 minutes and using an extinction coefficient of 6.22 mM^{-1} cm^{-1} (Ernster, 1967). The reaction was linear over this period.

(iii) Assay of hepatic microsomal NADPH cytochrome c reductase

NADPH cytochrome c reductase activity was determined by following the absorbance change at 550 nm, reflecting the appearance of reduced cytochrome c after addition of NADPH as described by Phillips and Langdon (1962). The reaction was carried out at 37°C in a mixture containing 0.15 µmoles of cytochrome c, 36 µmoles of $MgCl_2$ and 25 µlitres of

microsomal suspension in a final volume of 2.1 ml of 0.1 M phosphate buffer (pH 7.4). The reaction was started by the addition of 1.0 µmole of NADPH in a volume of 0.1 ml to the experimental cuvette. A corresponding volume of buffer was added to the reference cuvette. The rate of appearance of reduced cytochrome c was measured in a Beckman DBGT recording spectrophotometer and specific activity of NADPH cytochrome c reductase was determined from the tangent to the curve, representing the rapid phase of reduction (duration approximately 20 seconds). An extinction coefficient of 19.1 mN^{-1} cm^{-1} (Peters and Fouts, 1970) was used in calculating the absolute amount of cytochrome c reduced per minute per mg of microsomal protein.

(iv) Assay of hepatic microsomal cytochrome P-450 content

The reduced form of cytochrome P-450 combines readily with carbon monoxide to form a complex which is measureable spectrophotometrically through its absorption at 450 nm as described by Omura and Sato (1964a). A microsomal suspension containing 2-5 mg of protein per ml was obtained by dilution with 0.1 M phosphate buffer (pH 7.4). A few mg of sodium dithionite was added to the suspension in order to reduce the cytochrome P-450 present and following this reduction the suspension was divided equally between 2 cuvettes. Carbon monoxide was then bubbled gently through the sample cuvette for 30 seconds following which the cuvette was sealed. The sample cuvette was then read against the reference sample (unexposed to carbon monoxide) at 490 nm and 450 nm. The quantity of cytochrome P-450 was calculated from the difference in optical density (490 nm - 450 nm) using a molar extinction coefficient of 91 nM^{-1} cm^{-1} (Omura and Sato, 1964b). Content was expressed

as nmoles of cytochrome P-450 per mg of microsomal protein.

(v) Assay of hepatic microsomal NADPH cytochrome P-450 reductase

NADPH cytochrome P-450 reductase activity was determined by following the absorbance change at 450 nm (in a Beckman DBGT recording spectrophotometer) when carbon monoxide saturated microsomes were reduced by NADPH in a modification of the method described by Gigon et al (1968). Carbon monoxide which was deoxygenated by passage through an alkaline dithionite solution (0.05% sodium anthraquinone beta sulfonate and 0.5% sodium dithionite in 0.1 N sodium hydroxide) was bubbled for 5 minutes through a microsomal suspension containing 5 mg of protein per ml in 0.1 M phosphate buffer (pH 7.4). Three ml of the suspension was then transferred to an anaerobic Aminco cell (A1-65085) and a plunger assembly containing 2 μmoles of NADPH in 50 μlitres of buffer was fitted to the cuvette. Carbon monoxide was passed through the air space within the cell for a further 3 minutes (diffusion of CO in the suspension was enhanced by use of a small magnetic stirring bar) and placed in a spectrophotometer where it was allowed to equilibrate for 2 minutes at 37°C. The reference cell contained an untreated microsomal suspension of identical protein concentration. The plunger was depressed and the change in absorbance at 450 nm with the rapid appearance of a CO-reduced cytochrome P-450 complex was recorded on chart paper moving at 10 inches per minute. The velocity of the reaction was determined from the slope of the initial linear phase. In our experiments the initial linear phase was complete approximately 6 seconds after addition of NADPH.

(vi) Assay of aminopyrine-enhanced hepatic microsomal NADPH cytochrome P-450 reductase

The aminopyrine-enhanced rate of cytochrome P-450 reduction by NADPH was measured in a manner identical to that just described except that aminopyrine was added to the microsomal suspension to a final concentration of 1 mM. The protein concentration of the microsomal suspension was again 5 mg per ml.

(vii) Assay of hepatic microsomal aminopyrine N-demethylase

The activity of hepatic microsomal aminopyrine N-demethylase was determined by measuring the production of formaldehyde from aminopyrine as described by Cochin and Axelrod (1959). The reaction mixture contained 0.1 ml of microsomal suspension, 36 μmoles of $MgCl_2$, 24 μmoles of neutralized semicarbazide HCl, 40 μmoles of nicotinamide, 0.66 μmole of $NADP^+$, 16 μmoles of sodium isocitrate, 0.5 units of isocitric acid dehydrogenase and 10 μmoles of aminopyrine in final volume of 2 ml of 0.1 M phosphate buffer (pH 7.4). The concentration of nicotinamide used has been reported to inhibit hepatic microsomal N-demethylation of aminopyrine (Schenkman et al., 1967), but in these experiments addition of amounts up to 100 μmoles had no such effect. Samples were incubated for 20 minutes at 37°C in a Dubnoff shaking water bath and the reaction was terminated by the addition of 0.5 ml of 15% $ZnSO_4$ followed by vigorous mixing. After 5 minutes 0.5 ml of saturated BaOH was added and the samples were well mixed and allowed to stand for 5 minutes. After centrifugation for 5 minutes at 1500 x g the clear supernatant was assayed for formaldehyde by the method of Cochin and Axelrod (1959). The supernatant fraction (2.0 ml) was transferred to

a tube containing 1 ml of Nash reagent (0.2 ml of acetylacetone in 50 ml of an aqueous solution containing 15 g of ammonium acetate) and the mixture was incubated for 30 minutes at 60°C. After cooling to room temperature, optical density for each sample was determined at 415 nm in a spectrophotometer (Beckman model DBG or DBGT) and the quantity of formaldehyde formed was determined from a standard curve. The standard curve was prepared using concentrations of formaldehyde from 0.5 to 5 mg/ml in aqueous solution. The curve was linear throughout this range. Specific activity of microsomal aminopyrine N-demethylase was expressed as nmoles of formaldehyde formed per minute per mg of microsomal protein. The Nash reaction employed has been widely used since its original description (Nash, 1953). Formaldehyde which is formed during an incubation is trapped as its semicarbazone and measured colorimetrically utilizing a Hantzsch reaction in which a β-diketone (acetylacetone), an aldehyde (formaldehyde) and an amine (ammonia) react to form a coloured product, 3,5-diacetyl-1,4 dihydrolutidine which has an absorption maxima of 415 nm.

(viii) Assay of hepatic microsomal aniline p-hydroxylase

The activity of hepatic microsomal aniline p-hydroxylase was determined by measuring the production of p-aminophenol from aniline as described by Kato and Gillette (1965). The incubation mixture consisted of 0.1 ml of microsomal suspension, 36 µmoles of $MgCl_2$, 40 µmoles of nicotinamide, 0.66 µmoles of $NADP^+$, 16 µmoles of sodium isocitrate, 0.5 units of isocitric acid dehydrogenase and 10 µmoles of aniline, in 0.1 M phosphate buffer (pH 7.4) to a final volume of 2.0 ml. The mixtures were incubated in air for 20 minutes and were then

shaken with 20 ml of peroxide-free anhydrous ethyl ether and 0.8 gm of sodium chloride for 15 minutes. Fifteen ml aliquots of the ether phase were then transferred to tubes containing 3.0 ml of a 0.1 M sodium hydroxide solution mixed with 1% phenol. The p-aminophenol was returned to the alkaline aqueous phase after 15 minutes shaking, following which the ether phase was aspirated and discarded. The aqueous phase was then allowed to stand in a warm water bath (37^{o}) for 30 minutes in order to vapourize any remaining ether. When clear, the tubes were read spectrophotometrically at 620 nm and the p-aminophenol content was determined from a standard curve. The standard curve was prepared following extraction of para-aminophenol from the reaction mixture (minus aniline) in concentrations ranging from 1 to 10 µg/ml. The curve was linear throughout this range. Specific activity of hepatic microsomal aniline p-hydroxylase was expressed as nmoles of p-aminophenol formed per minute per mg of microsomal protein.

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