Environmental Health Effects Research Series

METABOLIC INTERACTIONS OF HORMONAL STEROIDS AND CHLORINATED HYDROCARBONS-Effects of Neonatal Treatment with o,p'-DDT on the Development of the Steroidogenic Endocrine System of the Male Rat

Health Effects Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina 27711

RESEARCH REPORTING SERIES

Research reports of the Office of Research and Development, U.S. Environmental Protection Agency, have been grouped into nine series. These nine broad categories were established to facilitate further development and application of environmental technology. Elimination of traditional grouping was consciously planned to foster technology transfer and a maximum interface in related fields. The nine series are:

1. Environmental Health Effects Research
2. Environmental Protection Technology
3. Ecological Research
4. Environmental Monitoring
5. Socioeconomic Environmental Studies
6. Scientific and Technical Assessment Reports (STAR)
7. Interagency Energy-Environment Research and Development
8. "Special" Reports
9. Miscellaneous Reports

This report has been assigned to the ENVIRONMENTAL HEALTH EFFECTS RESEARCH series. This series describes projects and studies relating to the tolerances of man for unhealthful substances or conditions. This work is generally assessed from a medical viewpoint, including physiological or psychological studies. In addition to toxicology and other medical specialities, study areas include biomedical instrumentation and health research techniques utilizing animals — but always with intended application to human health measures.

METABOLIC INTERACTIONS OF HORMONAL STEROIDS

AND CHLORINATED HYDROCARBONS

Effects of Neonatal Treatment with o,p'-DDT on the Development

of the Steroidogenic Endocrine System of the Male Rat

by

Kenneth Lyle Campbell
The University of Michigan Medical School
Ann Arbor, Michigan 48104

Principal Investigator

Merle Mason, Ph.D.
Associate Professor of Biological Chemistry
The University of Michigan Medical School
Ann Arbor, Michigan 48104

DISCLAIMER

This report has been reviewed by the Health Effects Research
Laboratory, U.S. Environmental Protection Agency, and approved for
publication. Approval does not signify that the contents necessarily
reflect the views and policies of the U.S. Environmental Protection
Agency, nor does mention of trade names or commercial products
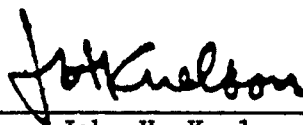constitute endorsement or recommendation for use.

FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The study described herein was initiated to meet program requirements concerning the improvement of in vivo measurements involved in determining the potential toxicity of xenobiotics on reproduction. The information gained from this study has permitted the evaluation of the effects on the developing endocrine system of a rat following neonatal exposure to a model compound, o,p'-DDT. It is anticipated that the model developed in this study can be applied to other compounds and species and thus serve as a useful adjunct to toxicology research.

John H. Knelson, M.D.
Director,
Health Effects Research Laboratory

iii

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

TABLE OF CONTENTS (Cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Cont.)

x

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND COMMON NAMES

ACTH	adrenocorticotrophic hormone
Aldrin	1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-exo-1,4-endo-5,8-dimethano-naphthalene
$\triangle^4$-	$\triangle^4$-androstenedione; 4-androsten-3,17-dione
ANOVA	analysis of variance
BSA	bovine serum albumin
C	control
cAMP	cyclic 3',5'-adenosine monophosphate
Cholesterol	5-cholesten-3α-ol
CBG	corticosteroid binding globulin
Ci	Curie
CPE	5-cholesten-3α-ol-3-propyl ether
CRH	corticotrophin releasing hormone
DDA,DDE,DDD,DDT	either the o,p'- or p,p'-isomer
o,p'-DDA	2-(2-chlorophenyl)-2-(4-chlorophenyl)-acetic acid
p,p'-DDA	2,2-bis-(4-chlorophenyl)-acetic acid
o,p'-DDD	2,2-dichloro-1-(2-chlorophenyl)-1-(4-chlorophenyl)-ethane; 1,1-dichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)-ethane
p,p'-DDD	2,2-dichloro-1,1-bis-(4-chlorophenyl)-ethane; 1,1-dichloro-2,2-bis(4-chlorophenyl)-ethane
o,p'-DDE	2,2-dichloro-1-(2-chlorophenyl)-1-(4-chlorophenyl)-ethylene; 1,1-dichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)-ethylene

p,p'-DDE	2,2-dichloro-1,1-bis-(4-chlorophenyl)-ethylene; 2,2-bis-(4-chlorophenyl)-1,1-dichloroethylene
p,p'-DDMU	2-chloro-1,1-bis(4-chlorophenyl)-ethylene
o,p'-DDT	1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)-ethane
p,p'-DDT	1,1,1-trichloro-2,2-bis-(4-chlorophenyl)-ethane
DHA	dehydroepiandrosterone; 5-androsten-3α-ol-17-one
DMSO	dimethyl sulfoxide
dpm	disintegrations per minute
EST	Eastern standard time
estradiol	17β-estradiol; 1,3,5(10)-estratriene-3,17β-diol
estriol	1,3,5(10)-estratriene-3,16α,17β-triol
EV	17β-estadiol-17-valerinate
FID	flame ionization detector
FSH	follicle stimulating hormone
GC	gas chromatography
GnRH	gonadotrophin releasing hormone
G-6-P	glucose-6-phosphate
G-6-PDH	glucose-6-phosphate dehydrogenase
hCG	human chorionic gonadotrophin
17α	17α-hydroxyprogesterone; 4-pregnen-17α-ol-3,20-dione
ICSH	interstitial cell stimulating hormone (LH)
i.p.	intraperitoneal
LD_{50}	lethal dose to 50%

LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
Mirex	dodecachlorooctahydro-1,3,4-metheno-2H-cyclobuta [cd] pentalen-2-one
MTS	Michigan Terminal Systems
NADH	reduced nicotinamide adenine dinucleotide
NADP$^+$	nicotinamide adenine dinucleotide phosphate
OAAD	ovarian ascorbic acid depletion
oLH	ovine luteinizing hormone
P	progesterone; 4-pregnen-3,20-dione
PB	phenobarbital
PBS	phosphate-buffered-saline
PCB's	polychlorinated biphenyls
pg	picogram
POPOP	1,4-bis-[2-(5-phenyloxazolyl)] -benzene
ppm	parts per million
PPO	2,5-diphenyloxazole
psi	pounds per square inch
$\triangle^5$-	$\triangle^5$-pregnenolone; 5-pregnen-3α-ol-20-one
RIA	radioimmunoassay
rpm	revolutions per minute
s.c.	subcutaneous
SKF-525A	2'-diethylaminoethyl-2,2-diphenylpentanoate
T	testosterone; 4-androsten-17β-ol-3-one
technical DDD	90% p,p'-DDD + 10% o,p'-DDD

LIST OF ABBREVIATIONS AND COMMON NAMES (Cont.)

technical DDT   80% p,p'-DDT + 15-20% o,p'-DDT

TLC   thin layer chromatography

TP   testosterone-17-proprionate

TVBE   tail vein-bleeding under ether anesthesia

CHAPTER 1

INTRODUCTION

This investigation was undertaken to determine the
feasibility of using the measurement of specific endocrine
functions as a general screen for the effects of foreign
compounds on reproduction and viability. Its primary
purpose was to generate approaches which might be utilized
to shorten current toxicological screening procedures by
augmenting or supplanting the multigenerational tests now
used to measure effects on reproduction. Secondarily, its
goal was to establish whether 1,1,1-trichloro-2-(2-chloro-
phenyl)-2-(4-chlorophenyl)-ethane (o,p'-DDT) has any effect
on the steroidogenic endocrine tissues of the developing
male rat and if so, what mechanism was involved in
producing the effect.

The experiments to be described do shed some light on
the secondary questions and as a result imply the utility
of at least a portion of the approach for use in screening
protocols. Of equal importance, perhaps, they indicate
the potential for utilization of xenobiotics, such as
o,p'-DDT, to produce pathologic states which can provide
information about the normal development and function of
the endocrine system that is not easily obtained by known
procedures. Finally, these experiments indicate both a
direction for future investigations on the mode of action
of o,p'-DDT in the steroidogenic endocrines and a crude

estimation of how much data of this type must be amassed to generate conclusions of use in the legal regulation of synthetic chemicals.

In order to understand the experiments detailed in Chapters 3-5 and their relation to the objectives an exposition of some of the background literature and the resultant experimental approaches and reasoning is necessary. To that end I will briefly discuss the present models of steroidogenic endocrine function and development and the known metabolism and biological effects of DDT.

I. Steroidogenic Endocrine Function:

The physiologic "purpose" of the steroidogenic endocrines appears to be the production of the steroid hormones; the major synthetic paths are indicated in Figure 1. The active forms of these hormones typically show effects on target tissues possessing intracellular steroid receptor proteins (1,2). Usually these effects are trophic in nature and lead to growth, proliferation and/or differentiation of the target tissue. However, in the case of the hypothalamic centers and at least in some of the pituitary cell types (3) the steroids act by suppressing release of the releasing hormones (-RH, -RF) or the trophic hormones. In this latter manner they indirectly control their own production. Likewise, by effecting the functions of the adrenals and/or kidneys and liver (4,5), they may ostensibly exert some control over their own inactivation as well.

Figure 1. Major Steroid Pathways in the Rat ·

Shown is a composite of the major pathways found in the rat testis, adrenal cortex and ovary. It summarizes the probable sites of action for both trophic hormones (luteinizing hormone - LH, adrenocorticotrophic hormone - ACTH) and $3',5'$-cyclic adenosine monophosphate (cAMP) as well as the various enzymes involved in processing the precursor cholesterol. The enzymes and enzyme complexes shown are: $0 =$ cholesterol-acyl esterase; $1 = 20\alpha$-hydroxylase + 22R-hydroxylase; $2 = C_{20-22}$ lyase; $3 = \Delta^5$-3β-hydroxysteroid dehydrogenase; $4 = \Delta^5 \rightarrow \Delta^4$ isomerase; $5 =$ 21-hydroxylase; $6 = 11\beta$-hydroxylase; $7 = 17\alpha$-hydroxylase; $8 = C_{17-20}$ lyase; $9 =$ aromatizing enzyme complex; $10 = 17\beta$-hydroxysteroid dehydrogenase. Substrate specific forms of many of these enzymes occur in the various tissues.

Those pathways occurring in the testis include the Δ^4- and Δ^5-pathways proceeding from cholesterol and pregnenolone to testosterone and Δ^4-androstenedione using enzymes 1,2,3,4,7,8 and 10. In the adrenals the same pathways occur but utilize enzymes 5 and 6 to produce corticosterone as the major active product. In the ovary the major androgen pathways are still present but testosterone and Δ^4-androstenedione undergo aromatization, 9, to form the major estrogenic products 17β-estradiol and estrone.

FIGURE I

MAJOR STEROID PATHWAYS IN THE RAT

Subcellularly, steroids are formed in the adrenals and gonads from cholesterol or cholesterol esters by the action of enzymes localized in the lipid droplets (cholesterol-acyl esterase), mitochondria (C_{20-22} lyase, 18- and 11β -hydroxylases), endoplasmic reticulum (3β - and 17β -hydroxysteroid dehydrogenases, $\Delta^5 \rightarrow \Delta^4$ -isomerase, C_{17-20} lyase, 17α - and 21-hydroxylases and aromatizing enzymes) and cytosol (20α -hydroxysteroid dehydrogenase) (6). These multienzyme pathways and the indicated compartmentalization allow subcellular modulation of steroid synthesis. They also impose numerous potential sites for the action of modulators, e.g., specific enzyme regulation, membrane modification, blockage or stimulation of cosubstrate (reduced nicotinamide adenine dinucleotide phosphate - NADPH) production, etc. This latter point becomes particularly important and complex when the precise mode of action of an extracellular agent is in question.

The rate of synthesis of the steroids appears to be largely dependent on circulating trophic hormone levels and the state of activation of intracellular adenylate cyclase which is proximally associated with the trophic hormone receptors. Stimulation of the cyclase, or inhibition of 3',5'-cyclic nucleotide phosphodiesterase (7), ultimately leads to protein synthesis generally and subsequently to steroidogenic enzyme synthesis. In addition, direct activation of cholesterol-acyl esterase and the cholesterol side-chain cleavage complex occurs acutely

upon stimulation of the cyclase (8). Thus, the rate of steroidogenesis is both acutely and chronically controlled by enzyme levels and activation states which are in turn determined by trophic hormone levels.

Serum trophic hormone levels are controlled by the rate of synthesis and release of hypophysial trophic hormone stores. In their turn, the stores of these protein hormones depend on the activation of cellular protein synthesis and on cAMP concentrations which are controlled by the level of certain peptides, the trophic hormone releasing hormones (factors), in the hypophysial portal blood (3,9). Further modulation occurs by the direct feedback actions of the steroids as mentioned above. Once more, as in the case of the trophic hormones acting on the adrenals or gonads, an extracellular signal, -RH, controls the synthesis and release of a hormone.

Finally, the release of the releasing hormones is controlled by neurons which contain steroid receptors and which are associated with the neurons which produce and release the releasing hormones into the portal vessels. At this point, however, the modulation by steroid hormones is inverse, and the negative feedback loop of classic endocrinology is completed (Figure 2) (3,9,10).

Throughout this complex feedback system the ultimate effects on steroid target tissues are subject to modulation by parameters such as receptor levels, Ca^{++} levels, blood flow, multiple hormone synergisms, etc., which are in turn

Figure 2. Schematic Diagram of the Steroidogenic

Endocrine System of the Male Rat

The products of the hypothalamus, the releasing hormones (luteinizing hormone releasing factor - LHRH, or gonadotrophin releasing hormone - GnRH, and corticotrophin releasing hormone - CRH), are shown as being transported to the anterior pituitary where they induce release (and synthesis), ⊕, of the trophic hormones (LH or interstitial cell stimulating hormone - ICSH, follicle stimulating hormone - FSH, and ACTH). These hormones are, in turn, transported via systemic circulation, →, to their respective target organs, the steroidogenic glands. These glands are stimulated, ⊕, by the trophic hormones to synthesize their steroid products which are then circulated throughout the body, →. These steroids produce trophic effects, ⊕, on their own respective target organs and provide feedback controls on the hypothalamus, ⊖. Simultaneously the steroids themselves are catabolized, --→, by the liver and other tissues to less active compounds which may be eliminated.

Sperm production appears to be controlled by an interplay of both the pituitary and steroid hormones; FSH, LH and testosterone all exhibit a stimulatory influence on the process.

More complex relationships may exist among the steroidogenic glands themselves, ⦸, but these remain to be clarified.

FIGURE 2
SCHEMATIC DIAGRAM OF THE STEROIDOGENIC ENDOCRINE SYSTEM OF THE MALE RAT
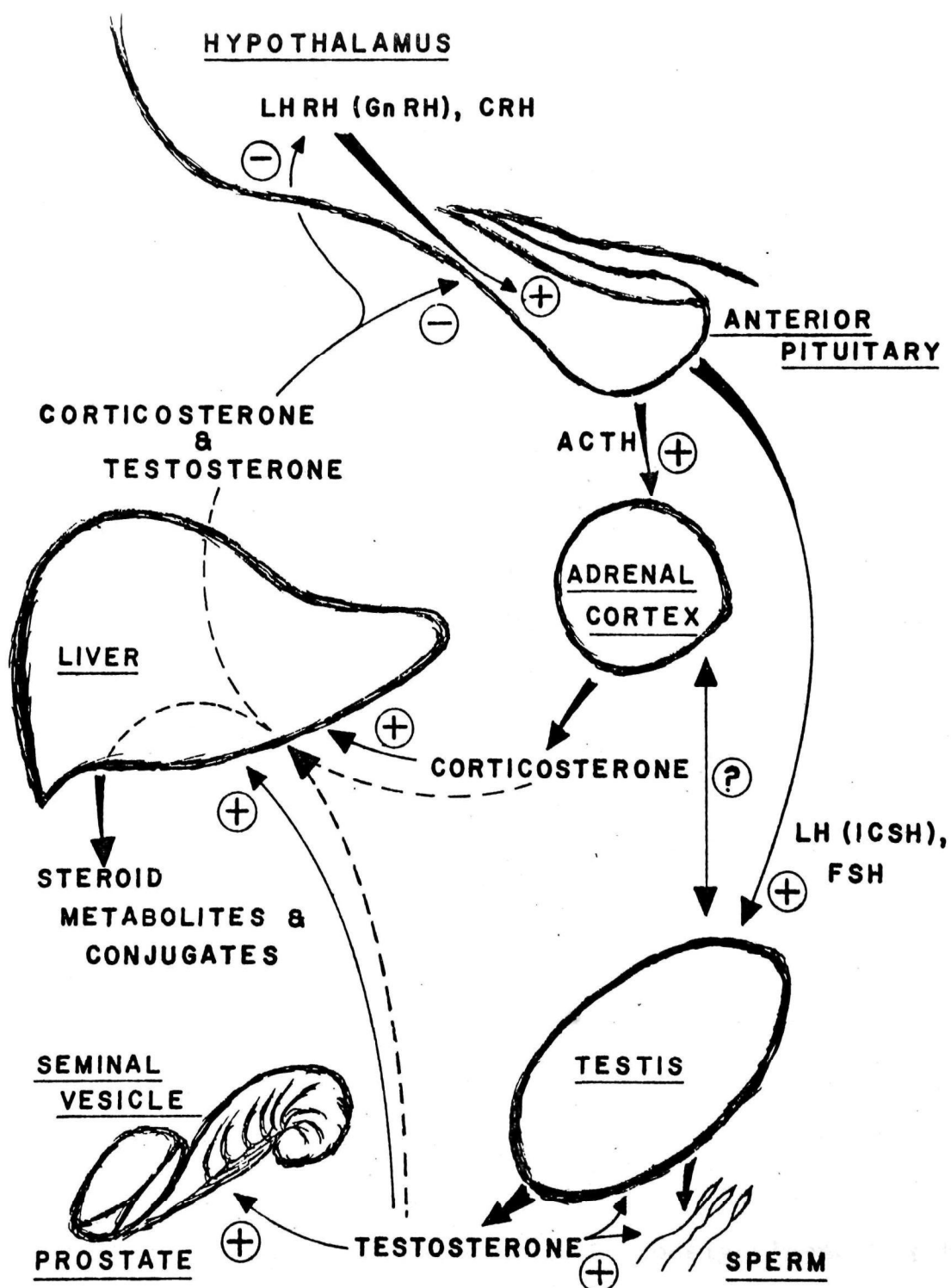
determined by age, sex, nutrition, health, other physiological states and/or the external environment. The feedback loops, which help maintain organismic homeostasis, and the sensitivity of these loops to large numbers of diverse modifying influences appear to be quite suitable as indicators of the unknown effects of exogenous agents on an organism. Since the initial phenomenological question is always whether an agent does or does not exhibit a measurable effect, the fact that exact etiology of an elicited alteration in a hormone feedback loop is unclear is immaterial. If the elicited change is potentially capable of altering the viability and/or fertility of the animal involved the regulatory screening question has been answered. The complexity of endocrine control systems does present problems in defining exact modes of xenobiotic action but it also provides experimentally exploitable elements not found in systems contained within single cells or organs.

In addition to allowing experimental manipulation of these feedback loops the possibilities of exploiting temporal changes is also available. Studies of the time course for the maturation of these loops, in both sexes, and of the cyclical alteration of the loops caused by pregnancy, menstruation or estrus, provide opportunities to explore stages of varying sensitivity to environmental influence. They thus allow identification of the stage(s) in life during which the exogenous agent being tested produces the most profound and/or lasting effects.

Previous work in the general area of toxicity on the steroidogenic endocrines has concentrated on the adult rat and its responses to long term feeding or acute injection of a variety of drugs, pesticides and other synthetic chemicals. Conney et al. concentrated on the effects of barbiturates and pesticides on the clearance of steroids from systemic circulation (11-18) and on the capacity of target organs (uterus and prostate) to bind steroids in the presence of the synthetic chemicals (19-23). Their results have indicated that steroid metabolism and function is indeed sensitive to agents which induce hepatic microsomal metabolism; the steroids are metabolized more quickly and more extensively than normal in treated animals. Further, this same group along with several others (24-27), have shown that some chemicals, e.g., o.p'-DDT and polychlorinated biphenyls (PCB's) are capable of specifically blocking the uptake of steroids by their target organs even in the absence of hepatic induction. Rybakova et al. (28-30) have shown that the pesticides DDT and Sevin are capable of increasing pituitary levels of gonadotrophins in male rats fed contaminated food for long periods of time. Ottoboni has demonstrated lengthened reproductive life span in female rats fed DDT (31) while Ware and Good and Harr et al. have shown a decrease in fertility and fecundity in mice and rats treated with Mirex and dieldrin (32,33). Acute blockage of the ovulation surges of both LH and estrogen in the rat have been

shown with barbiturates (34) given on the morning of proestrus. Ethyl ether acutely raises the serum levels of FSH and LH in rats (35,36). An extensive literature exists on the inhibitory action of DDT and its analogs (especially 2,2-dichloro-1-(2-chlorophenyl)-1-(4-chloro-phenyl)-ethane (o,p'-DDD)) on the adrenal cortices of man (37-41), the dog (42-47) and guinea pig (48,49) and on the lack of this effect on the adult rat (50,51). ACTH release in the rat was increased by dieldrin in a chronic manner unaccounted for by stress (52). Finally, Heinrichs and Gellert have demonstrated cystic ovarian development, persistent vaginal estrus and decreased serum LH levels in response to adult castration in female rats injected neo-natally with o,p'-DDT (53,54).

These last investigations (53,54) have a unique and important character. They demonstrate a permanent altera-tion of a steroidogenic endocrine control system and, together with the "imprinting" experiments discussed below, imply the acute sensitivity of these control systems early in life, either perinatally in the rat and mouse or in utero in most other species. Of equal importance, this sensitive point occurs coincidentally with complete maternal dependence and with the time of most effective transfer of diffusable and lipid soluble chemicals from mother to offspring (55,56). Since the functioning of this system after perinatal exposure may be one of the most sensitive indicators of the potential toxicity

11

affecting the reproduction and the viability of species and individuals, the coincidence of these two conditions, impressionability and dependence, indicate the need to examine endocrine function subsequent to such exposure.

II. Steroidogenic Endocrine Development: Imprinting

Because the immature steroidogenic endocrine system appears in the above case (53,54) to be so vulnerable to the presence of an exogenous agent, demonstrating permanent changes in endocrine status or response, the need to review steroidogenic development becomes clear. Since most of the studies have been done on the rat I shall concentrate there.

Some of the most classical studies concerning the development of the sex steroid systems relate to the development of the brain, its sex specificity and control properties. These studies, reported by Harris (57) utilized the technique of ectopic implantation. Ovarian grafts were made to the anterior chamber of the eye and vaginal grafts to subcutaneous sites. By surgically and chemically manipulating the steroid status of the host animal, e.g., neonatal or adult castration plus steroid injection, etc., it was possible to determine the basic outline of hypothalamic sex differentiation. As a result of these studies and those reported by Barraclough (58-60) and Gorski (61) it became established that there are two hypothalamic centers which control gonadotrophin release

and which, in turn, are affected by the presence of
circulating sex steroids.

First, there is a tonic center, anatomically located
in the arcuate-ventromedial nuclear area, which in adult-
hood controls the tonic release of GnRH, and, subsequently,
LH and FSH. This center is subject to negative feedback
inhibition by circulating testosterone (in males) or
17β-estradiol (in females). Additionally, it remains
largely intact even if doses of exogenous estradiol or
testosterone are given during the perinatal period, 1-10
days of age.

The second center, the cyclic center, is associated
with the preoptic area of the hypothalamus and controls
the gonadotrophin surges seen near the time of ovulation
in the mature female. It differs from the tonic center
both in that it apparently responds positively to circu-
lating estrogen levels above an unknown threshold level by
increasing release of GnRH, LH and FSH and in that it is
effectively eliminated by either perinatal administration
of exogenous steroids or by the testosterone endogenously
produced by the intact neonatal male. It is this elimina-
tion of the cyclic center by exposure to androgens or
estrogen during the critical perinatal period which has
led to the concept of "imprinting". A multitude of manip-
ulations of the castrate and intact state with steroids,
neuronal lesions and surgery now support the imprinting

13

concept (61-63). A summary of some of them is presented for comparative purposes in Table 1.

Since perinatal steroid status manipulations, such as those above, alter all those steps controlled by the hypothalamus as well as the hypothalamus itself imprinting has obvious pleotropic effects. Most prominent of these effects are phenotypic changes in external genitalia, growth and sexual behavior (57-61), all of which are associated with varying degrees of sterility. But imprinting is not necessarily a clear-cut phenomena; the degrees of abberation are quite dependent on the steroid dosages administered and the timing. For example, 100% of a group of female rats injected with 1250 ug of testosterone propionate on day 5 postpartum showed sterility as adults while 70% showed sterility with a dose of 10 ug (59). Or, as another example, castration of male rats beyond 5 days of age blocks normal estrus cycles in implanted ovaries while similar males bearing implants after castration prior to day 5 do demonstrate normal estrus cycles. The implications are that endocrine manipulations may alter both the cyclic and tonic centers (63), the differences in end result being a matter of the comparative degree of alteration of the two sites.

More subtle changes which may involve sites other than the two centers defined thus far also take place as has been shown by Gustafsson et al. in their studies of specific steroid metabolic enzymes in the liver (64-68).

Table 1

Description of Neonatal "Imprinting"[1] of the Hypothalamus[2] of the Rat

Gonadal Status	Age	Sex (at birth)	Functional Cyclic Center?	Functional Tonic Center?	Normal Development?
I. Uninjected Pups					
Intact	0-15 Days	Male	$+^3$	+	+
		Female	+	+	+
Intact	5-15 Days	Male	−	+	+
		Female	+	+	+
Intact	Adult	Male	−	+	+
		Female	+	+	+
Neonatally Castrated	Adult	Male	+	+	−
		Female	+	+	−
II. Neonatally Injected with Estrogen or High Levels of Testosterone					
Intact	Adult	Male	−	+ ?	$-^4$
		Female	−	±	−
Neonatally Castrated	Adult	Male	−	±	−
		Female	−	±	−

Table 1 (Cont.)

III. Neonatally Injected with o,p'-DDT

Intact	Adult	Male	±	+ ±	+ ?
		Female	−	−	−
Neonatally Castrated	Adult	Male	?	+ ?	−
		Female	?	?	−

---

[1] "Imprinting" is defined as fixation of a biochemical or physiological state or developmental direction; depending on the parameter measured, it may be observed at any time between 5 and 15-30 days of age.

[2] The tonic and cyclic centers of the hypothalamus are those neurons or nuclei which control tonic or cyclic release of gonadotrophins.

[3] Symbols used indicate established normality, = +, or abnormality, = −, and uncertain functionality = ±. Unknown functionality or conclusions based only on the results in this thesis are indicated as ?.

[4] The degree of abnormality is dependent on dosage and time of administration after birth.

Perinatal treatment of females or neonatally castrated males with high doses of testosterone, 5α-dihydrotestosterone, 5α-androstanediol or estradiol all yielded hepatic alterations tending toward the intact male phenotype. Similar treatments of the castrate adult male, however, offer a more complex picture, some activities being altered toward the female or castrate phenotypes, others being unaffected (64,68). The data are consistent with enzymatic activities being classified into groups in which control is: (1) not exerted by the presence of steroids, (2) actively exerted by the presence of androgens, and/or (3) imprinted by the presence of neonatal steroids.

Beyond these controls by hypothalamic mechanisms - which may include alterations in neuronal growth and association (60) - imprinting by steroids may also occur by direct effects on peripheral tissues. Diminished specific uptake of labelled estradiol into pituitary and uterus of adult rats neonatally injected with androgens or estrogens was shown by Flerkó et al. (69) and McGuire and Lisk (70). Similar decreases in testosterone uptake into the pituitary were found by Simmons (71) in adult male rats neonatally injected with estradiol and castrated two days prior to testing. These experiments seem reminiscent of the findings in the genetically determined androgen-insensitive rat described by Bullock and Bardin (72). This rat lacks the steroid receptor proteins

necessary for effective specific uptake of androgens into target tissues and consequently exists as a pseudo-hermaphrodite. Since changes like these, or others involving receptors for trophic hormones (73), may exhibit temporal and dosage dependencies somewhat different from those of hypothalamic imprinting per se, they may well be the biochemical mechanisms ultimately responsible for the broad spectrum of morphological and physiological changes seen in adult animals neonatally exposed to androgenizing or estrogenizing influences at differing times and dosages.

III. Steroidogenic Endocrine Development: The Normal Time-Course

Morphologically the testis demonstrates two generations of testosterone-producing cells (the interstitial or Leydig cells). These cells, occupying the interstitium between the tubular (sperm-producing) elements of the testis, exist at birth and are histochemically demonstrable until roughly 5-7 days of age. They largely disappear at this time and either regenerate or become reactivated at approximately 3 weeks of age. Production of potent spermatozoa in the tubules begins to occur at roughly 45 days of age; sexual maturity as measured by sexual potency, peripheral organ enzyme activities and serum hormone levels is reached by roughly 50-60 days of age (74,75).

Steroid production in the first generation of Leydig cells appears quite similar to that occurring in the adult and is sensitive to exogenously administered human chorionic gonadotrophin (hCG), i.e., LH (76). The testis which exists between the first and third week of life appears steroidogenically inactive and insensitive to exogenous LH because testosterone levels fall even in the continuous presence of serum LH (77). This may, in fact, be due to the diminished number of Leydig cells present (76) although other schemes involving the coincident absence of the glucocorticoids (see below) have been advanced in the face of the constitutivity of the testicular LH receptors (78,79). Full adult steroidogenic capacity is gradually reached during the period following weaning (at roughly three weeks of age) and prior to maturity (80,81), the rise proceeding most rapidly from about 40 to 60 days of age. Accessory gland development closely correlates with the increasing serum levels of active androgens produced during this period of maturation as do the levels of certain enzyme activities found in peripheral tissues such as the liver (4,5).

Serum hormone levels during the developmental period have been measured by several groups (77,82,83). Levels of LH appear highly variable prior to approximately three weeks of age after which they stabilize slightly below adult levels until the onset of maturity at which the slight rise to adult levels occurs. FSH demonstrates a

stable plateau at roughly adult levels until about three weeks of age. At this time the levels rise about two-fold and plateau until 40-50 days of age after which they fall back to adult levels. On the hypothalamic level, GnRH concentrations begin to slowly rise immediately after birth and finally reach the adult plateau at about 50-60 days of age (84). Throughout development the feedback loop remains sensitive to exogenous LHRH (82) or castration (85-87) implying that it is functional and is only modified or modulated differently during the adult period, the change taking place during the transitional state, puberty.

Development in the female exhibits a distinctly different pattern. Within about 10 days after birth the ovaries demonstrate aggregation of several follicular cells with each egg cell and progression of the initial meiotic division of the egg cells themselves to the dictyate stage of prophase I. The oöcytes appear to randomly begin enlargement, apparently initiating the events which would eventually lead to ovulation in the adult ovary. Prior to the cyclical events which occur during the estrus cycle, however, those oöcytes which began development and stimulated their associated follicular cells to divide and form small follicles undergo degeneration long before reaching the ovulatory state. This atresia seems to be associated with unfavorable concentrations of estradiol, LH and/or FSH (88,89) and may reflect immaturity of the thecal portion of the ovary.

Slow maturation of the thecal, i.e., interstitial or
stromal, elements of the ovary ensues over the 5-6 weeks
after birth. These steroidogenic elements show sensitivity
to endogenous LH and FSH producing serum estradiol peaks
subsequent to serum LH peaks (77) and supporting uterine
weight gain (90). Near 35-40 days of age the theca
reaches full maturity and is capable of playing its role
in the cyclic events of normal estrus. Its production of
estradiol, perhaps as a result of the presence of adult
titers of gonadotrophins, becomes sufficient to cause full
differentiation of the female accessory tissues. This
results in the opening of the vagina, the maturation of
the uterus and the initiation of female sexual behavior.

Steroid synthesis during the immature phase is local-
ized in the thecal tissue, estrogen being the major
product. After the first ovulation, however, the post-
ovulatory follicle forms a temporary gland and site of
progesterone production, the corpus luteum; similar
temporary glands subsequently generate the cyclic changes
in serum progesterone seen during the female cycle.

Trophic hormone production by the female hypophysis
differs from that of the male. FSH is markedly elevated
at birth and remains so until roughly three weeks of age
when it drops to a plateau of 1/3 to 1/4 the early level.
It continues there until the periodic rises associated with
ovulation begin to occur at about 5-6 weeks of age (77).
LH, on the other hand, shows nearly the same picture as in

the male; highly variable levels exist until three weeks
of age followed by a plateau near adult diestral (basal)
levels until the onset of cyclicity near 40 days of age
(77). Hypothalamic GnRH rises slowly from birth until 4-6
weeks postpartum when the plateau of maturity is reached
(84). The feedback controls are all operative as can be
seen by the abrupt response of serum LH to perinatal
ovariectomy or steroid injection (85,87) or by the halt in
ovarian and uterine growth caused by administration of
anti-gonadotrophin anti-sera (90).

In the female, puberty and final maturity seem closely
associated with ovarian development and/or ovarian
mediated changes in the neural-hypophysial control centers
(91). Conversely, in the male, changes in testicular
sensitivity to LH caused by high FSH levels appear to play
the dominant role (92,93). Though they begin similarly,
the control systems for reaching maturity appear quite
different for the two sexes.

Adrenal cortical development in the rat follows a
similar time course in both sexes for roughly the first
three weeks postpartum. The glomerulosal layer is well
defined at the exterior of the cortex and appears stable
throughout the developmental timecourse. The cortical
tissue within the glomerulosa appears compact and undif-
ferentiated at birth with the reticular and fasicular
zones being morphologically indistinguishable (94). No
definitive perimedullary fetal or x-zone is present, as it

is in the mouse, but steroidogenesis does occur as has been shown by Milković and Milković (95). The response to stress appears blunted for several days following birth but at no time is it entirely absent (96). Further, this response is sensitive to hypothalamic control by CRH and to hypophysial control by ACTH from the 18th fetal day onward as has been shown in experiments using natural and experimental anencephally and hypophysectomy (97). In spite of this functional intactness the adrenal cortex exhibits a regression in volume, cell size and steroid content immediately after birth and does not regain mature functionality until sometime between 2-3 weeks of age (96). Beyond this point development in the sexes begins to differ because estrogen produces a positive trophic action on the cortex resulting in larger adrenals with higher corticosterone output in the female. This difference has been implicated in the onset of female puberty through a direct influence of the adrenal on the ovary (98) although the exact mechanism is at yet unclear. During the period of 3-5 weeks postpartum the fasicular and reticular zones become differentiated, formation of vascular elements and tissue cords becoming visible histologically. Finally, by 5-6 weeks of age the adult diurnal fluctuations of corticosterone output become established (98) and maturation of this system is also completed.

IV. Metabolism and Physiological Effects of DDT:

The rat was chosen as the animal model for this work because of the wealth of information available on its normal steroidogenic functioning and development and because of its potential susceptibility to perturbation of those normal conditions. Choice of the model compound was based on a review of the known properties of widely used synthetic chemicals. DDT stands out as one of the few chemicals which has been extensively examined from essentially all facets: chemical, toxicological, pharmacological and ecological. And yet, the extent of its biological effects, to say little of its mode of action, remain incompletely defined, as do many of the factors which cause it to demonstrate specific toxicities, e.g., reproductive toxicity.

In speaking of DDT it must first be borne in mind that the commercial chemical, technical DDT, is actually a mixture. Most analyses are in general agreement with those of Haller et al. (99), finding 70-80% 1,1,1-tri-chloro-2,2-bis-(4-chlorophenyl) ethane (p,p'-DDT), 15-20% o,p'-DDT plus small amounts of 2,2-dichloro-1,1-bis(4-chlorophenyl) ethane (p,p'-DDD), o,p'-DDD and other synthetic-reaction byproducts. Because of this predominance of p,p'-DDT in the mixture a large proportion of the literature on the insecticide has been generated using pure preparations of p,p'-DDT. Still, it must be said that

all the major components have been tested for, and found
to have, biological effects.

Metabolically, in mammals, p,p'-DDT has been found to
undergo either dehydrohalogenation of the side-chain to
form 2,2-dichloro-1,1-bis-(4-chlorophenyl) ethylene
(p,p'-DDE) or dechlorination of the side-chain to form
p,p'-DDD and subsequently side-chain oxidation to form 2,2-
bis-(4-chlorophenyl) acetic acid (p,p'-DDA) which may be
conjugated (100,101). It has also been shown to be sus-
ceptible to nonenzymatic reductive dechlorination to
p,p'-DDD catalyzed by agents such as lake water, reduced
porphyrins, water-logged soil and boiled pigeon liver
(102-104). Yet even though conversion to p,p'-DDD and
then to p,p'-DDA seems to be a ubiquitous possibility the
conversions occur slowly enough for both DDT and its
metabolites to accumulate, sometimes drastically, in
biological tissues. This accumulation, which is respon-
sible for the large majority of the effects seen in
non-target species, is due not only to DDT's slow
metabolic conversion to excretable products, but also to
its high lipid solubility, a property which it shares with
its two major neutral metabolites DDD and DDE.

The proportions and levels of the DDT derived
residues found in mammalian tissues is highly dependent on
factors determined by the species, age, sex, nutritional
status and drug-history of the animal involved (105-112).
These factors not only determine the distribution of body

fat, the major storage tissue for the residues, but also, more importantly, the activity of the hepatic microsomal enzymes. These enzymes, which have been demonstrated to be involved in the active metabolism of p,p'-DDT (100,113, 114), are similar to, if not the same as, those involved in the breakdown of drugs such as phenobarbital or steroids such as testosterone (12-15, 115-122). Furthermore, in most mammalian and avian species studied the enzymes are inducible by drugs (barbiturates) or by DDT itself (11, 13-15,19-21,115-122). Such induction leads to decreases in the total pesticide load (123,124) and in the DDE/DDD ratio but it also increases metabolism of normal body constituents such as steroids and vitamin D (125-129). It is this latter consideration which is particularly worrisome in non-target species since it can result in serious hormonal and nutritional imbalances.

Another major factor influencing the form and levels of the stored insecticide residues is the presence of gut flora. McCully et al. established the differences in DDE/DDD ratios in rats, sheep, chickens, rabbits and guinea pigs given DDT by several different routes (130). Their conclusions agree well with other investigations which have found that DDT metabolism involves extensive enterohepatic circulation and is highly sensitive to the presence of gut microflora (105,131). These microbes function under largely anaerobic conditions and, like those in anaerobic soils, catalyze the formation of DDD from DDT

(132,133). This catalysis overcomes what appears to be a rate limiting step in DDT breakdown, allowing more rapid catabolism to DDA and shunting DDT away from the formation of the inert storage residue, DDE.

Finally, levels of residue retention are, of course, dependent on the extent of exposure. In the case of low, 1 -50 ppm, exposures in the food of rats, the quantity of total DDT residues climbs to a plateau over the course of 17-23 weeks and decreases over the course of about 25-30 weeks after exposure has been discontinued (134). This pattern of residue levels is paralleled, as expected, by microsomal drug metabolizing activity and is sensitive to agents which further induce or surpress hepatic microsomal activity (106,107,129) or which mobilize stores of body fat (108).

Most of the previous statements concerning metabolism are also applicable to the other chemicals found in technical DDT. The normal metabolite p,p'-DDD is broken down to p,p'-DDA, as mentioned above, and yields no residues of p,p'-DDE (100,132,133,135,136). On the other hand, o,p'-DDT, as is shown in Figure 3, is not only subject to side-chain decomposition but is also degraded by way of ring hydroxylation reactions (51,137,138). These latter reactions are predictable on the basis of the open para-position in the ortho-substituted ring. This extra metabolic "handle" and the higher polarity of the resultant hydroxylated metabolites can largely explain the higher

Figure 3. Major Metabolic Routes of o,p'-DDT

The known routes of DDT metabolism are depicted as separate and parallel pathways. In mammalian systems pathways I) and II) cross in such ways as to yield a variety of hydroxylated intermediates with the side-chain in various stages of oxidation or conjugation. Pathway III) appears to be independent for the p,p'-isomer of DDT producing a major storage form of the pesticide; however, for the o,p'-isomer hydroxylation and other routes seem to be favored since o,p'-DDE residues are detectible only in minor quantities.

The specific enzymes involved in the pathways are largely unknown. The P-450 complex does play some role since it is induced by DDT and stimulates the elimination of DDT residues after induction by other agents.

FIGURE 3

MAJOR METABOLIC ROUTES OF o,p'-DDT

I) SIDE CHAIN OXIDATION

DDT → DDD → DDMU →

DDMS → DDNU → DDOH →

DDA → CONJUGATES

X= SERINE, GLYCINE, ASPARTIC ACID
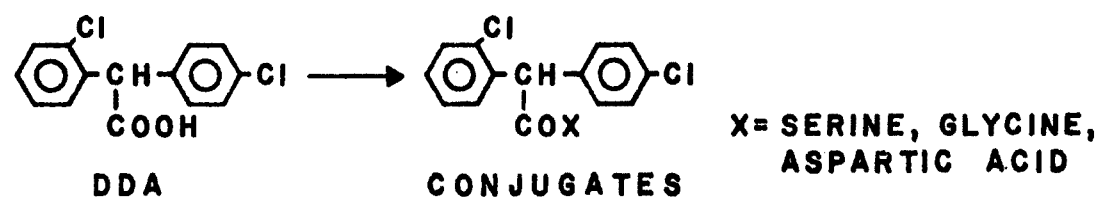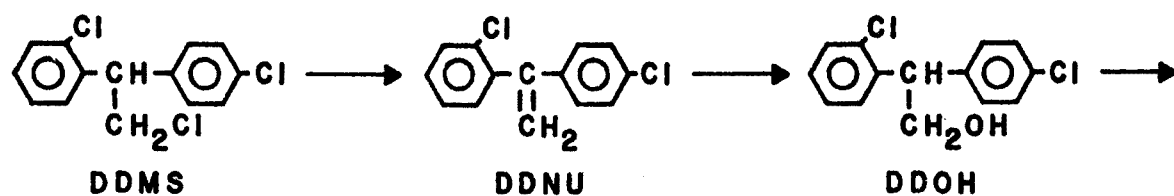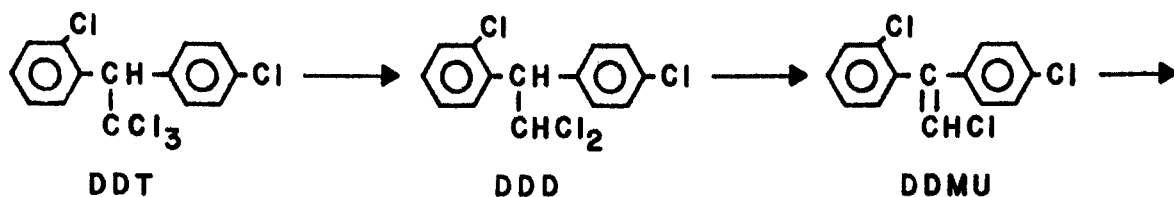
II) RING HYDROXYLATION

DDT →
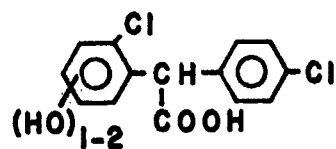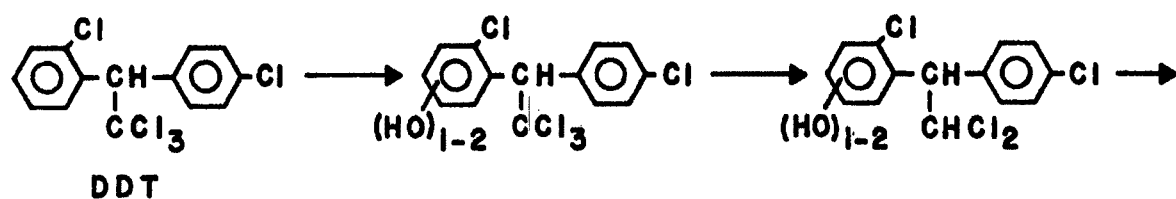
III) DEHYDROHALOGENATION

DDT → DDE
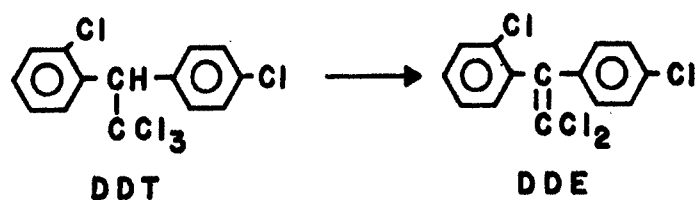
metabolic rate of o,p'-DDT and o,p'-DDD in comparison with the p,p'-isomers. Furthermore, they can also explain the lack of 2,2-dichloro-1-(2-chlorophenyl)-1-(4-chlorophenyl) ethylene (o,p'-DDE) in body residues following administration of either technical DDT or pure o,p'-DDT (131,139). They may not, however, be able to explain the differences in biological actions found with o,p'-DDT and o,p'-DDD in comparison with p,p'-DDT and p,p'-DDD, respectively.

Though all the compounds are capable of causing hepatic inductions similar to those seen with p,p'-DDT, the o,p'-isomers differ in their ability to produce neurological and steroidal effects. Comparison of the acute toxicities, reflecting neural toxicity, illustrate these differences rather dramatically. Oral dosages in rats required to kill 50% of the animals dosed ($LD_{50's}$) were 100-250 mg/kg for p,p'-DDT and 800-2000 mg/kg for o,p'-DDT (140,141). Furthermore, the muscular tremors and convulsions elicited by a toxic dose of technical DDT have been shown by Hrinda et al. (142,143) to be associated with p,p'-DDT; o,p'-DDT showed no neurotoxicity.

As regards steroidal effects, the situation is nearly the reverse. As early as 1949 Nelson and Woodard described the adrenolytic activity of technical DDD (90% p,p'-DDD, 10% o,p'-DDD) in dogs (42). Within nine years the technical material had been fractionated and the o,p'-isomer associated with the observed involution of the fasicular and reticular cortical zones and with the associated

decrease in urinary and cirulating 17-hydroxy- and
17-keto-steroids (45). After similar activity was shown
in several species including man and the purified material
became available, o,p'-DDD began to be used for the treat-
ment of adrenal hyperfunction as it occurs in Cushing's
syndrome and adrenal carcinoma.

The other outstanding example of steroidal effect is
the innate estrogenicity of o,p'-DDT. In a study of DDT
effects on White Leghorn cockerels published in 1950
Burlington and Lindeman (144) demonstrated what appeared
to be an estrogen-like effect of prolonged exposure to
technical DDT. They found a marked decrease in testicular
growth and in development of secondary sex characteristics
and attributed the effects to a configuration of p,p'-DDT
which superficially resembles the synthetic estrogen
diethylstilbestrol. A conflicting report based on work
with female rats appeared in 1952 (145), but it was not
until 1968 that Bitman et al. (146) and Welch et al. (22)
conclusively demonstrated that only o,p'-DDT was intrin-
sically estrogenic. These later studies showed that not
only did o,p'-DDT have a trophic effect on the oviducts
of injected chickens and quail (146) but it also was
capable of increasing wet weight, glycogen content and
^{14}C-glucose uptake into lipid, proteins and RNA in
immature or ovariectomized adult rat uteri (22, 147).
Subsequent studies by Singhal et al. (148) showed that the
uterotropic response in ovariectomized rats also involved

enhancement of gluconeogenic and hexosemonophosphate shunt enzymes; the enzymatic enhancement was inhibited by actinomycin D or cycloheximide. This report also indicated that acute administration of o,p'-DDT combined with 17β-estradiol produces a somewhat additive effect. Notwithstanding this last result, other studies (26,27) have shown that o,p'-DDT is capable of effectively competing for uterine cytosolic estradiol receptors _in vitro_ at levels of 1-10 ppm; such levels are within the reported tissue levels of DDT under normal environmental exposure (149). Still later experiments by Gellert _et al_. (53,54,150,151) have demonstrated that o,p'-DDT can decrease serum LH in ovariectomized adult female rats and is even capable of causing appearance of persistent-vaginal-estrus in adult rats injected with 3 mg neonatally. This last effect, perhaps the most striking, has been demonstrated to be dose-dependent and specific to o,p'-DDT (if all of its aspects are included). It represents a starting point for the studies described in this thesis.

It must also be noted that a large body of literature, coming mostly from the laboratories of A. H. Conney, has outlined the complicating factor in any _in vivo_ studies concerning the steroidal actions of DDT and its analogs, i.e., the induction of hepatic metabolism (11,14-21,23,39, 118,120,122,127-129,143). This series of papers has demonstrated that chronic doses of pesticides or drugs are both capable of inducing the hepatic enzymes responsible

for catabolizing circulating steroids of all classes. The work of Peakall (152), albeit in ring doves, directly demonstrates reductions in circulating estradiol levels upon chronic feeding with p,p'-DDT. That such is also the case in the rat and mouse has been indirectly shown by the fact that uterine ^{3}H-17β -estradiol uptake and the uterotropic response to estradiol are both blocked by chronic exposure to halogenated hydrocarbon insecticides (18). Similar results have also been shown for androgen uptake and response in rat seminal vesicles (23) or mouse prostate (25). That microsomal induction may occur throughout the postnatal life of the rat has also been demonstrated (109,153). All the above observations taken together require that in vivo experiments on the mode of action of pesticides, especially in regard to their actions on steroidogenesis, be interpreted with the possibility in mind that indirect effects mediated by hepatic steroid metabolism are responsible for the effects seen.

The mode of toxic action of DDT and its analogs in mammals may well be related to its demonstrated actions in insects. Holan (154) briefly described a molecular model of the membrane site to which DDT binds in insect nerve. This model was based on molecular-dimension calculations and on an extensive series of structure-function relationships including at least 57 analogs of p,p'-DDT. The final picture of the binding of DDT involved insertion of the trichloroethyl-tail into a pore in the lipid portion

of the membrane with the aromatic rings extending into the protein layer and forming π-bonds to the protein molecules. The pore to which DDT was bound is associated with Na^+ transport; treatment with the insecticide results in atypical and continuous nerve impulses indicative of perturbed Na^+ efflux. Other work has indicated coincident or subsequent changes in K^+ and/or Ca^{++} transport in both insect and mammalian species and in both nerve and muscle preparations (155). Obviously, if the compound effects such universal elements of membrane function as membrane potential and cation transport it is not surprising that Hrinda et al. (143) have demonstrated increased levels of cAMP in tissues taken from rats treated with DDT or its analogs. This is especially so in view of the postulations of cation involvement in the production and actions of cAMP put forward by Rasmussen (9).

The foregoing discussion has emphasized how much we know and implied how much we do not know about the DDT complex (technical DDT), one of the best characterized of the synthetic chemical mixtures. In spite of the great humanitarian services DDT performed after its rediscovery in 1939, a great public outcry arose concerning DDT's safety after the publication of Rachel Carson's Silent Spring in 1962 (156). This outcry, combined with the professional questions which stemmed from the papers listed in the above discussions and from ecological studies such as Hickey's Peregrine Falcon Populations,

Their Biology and Decline (157), resulted in the banning
of DDT for use in the United States in 1972. Still, its
environmental persistence (158), global mobility (159,160)
and extensive use outside of the U.S. continue to make it
a material of some ecological importance. Because
o,p'-DDT was implicitly included in the ban and the furor
which brought it about and because it has known effects
which include precisely the type of alterations which
could best be probed by the approaches I have proposed for
screening other materials, I chose to use o,p'-DDT in these
investigations.

Having chosen the endocrine system of the rat and
o,p'-DDT as objects of the experimentation, more specific
questions arose. First, the phenomenological question of
the presence of a measurable effect begged a second ques-
tion which had mechanistic ramifications; what part of
the endocrine system or its control loop is affected?
This question forced an experimental design capable of
answering botn the question of the existence of an effect
and the question of the focus of action of the chemical
under study. Therefore, the object of the experimentation
had to be more fully defined. Being that earlier reports
have generally indicated little reproductive effect of DDT
on the adult rat (161,162) and that several studies had
been published on the effects of neonatal injection with
o,p'-DDT in the female rat (53,54,150,151), the choice was
made to examine the results of neonatal exposure on the

function of the endocrine system of the male rat. The
somewhat simpler adult hormone pattern of the male as
compared to the female also made the male system more
attractive than the female. But the question, "On what?"
required even further definitions. It must be understood
that the question of looking for an effect on the organism
as a whole or on a specific subsystem within the organism
required a broad investigation employing a variety of
measurements. Furthermore, the measurements had to probe
both structure and function in order to ascertain if
subtle but still consequential changes had taken place.
In regard to the endocrine system, this emphasis on scope
required that a variety of measurements be made on both
the gonadal-hypothalamo-hypophysial and the adrenal-hypo-
thalamo-hypophysial systems. That is, observations
including morphology, histology and hormone secretions had
to be coupled with function tests, akin to glucose-toler-
ance tests, such as responses to castration or exogenous
LHRH. Obviously, though the investigation was undertaken
to determine the existence of an effect it may also give
insight into the later question of mechanism of action.
The question of an effect "On what?" was therefore
answered in these studies by noting any differences in the
results of the measurements used in treated and untreated
animals.

It is evident from the previous discussions that the
perinatal period is, at least in the rodent, a period of

either ambiguity or preparation in the steroidogenic control systems. It is therefore a period of great sensitivity to outside influences in both the male and female. Because of this, and because the neonatal detoxification systems are not as active as they are in the adult (5), the potential for exogenous agents such as o,p'-DDT to influence reproduction and/or viability seems greatest if exposure occurs perinatally as was proposed in these experiments. It seemed reasonable that if a model of interference with the steroidogenic control loops was to be used in screening for the effects of foreign compounds, direct introduction of the materials into test groups could generate the desired answers. If, however, the specific effects of natural exposure was to be used or questioned, introduction must occur via the mother, since neonates are normally completely dependent on her. In these investigations both routes of exposure were examined.

In the case of either exposure, measurements were made in both intact and neonatally castrated rats to ascertain if some of the changes found in the female (53,54,150, 151) could be found in the proportedly neurologically similar neonatally castrated male (57-61). Furthermore, it was hoped that such parallel experiments might elucidate the role of the testes in any changes observed in the intact rat. The measurements performed included body and organ weights, organ histologies, serum hormone levels under normal and stressed conditions and pituitary hormone

levels. Studies on in vitro testicular steroid trans-
formations were also initiated and spawned the generalized
techniques and computer program detailed in Chapter 6 and
Appendix I. A general schematic of the approach and treat-
ments used is shown in Figure 4.

The observations described in this thesis are meant
to answer the experimental question posed: "What is the
effect, if any, of neonatal exposure to o,p'-DDT on the
steroidogenic endocrine systems of the male rat?" Some
of the later studies may also shed some light on the locus
of action of the chemical because they were designed to
fix the actual location of the effects of o,p'-DDT more
firmly than the initial studies; they attempted to
answer the question "Exactly where does it act?" Even the
question of when the changes occur was partially addressed
by studies which examined development in conjunction with
function. It is with these questions in mind that the
experiments will be described and related to the primary
objective, i.e., design of a general procedure for
screening foreign compounds for effects on reproduction
and viability.

Figure 4. Schematic of Approach and Treatments

     Treatment was either via direct s.c. injection into
1-5 day old pups or via the residues carried into the
milk of dams injected daily, i.p., with large quantities
of o,p'-DDT. In both situations growth was measured as
were endocrine tissue development, histology and function.
Tests for subtle alterations of endocrine function were
conducted in surgically modified animals, e.g., neonatal
castrates, $\varnothing$, or those stressed by other treatments so
as to exaggerate otherwise masked biochemical or physio-
logical changes.

FIGURE 4

SCHEMATIC OF APPROACH AND TREATMENTS

VIA THE INJECTED DAM

TREATMENTS

DIRECT INJECTION

MEASUREMENTS

PHYSICAL GROWTH AND DEVELOPMENT

TISSUE FUNCTION

NORMAL   STRESSED
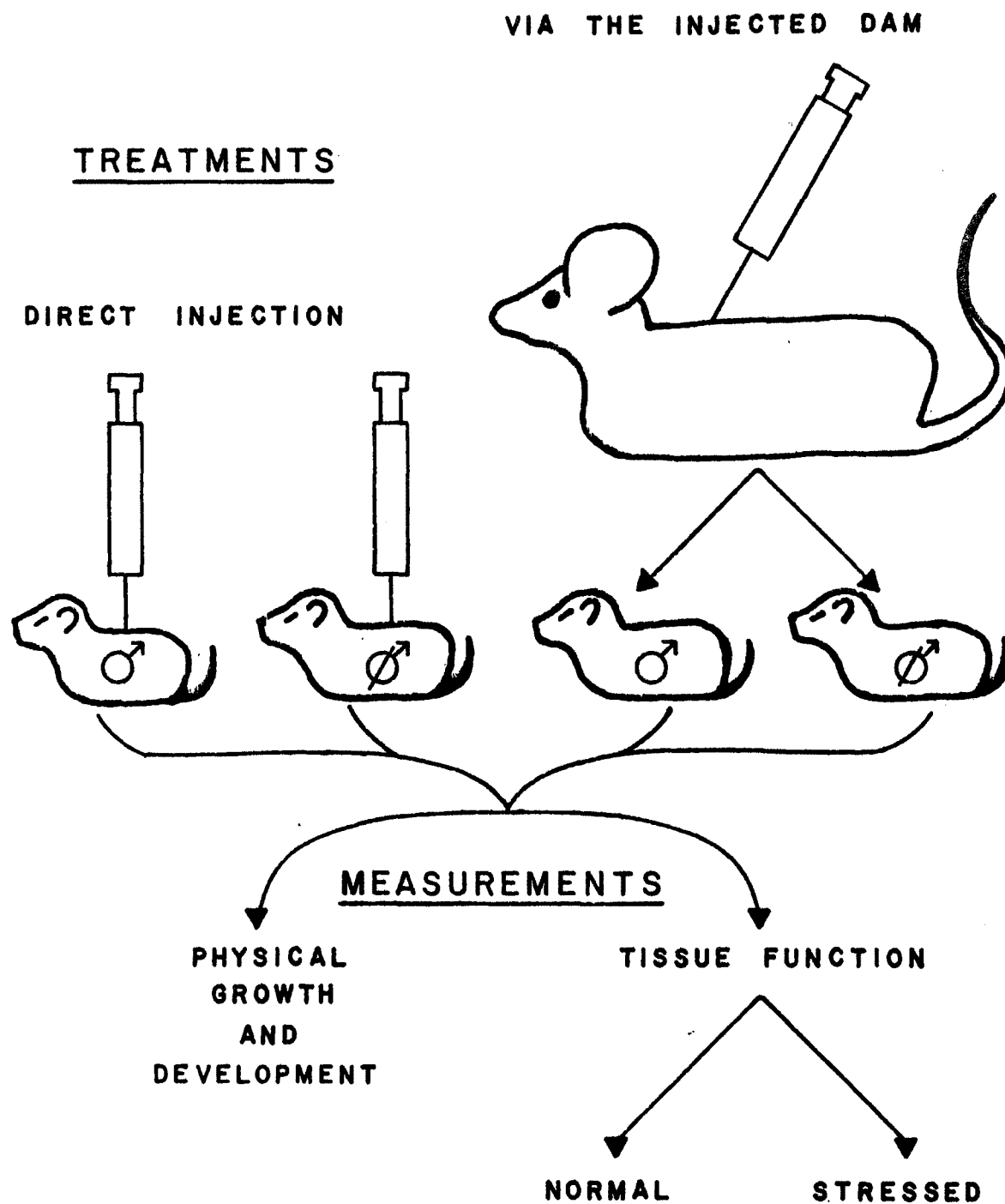
CHAPTER 2

MATERIALS AND METHODS

I. <u>Animals</u>:

All rats used in these experiments were Sprague-Dawley
purchased from Spartan Laboratories, Hazlett, Michigan.
They were housed in 30 cm x 30 cm x 15 cm nesting boxes on
wood shavings from birth until weaning and thereafter in
35 cm x 60 cm x 15 cm cages with wire mesh floors. Caging
was in groups of 6-8 dependent on age. All animals were
allowed free access to water and food (Purina Rat Chow).
A light-dark cycle of 12/12 (6.00 - 18.00 EST) was main-
tained. Room temperature and humidity varied somewhat with
the season, but normal values were approximately $25^{o}C$ and
60%. Cages were cleaned every 2-3 days. Medication was
only used when serious outbreaks of respiratory infection
occurred in or near the experimental groups or when
animals were exposed to heavy or repeated ether anesthesia.
In any cases in which medication was used, all test and
control groups were treated equally with Terramycin dis-
solved in the drinking water (50 mg/ml).

When neonates were to be used, the pregnant females
were placed in nesting boxes roughly one week before
delivery. After delivery litter sizes were adjusted to
meet the needs of the experiment, usually 6 or 8 pups per
dam. Litters were maintained, by addition of pups if
necessary, as near to the original number as possible

until weaning. Pups were allowed access to the dams until 25 days of age by which time natural weaning was complete in all litters (163).

Neonatal castrations were performed under cold anesthesia according to the procedure of Pfeiffer (164). Following the surgery and warming to adult body temperature, the pups were returned to their dams. Adult castrations were performed under ether anesthesia. A short midventral-scrotal incision was made and followed by two small lateral incisions through the tunica vaginalis of each testis. The testes were expressed through the incisions and the spermatic artery, vein and the vas deferens were tied off with sterilized cotton thread. The testes were severed below the constriction and the wound closed with wound clips. Adult castrates were placed on Terramycin for at least 3-5 days after the surgery.

Blood samples were obtained from animals either after decapitation, in which case trunk blood was collected, or under light ether anesthesia by tail-vein bleeding (TVBE). The latter procedure was chosen over heart-puncture, catheterization or tail-vein bleeding without anesthesia for the following reasons. First, I found heart puncture, during which many animals were killed by faulty punctures, to be a less predictable process. Since many of these animals required 2-3 months to obtain I was conservative in their use. Second, catheterization is normally only acceptable for bleedings carried out for less

than 3-5 days and requires a moderate amount of surgery to insert the cather. Because a large number of animals were to be bled during periods which exceeded one week catheterization was discarded. Finally, after several attempts at bleeding by tail-vein without anesthesia the ability of the rat to constrict blood flow to the tail became obvious. Because blood samples of 1.0 ml or more were needed for the measurements to be made anesthesia was necessary.

TVBE was carried out by placing the rat on an ether-moistened cotton pad in a metal can equipped with a glass cover. After 45-60 seconds the rat was sufficiently anesthetized to be placed in a restraint cage, to have a small (about 5 mm) fragment of the tail cut off with a razor blade, and to have 1-3 ml of blood collected in a test tube prior to regaining full consciousness. Including the tying off of the tail with a loop of cotton cord, the time from introduction into the ether chamber to placing the animal into a holding cage was 3-5 minutes.

Tissue samples were collected after the animals were weighed, stunned, decapitated and bled. If they were to be examined histologically they were weighed; divided and placed into 10% neutral-buffered formalin (165).

Animal weights were determined on a triple beam balance to within $\pm$ 0.5 g. The adrenals and pituitaries of all of the animals and the testes and accessory glands of young or steroid-treated rats were rinsed in a modified

Ringer's solution (Buffer I, Table 3), blotted and
weighed on a Sartorious semi-automatic milligram balance
accurate to $\pm$ 0.03 mg. Kidneys and livers from all the
animals and the testes and accessory glands from older
rats were rinsed and blotted as above and weighed on a
Mettler top-loading balance accurate to $\pm$ 0.01 g.

II. Chemicals:

All chemicals utilized were obtained from commercial
sources. Miscellaneous solvents, salts, acids and bulk
chemicals were obtained from Mallinckrodt, Inc., Fisher
Scientific Co., Merck Chemical, J. T. Baker Chemical Co.
and Matheson, Coleman and Bell Manufacturing Chemists.
All water used in solutions or washes, etc., was
double-distilled.

DDT analogs were purchased as $99^+\%$ pure from Aldrich
Chemical Co., Inc. Subsequent analysis by thin layer
chromatography (TLC) demonstrated single spots for all of
these chemicals; however, gas chromatography (GC) later
showed impurities in the o,p'-DDT, and p,p'-DDD. Bitman
et al. had analyzed Aldrich o,p'-DDT in 1971 as being
98.7% pure, with 1.1% p,p'-DDT contamination (166).
Analysis of the o,p'-DDT used in these experiments showed
98.5% o,p'-DDT and 1.5% p,p'-DDT even after recrystalliza-
tion from several solvent systems (acetone/water, ethanol,
hexane). Contamination of p,p'-DDD was with minor (< 1%)
amounts of o,p'-DDD.

Labelled steroids, ^{3}H- and ^{14}C-, were purchased from Amersham/Searle Corp. Cold steroids were obtained from Sigma Chemical Co. and Schwartz-Mann. Purity was verified for all labelled steroids by TLC followed by radioscanning at high sensitivity on a Vanguard Model 880 strip scanner. Three solvent systems were used: benzene, 8:2-benzene: acetone, and chloroform. Labelled steroids were used at the original specific activity determined by the manufacturer: 18.6 Ci/mmole, [7 n-^{3}H] - Δ^5-pregnenolone; 52 mCi/mmole, 4-^{14}C-dehydroepiandrosterone; 59.4 mCi/mmole, 4-^{14}C-testosterone; 60 mCi/mmole, 4-^{14}C-Δ^4-androstenedione; 53 mCi/mmole, 4-^{14}C-Δ^5-pregnenolone; 61.0 mCi/mmole, 4-^{14}C-progesterone; 61 mCi/mmole 4-^{14}C-17α-hydroxyprogesterone. They were diluted to appropriate volumes, i.e., the correct number of counts per volume, with 1:1-benzene:ethanol or ethanol and stored under refrigeration. Unlabelled steroids were similarly checked for purity by TLC and subsequently recrystallized from ethanol and/or acetone; purity was then checked by TLC, GC and melting point. Cold steroids were stored at room temperature in brown glass, and their solutions in ethanol or benzene-ethanol were refrigerated. Corticosterone solutions were made up in redistilled ethanol and water and stored for no more than one week under refrigeration. Cholesterol-3-propyl ether (CPE) was purchased in highly purified form (verified by GC) from Sigma and was stored at -20°C. CPE solutions were made up in 1:1

benzene:ethanol and stored under refrigeration. Testosterone-17-propionate and 17β-estradiol-17-valerinate were also purchased from Sigma and were used as supplied.

Aldrin was obtained from K & K Laboratories, Inc. and purified by recrystallization from ethanol and hexane after washing with methanol and acid. Purity was verified by appearance of a single peak upon GC.

Sodium phenobarbital and 2'-diethylaminoethyl-2,2-diphenylpentanoate (SKF-525A) were donated by the laboratories of Dr. M. J. Coon and Dr. H. H. Cornish, respectively. The chemicals were dissolved in physiological saline by addition of acid or base and titrated to pH 7-8 prior to use in injections.

Pyridine nucleotides (NADP$^+$ and NADH), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase from Baker's or Torula yeast (G-6-P DH) and bovine serum albumin (BSA) were purchased from Sigma. Synthetic LHRH was obtained from Calbiochem. All these biologicals were diluted in modified Ringer's solution or physiological saline immediately prior to use. Storage of the crystalline materials was at -20°C.

Refined sesame oil and dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific and used as obtained. Glass-distilled hexane purchased from Burdick and Jackson Laboratories, Inc. was also used as received. To remove fluorescent impurities, dichloromethane from Mallinckrodt or Fisher Scientific was allowed to stand over

concentrated sulfuric acid for several days, then washed with sulfuric acid, bicarbonate and water. The wet solvent was dried over anhydrous sodium sulfate and distilled through a Vigreaux column (the 39°C fraction was collected). Similarly, ethanol was distilled after refluxing with 2,4-dinitrophenylhydrazine and hydrochloric acid to remove fluorescing impurities.

Precoated silica gel G TLC plates were purchased from Brinkman Instruments, Inc. and thoroughly washed with methanol prior to use. Gas Chrom Q and silicone GC coatings were purchased from Applied Science Laboratories. GC column packings were prepared using filtration and fluidizer techniques (167).

Scintillation chemicals, 2,5-diphenyloxazole (PPO) and 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP), were obtained from Research Products International, Corp. The scintillation cocktail used was 6 g PPO plus 0.25 g POPOP per liter of toluene and was based on the data of Bush and Hansen (168) for nonquenched systems.

The ovine-LH (oLH), radioiodinated oLH and anti-rabbit gamma globulin utilized in the radioimmunoassay (RIA) procedures were obtained from the Reproductive Endocrinology Program of the Department of Pathology, The University of Michigan. The anti-oLH used was a dilution of the Niswender anti-serum (169) and was also obtained from the Pathology Department. These biologicals were used as supplied.

III. Treatments:

All direct chemical treatments were by injection.
Steroids and o,p'-DDT were dissolved in sesame oil for
subcutaneous (s.c.) injection into neonatal animals. Such
injections were done on the day of birth and repeated on
the following four days; all these injections were volumes
of 0.05 ml. The concentrations used are given in the
experiments. Injections of o.p'-DDT into nursing dams, to
indirectly administer the chemical to the pups, were given
intraperitoneally (i.p.). These injections were begun on
the day of parturition and were continued daily until the
day of weaning (day 25). All injections were either 0.1
ml of 50 mg of o,p'-DDT dissolved in DMSO or 0.1 ml DMSO
alone. The solutions of DDT in DMSO were nearly saturated
and were warmed prior to injections.

The quantities of steroids given to neonates were
based on the results of imprinting experiments previously
conducted (59) and were chosen to be near the lowest effec-
tive single doses. These doses were divided into five
equal parts, one part of which was given in 0.05 ml of
sesame oil on each of days 1-5 of age; daily doses were
40 ug 17β-estradiol-17-valerinate and 200 ug testoster-
one-17-propionate. The amounts of DDT injected into neo-
nates was meant to cover a range from nearly environmental
exposure levels to levels near those found effective by
Gellert et al. (54) in imprinting the neonatal female.

The daily dose of o,p'-DDT given the dams was estimated to be approximately $1/10^{th}$ of an LD_{50} dosage.

In later experiments involving the response of serum-LH to adult castration, a test of the negative feedback loop was performed by injecting testosterone several hours prior to bleeding. The dose of testosterone administered, s.c., in 0.1 ml of sesame oil was determined, on the basis of the work of Hutchison and Goldman (170), to be capable of partially suppressing castrate levels of serum LH without driving them down to normal intact levels.

The response to LHRH was measured by injecting a large (171), 1 ug, dose of synthetic LHRH into intact rats. The injection was in 0.1 ml of physiological saline and was performed 20-60 minutes prior to bleeding. This period has been shown to coincide with maximal serum-LH concentrations by previous workers (9,172-174).

The hepatic microsomal inducer, phenobarbital, and the inhibitor of microsomal induction, SKF-525A, were administered to neonatal rats i.p. in 0.05 ml of physiological saline. The doses used were based on the work of Levin et al. (17) and Harbison (175). Phenobarbital was given daily in two injections of 100 ug each, spaced 12 hours apart, for the first five days of life. SKF-525A was given as a single daily injection of 125 ug over the same five day period.

The amount of DDT actually absorbed by rat pups suckling injected dams was determined during these studies and is discussed in Chapter 4.

IV. Measurements:

A. Organ Weights:

Body and organ weights were measured as above and the percentage of body weight attributable to each organ was calculated for each animal. Means, standard deviations and standard errors were then·computed for both absolute weights and percent of body weight for each treatment group and each organ. Mean values were then compared statistically by applying unpaired Student's t-tests. Significance throughout this investigation was considered to be attained only if the chance probability of the observation in question was less than 5%.

B. Organ Histologies:

Tissue fragments fixed in 10% neutral buffered formalin were prepared for observation by the Histology Laboratory of the Department of Pathology, The University of Michigan. Common histological preparation was followed, i.e., alcohol/xylene dehydration, paraffin embedding, sectioning, staining, xylene/alcohol clearing and mounting (165). The tissues were sectioned at 7-10 mu and stained with hematoxylin-eosin. Observations were made at 5-100 power using a Zeiss photomicroscope with a polarizing light source and Nikon objectives. Subjective visual observations were recorded for each tissue and slide.

When differences between treatment groups became apparent
the slides were reexamined to verify the differences. The
adrenals, which showed the most marked changes due to
o,p'-DDT administration, were photographed using the same
microscope and Eastman Kodak Co. color photomicrography
film (ASA 16/13 DIN). The resulting slides were processed
into prints by Kodak. Between-group comparisons of the
frequencies of a given histologic observation were made
by using the X^2 test for a 2 x 2 table.

C. Serum Corticosterone Measurements:

Serum corticosterone was measured by a modification of
the procedures of Mattingly (176) and Silber et al. (177).
Figure 5 shows a schematic diagram of the protocol
utilized.

Upon decapitating an animal a trunk-blood sample was
collected into a 10 or 20 ml beaker. This was allowed to
clot on ice before being transferred to a tube and centri-
fuged for 20 minutes at approximately 5000 rpm (Variac
setting 50) in a Servall tabletop centrifuge. The serum
was decanted and stored at -20°C. Just prior to extrac-
tion the serum was thawed and 1.0 ml was mixed with 2.0 ml
of purified ethanol (all solvents were purified - see II.
Chemicals). The protein precipitate was removed by
centrifugation under the same conditions as previously.
To a measured volume of 0.5-1.0 ml of supernatant in a
screw-capped test tube, 1.0 ml of doubly-distilled water
and 5.0 ml of glass-distilled hexane were added. This

Figure 5. Protocol for Measuring Serum Corticosterone

Levels, Modified from Mattingly and Silber

Tests of both the Mattingly (176) and Silber (177)
procedures for serum corticosterone led to the outlined
protocol. The alkali wash used by Silber was found
unnecessary if the hexane wash was retained and the
reagents were all purified prior to use in the assay.

Figure 5

Protocol for Measuring Serum Corticosterone Levels,

Modified from Mattingly and Silber

Fresh Blood Sample

Centrifuged 20 min at 5000 rpm in a tabletop centrifuge;
serum decanted

Serum

Frozen

1.0 ml Thawed Serum

2.0 ml EtOH added, mixed then centrifuged as above;
aqueous EtOH decanted

0.5-1.0 ml Aqueous EtOH

1.0 ml H_2O and 5.0 ml hexane added, mixed, then centrifuged
as above; hexane aspirated off

Washed Aqueous EtOH

5.0 ml CH_2Cl_2 added, mixed and centrifuged 10 min as above;
aqueous EtOH aspirated off;
CH_2Cl_2 dried with anhydrous Na_2SO_4 or $MgSO_4$

Dry CH_2Cl_2 Extract

Stored for 1-2 days under refrigeration before reaction or
reacted immediately by adding 3.0 ml of $7:3-H_2SO_4:EtOH$,
mixing for 20 sec, centrifuging for 30 sec and
aspirating off the CH_2Cl_2 extract

Fluorescent Mixture

13 min after addition of the $H_2SO_4:EtOH$ emission read at
535 nm using 475 nm excitation and a C-70 filter
(cutoff = 510 nm)

solution was mixed for at least 20 minutes on a test tube
rotator or a metabolic shaker before being centrifuged as
before. The upper, hexane, layer was carefully aspirated
off and the lower, alcoholic, layer reextracted with 5.0
ml of dichloromethane. Again mixing was for at least 20
minutes on a tube rotator or a metabolic shaker; centri-
fugation was for 10 minutes at 5000 rpm. The upper,
alcoholic, layer was carefully aspirated off and the lower,
dichloromethane, layer was dried with anhydrous Na_2SO_4 or
$MgSO_4$. After drying, the tubes could be tightly covered
with Parafilm or teflon and stored under refrigeration
for up to 48 hours prior to assay without altering the
results. Though this short-term storage was possible
assays were completed as soon as possible after extraction.

The actual measurement was as follows. The dry
dichloromethane extract was added to 3.0 ml of a 7:3 mix-
ture of concentrated sulfuric acid:ethanol in a glass
stoppered conical centrifuge tube. This was Vortexed for
20 seconds and centrifuged for 30 seconds on a Clay Adams
tabletop centrifuge operated at a speed setting of 1-2.
The upper, dichloromethane, layer was aspirated off. At
13 minutes $\pm$ 30 seconds the fluorescence at 535 nm of the
alcoholic sulfuric acid solution was measured in a 1.0 cm
quartz fluorescence cuvette using 475 nm excitation light
and a 510 nm cutoff, exit filter (Corning C-70). The
instrument used was an Aminco-Bowman fluorimeter. The
measured fluorescence was recorded and later corrected for

the background fluorescence of blanks run simultaneously.
Calculation of concentration was based on the back-
ground-corrected fluorescence of standards run simultane-
ously with the unknowns.

Initial assays demonstrated that the use of an alkali
wash of the dichloromethane extract was unnecessary since
similar results were obtained in assays done on the same
sera without the wash. The correctness of the use of
corticosterone standards diluted in water was verified by
showing that a strictly additive relationship occurred in
samples measured with and without the addition of a
standard sample of corticosterone. It was also shown by
the parallelism of a standard curve with a set of serial
dilutions of a serum pool, Figure 6. A water blank was
used in all assays; the stock standard solution was 2 ug
of corticosterone per milliliter in water.

The mean index of precision of the assay, λ, was
$\pm13.45\%$; mean sensitivity was 0.969 ± 0.027 log units of
fluorescence per log unit of concentration, in ug/ml; the
mean limit of detection, defined as blank plus λ, was
0.0062 ug/ml. A measurement of recovery done in pentupli-
cate on extracted and unextracted standards indicated total
recovery to be $74 \pm 2\%$. It should be noted that the
parameters given are means and that the values have
improved during the course of experimental repetition so
that the value of λ, particularly, may be better reflected
by the value of $\pm9.07\%$ obtained in the last assay.

Figure 6.   Verification of the Sulfuric Acid Fluorescence

Assay for Serum Corticosterone: Parallelism of

the Standard with Serially Diluted Rat Serum

Log emission at 535 nm versus log concentration for
the standard or log relative concentration for serially
diluted serum yield straight lines with slopes of
0.995 ± 0.022 and 1.034 ± 0.029, respectively.   The
standard curve is plotted as mean log emission $\pm$ 1 standard
deviation (where errors are covered by the circle they are
not shown);   the serum curve shows the individual measure-
ments in a duplicate determination.   The serial dilutions
were made starting with a 2.0 ml volume of serum.

Since the standard contained only corticosterone
diluted in water the parallelism with the serially diluted
serum implies that the assay is specific over this con-
centration range (178).   Any contaminant would have to
demonstrate 2 different partition coefficients equalling
those of corticosterone and have a fluorescence intensity
at 535 nm similar to that of the standard.

FIGURE 6

VERIFICATION OF THE SULFURIC ACID FLUORES-
CENCE ASSAY FOR SERUM CORTICOSTERONE:
PARALLELISM OF THE STANDARD WITH
SERIALLY DILUTED RAT SERUM

STANDARD CURVE: LOG CONCENTRATION (UG/ML)
SERUM: LOG RELATIVE CONCENTRATION (LOG∠VOLUME/2.0 ML⅂)
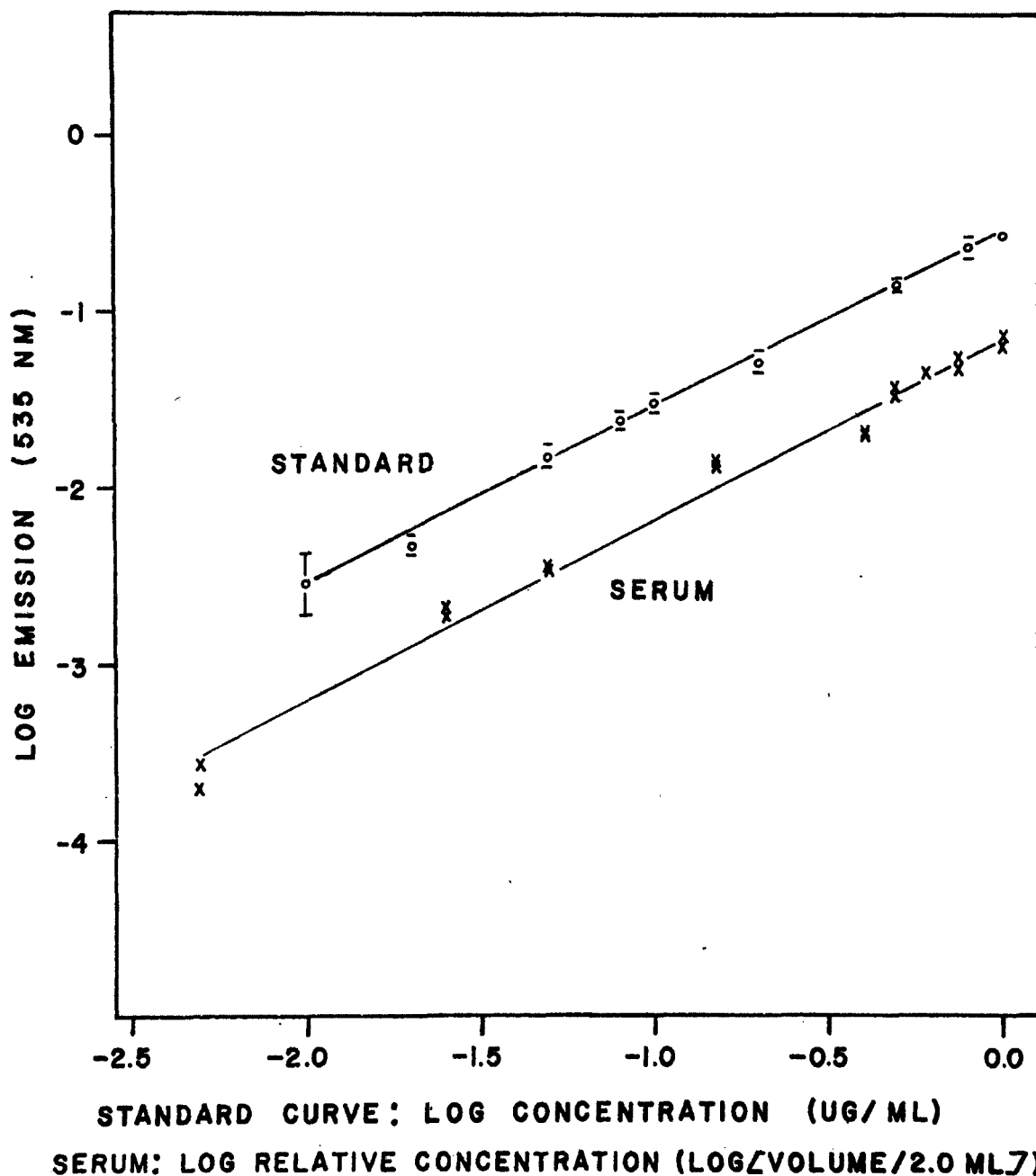
The specificity of the assay has been previously shown by Silber et al. (177) and is supported by the parallelism (178) of the standards and serially diluted unknown shown in Figure 6. Interference of DDT metabolites in treated animals was also eliminated as a source of error when measurements of fortified blanks showed no fluorescence above background.

The ability to store extracted samples for brief periods of time allowed processing of large numbers of samples in single assays. By doing this, using four matched cuvettes and having assistance during the measurement phase of the assay, it was possible to process over 200 samples in four working days or less. This represents an advantage over the previous manual procedures (176,177) and an alternative to automated assays which may be of use in some clinical and laboratory settings.

D. Radioimmunoassays:

1. The Assay

All measurements of LH in serum and pituitary extracts were made using the double-antibody procedure of Niswender et al. (169). All assays were performed in the laboratories of the Reproductive Endocrinology Program, Department of Pathology, The University of Michigan.

The assays utilized the rat pituitary extract, B-640, as the primary intra-laboratory standard for comparison. Previous bioassays (ovarian ascorbic acid depletion test, OAAD) and other radioimmunoassays have established that

B-640 has a potency of 0.03 times that of the National Institutes of Health LH standard S1 (NIH-LH-S1), i.e., each nanogram of B-640 is equivalent to 0.03 ng NIH-LH-S1. Secondary local standards consisting of pooled rat sera such as B-873 were run in each assay. The potency of these secondary standards relative to B-640 has been established by comparisons of the 50% inhibition points on plots of percentage of ^{125}I-oLH remaining bound versus log mass per tube. Even if the remaining portions of the RIA curves are nonparallel, and the samples used in generating them are thereby inferred to be dissimilar, the 50% points will yield a valid approximation of the relative potencies of the two samples (179). Such estimates gave B-873 a value of 212.0 ng B-640/ml or 6.36 ng NIH-LH-S1/ml.

There have been several (180-182) reports that the LH values measured in sera under varying physiological conditions show nonparallelism with the pituitary standard in this RIA. To ascertain that gross errors of measurement had not been introduced into the LH measurements made for the experiments to be described later, a check on the parallelism of the curves obtained with a rat pituitary extract, B-640, B-873 and a pool of rat serum generated during the studies for this thesis was performed. The percentage of ^{125}I-oLH remaining bound was plotted versus the log of the volume of sample taken for assay (which is proportional to the log of mass per tube). The curves were displaced on the plot by an arbitrary constant, α,

to prevent their overlap, and were examined visually for parallelism. The rat pituitary extract and B-640 curves were markedly similar. The curve generated by B-873 was noticeably nonparallel with B-640 but was quite similar to that given by the pool of rat serum. The curve given by the pooled serum also showed some similarity to the curve generated by B-640 but, in fact, seemed not to be strictly parallel throughout its length with either the B-873 or B-640 curves. These results, shown in Figure 7, indicated the use of B-640 as the standard for pituitary extracts and B-873 as the standard for serum samples. The latter guideline was not always followed if a logit plot of ^{125}I-oLH remaining bound versus volume of unknown serum added demonstrated better parallelism with B-640 or if the value of the percentage of ^{125}I-oLH bound fell within 43-57%. In those instances direct comparison with B-640 was made. Usually, the curves of serum derived from castrated male rats appeared to resemble those from B-873 more than those from B-640; serum from intact male rats yielded curves resembling those given by B-640. Whether the direct comparison was made with B-640 or B-873 the final results for the assay were calculated in units of mass of B-640 per milliliter of unknown and are reported as such with the potency of B-640, relative to NIH-LH-S1, noted.

Serum for use in the LH assays was obtained either from trunk-blood, taken at the time of decapitation and

Figure 7. The Relationships Between the Response Curves

for Rat Serum and Pituitary Extract and Those

of Radioimmunoassay Standards B-640 and B-873

Four single representative curves - displaced to the right or left by a constant, α, for purposes of clarity - of percent of ^{125}I-oLH remaining bound versus log of volume taken, i.e., LH mass added, are plotted. They illustrate the analytical relationships between the primary standard preparation B-640 (= 0.03 x NIH-LH-S1 by OAAD), the secondary standard preparation B-873 and the serum and pituitary extracts derived from the experimental animals. Parallelism between B-640 and the pituitary extracts, and between B-873 and sera prompted the use of those standards for the respective tissue preparations. Weighting of all data toward the 50% binding level did, however, allow the direct comparison of results from both tissues to each other even though their radioactivity displacement curves are nonparallel.

FIGURE 7

THE RELATIONSHIPS BETWEEN THE RESPONSE CURVES FOR RAT SERUM AND PITUITARY EXTRACT AND THOSE OF RADIOIMMUNOASSAY STANDARDS B-640 AND B-873
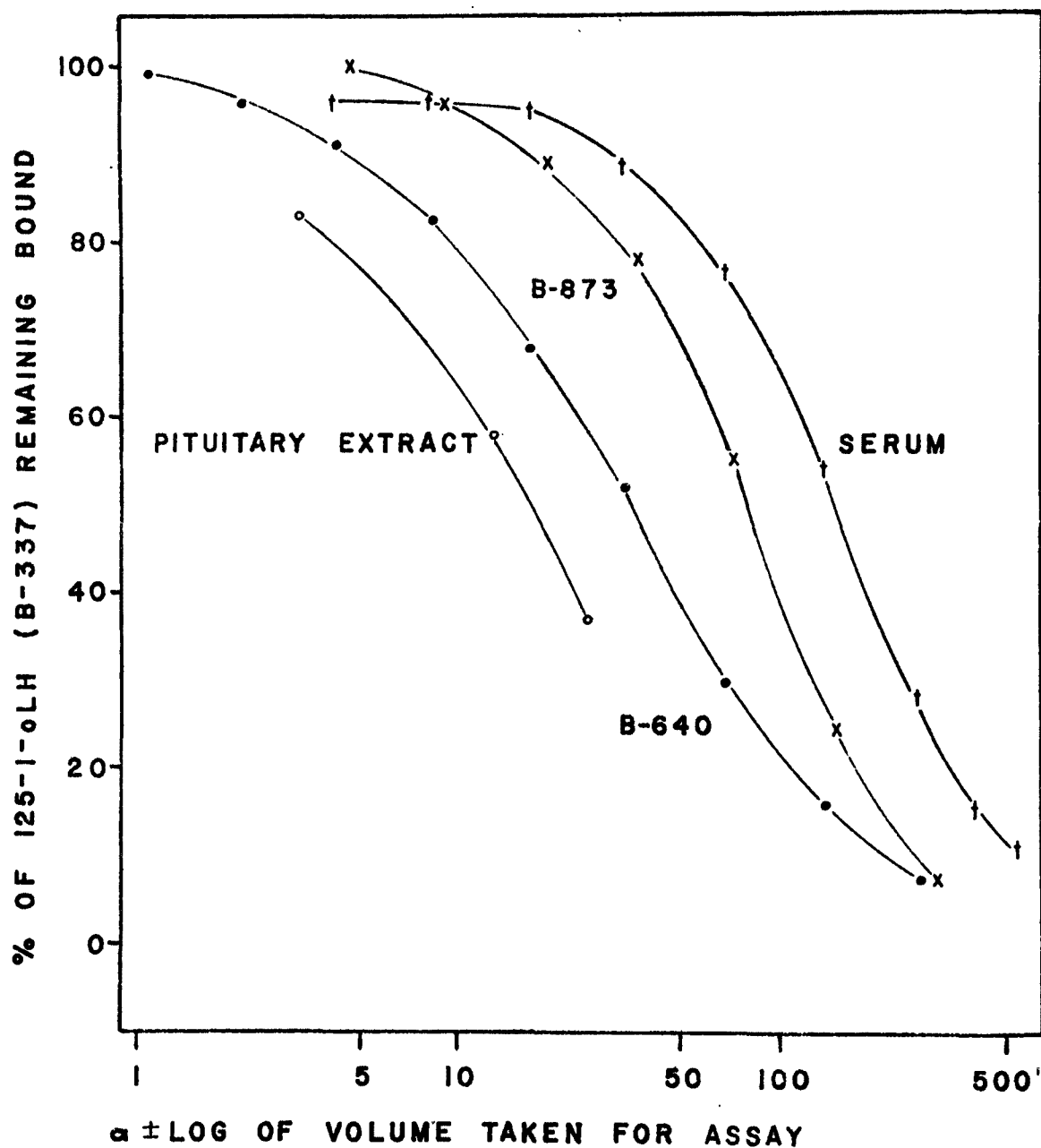
prepared as discussed previously in regard to corticoster-
one, or from samples obtained by TVBE (see Section I.
Animals). Blood obtained by TVBE was allowed to clot on
ice and then centrifuged for 20 min at 5000 rpm. The
serum from either bleeding procedure was decanted off
after centrifugation and stored at $-20^\circ C$ prior to assay.

Pituitary extracts were prepared from weighed tissues
either immediately after weighing or subsequent to storage
of the whole tissue at $-20^\circ C$. The whole pituitaries were
individually homogenized in phosphate-buffered saline, pH
7.4 (PBS) (169) using six strokes of the Teflon pestle
(driven by a Tri-R Stir-R Model 563C at settings of 5-8)
of a Potter-Elvehjem homogenizer. The final volume of PBS
diluent varied from 10 to 100 ml and was dependent upon
the size of the gland and whether the rat was intact or
castrated; pituitaries from castrated adults were pre-
pared in 100 ml of PBS while those of weanling intact
animals were homogenized in 10 ml of PBS. Extracts were
stored at $-20^\circ C$ until assayed.

The volume of the duplicate sample aliquots taken for
assay of LH also depended on the previous treatment of the
animal; sera from intact, untreated rats were assayed by
using aliquots of 200 ul; sera from long-term castrated
rats or animals injected with LHRH were assayed by using
aliquots of 10, 20, and/or 50 ul. The number of duplicated
measurements was determined by the volume of the sample
available. Single duplicates were done on sera from intact

animals while three duplicates of various sizes were done on pituitary extracts.

Since the lower limit of reliability for the assay (the maximum of ^{125}I-oLH remaining bound minus two times the standard deviation of its estimation) was normally 2-10 ng of B-640/ml, values which were computed to lie below that level, although distinctly above zero, were not necessarily accurately determined. Such values are reported as they were calculated; the potential inaccuracy will be taken into account in the interpretation of experimental results. Samples which generated values which exceeded the upper limit of the standard curve of a particular assay were reassayed using smaller aliquots. The other assay variables for these assays were as follows. The mean index of precision was -0.0173, i.e., $\pm$3.9%; the mean limit of detection (the maximum ^{125}I-oLH remaining bound minus the standard error of its estimation) was 215 $\pm$ 166 pg per assay tube; the mean sensitivity (the slope of the curve derived for the B-640 standard on the plot of percentage of ^{125}I-oLH remaining bound versus log mass of LH per tube) was -2.55 $\pm$ 0.15.

2. Effect of Sampling Time and Ether

Several authors have reported 24-hour variations in the titers of serum LH in male rats (183,184). Others have demonstrated elevations in LH levels in the serum due to ether anesthesia (36,183,185). Because the blood samples for several of my experiments were taken under

64

ether (TVBE) and because 24-hour periodicity was briefly

examined for animals treated via their o,p'-DDT-injected

dams, a short control experiment was conducted. This

experiment examined the effect of serial TVBE on the

24-hour periodicity of serum LH in normal adult (300-325 g)

male rats.

After allowing the animals to acclimatize to the

day/night cycle for four days the following sampling pro-

cedure was carried out. Beginning at 12.30 (EST) a group

of 5 rats were bled under ether by tail-vein at intervals

of 6 hours for 24 hours, i.e., 5 times. A second group of

15 rats was divided into 5 subgroups of 3 animals each.

One of these subgroups was sacrificed by decapitation each

time the ether-bled test animals were bled. Ether-bleeding

and decapitation were alternated so no time bias would be

introduced into the sampling for either group. All animals

were maintained under normal day/night conditions except

for the two short nighttime sampling periods to which the

ether-bled animals were exposed. The sera which were

collected at each sampling time were stored at $-20^{\circ}C$ until

assayed.

The serum LH levels ($\pm$ 1 standard deviation of the

mean) were calculated for each group and time. These were

plotted versus time of day. The individual means from the

ether-bled rats were tested against the individual means

of the decapitated controls by use of Student's t-tests.

The values at two of the time points differ with

probabilities of more than 95%; at 18.00 the ether-bled animals show serum levels approximately twice the basal levels and at midnight the decapitated controls show levels which are twice those of the ether-bled animals and twice those of the basal levels seen at any other time of day. These findings are shown in Figure 8.

The results support those of Dunn et al. (183) and Hefco and Lackey (184) in regard to the normal 24-hour periodicity of LH in unetherized male rats. They also support the findings of Dunn et al. (183), Howland et al. (36) and Krulich and Illner (185) in regard to an ether-induced rise in serum LH during the daytime hours and the lack of such a rise at night. The plot of the 24-hour periodicity of the serum LH in ether-bled animals is nearly superimposable on similar curves generated from rats suckled by vehicle-injected dams (see intact controls, Figure 31) and other vehicle injected adult male rats. The observed pattern is also very similar to the one Lawton and Smith (186) generated using etherized male rats and an LH bioassay. Together the reported and present data indicate a variable sensitivity of the male rat hypothalamo-hypophysial axis to ether. The peak of normal LH secretion occurs during the period of peak physical activity and food intake. This period may represent a time of passive physiological resistance to stress resulting in the diminished response to ether at midnight. This low value may merely represent a spuriously low mean

Figure 8. The Effect of Repeated Tail-Vein Bleeding Under

Ether on the 24-Hour Periodicity of Serum LH in

Normal Adult Male Rats

Serum LH measured by the radioimmunoassay of Niswender
et al. (169), as ng of primary standard, B-640 (=0.03 x
NIH-LH-S1 by OAAD), is plotted versus time of day. At
each time point indicated the number of animals shown in
parentheses were either bled immediately after brief (45
sec) exposure to ethyl ether or immediately after decapita-
tion. The same animals were bled under ether throughout
the experiment; missing values were due to technical
problems during sample collection or analysis. The
light-dark schedule was 06.30 to 18.30 as shown. All ani-
mals were fed and watered ad libidum throughout the experi-
ment. Values shown are means $\pm$ 1 standard deviation;
statistical difference from the decapitated controls was
tested at each time point by Student's t-test (* $p < 0.005$).

FIGURE 8

THE EFFECT OF REPEATED TAIL-VEIN BLEEDING UNDER ETHER ON THE 24-HOUR PERIODICITY OF SERUM LH IN NORMAL ADULT MALE RATS
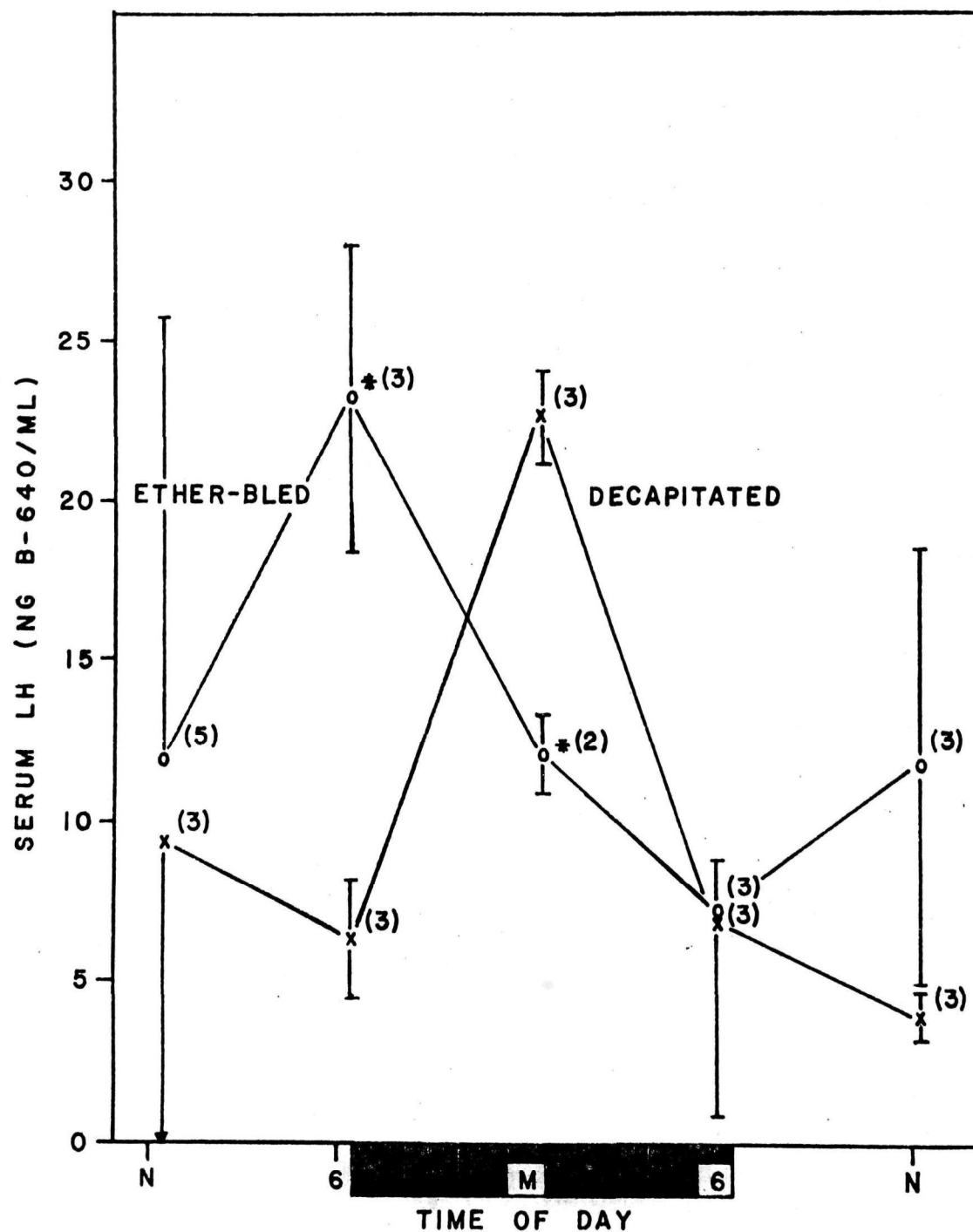
(n = 2), however, since the similar data in Figure 31 show
no difference between the serum LH levels of the intact
controls near midnight and the value obtained from the
unetherized control rats in the present experiments
(Figure 8). If the ether-bled and control values at mid-
night are, in fact, similar, the data are consistent with
the claim of Dunn et al. (183) that the serum LH levels
cannot be elevated further at the nighttime peak due to
the normal presence of an optimal LHRH output at that time.
The explanation probably combines facets of both increased
physical activity, respiration, etc., and a normal elevated
LHRH output.

Because of the temporal positions of the serum LH
elevations under normal or ether-stressed conditions,
later experiments were designed to take blood samples
under the most appropriate conditions. If basal levels
were of interest samples were taken by decapitation near
noon; whereas, if the difference between serum LH levels
was to be tested in DDT treated and untreated groups,
samples were taken near 18.00 (EST). In the second
example the time was chosen so as to maximize any differ-
ential effect ether might have on these two groups (see
also intact rats, Figure 31).

E. Estimation of o,p'-DDT Derived Residues in Rat
   Pups

The indirect treatment of rat pups, accomplished by
allowing them to suckle o,p'-DDT-injected dams, brought up

the question of administered dosage. To answer that
question and to allow analyses of the insecticide used in
these studies the following protocols were adapted.
Neutral hydrophobic compounds similar to DDT form the bulk
of the metabolites normally found in tissues (137,158,187).
Therefore, the "cleanup" protocol was designed to maximize
the recovery of these materials while eliminating the large
bulk of contaminating, nonrelated hydrophobic compounds.
It was suggested in large part by the sulfuric acid
purification of hexane extracts containing PCB detailed
by Widmark (188).

To measure the dam's secretion of neutral DDT
metabolites into her milk and the pup's subsequent uptake
of those metabolites, male pups were removed at various
ages from the litters of both DDT-injected and vehicle-in-
jected dams. The litter sizes were maintained by
replacing the males removed with females of similar age.
Within 10 min of removing the pups from their litters they
were killed by asphyxiation with carbon dioxide. Their
stomachs, containing milk curds, were then removed and the
stomachs and carcasses were frozen and stored separately
until the time of extraction and analysis.

A flowchart for the "cleanup" method is shown in
Figure 9. After weighing the biological sample of interest
it was homogenized with 2-5 weight-volumes of saturated
salt (NaCl) solution and 4-10 volumes of acetone. The
homogenization was done with a Potter-Elvehjem homogenizer

Figure 9.   Generalized Protocol for Estimation of DDT

              Analog Residues

       The flowchart outlines the extraction and purification procedures which were used on each milk curd or rat pup carcass utilized in the determination of the uptake of DDT residues by pups suckling o,p'-DDT injected dams.   Extraction and wash volumes used for processing the milk curds were constant due to the similarity in size of the curds. However, the large variability in the carcass weights forced the use of the smallest volume multiples listed in order to maintain workable total volumes.   Even in light of this problem recovery of the internal standard, aldrin, remained uniform within each category of samples.

Figure 9

Generalized Protocol for Estimation of DDT Analog Residues

Stomach Curd or Carcass
(Parallel Estimations)

1. Weighed;

2. Homogenized with 2-5 volumes of saturated NaCl
   and 4-10 volumes of acetone;

3. Internal Standard added;

4. Rehomogenized

Organic Layer ——————⟨ ⟩—————— Aqueous Residue

5. Reextracted with 8-20
   volumes of benzene and
   2-3 times with 0.3-20
   volumes of hexane

Combined Extracts ——————————— Aqueous Residue

6. Washed 2 times with   Discarded
   0.05-0.15 volumes
   of 80% MeOH

MeOH Wash ————————————— Organic Layer

Discarded   7. Volume measured;

                        8. Washed 1 time with
                           0.05-0.15 volume of
                           H_2SO_4 (conc.) and 2
                           times with 0.1 volume
                           of water

Acid and Water Washes ——————— Organic Layer

Discarded   9. Dried with anhydrous
                           $MgSO_4$ and evaporated
                           to appropriate volume

                        Dry Organic Extract

                        10. Gas chromatography

at high speed if the curd was being analyzed or with a Waring blendor equipped with an explosion-proof chamber if whole pups were being extracted. After the initial homogenization a known quantity of the internal chromatography standards used for pesticide analysis, aldrin and p,p'-DDT, were added (0.5 ug of each were added in curd extractions; 50 ug of each were added in whole-body extractions). The solution was then rehomogenized and placed either into a centrifuge tube or a separatory funnel. When the organic and aqueous phases had settled or been separated by centrifugation the organic layer was removed and saved. The aqueous residue was reextracted once with 8-20 volumes of benzene and 2 or 3 times with 0.3-20 volumes of hexane. The aqueous residue was discarded. All the organic phases were combined and washed twice with 0.05-0.15 volume of 80% aqueous methanol. The alcoholic washes were discarded and the volume of the organic layer measured. This solution was then washed with 0.05-0.15 volume of concentrated H_2SO_4. Following the acid wash the organic layer was washed twice with 0.1 volume of water. Acid and water washes were both discarded. This step, 8, removed the large majority of the hydrophobic impurities left in the solution. The wet organic phase was dried over anhydrous $MgSO_4$ and then evaporated on a steam-cone under nitrogen to near 5 ml (curd samples) or 50 ml (whole pups). Final volumetric adjustments were made by adding hexane. Aliquots of 1-8 ul

of these dry extracts were injected directly into the gas chromatograph for qualitative and quantitative analyses.

The precise volumes used in extractions and washes varied with sample weight. When dealing with whole body residues a ceiling of 300 ml was used for the addition of benzene and 600 ml for a total extraction volume. Other extraction and wash volumes were scaled up or down within the limits shown to prevent the sample preparation from becoming unwieldy. For small to moderate size samples, however, the upper limits of the volumes shown were favored to minimize problems with emulsions and to maximize clean recoveries. For curd samples standardized volumes were used throughout the preparation: 5 ml of saturated salt solution and 10 ml of acetone, 20 ml of benzene and 2 times 20 ml hexane, 2 times 10 ml of 80% methanol, 10 ml concentrated H_2SO_4, 10 and 5 ml of water. When calibration standards were processed by this procedure all steps followed those used for the preparation of stomach curd samples.

All concentrated extracts were analyzed by a Varian 2100 GC system which incorporated a ^{3}H-Scandium foil electron capture detector. The chromatography was performed using a 180 cm x 2 mm glass column packed with 80/100 mesh Gas Chrom Q coated with 6% QF-1 and 4% SE-30. Nitrogen carrier gas was used at a flow rate of 63 ml/min under a pressure head of 70 psi. The injector temperature was 245°C; the detector was held at 295°C. Chromatograms

74

were run under a shallow temperature program, $175°$ to $195°C$ at $0.5°$/min, which had been demonstrated not to interfere with peak resolution or baseline stability. Attenuator settings varied slightly but were usually at 16×10^{-10} amp/mv; detector standing current was normally 2×10^{-9} amp. Chart speed was 0.5 cm/min. The column packing was suggested by the work of McCully and McKinley (189) and Abou-Donia and Menzel (190); the retention times observed agree fully with their findings. Relative retention times with respect to aldrin were: 1.45, o,p'-DDE; 1.84, p,p'-DDE; 2.04, o,p'-DDD; 2.39, o.p'-DDT; 2.57, p,p'-DDD; 3.06, p,p'-DDT. Three typical chromatograms are shown in Figure 10.

Since the o,p'-DDT was contaminated with 1.5% p,p'-DDT (see II. <u>Chemicals</u>) the knowledge that p,p'-DDT is biologically stored preferentially to o,p'-DDT (166) militated against any reliance on the use of p,p'-DDT as an internal standard. Therefore, all analyses were performed using only aldrin as the standard for internal comparison and deleted quantitation of o,p'-DDT. The sharpness and form of most of the peaks in the chromatogram indicated that plots of peak height versus mass, or some transformation of these measurements, would be suitable for quantitation. Calibration curves were generated by chromatographing mixtures containing various amounts of o,p'-DDE, p,p'-DDE, o,p'-DDD, o,p'-DDT and p,p'-DDD both before and after extraction. The linearity of the curves of DDT analog

Figure 10. Typical Gas Chromatograms Obtained During

Analyses of o,p'-DDT and Its Analogs

Profiles of detector response versus retention time
are shown for 3 typical samples. Sample A, ——, is a 1.2
ul injection of an unextracted standard solution containing
1.5 ng/ul of each of o,p'-DDE (II), p,p'-DDE (III),
o,p'-DDD (IV), o,p'-DDT (V), p,p'-DDD (VI) and 1.0 ng/ul
of both aldrin (I) (internal standard) and p,p'-DDT (VII).
Sample B, - - -, is a 2.2 ul injection of the residues
extracted from a 15 day old rat pup which had nursed an
o,p'-DDT injected dam; 1.0 ng/ul of internal standard has
been added. Sample C, — —, is a 1.75 ul injection of the
hexane extract of a milk curd from the stomach of a 10 day
old rat pup which had nursed an o,p'-DDT injected dam;
0.1 ng/ul of internal standard has been added.

All samples were run on a 2 m x 2 mm column of 6%/4%
QF-1/SE-30 on 80/100 mesh Gas Chrom Q under a temperature
program of 0.5°/min from 175° to 195°C. Injector and
detector temperatures were 245° and 295°C, respectively.
Gas, N_2, flow rates were 63 ml/min under a pressure head
of 70 psi. An electron capture detector was used at an
attenuator setting of 16 x 10^{-10} amp/mv; chart speed was
0.5 cm/min.

FIGURE 10
TYPICAL GAS CHROMATOGRAMS OBTAINED DURING
ANALYSES OF o,p-DDT AND ITS ANALOGS
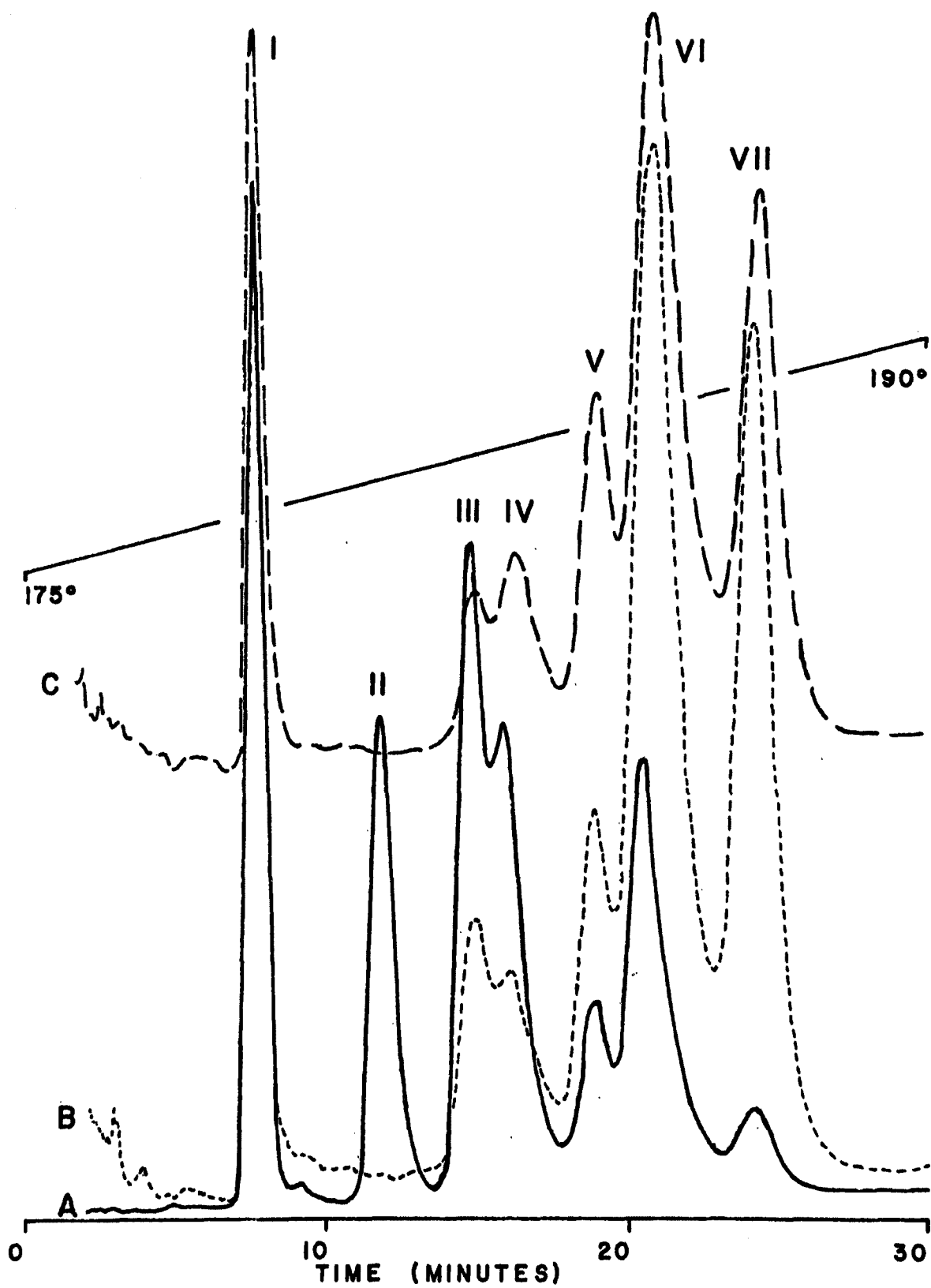
peak height/aldrin peak height versus DDT analog concen-
tration was tested by least-squares regression. Though the
curvature of these plots was not great the best lines were
obtained if a log-log transformation was applied. There-
fore, all unknowns were calculated from standard curves of
$\log_{10}$ (DDT analog peak height/aldrin peak height) versus
$\log_{10}$ (concentration of DDT analog). Two overlapping
standard curves were generated using high, 1 ug/ml, and
low, 0.1 ug/ml, final concentrations of aldrin. This was
done to allow more precise measurement of the peak height
ratios and thus to allow more precise measurement of the
residue levels of the unknowns. These two curves were
strictly parallel and their intercepts differed after cor-
rection for aldrin concentration only because the recovery
of aldrin was proportional to the amount added prior to
extraction. The characteristics of each of the high
aldrin concentration curves are shown in Table 2 along
with the extraction recoveries calculated from extracted
and unextracted standards. The somewhat high variability
of the curves most likely reflects both the small number
of replicates, 2-3, done on each sample and departures from
good peak symmetry. Duplicates of unknowns were normally
within $\pm$ 10-15% and thereby indicate that λ is probably a
pessimistic estimate of precision.

Table 2

Standard Curves for Determination of DDT Analogs by Electron-Capture Gas Chromatography

Variable	Analog				
	o,p'-DDT	o,p'-DDD	o,p'-DDE	p,p'-DDD	p,p'-DDE
Slope[1] $b \pm SD$	0.986 ± 0.032	0.846 ± 0.024	0.876 ± 0.021	0.488 ± 0.029	0.836 ± 0.024
Intercept[2] $a \pm SD$	-0.781 ± 0.020	-0.434 ± 0.017	-0.469 ± 0.014	-0.324 ± 0.017	-0.346 ± 0.017
Correlation Coefficient R	0.994	0.993	0.995	0.975	0.993
SE_y[3]	0.065	0.068	0.061	0.065	0.070
λ [3]	0.066	0.080	0.070	0.133	0.084
Useful Range[4] (ug/ml)	0.008-9.0	0.009-9.0	0.019-9.0	0.012-9.0	0.007-9.0
% Recovery[5]	97.2	106.3	102.8	103.0	102.7

[1] The standard curve is a plot of Log_{10} (Peak Height Analog/Peak Height Aldrin) versus Log_{10} (Analog Concentration) and is of the form $Log\ y = a + b\ Log\ x$.

[2] The intercept is given for the standard curves used at higher analog concentrations which utilized an aldrin (internal standard) concentration of 1 ug/ml; at lower analog concentrations an aldrin standard of 0.1 ug/ml was used and the intercept increased.

Table 2 (Cont.)

[3] The error in the predictability of Log y given Log x is the standard error of the estimate, SE_y; the error in the predictability of Log x given Log y is the index of precision, λ, and equals SE_y/b.

[4] The useful range is calculated from the linear portion of the parallel curves generated for both high and low concentrations of analogs.

[5] Recovery of extracted versus nonextracted standards; aldrin recovery was 66% at 0.1 ug/ml and 84% at 1 ug/ml.

F. Testicular Incubations:

    1. Protocol

Measurements were undertaken to investigate the
existence, and possible site, of subcellular interactions
between o,p'-DDT and the testicular steroids. Incubations
of $[7\ n\text{-}^3H]\text{-}\Delta^5$-pregnenolone were conducted in the presence
or absence of o,p'-DDT in testicular homogenates. The
protocol, described below, was so designed that it also
showed the presence or absence of o,p'-DDT metabolism in
the same homogenates.

Testes, obtained during castrations or after animal
sacrifice, were weighed, decapsulated and homogenized at
$4^\circ C$ in 6 weight-volumes of Buffer I. Buffer I is a
modified Ringer's solution suggested by Moldeus et al.
(191) which resembles buffers previously used both in
steroid incubations with testes (182,193) and in drug
incubation with hepatic microsomes (191). Buffer I is
125 mM NaCl, 6 mM KCl, 5 mM $MgCl_2$, 15 mM Na_2HPO_4, 10 mM
succinate adjusted to pH 7.4. The homogenization was
achieved with 6 slow strokes of a Potter-Elvehjem homogen-
izer fitted with a Teflon pestle (driven by a Tri-R Stir-R
Model 563C at settings of 5-8). The homogenate volume was
measured and poured into 50 ml centrifuge tubes. These
were centrifuged at $4^\circ C$ at 500 x g for 10 minutes in a
Lourdes model LRA centrifuge. After centrifugation the
supernatants were decanted into flasks and held on ice
prior to incubation.

Incubations were performed in 20 ml beakers to which
200 ul of propylene glycol, measured amounts of ethanol or
ethanolic solutions of Δ^5-pregnenolone and/or o,p'-DDT,
cofactors and 1.90 ml of Buffer I were added. The cofac-
tors included an NADPH-generating system and NADH; their
concentrations in the final incubation volume were 0.825 mM
$NADP^+$, 0.4125 mM G-6-P, 1 unit of G-6-P DH/ml and 0.4125 mM
NADH. Substrate concentrations were 1 nmole/ml in the final
incubation volume for both Δ^5-pregnenolone (including 5 uCi
of $[7 \text{ n-}^3H]-\Delta^5$-pregnenolone) and o,p'-DDT. Incubations
were initiated by vigorously adding 4.0 ml of supernatant
(40-60 mg of protein) or Buffer I to the chilled cofactor-
substrate solution. Immediately after this addition a 1.0
ml aliquot was removed for a zero-time determination of ste-
roids and/or DDT metabolites. The remaining solution was
quickly placed into a Dubnoff metabolic shaker set at 20
rpm. The incubation proceeded under air at 37^oC. At 5, 10,
30 and 60 minutes of incubation additional 1.0 ml aliquots
were taken. After incubation the residual volume of solu-
tion was retained for determination of protein and to check
the number of tritium counts initially added to the incuba-
tion. Protein was measured by the Biuret method (194) using
BSA as a standard. Counts were determined on a 0.1 ml ali-
quot which was allowed to air dry prior to addition of
scintillation fluid. The protocol is summarized in Table 3.

Each of the incubations, 1-5, was designed to probe a
different facet of the potential differences between

Table 3

Incubations Performed[1]

| Addition | Volume of Solutions Added to Each Flask | | | | |
| | Incubation Number | | | | |
	1	2	3	4	5
Buffer I[2] (ml)	1.90	1.90	1.90	1.90	5.90
Cofactors[3]	+	+	+	+	+
Propylene Glycol (ul)	200	200	200	200	200
Supernatant[4] (ml)	4.00	4.00	4.00	4.00	0
$[7\ n\text{-}^3H]$-Δ^5-Pregnenolone[5] (ul)	5	5	0	0	5
cold Δ^5-Pregnenolone[6] (ul)	5	5	0	0	5
o,p'-DDT[7] (ul)	8.2	0	8.2	0	0
Ethanol (ul)	0	8.2	10.0	18.2	8.2
Total Volume (ml)	6.20	6.20	6.20	6.20	6.20

[1] Flasks were incubated in a Dubnoff metabolic shaker at 20 rpm under air at $37^\circ C$ and samples taken at 0, 5, 10, 30 and 60 minutes of incubation. The residual volume of each flask was retained for determination of protein by the Biuret procedure (BSA standard) and for determination of the total initial content of ^3H counts.

[2] Buffer I is 125 mM NaCl, 6 mM KCl, 5 mM $MgCl_2$, 15 mM Na_2HPO_4, 10 mM succinicate at pH 7.4.

Table 3 (Cont.)

[3] Final cofactor concentrations in the total incubation volume were 0.825 mM $NADP^+$, 0.4125 mM G-6-P, 0.4125 mM NADH and 1 unit of G-6-P DH/ml.

[4] The 500 x g supernatant of a 6:1 homogenate of testicular tissue in Buffer I was used.

[5] Concentration of the ethanol solution added was 1 uCi/ul.

[6] Concentration of the ethanol solution added was 1.18 nmoles/ul.

[7] Concentration of the ethanol solution added was 0.75 nmole/ul.

supernatants derived from treated and untreated animals. Number 5 serves as a control for thermal or nonenzymatic steroid transformations and therefore as the baseline for steroid metabolism in supernatants from both treated and untreated animals. Number 4 provides a control for the endogenous presence and/or conversion of o,p'-DDT. Number 3 allows the measurement of the metabolism of DDT in the absence of exogenously added steroid while number 2 allows the measurement of the metabolism of Δ^5-pregnenolone in the absence of exogenously added o,p'-DDT. Number 1 provides a direct test of the interactions of steroid metabolism and DDT metabolism by allowing simultaneous measurement of both sets of metabolites.

To allow this simultaneous measurement of both sets of metabolites an extraction and purification scheme was devised; it is summarized in Figure 11. The aliquots taken during incubation were forcefully expressed into 5 ml of ethanol held in a 15 mm x 120 mm ignition tube. This stopped any metabolic conversions and began the extraction process. If further processing was not begun immediately the solutions were stored at -20°C. A recovery marker (60,000 dpm ^{14}C-steroid) and carrier steroid (100 ug) were then added for each of the several steroids of interest. An internal standard, 5 ug of p,p'-DDT, was also added to monitor the recovery of DDT metabolites. This solution was Vortexed thoroughly and then centrifuged for 10-15 minutes at top speed in a Clay-Adams tabletop

Figure 11. Protocol for Extraction and Partial Purifica-

tion of DDT and Steroid Metabolites in

in vitro Incubations

The 1.0 ml samples taken from the in vitro incuba-
tions carried out under conditions described in Table 3
were prepared for analysis by this procedure. Steroids
added to aid in recovery and estimation included 100 ug
cold steroid plus 60,000 dpm $4\text{-}^{14}\text{C}$-steroid each for:
Δ^5-pregnenolone, progesterone, 17α-hydroxyprogesterone,
dehydroepiandrosterone, Δ^4-androstenedione, and tesoster-
one. The p,p'-isomer of DDT was also added (5 ug) as an
internal standard and recovery tracer for the DDT analogs.
The internal standard for the steroids, cholesterol-3-pro-
pyl ether (CPE), was added just prior to gas chroma-
tography in a system which split the column effluent in
approximately a 3:1 ratio between a collection capillary
and the mass detector of the chromatograph. Specifics of
the measurements are discussed in Methods Section III.F.2.
and in Chapter 6.

Figure 11

Protocol for the Extraction and Partial Purification of DDT

and Steroid Metabolites in <u>in vitro</u> Incubations

<u>1.0 ml Aliquot</u>
|

Added to 5 ml of ethanol in a 15 mm x 120 mm ignition
tube; ^{14}C-steroid plus cold carrier steroid added;
5 ug of p,p'-DDT added;   Vortexed and centrifuged
|

<u>Alcoholic Extract</u>
|

Extracted with 2 x 2 ml of hexane
in 60 ml separatory funnel

<u>Alcoholic Solution</u> ———————— <u>Hexane Extract</u>
| |

Extract with 20 ml CH_2Cl_2 Washed with 10 ml conc.
                                        H_2SO_4 and 10 ml H_2O;   dry
Aspirate   <u>CH_2Cl_2 Extract</u>   over anhydrous $MgSO_4$
off | |
alcohol   Wash with 5 ml 1 N   Evaporate in vial under
            HCl, 5 ml 5% $NaHCO_3$, N_2 for storage at -20°C
            2 x 5 ml NaCl solu- |
            tion;   dry over   Reconstitute in hexane
            anhydrous $MgSO_4$; |
            evaporate to dryness   <u>Gas Chromatography</u>
            under N_2 in 10 mm x 75 |
            mm test tube   <u>DDT Data</u>
                    |

Store in pyridine at -20°C
                    |

Evaporate pyridine;   add 200 ul
of 1:1-ethanol:benzene and 200
ug CPE
                    |

<u>Gas Chromatography</u>————————
        | <u>Collected Effluents</u>
        | |
   <u>Area Data</u>   <u>Count Data</u>
                <u>Quantitation Program</u>
                        |
                <u>Steroid Data</u>

centrifuge. The extract was carefully decanted off into a 60 ml separatory funnel. The pellet was reextracted with another 2 ml of ethanol. The combined ethanol extracts were extracted twice with 2 ml of hexane. These hexane extracts were combined and washed with 10 ml of concentrated H_2SO_4 followed by 10 ml of water. The purified hexane extract was dried over anhydrous $MgSO_4$. Before storage at $-20^{\circ}C$ prior to assay, the DDT extract was evaporated to dryness under nitrogen in a $\frac{1}{2}$ dram vial.

The hexane-extracted ethanol solution was extracted further with 20 ml of dichloromethane. The phases were allowed to separate and the upper alcoholic phase was aspirated off. Then the dichloromethane, which contained the majority of the steroids, was washed with 5 ml each of 1.0 N HCl and 5% $NaHCO_3$ and twice with 5 ml of a concentrated NaCl solution; each wash was aspirated off. The dichloromethane extract was then dried over anhydrous $MgSO_4$ and evaporated to dryness under nitrogen in a 10 mm x 75 mm test tube. Pyridine, 0.1 ml, was added and the tube was sealed with Parafilm; the extract was stored at $-20^{\circ}C$ prior to assay.

Just before assay of the DDT extract, 100-1000 ul of hexane were added. Then a 1-5 ul aliquot of the sample was quantitated by GC using the conditions mentioned in Methods Section E., Estimation of o,p'-DDT Derived Residues in Rat Pups. Before assay of the steroid extract the pyridine was evaporated off with nitrogen and 200 ul of a 1:1 solution

of benzene and ethanol containing 200 ug of the chosen

internal standard for GC of steroids, cholesterol-3-propyl

ether (CPE), was added to make up the assay solution.

2.   Estimation of Steroids by Gas Chromatography

and Scintillation Counting

Aliquots of the final steroid assay solution, 1-5 ul,

were injected onto a 350 cm x 2 mm glass column equipped

with an effluent splitter. The column was packed with

80/120 mesh Gas Chrom Q coated with 1.5% OV-7 and 1.5%

SE-54. The effluent splitter favored an effluent-cap-

ture-tube over the flame-ionization detector by 3 to 1,

i.e., 75% of the effluent was captured and 25% was burned

for qualitative and quantitative analysis. Chromatograms

were run using nitrogen as the carrier gas at a flow rate

of 25 ml/min (head pressure was 70 psi); hydrogen and

oxygen were used as the flame support gases at flow rates

of 30 and 60 ml/min, respectively. Injector and detector

temperatures were 250° and 295^OC, respectively. Column

temperature was programmed on a linear gradient of 1^O/min

from 175° to 275^OC. Chart speed was 10 cm/hour. Two

sample chromatograms are shown in Figure 12.

The use of acetate and methyloxime-silyl derivatives

(195) on this and several other columns had been explored

with varying degrees of success, in terms of efficient

effluent capture and adequate resolution of the main

steroid peaks of interest. Methyloxime-silyl derivatives

were chromatographically more stable and volatile than the

Figure 12. Typical Gas Chromatograms Obtained During

Analysis of Testicular Δ^5-Pregnenolone

Metabolites

Two sample chromatograms obtained during analyses of
testicular metabolites of Δ^5-pregnenolone are shown.
Plot A was taken from a 7.75 ul injection of a calibration
standard containing 2.0 ug/ul each of dehydroepiandroster-
one (II), Δ^4-androstenedione (III), testosterone (IV),
Δ^5-pregnenolone (V), progesterone (VI), 17α-hydroxypro-
gesterone (VII) and 200 ng/ul cholesterol-3-propyl ether
(internal standard) (IX). Plot B was obtained from a
6.05 ul injection of an extract prepared via the protocol
of Figure 10 from a sample taken at 30 minutes of incuba-
tion of a testicular homogenate. The homogenate was pre-
pared from the testes of an adult male rat which had been
injected daily on days 1-5 of age with 0.05 ml of sesame
oil containing 40 ug estradiol-17-valerinate. In addition
to the peaks II-VII and IX indicated in the calibration
mixture two unidentified peaks, I and VIII, are also
evident. It should be noted that in plot B the bulk of
the steroid mass responsible for peaks II-VII is due to
the added carrier steroids; the similarity of the
inter-peak relationships between Plots A and B is a reflec-
tion of the similarity of the inter-steroid mass ratios in
the standard solution and the unknown solution containing
the added carrier steroids. See <u>Materials and Methods</u>
Section IV.F.2. for chromatographic details.

FIGURE 12

TYPICAL GAS CHROMATOGRAMS OBTAINED DURING ANALYSES OF TESTICULAR Δ^5-PREGNENOLONE METABOLITES
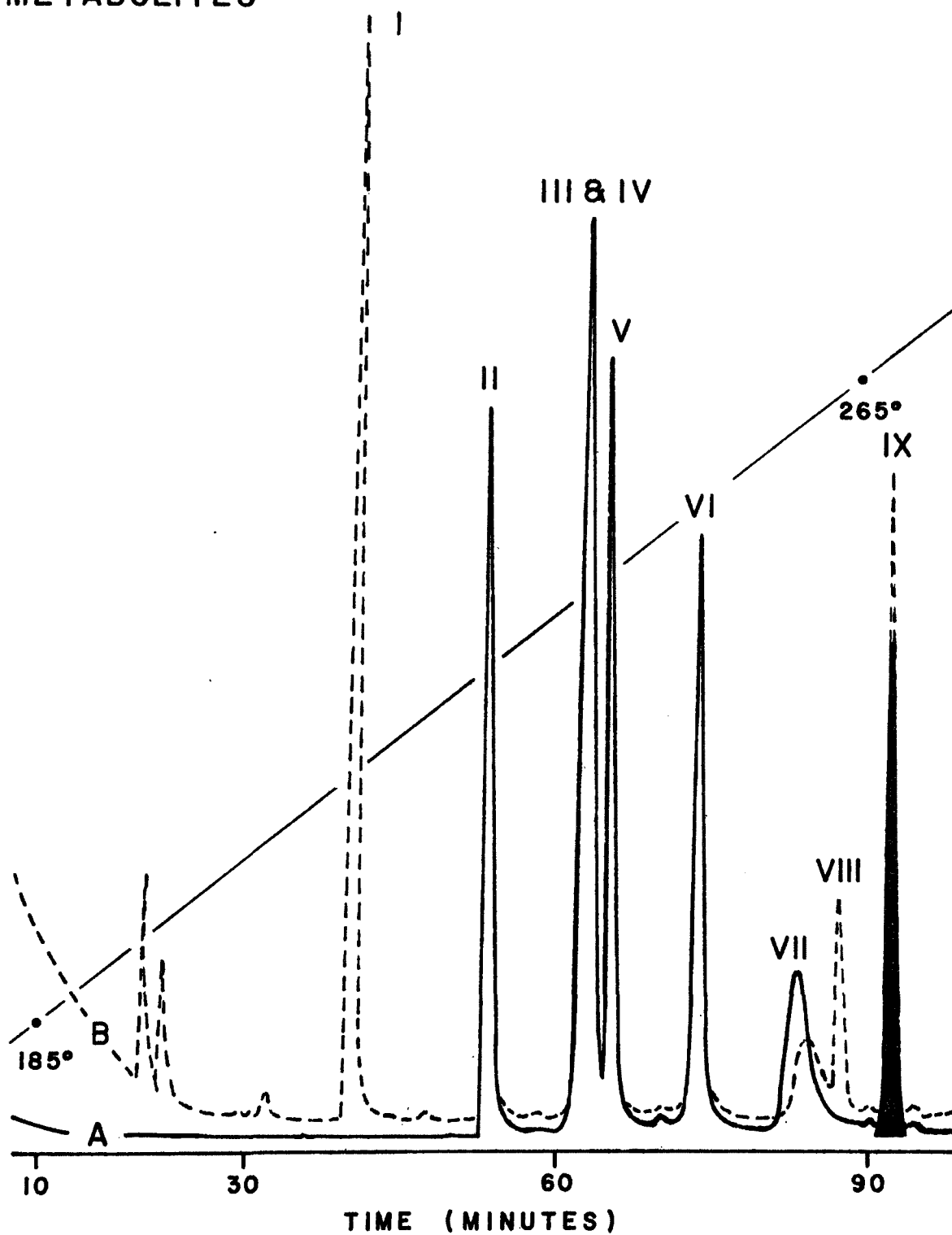
parent compounds but their capture efficiency was poor due to their tendency to form stable aerosols when cooled (195) rather than condensing onto the capillary capture tubes. Acetate derivatives worked well also but they had increased retention times and the peaks for dehydroepiandrosterone acetate and Δ^4-androstenedione overlapped. Though the peaks for testosterone and Δ^4-androstenedione overlap in the present system their metabolic status as end-products of both major androgenic pathways (see Figure 1) provides a more logical reason to measure them together than would be the case for measuring dehydroepiandrosterone and Δ^4-androstenedione concurrently. Although both this peak overlap and the thermal breakdown of 17α-hydroxyprogesterone were recognized problems, the use of parent compounds obviated the need for derivatization time and the problems associated with the derivatives.

The components eluted coincidentally with each of the major peaks of interest, II-VII and IX in Figure 12, were captured in dry-ice chilled capillaries which were then flushed into scintillation vials with 10 ml of scintillation fluid (6 g PPO and 0.25 g POPOP/1 in toluene). These individual samples were counted 3-4 times for 10 minutes each time in a Packard Tri-Carb Model 3320 scintillation counter. Tritium was consistently counted at 48-49% efficiency in this system while carbon-14 was counted at 66-69% efficiency; no quenching was encountered. Counts were converted to ^{3}H and ^{14}C dpm by using a program

written for a Wang 614 calculator which was based on the discriminator-ratio method of Okita et al. (196). Counting errors were normally less than 1.2% for ^{3}H and less than 1.4% for ^{14}C.

Initial qualitative identification of the peaks was based on the retention times relative to CPE of known steroids. Solutions of various known concentrations of dehydroepiandrosterone, Δ^4-androstenedione, testosterone, Δ^5-pregnenolone, progesterone and 17α-hydroxyprogesterone, each of which contained 1 ug/ul of CPE, were chromatographed to identify the various peaks and to calibrate the system for the later quantitation of unknowns. The retention times relative to CPE were: 0.57, dehydroepiandrosterone; 0.67, Δ^4-androstenedione and testosterone; 0.69, Δ^5-pregnenolone; 0.79, progesterone; and, 0.91, 17α-hydroxyprogesterone. Peak area data for these chromatograms were calculated both by triangulation and by an automatic integration system incorporating an Adams-Smith interface and an experimental program written for a Wang 614 calculator; the results from both methods compared favorably for all but low, broad peaks which the automatic system failed to detect. The logarithms of peak areas, normalized by the peak area of CPE, were plotted versus the log of the concentration of steroid injected. These standard calibration curves along with the mean percentage of steroid captured for counting are described in Table 4. Overall recoveries for the steroids, including

Table 4

Standard Curves for Determination of Steroids by Gas Chromatography

Variable	Steroid[1]				
	DHA	Δ^4 + T	Δ^5	P	17α
Slope[2] $b \pm SD$	1.005 ± 0.028	0.988 ± 0.024	1.047 ± 0.031	1.004 ± 0.031	0.994 ± 0.025
Intercept[2] $a \pm SD$	0.183 ± 0.018	0.440 ± 0.013	0.153 ± 0.020	0.086 ± 0.019	-0.023 ± 0.018
Correlation Coefficient R	0.993	0.995	0.992	0.992	0.995
SE_y[3]	0.066	0.049	0.073	0.072	0.061
λ[3]	0.066	0.050	0.070	0.072	0.061
Useful Range[4] (ug/ml)	0.02-25	0.04-50	0.02-25	0.02-25	0.02-25
% Recovery[5]	68.6	59.0	88.5	78.1	49.1

[1] DHA, dehydroepiandrosterone; Δ^4, Δ^4-androstenedione; T, testosterone; Δ^5, Δ^5-pregnenolone; P, progesterone; 17α, 17α-hydroxyprogesterone.

[2] The standard curve is a plot of Log_{10}(Peak Area:Steroid/Peak Area:Cholesterol-3-propyl ether) versus Log_{10}(Steroid Concentration) and is of the form $Log\ y = a + b\ Log\ x$.

[3] The error in the predictability of Log y given Log x is the standard error of the estimate, SE_y; the error in the predictability of Log x given Log y is the index of precision, λ, and equals SE_y/b.

Table 4 (Cont.)

---

[4] The least concentration detectibly greater than zero + 2 λ to the maximum measured.

[5] Recovery of ^{14}C-steroids injected onto the column and trapped with a chilled capillary tube divided by the actual fraction of chromatographic effluent flowing to the capillary.

extraction, GC and capillary recovery (which is shown in Table 4) ranged from 37% for 17α-hydroxyprogesterone to 66% for Δ^5-pregnenolone. Due to the somewhat high values of λ all measurements on unknowns were done at least in duplicate.

To further support the identities of the steroids with the peaks involved in the chromatograms, $^3H/^{14}C$ ratios were generated for each GC peak of interest both before and after each of a series of further purification steps. Peak effluents were combined from 5 chromatograms of an assay sample generated from the aliquot taken after 60 minutes of incubation of a testicular supernatant from a sesame oil-injected rat. The combined effluents for each peak were eluted from the capillaries with 3 ml of acetone and evaporated to dryness. They were taken up in 200 ul of ethyl ether and 10 ul were counted. Fifty micrograms of each of Δ^4-androstenedione and testosterone were added to the solution containing the effluent of peak III and IV (Figure 12). This solution was applied to a TLC plate and run twice in 7:3-hexane:acetone. The spots were located under UV light, scraped and then eluted with 1.5 ml 1:1-methanol:chloroform. Aliquots of these eluents were counted.

After the addition of 5 mg of the putative steroid each peak effluent was submitted to derivatization with either acetic anhydride or methoxylamine hydrochloride. GC effluents corresponding to peaks II, IV, V and VII were

acetylated while the methyloximes were formed for those corresponding to peaks II and VI. After derivatization the steroids were recrystallized from ethanol/water. The crystals were collected and dissolved in acetone; aliquots were again counted. Recrystallization of the derivatives was carried out twice more, first from acetone/water and then from ethyl ether/hexane. In both cases aliquots of the collected crystals were counted. The $^3H/^{14}C$ ratios obtained are given in Table 5. Most of the steroids initially lose some ^3H counts before the ratio stabilizes, indicating the inclusion of some other labelled metabolites within the captured effluent or the inclusion of column-derived background counts for which corrections have not been made. (Based on effluents corresponding to the unlabelled CPE peak, column effluents include nonspecific counts amounting to 1-2% of the total number of counts injected onto the column for a particular chromatogram for both ^3H and ^{14}C.) The peak containing Δ^4-androstenedione and testosterone appears reasonably pure as does 17α-hydroxyprogesterone. The probable tailing of testosterone into the Δ^5-pregnenolone peak explains the initial loss of ^3H for this peak. The progesterone peak obviously contains at least one other product and the dehydroepiandrosterone peak appears to be grossly contaminated by another product or products. Both these last peaks do, however, show an approach to a stabilized $^3H/^{14}C$ ratio by the third crystallization and therefore

Table 5

$^3H/^{14}C$ Ratios[1]: Evidence Supporting Most of the Steroid Identities Assigned to the Gas Chromatographic Peaks Obtained During Analysis of Steroid Metabolite Mixtures

Treatment	Steroid[2] and Assigned Peak					
	DHA/II	Δ^4/III	T/IV	Δ^5/V	P/VI	17α/VII
Captured Peak	4.333	----- 9.700 ------		1.125	1.189	1.190
TLC (7:3-Hexane:Acetone)	-----	3.076 ----- 9.259 ------	15.766	-----	-----	-----
Derivatization[3] and Crystallization I (EtOH/Water)	2.763	2.980 -----10.397------	19.369	0.372	0.672	0.953
Crystallization II (Acetone/water)	1.790	2.770 -----11.174------	20.168	0.186	0.506	0.958
Crystallization III (Ethyl Ether/Hexane)	1.398	3.130 -----10.583------	20.118	0.219	0.484	0.937

[1] Five injections (34.6 ul) of an extract from a sample taken after 60 minutes of incubation of a testicular homogenate with [7 n -3 H]-Δ^5-pregnenolone and to which ^{14}C-carrier steroids had been added were submitted to gas chromatography; the indicated peaks (Figure 32) being trapped and analyzed. All ratios represent single determinations; 40 minute counts, giving counting errors of less than 2% for either ^3H- or ^{14}C-, were done in all cases.

[2] DHA, dehydroepiandrosterone; Δ^4, Δ^4-androstenedione; T, testosterone; Δ^5, Δ^5-pregnenolone; P, progesterone; 17α, 17α-hydroxyprogesterone.

Table 5 (Cont.)

[3] Prior to derivatization 5 mg of the putative steroids were added. Acetates of DHA, T, Δ^5 and 17α were made by reaction with 2:1-pyridine:acetic anhydride; for Δ^4 and P methyloximes were formed by reaction with a 2% solution of methoxylamine HCl in pyridine.

support the presence of the putative steroids within the captured effluents. It should also be noted that these data were generated by a single set of recrystallizations, each sampled only once for each step of purification; the variability of the ratios between crystallizations would likely have been smaller if duplicates or triplicates had been run. The results support the identity of the captured radioactivity with the peak assignments given in Figure 12 with the possible partial exceptions of dehydro-epiandrosterone and progesterone. (Other data, reported in Chapter 6 and the Appendix tend to mollify even these exceptions.)

Counting and GC data for the metabolism of Δ^5-pregnenolone were combined and analyzed by the use of the procedures discussed in Chapter 6 and the computer program detailed in the Appendix.

CHAPTER 3

EXPERIMENTS ON THE EFFECTS OF DIRECT INJECTION OF

o,p'-DDT INTO NEONATAL MALE RATS

The approach summarized in Figure 4 implied several
possible divisions of experimental results: by route of
administration, by castration state or by measured vari-
able. The most coherent discussion of the findings seemed
to be based on the route of administration. Therefore,
the next two chapters will describe the results of the
physiological measurements made on animals dosed by direct
(Chapter 3) and indirect (Chapter 4) means. A coordinating
discussion including conclusions and postulations for
future experimental directions comprises Chapter 5. Due
to the different nature and development of the studies
done on testicular incubations they will be discussed
separately in Chapter 6.

I. Organ Weights in Intact Male Rats and in Neonatally
     Castrated Adult Male Rats Neonatally Injected with
     o,p'-DDT:

The initial experiment in these studies was an
attempt to determine the existence in male rats of a hypo-
thalamic imprinting by o,p'-DDT which was similar to that
which had been reported in females (53). It was decided,
on the basis of observations made on animals imprinted with
steroids (57-61,63-67), that alterations of growth and/or
organ weight should be demonstrable if steroidogenic

derangement occurred. This would be true if the derange-
ment was caused by either a direct action on the steroido-
genic endocrine glands or by an indirect action via the
hypothalamus or the liver. Neonatally castrated animals
were also included in the experiment to investigate the
possibility that endogenous testosterone was involved in
either the production or the amelioration of any observable
effect of neonatally administered o,p'-DDT. Animals
treated with 17β-estradiol-17-valerinate (EV) were also
included. They served as positive controls, confirming
the sensitivity of the animals used to imprinting and pro-
viding a comparison of the extremes seen in normal and
imprinted animals. Estradiol was chosen as a control over
testosterone because previous reports supported the idea
that o,p'-DDT acted as a weak estrogen (22,24,26,27,146-
148,150,162).

Within 6 hours after delivery pups were placed into
litters of 8 pups each and about 1/3 of the males were
castrated. The castrated and intact litters were then
placed into 3 treatment groups and injected on each of
days 1-5 of age. Controls received injections of 0.05 ml
sesame oil; DDT-treated animals received injections of
0.05 ml of sesame oil containing 400 ug o,p'-DDT; EV
treated animals received injections of 0.05 ml sesame oil
containing 40 ug 17β-estradiol-17-valerinate. Weights
were determined at regular intervals throughout develop-
ment. The rats were sacrificed between 81 and 110 days of

age at which time the body and organ weights were recorded along with any obvious morphological abnormalities.

Body weights measured during development did not differ noticeably among the groups until after 35-40 days of age. At this time the growth of estrogen-treated animals began to lag behind the other groups; castrates lagged behind intact animals in all groups after 45-55 days of age. In neither the intact groups nor the castrated groups did neonatal treatment with o,p'-DDT alter the course of weight gain. The effect of estrogen treatment in both intact and castrate groups was marked, in accord with the observations of Oštádalová and Pařízek (197).

The final body weights and organ weights, normalized by body weight, of all the groups are shown in Table 6. Body weights for intact control and intact DDT-treated animals were statistically indistinguishable. Neonatally castrated control and DDT-treated groups were likewise similar to each other though the influence of castration was marked. In both intact and castrated states EV caused a very significant (p < 0.0005) decrease in body weight, roughly 35% in both cases.

The glands directly involved in the production of trophic and steroid hormones, the pituitary, adrenals and testes, demonstrated no differences between the control and DDT-treated groups in either the intact or castrated states. Estrogen on the other hand, acted on the adrenals

Table 6

Effects of Neonatal Injection with o,p'-DDT or Estradiol[1] on Body[2] and Organ Weights of Intact and Neonatally Castrated Male Rats

Percent of Body Weight[3]	Intact			Castrated		
	Control(11) (422 ± 22)	DDT(5) (404 ± 20)	EV(11) (298 ± 20)‡	Control(5) (240 ± 23)	DDT(7) (249 ± 19)	EV(6) (222 ± 11)‡
Pituitary x 10^3	2.75 ± 0.20	2.59 ± 0.11	2.81 ± 0.18	3.76 ± 0.18	4.12 ± 0.70	3.42 ± 0.46
Adrenals x 10^2	1.27 ± 0.10	1.23 ± 0.09	1.99 ± 0.30‡	1.83 ± 0.29	1.95 ± 0.15	2.46 ± 0.24#
Testes	0.84 ± 0.07	0.87 ± 0.06	0.25 ± 0.06‡	---	---	---
Seminal ⎫ x 1	0.28 ± 0.02	0.30 ± 0.03*	0.04 ± 0.02‡	---	---	---
Vesicles ⎭ x 10^3	---	---	---	6.71 ± 2.14	4.84 ± 1.08	32.9 ± 5.7‡
Ventral ⎫ x 1	0.11 ± 0.02	0.12 ± 0.01	0.02 ± 0.02‡	---	---	---
Prostate ⎭ x 10^3	---	---	---	3.10 ± 0.71	2.57 ± 0.59	3.06 ± 0.17
Liver	3.71 ± 0.30	3.74 ± 0.32	3.39 ± 0.21t	3.35 ± 0.25	3.25 ± 0.27	3.92 ± 0.10#
Kidneys	0.76 ± 0.05	0.73 ± 0.05	0.66 ± 0.05‡	0.62 ± 0.06	0.60 ± 0.03	0.68 ± 0.03*

[1] Injected s.c. with 0.05 ml sesame oil, 400 ug o,p'-DDT in 0.05 ml sesame oil or 40 ug estradiol valerinate in 0.05 ml sesame oil on days 1-5 of age. Reared in litters of 8 pups each and sacrificed at 81 to 110 days of age.

104

Table 6 (Cont.)

[2] Body weights given in grams in parentheses under each heading.

[3] All data are given as mean weights or mean % of body weight ± 1 standard deviation; numbers in parentheses after the treatment designation are the numbers of animals examined; comparisons are all versus the appropriate control groups and are made by unpaired Student's t tests, * $p < 0.05$, t $p < 0.01$, # $p < 0.005$, ‡ $p < 0.0005$.

of both intact and castrated animals to cause elevations
of weights to above control group levels. While neonatal
castration itself caused marked hypertrophy to above intact
weights of both the adrenal and pituitary in both control
and DDT-treated groups, the effects of neonatal castration
and EV were nearly additive. This could be seen by com-
paring the intact EV-treated group with both the castrated
control group and the castrated EV-treated group. Estrogen
also markedly diminished testicular weight suppressing it
roughly 70% below control values.

The accessory sex tissues, the seminal vesicles and
ventral prostate, should reflect the output of hormonally
active gonadal androgens (198). DDT only slightly
increased (p < 0.05) the weight of the seminal vesicles
in intact animals in comparison to controls; it did not
effect the seminal vesicles of castrated rats when com-
pared to similarly castrated controls. Prostate weight in
both intact and castrated animals was unaffected by DDT
administration. Castration alone produced a marked
decrease in sex-accessory weights in both DDT and control
groups. In the estrogen treated groups, however, the con-
sequences of EV treatment and/or castration were more
complex. First, estrogen suppressed intact prostate
weights into the range seen in control castrates; super-
position of castration and EV did not effect prostate
weights more than castration alone. Second, estrogen
decreased (p < 0.0005) the seminal vesicle weights of

intact animals into the range seen in EV-treated neonatally castrated rats. Third, the seminal vesicular weights of neonatally castrated animals was increased by EV treatment 5-6 fold (p < 0.0005) over the weights observed for castrated controls. These seminal vesicles (from EV-treated neonatal castrates) are, however, morphologically abnormal, being more muscular than those in the other castrates and retaining an integral association with what are probably the vestiges of parts of the Müllerian ductal system. These tissues in the other castrated groups appear merely immature in comparison to intact controls.

Liver and kidney weights are also affected by steroidal status (199) and are, in addition, sensitive to potential hepatic inducers (5). They were unaffected by the administration of o,p'-DDT to either intact or castrated animals. Again castration removed what is an anabolic influence since the liver and kidneys of castrates were slightly lighter than those of the intact animals. Neonatal estrogen again exerted paradoxic effects by diminishing the organ weights in intact animals into the control castrate range while increasing the organ weights in castrated animals toward, or even in excess of, intact control levels.

Changes due to estrogen treatment were marked, as anticipated. Inconsistencies of the changes in intact animals with those seen with castration alone should not,

however, allow the misinterpretation that estrogen treatment of intact males is nothing more than chemical castration (200); body, pituitary and seminal vesicle weights in intact EV-treated animals do not concur with those found in control castrates. The issue is confused by the changes seen in EV-treated neonatal castrates where body, adrenal, seminal vesicular, liver and kidney weights are increased rather than suppressed. The continued presence of injected estrogen at levels sufficient to suppress body weight and to stimulate adrenal weight in both intact and castrated animals, to suppress gonadal function in intact animals and to induce preservation of Müllerian structures serves as an obvious alternative to imprinting as an explanation for the results observed.

Clearly, the changes due to neonatal treatment with a total of 2 mg of o,p'-DDT were minimal. Several interpretations were therefore possible: no effects occurred; effects were too subtle to be detected by such gross methodology; or, effects occurred earlier in development and were not measurable at the time of this experiment. The first interpretation was presumptuous at this point and finalistic, in addition; it could lead to no further experimentation. The second interpretation dictated the use of more sensitive measurements in subsequent experiments. The third interpretation implied the need for the examination of some sort of developmental time course.

These last two suggestions formed the basis for the next experiment.

II. Developmental Time Course and Dose-Response to

  Neonatally Administered o,p'-DDT:

In addition to probing body and organ weights as a function of development, this experiment included examination of organ histology and measurements of serum corticosterone, to monitor adrenal output, and serum LH, to measure pituitary function. It also included several levels of o,p'-DDT exposure. The inclusion of more than one dose level was meant to generate dose-response data on any effects encountered. It was also meant to minimize the potential effect of multiple antagonistic responses on the conclusions drawn from the experimental results, e.g., to prevent hepatic induction from completely eliminating a form of DDT which imprints the hypothalamus. Positive control groups were injected with either EV or testosterone-17-propionate (TP). Estrogen was included for the reasons cited previously while testosterone was included to provide an apparently less drastic though similar form of imprinting (59) which has been postulated to be mediated by the estrogen formed by localized hypothalamic aromatization of testosterone (87). Due to the size of the proposed experiment neonatal castrates were not included.

Following parturition, the intact neonatal males were placed into litters of 8 pups each. Thirty-six litters

were divided into 9 groups, 6 vehicle-treated control (C) litters, 6 EV-treated litters, 6 TP-treated litters and 18 litters treated with o,p'-DDT - 3 litters at each of 6 dose levels. The chemicals were administered s.c. in 5 equal daily doses, each contained in 0.05 ml of sesame oil, given from the day of birth until 4 days after birth. Dosages were as follows:

Group	Chemical	Daily Dose (ug)	Total Dose (ug)
C	None	0	0
I	o,p'-DDT	400	2000
II	o,p'-DDT	200	1000
III	o,p'-DDT	100	500
IV	o,p'-DDT	20	100
V	o,p'-DDT	10	50
VI	o,p'-DDT	2	10
EV	Estradiol	40	200
TP	Testosterone	200	1000

After weaning, animals were caged 6 to a cage. Body weights were recorded throughout development. On days 25, 50, 75 and 110 five to six animals from each DDT-treatment level and 8-11 animals from each of the control, EV and TP groups were decapitated between 12.00 and 16.00 (EST).[1,2]

---

[1] Since the experiment was actually conducted in two halves started 5 days apart, only 5-6 animals from each of 3 DDT-treated groups and 5-6 animals from each of the control, EV and TP groups were processed on any one day.

[2] On day 110 groups C and I and ½ of groups EV and TP were

Animals from all the groups were alternately sacrificed in order to minimize any time bias between groups. Tissues were rinsed in Buffer I (see <u>Materials and Methods</u> IV.F.1.), blotted, weighed, divided and placed into 10% neutral formalin.

Data for the halves (see footnote 1, p. 110) of each group of controls, EV-treated or TP-treated animals were checked for uniformity by applying Student's t-test to the two halves. After ascertaining uniformity, the values from the half-groups were combined. All organ weights were normalized against total live body weight before means or errors were calculated. Mean values for each individual group of treated animals were then tested by Student's t-tests against the means of the combined control group, C.

A. <u>Body and Organ Weights</u>:

Body weight gain in all groups prior to 32-35 days of age was uniform. By about day 40 estrogen treated animals began to lag behind in growth and by day 110 were fully 30% lighter than control rats. Testosterone-treated animals lagged slightly in growth between days 40 and 85 but were nearly normal by day 110. Body weight gain of DDT-treated rats was intermediate between that of the controls and that of the TP group from days 40 to 80. Beyond 80 days, groups I-VI showed a somewhat slower growth with groups

---

sacrificed at 10.00 while the remaining animals were killed at 19.00 (EST).

II, III and V demonstrating significantly lower body weights by day 110.[3] No relationship between DDT dosage and growth, as measured by body weight was evident. Body weights for days 25, 50, 75 and 110 are summarized in the graph in Figure 13.

The mean normalized organ weights of the animals described in Figure 13 are shown in Figures 14-20.

Normalized pituitary weights (Figure 14) decreased uniformly with time. Estrogen treatment caused a significant rise, above control levels, in pituitary weight which was only evident before day 110 of age. Testosterone did not alter pituitary weight gain. Although several isolated changes were seen in the DDT-treated animals those on day 110 may be explained by the lower body weight of the animals (the mean raw weights of the pituitaries were not different from controls). The significant value for group II on day 25 was probably by chance due to the size of the experiment. No consistent pattern of fluctuation of means about the control mean was evident for the DDT-treated animals.

Normalized testicular weights for pairs of testes (Figure 15) in both control and DDT-treated groups demonstrated the abrupt hypertrophy associated with puberty,

[3] Group III contracted respiratory infection shortly before the termination of the experiment; most of the actual weight loss seen on day 110 in that group and many of the deviations in normalized organ weights are undoubtedly due to illness.

Figure 13. Body Weights of Neonatally Injected Male Rats

Intact male rats were injected daily, s.c., on days 1-5 of age with 0.05 ml of sesame oil which contained 1/5 of the following total dosages: C = vehicle only, control; I = 2000 ug o,p'-DDT; II = 1000 ug o,p'-DDT; III = 500 ug o,p'-DDT; IV = 100 ug o,p'-DDT; V = 50 ug o,p'-DDT; VI = 10 ug o,p'-DDT; EV = 200 ug 17β-estradiol-17-valerinate; TP = 1000 ug testosterone-17-propionate. The animals were raised in litters of 8 pups and had free access to water and food at all times. Between 12.00 and 16.00 on the days specified the animals were weighed, decapitated and dissected; fresh organ weights were then determined. (Note: on day 110 groups C and I and $\frac{1}{2}$ of groups EV and TP were sacrificed at 10.00 while the remaining animals were killed at 19.00 (EST). This does not alter the interpretation of the results or the contrasts made except for the liver which shows marked diurnal weight fluctuations.)

The data shown are means +1 standard deviation; groups I-VI contained 5-6 rats each while groups C, EV and TP each contained 8-11 animals. Tests of statistical difference from the mean of the control group, C, on the given days were made by unpaired Student's t-tests. Probabilities of the differences seen occurring by chance are noted for those probabilities of less than 5%: a, $p <$ 0.05; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.0005$.

FIGURE 13
BODY WEIGHTS OF NEONATALLY INJECTED MALE RATS

Figure 14. Organ Growth in Neonatally Injected Male

Rats: Pituitary

The pituitary weights, expressed as 1000 x percent of body weight which are displayed are those of the animals listed in Figure 13. Organ weights were normalized by body weight for each individual animal prior to calculating the group means which are shown (+1 standard deviation). Group sizes were 3-6 for groups I-VI and 8-11 for groups C, EV and TP. Group means were tested individually by Student's t-test for differences from the control group, C, mean on each day listed. Statistical probabilities of less than 0.05 are noted: a, $p < 0.05$; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.0005$.

FIGURE 14

ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS

Figure 15.   Organ Growth in Neonatally Injected Male

Rats: Testes

The testes weights, expressed as percent of body
weight, of the animals listed in Figure 13 are shown.
Organ weights are for pairs of testes and were normalized
by body weight for each individual animal prior to cal-
culating the group means which are given (+1 standard
deviation).   Group sizes were 5-6 for groups I-VI and
8-11 for groups C, EV and TP.   Group means were tested
individually against the mean of the control group at
each age by Student's t-tests.   Statistical differences
with probabilities of less than 0.05 are noted: a, p
< 0.05;   b, p < 0.025;   c, p < 0.01;   d, p < 0.005;
e, p < 0.0005.

FIGURE 15

ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS
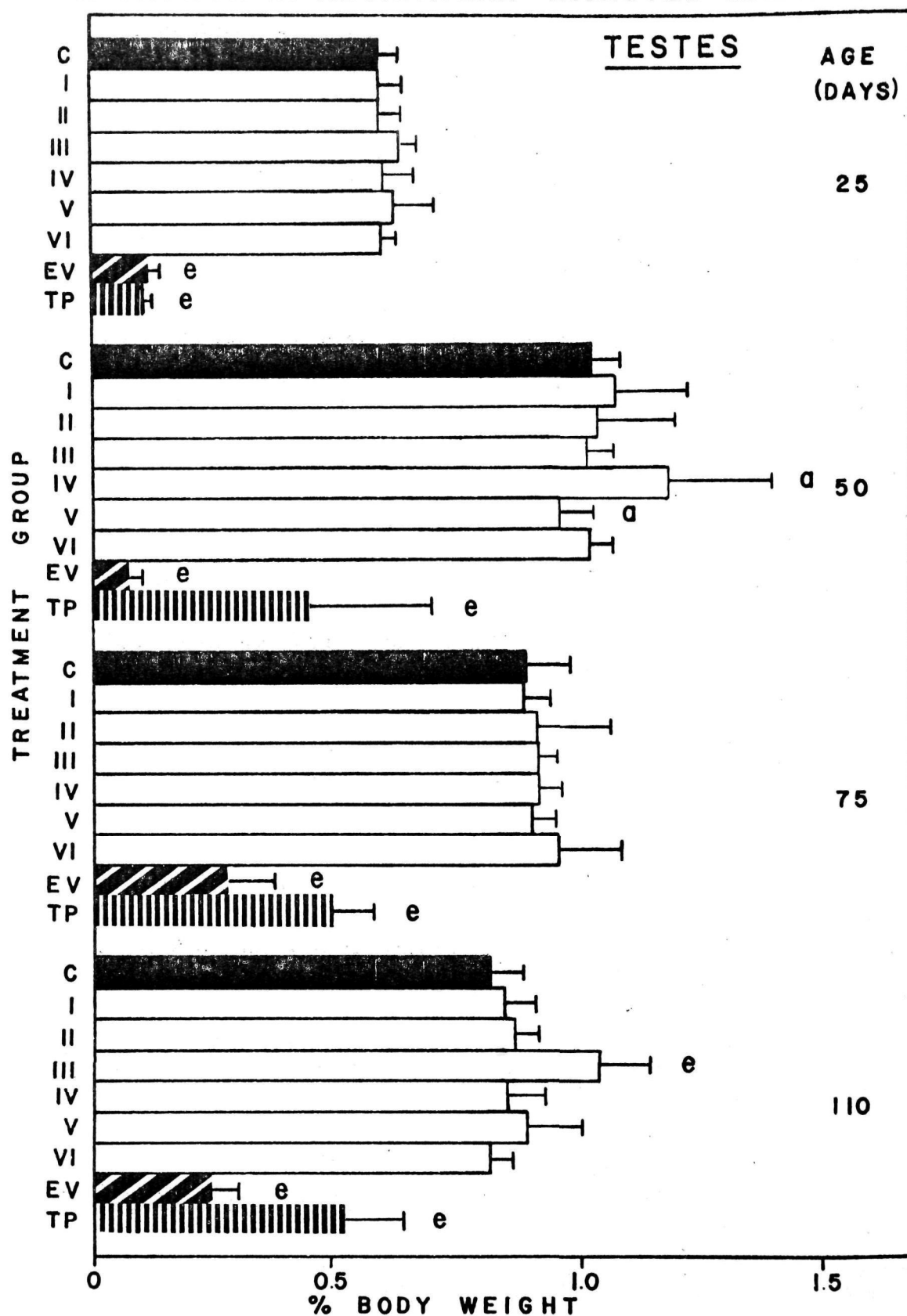
activation of steroidogenesis and active spermatogenesis,
between 25 and 50 days of age; thereafter body weight
rose proportionately faster than testicular weight and the
normalized organ weights slowly decreased. As in the case
of the pituitary, several isolated significant deviations
from the control mean were seen in the DDT-treated groups;
all could be explained by differences in body weights.
This was not true, however, for treatment with EV or TP.
Estrogen markedly suppressed testicular weight throughout
the period studied; only a slight alleviation of the
effect was seen between days 50 and 75. Testosterone
yielded marked suppression throughout the experiment.
Still, some sort of recovery, which coincided with the
increased body weight seen after 75 days of age, apparently
took place since normalized testicular weights for this
group slowly rose beyond 50 days of age. This apparent
recovery in TP-treated rats is in concert with similar
observations in mice (59).

Normalized adrenal weights for pairs of adrenals
(Figure 16) declined abruptly for all but the estrogenized
animals between days 25 and 50; they decreased in propor-
tion to total weight only slowly beyond day 50. Again
DDT appeared ineffective in altering weights although
group IV on day 25 did show an increase above control
levels which was not explicable on the grounds of dif-
ferences in total body weight. The majority of the dif-
ference seen with group III on day 110 was, however,

Figure 16. Organ Growth in Neonatally Injected Male

         Rats: Adrenals

     The adrenal weights, expressed as 1000 x percent of

body weight, of the animals listed in Figure 13 are

shown. Organ weights are for pairs of glands and were

normalized by body weight for each individual animal prior

to calculating the group means given (+1 standard devia-

tion). Group sizes were 5-6 for groups I-VI and 8-11 for

groups C, EV and TP. Group means were tested individually

against the mean of the control group at each age by

Student's t-tests. Statistical differences with probabil-

ities of less than 0.05 are noted: a, $p < 0.05$; b,

$p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.0005$.

FIGURE 16
ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS
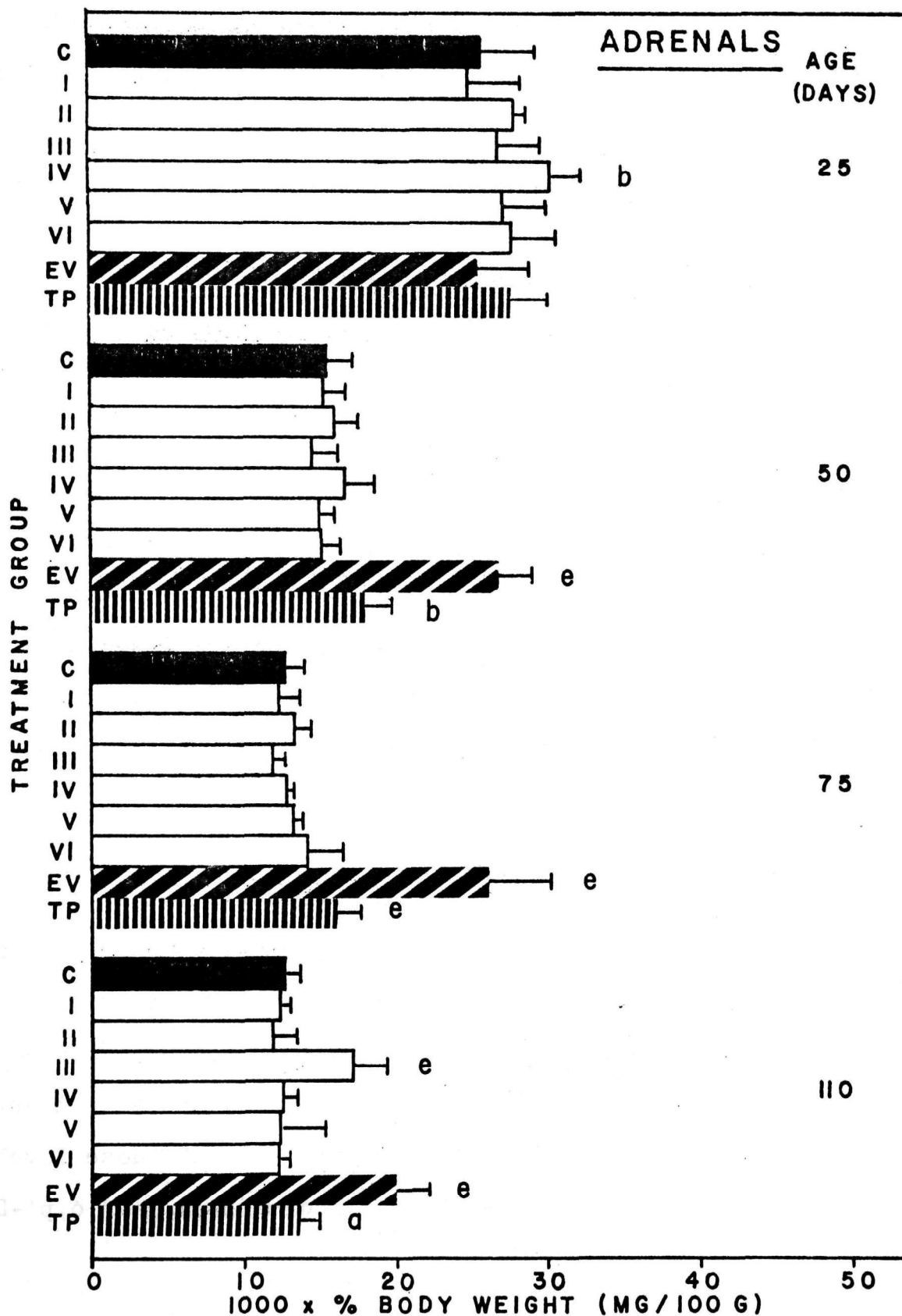
accounted for by a decreased body weight (see footnote 3, p. 112). The same explanation for the results in EV and TP treated animals was insufficient; these steroids produced an actual hypertrophy of this organ as has been shown previously in both rats (201) and mice (202). Interestingly, the hypertrophy was only expressed beyond day 25, i.e., during puberty (day 50) or maturity (days 75 and 110). Because the expression of this effect has this time lag and was similar in pattern for both TP and EV groups, but more marked with estrogen, it bears the appearance of an imprinting phenomenon. The slight suppressions of the weights in older TP-treated rats probably is another reflection of the slow recovery of testicular function in these rats.

The weights of pairs of seminal vesicles, normalized by total body weight, from groups C and I-VI (Figure 17) illustrate the accelerating weight gain between weaning and maturity seen in organs dependent on circulating androgens. The abrupt jump between day 50 and 75 and the plateau thereafter is assuredly a result of the final full maturation of testicular steroidogenesis. The deviations from mean control weights shown by the DDT-treated animals were not - except for group III on day 110 - accounted for by changes in total body weights. Since the slight deviations (increases) occur only at the high DDT dose levels they may be slight ($p < 0.05$) indications that o,p'-DDT

Figure 17.   Organ Growth in Neonatally Injected Male

                Rats: Seminal Vesicles

      The weights of pairs of seminal vesicles, expressed

as percent of body weight, are given for animals listed

in Figure 13.   The weights of the blotted pairs of

vesicles with the coagulating glands attached were nor-

malized by body weight for each individual animal prior

to calculating the group means given (+1 standard devia-

tion).   Group sizes were 4-6 for groups I-VI and 8-11 for

groups C, EV and TP.   Group means were tested individually

against the mean of the control group at each age shown by

Student's t-tests.   Statistical differences with probabil-

ities of less than 0.05 are noted: a, $p < 0.05$;   b,

$p < 0.025$;   c, $p < 0.01$;   d, $p < 0.005$;   e, $p < 0.0005$.

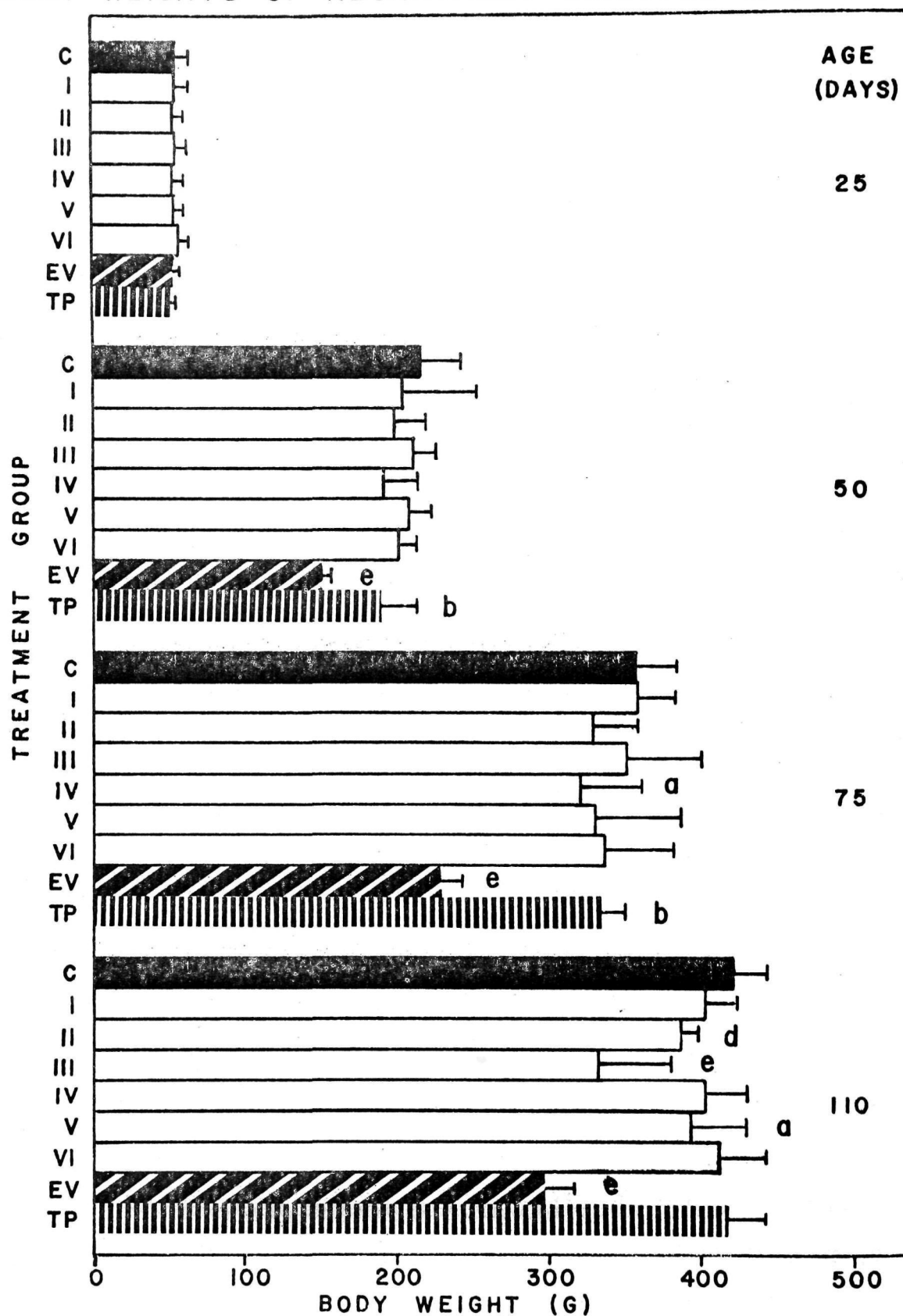# FIGURE 17
ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS

SEMINAL VESICLES

AGE (DAYS)

25

50

75

110

TREATMENT GROUP

% BODY WEIGHT
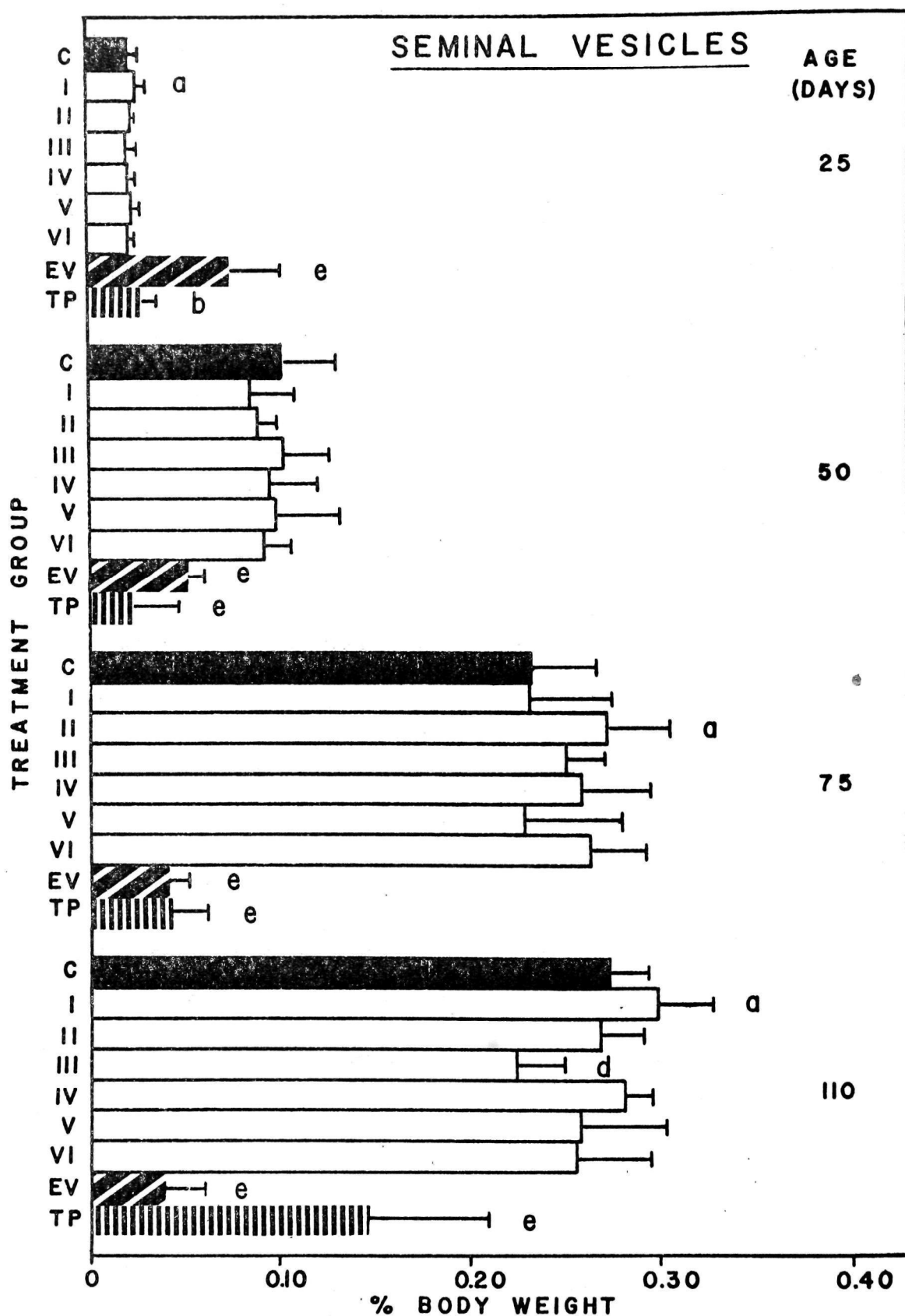
is a weak imprinting agent which, paradoxically, causes increased adult gonadal output.

The effect of estrogen was marked ($p < 0.0005$ on all days examined). On day 25 an hypertrophy similar to that seen in estrogen-treated neonatal castrates (experiment 1) and reported by Freud (203) was observed - again, abnormal morphology may hold the explanation for this result. The raw weight of the vesicles in these animals continue to increase slowly until sometime between 50 and 75 days of age after which it stabilizes; in no case, however, does it approach normal levels, indicating a lack of, or insensitivity to, circulating androgens.

Testosterone also exerts a marked effect on this tissue beyond 25 days of age (day 25 $p < 0.025$, days 50-110 $p < 0.005$). But, as in the cases of the testes and adrenals, it appears to be mainly a retardation of development, maturation being completed sometime after 110 days of age in these animals. The results were suggestive of residual titers of injected TP which only fall below values which suppress LH levels after 75-110 days of age. If these TP titers were, in addition, insufficient to support gonadal function (normal function would antagonize rises in adrenal weight (4,204)) they could explain the results discussed thus far for testosterone treated animals.

The ventral prostate, normalized weights of which are shown in Figure 18, would be expected to demonstrate the

Figure 18:   Organ Growth in Neonatally Injected Male

Rats: Ventral Prostate

The weight of the ventral portion of the prostate
gland, expressed as percent of body weight, is shown for
the animals described in Figure 13. The weight of the
tissue was normalized by body weight for each individual
animal prior to calculating the group means (+1 standard
deviation) depicted. Group sizes were 4-6 for groups I-VI
and 8-11 for groups C, EV and TP. Group means were tested
individually against the mean of the control group at each
age shown by Student's t-tests. Statistical differences
with probabilities of less than 0.05 are noted: a,
$p < 0.05$; b, $p < 0.025$;   c, $p < 0.01$;   d, $p < 0.005$;
e, $p < 0.0005$.

FIGURE 18
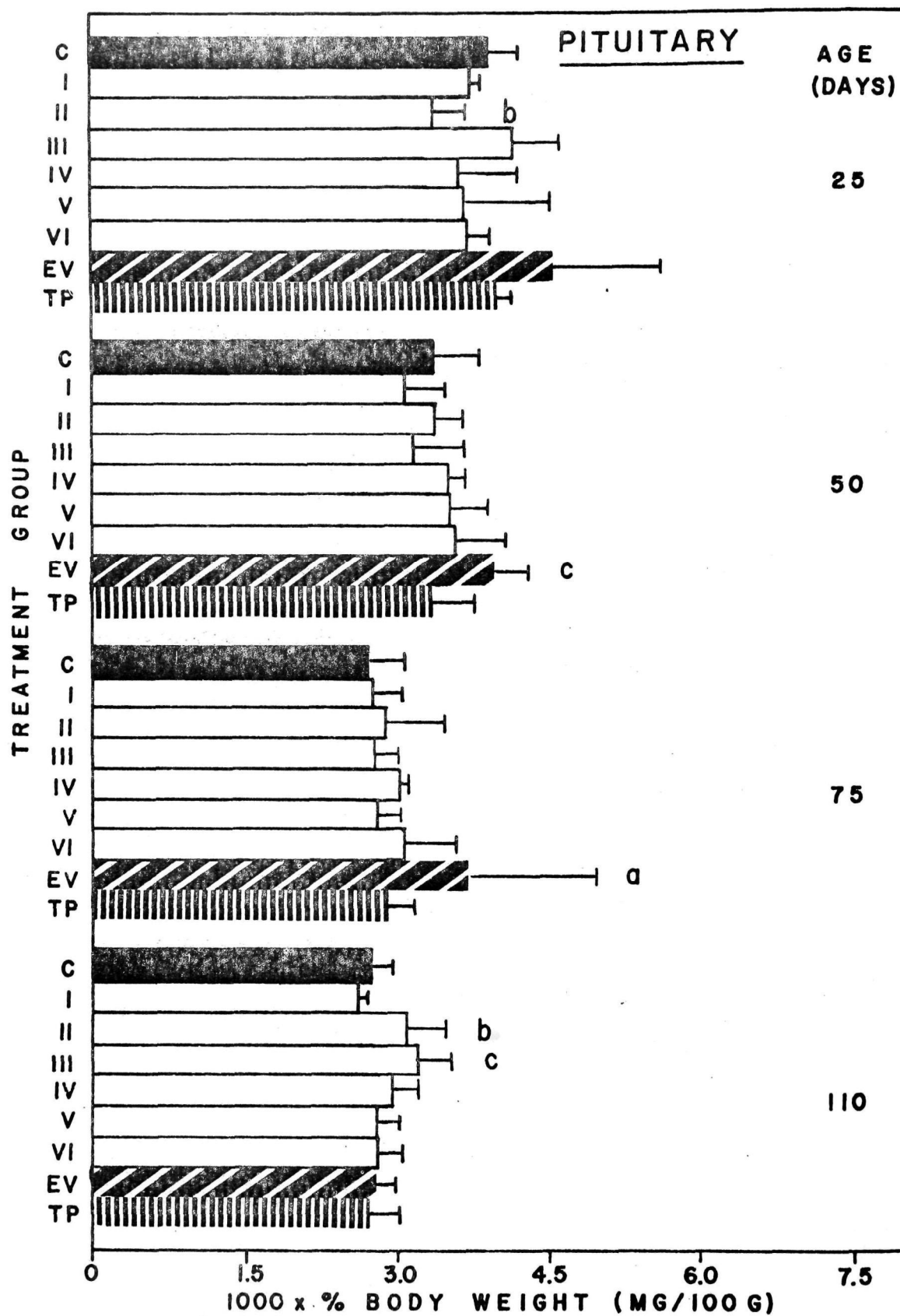## ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS

VENTRAL PROSTATE
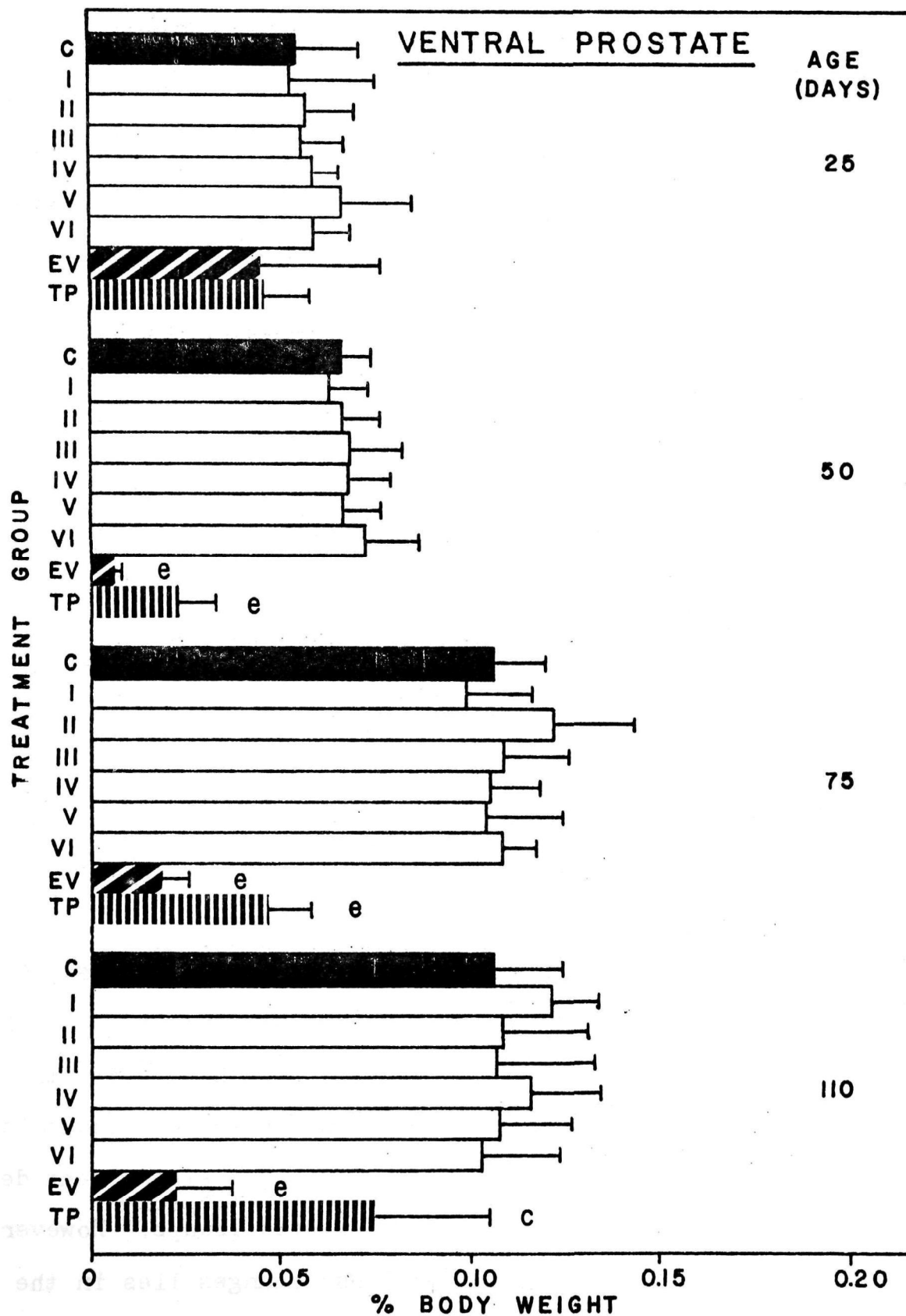
same patterns as the seminal vesicles. This was generally true for all groups but that treated with EV. The increments between weaning and immature and between immature and mature normalized weights were less marked than they had been for the seminal vesicles of groups C and I-VI. DDT treatment showed no significant effect on the organ although variation about the control mean generally agreed with that found for the seminal vesicles. The pattern of suppressive effects of TP was the same as that seen with the seminal vesicles. The same was true of the pattern seen in EV treated rats beyond day 50. Apparent tissue proliferation seen at 25 days of age in the seminal vesicles of estrogen-treated rats was absent here. This may reflect either the lack of a close association with Müllerian remanents or an innate difference in the sensitivity of the two sex-accessory tissues to estrogen as has been discussed by Sufrin and Coffey (205).

Normalized hepatic weights are graphed in Figure 19. The liver appears largely insensitive to neonatal treatment with DDT until at least 75 days of age; the unnormalized mean weight of the liver in group IV on day 50 was not different from the control. Even the effects of the steroids, EV and TP, were minimal through day 75; the significant deviations that were observed were inconsistent.

The values found for day 110 showed significant deviations for both DDT and steroid treated groups. However, the explanations for most of these changes lies in the

Figure 19.   Organ Growth in Neonatally Injected Male

            Rats: Liver

The mean weight of the liver of each of the treatment
groups described in Figure 13 is shown in terms of percent
of body weight at various ages. The organ weights were
normalized to body weight for individual animals prior to
calculation of the means (+1 standard deviation) shown.
Group sizes were 5-6 for groups I-VI and 8-11 for groups
C, EV and TP. Group means were tested individually
against the mean of the control group at each age shown
by Student's t-tests. Statistical differences with
probabilities of less than 0.05 are noted: a, p < 0.05;
b, p < 0.025; c, p < 0.01; d, p < 0.005; e,
p < 0.005.

The differences seen on day 110 may be more reflec-
tive of differences in time of sacrifice than actual
treatment effects as noted in Figure 13.

FIGURE 19
ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS
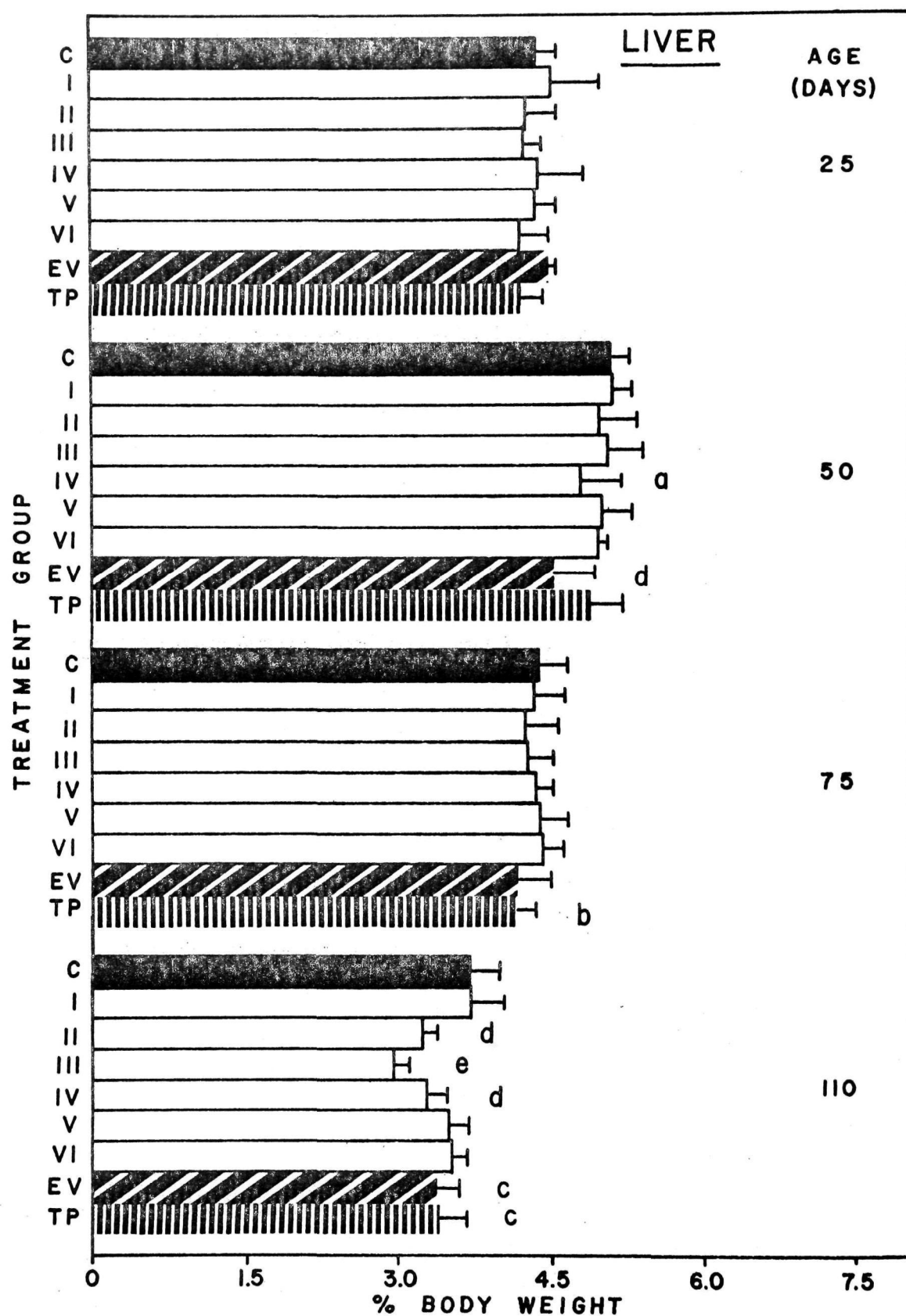
LIVER

altered experimental procedure followed on that day and
in the normal diurnal weight fluctuations demonstrated by
the liver (206). In order to allow incubations of the
testes to be done on these rats sacrifice times were
altered from the normal time of 12.00-16.00 (EST) used on
previous days (see footnote 2, p. 110). The data from the
halves of groups EV and TP were compared but failed to
yield significant t values; therefore, the data were
combined. The decreases of the normalized organ weights
to below the control mean in groups II-IV, EV and TP
probably best reflected the 4-9 hour difference in
sampling times rather than actual effects of DDT or steroid
exposures. The magnitude and time course of the change
agree completely with those observed by Potter et al.
(206) in studies of feeding and corticosterone secretion.

Finally, weights for pairs of kidneys, normalized to
body weight, are shown in Figure 20. A consistent decrease
with age in the normalized organ weight was demonstrable
in all treatment groups; the value for group III on day
110 was explained by decreased body weight. In fact, the
greatest portion of each of the deviations from the group
C mean before day 75 are explicable by differences in body
weights since data on raw organ weights indicate minimal
deviations from that of group C. Treatment with TP did
depress kidney weights after day 50 but the effect of
estrogen treatment was significant for both raw and nor-
malized weights only on day 110. Again, as has been the

Figure 20.   Organ Growth in Neonatally Injected Male

Rats: Kidneys

The mean weights of pairs of kidneys, expressed as percent of body weight, are shown for the treatment groups described in Figure 13 for various ages.   Pairs of organs were blotted and weighed, the weights being normalized by body weight for each individual animal prior to calculation of the group means (+1 standard deviation) given. Group sizes were 5-6 for groups I-VI and 8-11 for groups C, EV and TP.   The individual group means for each age were tested against the control group means by Student's t-tests;   statistical differences with probabilities of less than 0.05 are noted: a, $p < 0.05$;   b, $p < 0.025$; c, $p < 0.01$;   d, $p < 0.005$;   e, $p < 0.0005$.

FIGURE 20
ORGAN GROWTH IN NEONATALLY INJECTED MALE RATS

KIDNEYS
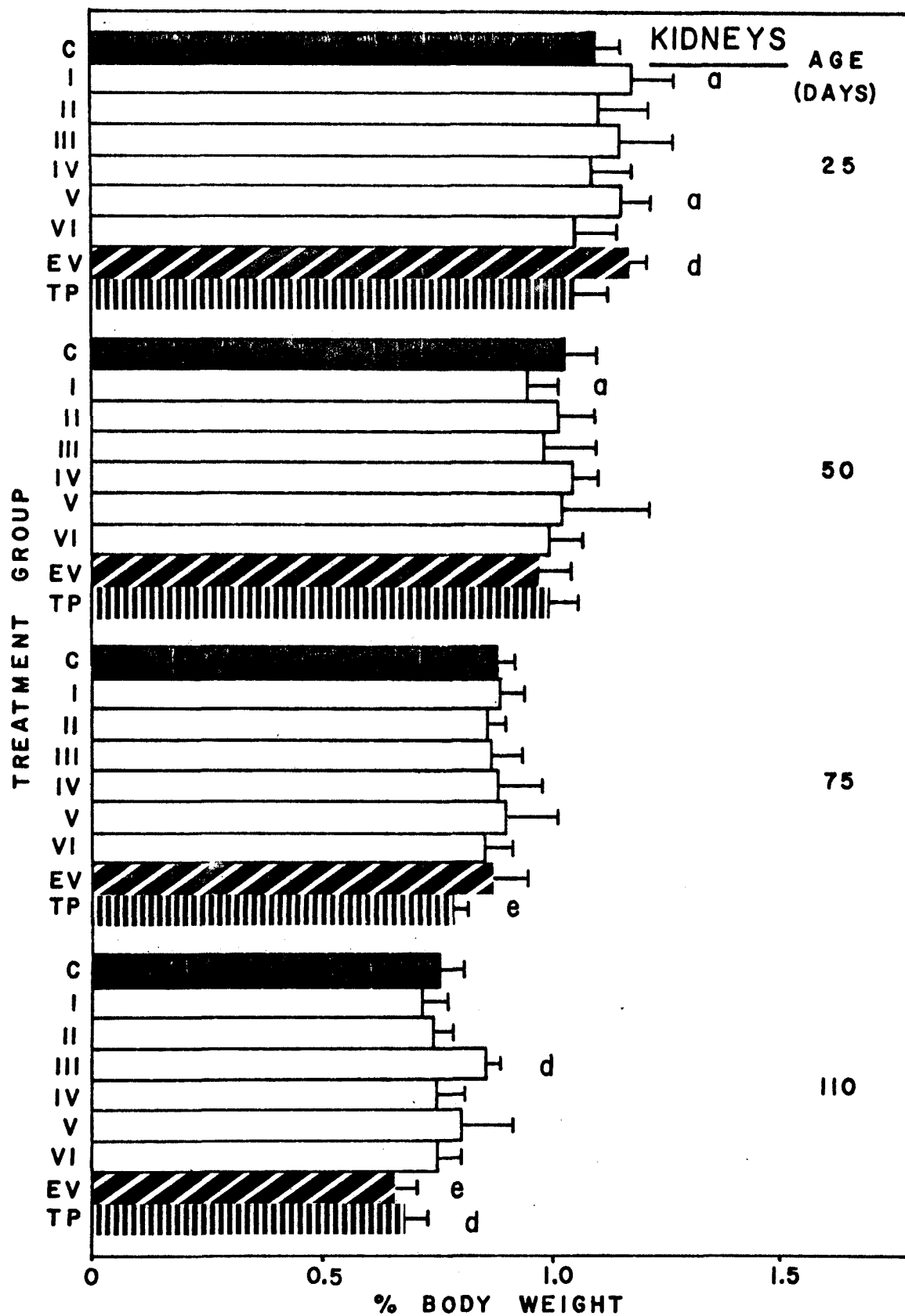
case for most of the organs examined, the actions of the steroids become manifest as alterations in mean normalized organ weight only after puberty, or full maturity, is reached.

In summary, neonatal injection with up to 2 mg of o,p'-DDT (total dose) yielded only minimal, inconsistent alterations in mean normalized organ weights at 25, 50, 75 and 110 days of age. No consistent correlations could be drawn between dose and effect on any single organ or combination of organs. Though applications of various forms of analysis of variance (ANOVA) might have been used in the data analysis it is doubtful, due to the inconsistency of the deviations from the control means, whether even these more powerful statistical methods could have extracted any further results from the data. In concordance with the results of the first experiment, but extending it in time, the gross measurement of organ weights failed to expose any effect of o,p'-DDT on the male rat.

Treatment with steroids gave results consistent with those obtained in other studies (59,207) in regard to alterations of adult organ weights. But they are also consistent, in the main, with the continued presence of a depot of estrogen or testosterone which acts to suppress gonadotrophin release until sometime between 75 and 110 days of age. The oil droplet which remains after neonatal treatment, vestiges of which still exist at 110 days of

age, can act as a depot (61). Still, in the absence of controlled measurements of serum steroids in the mature animals and in light of the extensive literature on steroidal imprinting, the present results must be viewed as being most probably due to neonatal imprinting phenomena rather than merely steroid feedback inhibition.

B. Organ Histologies:

Having established the lack of any easily located DDT effect on body weight or organ weights at any age up to adulthood, measurements for more subtle effects were undertaken. Organ histologies were done on liver, kidney, pituitary, testis and adrenal tissues chosen at random from the groups of treated animals and from the control group. From each prepared tissue 6-8 sections were examined; the subjective observations were recorded.

Pituitary, kidney and liver tissues were indistinguishable among the groups or ages; they did not differ overtly from the appearance of the controls under the general histology conditions used for preparation and staining.

Histology of the testes for groups C and I-VI were nearly identical at any given age. Sperm production was absent in animals of 25 days of age but was active at 50, 75 and 110 days. This agreed with the findings of Gellert et al. (54) of motile sperm in adult male rats neonatally injected with 3 mg of o,p'-DDT. Subjectively, the DDT-treated groups in my experiment showed some evidence

of oligospermia. This was not inconsistent with Gellert's findings since he did not do sperm counts. It did, however, reinforce the recent report of Krause et al. (208) who did histological counts of various testicular cell types in sections made from the testes of rats injected with massive doses of technical DDT on day 4 of life.

Estrogen treatment resulted in complete aspermia through day 75 and only minimal spermatogenesis by day 110, results which were in complete accord with a report by Maqueo and Kincl (209) on histo-morphology of rats injected with estradiol benzoate on day 5 of age. TP treatment caused a definite oligospermia until at least day 75; spermatogenesis on day 110, however, appeared normal. These observations were similar to those reported in mice by Barraclough (59).

Adrenal histology for all groups on days 50, 75 and 110 seemed qualitatively similar. At all these ages the glomerulosa layer of the cortex was well defined and histologically invariate; the medulla was well defined and highly vascular; the outer (fasicular) layer of the cortex represented 30-50% of the cortical area and con-sisted of fairly large cells containing lipid vacuoles; the inner (reticular) layer of the cortex appeared highly vascular with small dense cells organized into definite cords.

On day 25, however, a rather striking picture emerged. Control and testosterone-treated rats exhibited a lack of

the perimedullary vascularity and cellular organization typically found later. This observation was in agreement with the early observations of Howard (94). These adrenals also showed considerably less definition of the reticular and fasicular zones - cells were uniform throughout the cortex. In contrast, the glands from animals treated with o,p'-DDT or estradiol appeared very similar to normal animals at least 25 days older. The cortex had apparently aged precociously. Subjectively, the degree of aberration on the DDT-treated animals was dose-dependent.

Initial observations, summarized in Figures 21 and 22, were confirmed by examining similar slides prepared from the remaining control, DDT and EV-treated animal tissues (Table 7). Collective DDT and control observations were compared by X^2 analysis using the most vascular 25-day old control adrenal as a comparative standard. For the slides examined p was < 0.02, implying that DDT treatment with at least some dosages changed adrenal histology at 25 days of age. Histology itself cannot imply a mode of action, but similarities between EV and DDT-treated adrenals were plain.

Recapitulating, these histological examinations demonstrate that neonatal DDT treatment did indeed have an effect on the steroidogenic endocrine tissues, altering adrenal morphology and possibly sperm production as well.

C. <u>Serum Corticosterone</u>:

In view of the histological findings in the adrenal, the importance of measuring adrenal function became even

Figure 21.   Normal Adrenal Development in the Male

Rat: Reticular Zone Histology

These photomicrographs of normal rat adrenal glands at various ages are representative of the histological sections obtained from the control animals listed in Figures 13-20 (intact male rats injected s.c. with 0.05 ml sesame oil on each of days 1-5 of age).   All photographs were taken at the same magnification ($\sim$ 15x) with a Zeiss polarizing photomicroscope using Kodak color photo-micrography film (ASA 16;   13 DIN).   The histology slides were prepared from formalin fixed tissues using paraffin embedding and hematoxylin and eosin stains; sections were saggital and 7-10 mu thick.

The medullary region of the adrenals is located at the left hand or top of the photographs and forms a distinctive border with the reticular layer of the cortex. The glomerulosa forms a densely packed region near the outer edge of the organ, except on day 110 where it is not pictured.   The fasicular and reticular zones are undifferentiated at day 25, but by day 50 the organization of the vasculature and cords of the reticularis is evident. This area increases in extent at the expense of the fasiculata, at least until maturity.   This gradual expansion with maturity stands in contrast to the picture seen in estrogen or o,p'-DDT treated animals (see Figure 22).

FIGURE 21

NORMAL ADRENAL DEVELOPMENT IN THE MALE RAT: RETICULAR ZONE HISTOLOGY
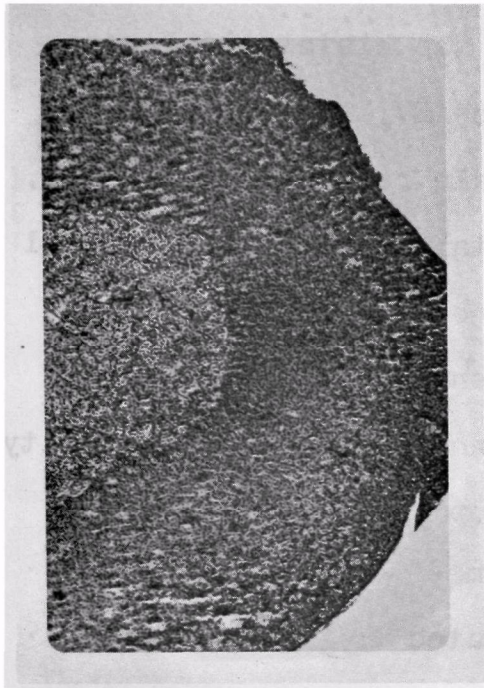
DAY 25
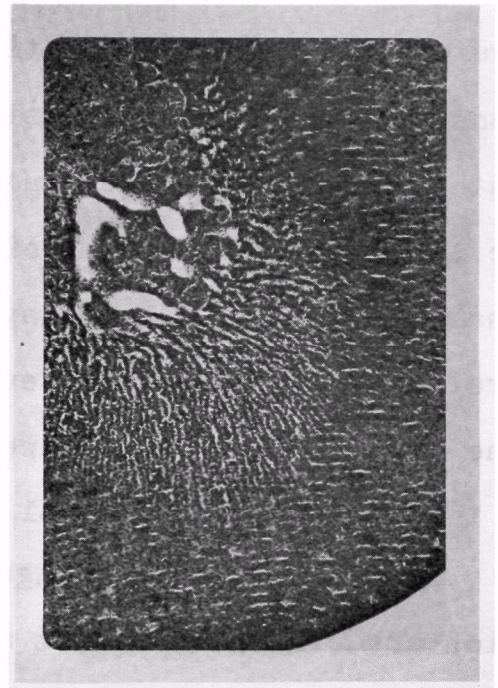
DAY 50
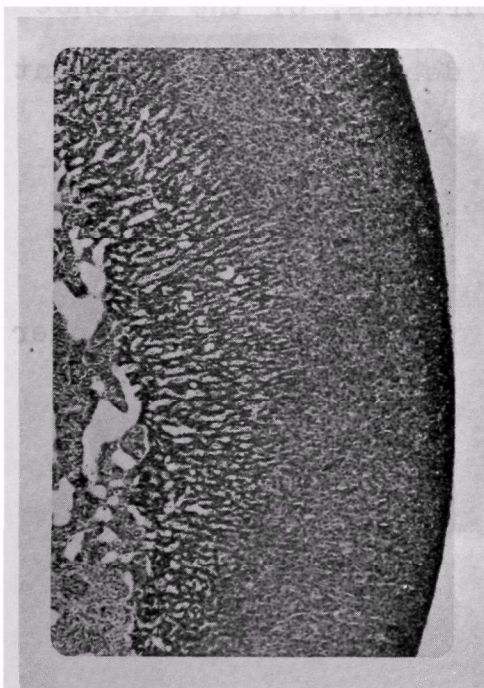
DAY 75
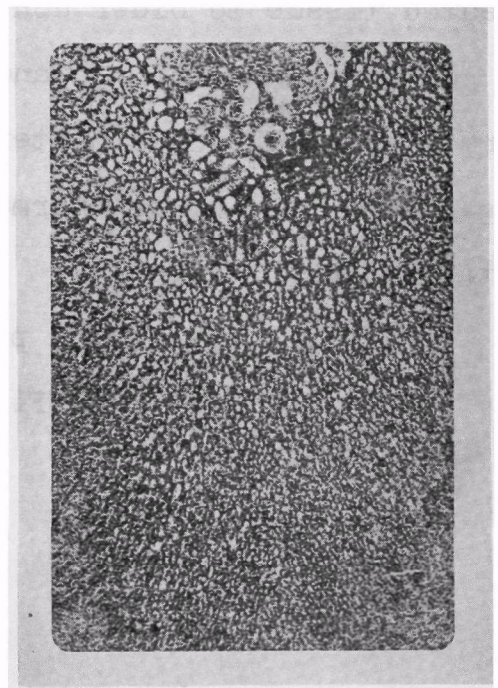
DAY 110

Figure 22.   Adrenal Reticular Zone Histology at 25 Days

of Age in the Neonatally Injected Male Rat

These photomicrographs of rat adrenal glands at 25

days of age are representative of the histological sec-

tions obtained from the test groups EV, II, III and IV

listed in Figures 13-20 (intact male rats injected daily,

s.c., with 0.05 ml sesame oil containing 1/5 of the total

dosages shown on each of days 1-5 of age). They demon-

strate marked precocious development of the reticular

zone at this age (see Figure 21) and point to a similarity

in the actions of estradiol and o,p'-DDT on this organ.

The histological changes from 25-day old controls

which are evident were not attributed to perimedulary

necrosis in view of the similarity of the histology of the

treated tissue to older control adrenals, of the absence

of polymorphonucleocytes and cell debris, of the apparent

normality of the corticosterone levels secreted (see

Figure 23), and of the normality of the adrenal weights

obtained (see Figure 16).

Histology slides and photographs were prepared under

the conditions listed in Figure 21.

FIGURE 22
ADRENAL RETICULAR ZONE HISTOLOGY AT
25 DAYS OF AGE IN THE NEONATALLY
INJECTED MALE RAT
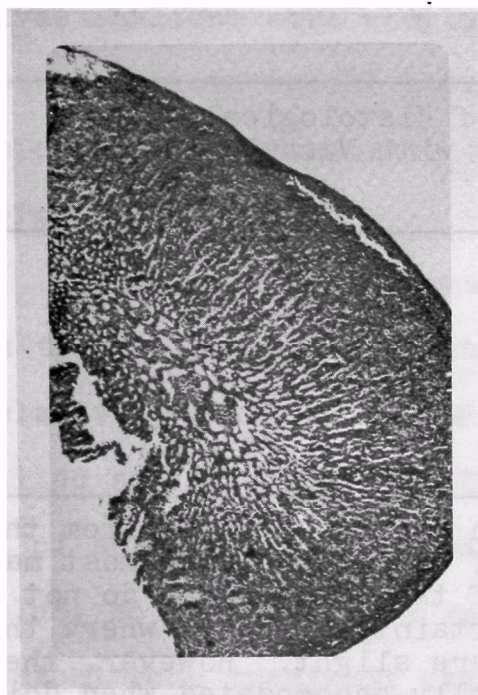
200 UG EV
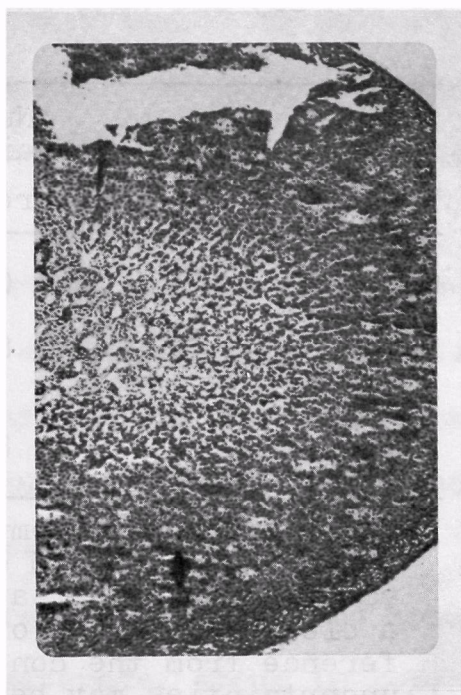
1000 UG o,p'-DDT

500 UG o,p'-DDT
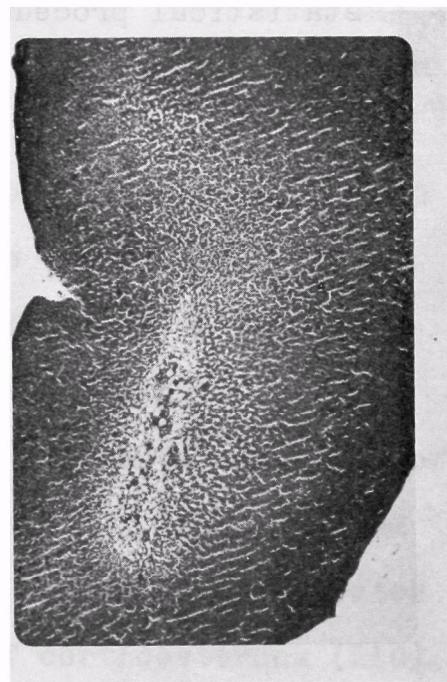
100 UG o,p'-DDT

TOTAL DOSE INJECTED BY DAY 5 OF AGE

Table 7

Frequency of Increased Reticular Vascularity[1] in

25 Day Old Rats Injected Neonatally with o,p'-DDT

	Number of Histological Preparations with Vascularity		
Treatment	Increased	Unchanged	Total
Control	0	10	10
DDT	8	12	20
Total	8	22	30

[1] The standard for comparison was the adrenal from the control group at 25 days of age showing the most marked vascularity. The nature of the comparisons do not allow a clear indication of uncertainty in cases where the difference from the control were slight. However, these uncertainties may be partially accomodated when doing comparisons, by the use of appropriate nonparametric statistical procedures such as X^2.

more apparent than when initially planned. Many of the serum measurements had been done before the histological investigations were complete but they tended to support both the histological observations and the organ weights which had been measured. They are presented in graphical form in Figure 23.

On days 50 to 110 the mean serum levels, determined on trunk-blood taken at the time of decapitation, showed relatively small variation; control means from the rats killed at 10.00-16.00 (EST) fluctuated from 20-25 ug/100 ml of serum. Likewise, except for an occasional high value, so did the serum values from the DDT-treated groups. Testosterone treatment caused a moderate, about 10-20%, elevation of the levels on day 50 and 75, then fell to, or slightly below, the control levels on day 110. Estrogen treatment caused marked elevations above the control levels observed, ranging from a 100% elevation on day 50 to approximately a 60% elevation on day 75. However, normal levels were evident in the EV group on day 110. On day 110 the effect of illness was clearly seen in Group III as was the effect of the diurnal fluctuation of serum corticosterone in groups EV and TP; the elevated levels seen in groups II and IV-VI were also interpreted in terms of the circadian rhythm of corticosterone (210).

Small volumes of serum on day 25 required that samples be pooled; although multiple determinations were made on the pooled sera only the single resultant values

Figure 23. Serum Corticosterone in Neonatally Injected
         Male Rats

Serum corticosterone, in ug per 100 ml, as measured
by sulfuric acid fluorescence is plotted versus age and
treatment group. The serum samples were obtained from
trunk-blood taken at the time of decapitation (12.00-16.00
(EST) except where otherwise indicated) of the animals
listed in Figures 13-20 and a group of intact male rats,
A, injected similarly with 5 daily doses of 2000 ug
o,p'-DDT in 0.05 ml sesame oil (total dose - 10 mg) on
days 1-5 of age.

Serum samples were small on day 25 and were pooled
except for groups C and A. Each serum was assayed in
duplicate; the means for groups were based on the means
of these duplicates. Means +1 standard deviation are
shown, n being 3-6 for groups A-VI and 7-12 for groups C,
EV and TP. Statistically significant differences from
the properly paired control groups were determined by
Student's t-tests (a, $p < 0.05$; b, $p < 0.025$; e,
$p < 0.0005$).

FIGURE 23
SERUM CORTICOSTERONE IN NEONATALLY INJECTED MALE RATS
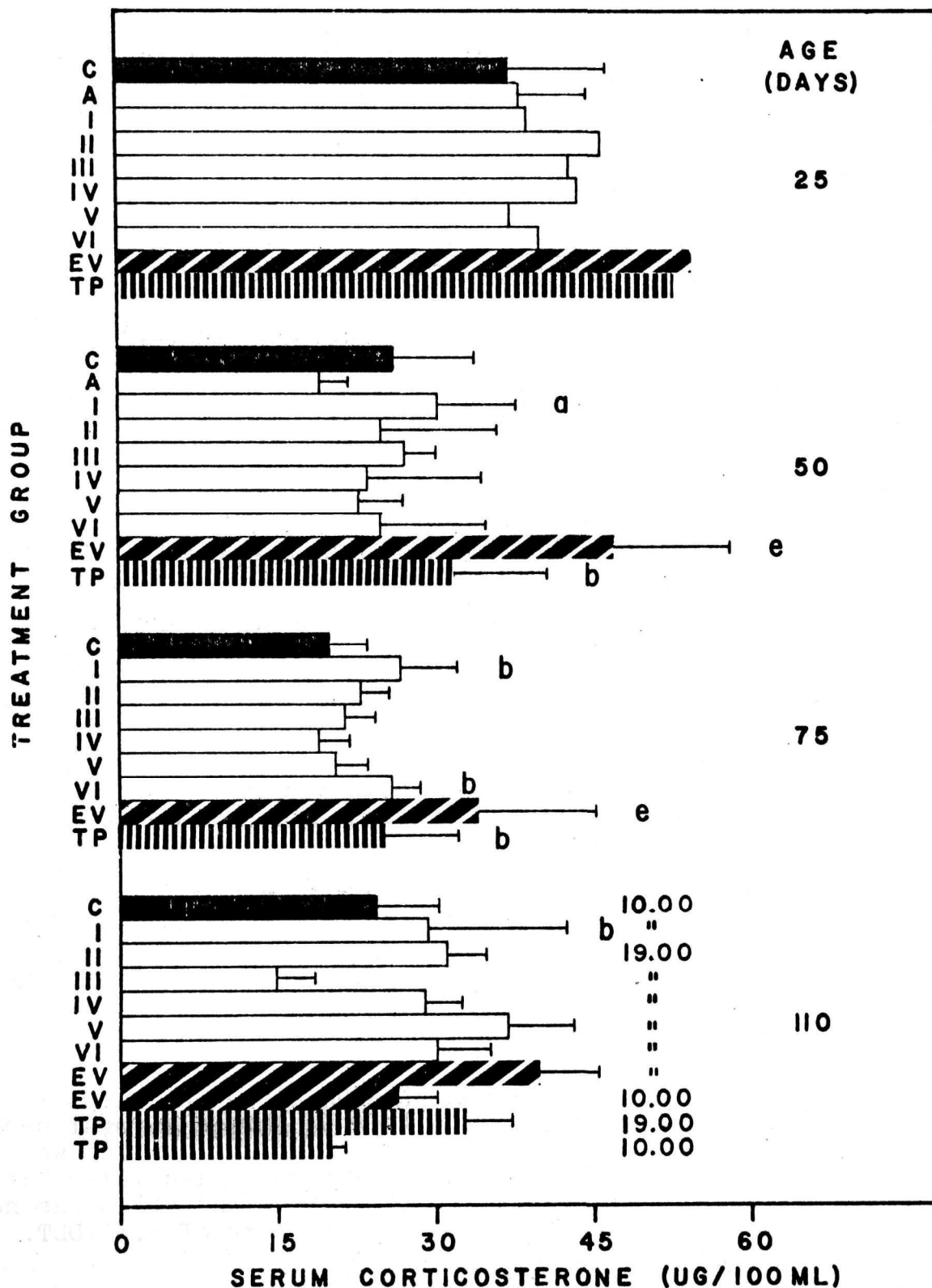
were available for between-group comparisons.[4] The serum
levels in weanlings in all the treatment groups were about
20-75% higher than those seen at 50 days when normal
maturation of cortical secretion should have occurred
(5-6 weeks) (98). Steroid treatment raised levels fully
45% higher than even this control mean while DDT treatment
caused less marked, and statistically insignificant,
elevations of 0-20%.

The suggestion of elevated corticosterone levels on
day 25 in DDT-treated animals and the clear rise caused by
estrogen treatment support interpretation of the histo-
logical findings as morphological expressions of increased
steroidogenic activity. However, an altered function need
not, necessarily, be correlated with an altered morphology,
as is shown by the fact that no morphological change was
seen in the testosterone-treated group yet it showed a
marked corticosterone elevation.

The visual suggestion of some correlation between
DDT dose and serum corticosterone level was evident at
both 50 and 75 days of age in groups I-VI. Therefore, a
series of linear regression analyses were done on the
results summarized in Figure 24. If all the values were
included in the calculations, either linear regression or

-----

[4] This was true with two exceptions in which samples were
not pooled: 1) the control group formed several serum
pools; and 2) a group of males, group A, which was
obtained from a small experiment conducted later but
included in the overall calculations and which was neo-
natally injected with a total of 10 mg of o,p'-DDT.

Figure 24.   Serum Corticosterone in 50 and 75 Day Old Male

Rats Versus Dose of Neonatally Injected

o,p'-DDT

The data from groups C, A and I-VI on days 50 and 75 given in Figure 23 are replotted.   Data are plotted as means ± 1 standard deviation with n for each point shown in parentheses.   All assays were run in duplicate.

Analysis of variance for either day 50 or 75 leads to the conclusion that no correlation between corticosterone level and o,p'-DDT dose can be found.   For doses between 100 and 2000 ug of o,p'-DDT on day 75, however, a weak correlation with dose does exist, $R = 0.637$; using regression analysis the slope of this line is non-zero by a t-test with a probability of < 0.01.

FIGURE 24

SERUM CORTICOSTERONE IN 50 AND 75 DAY OLD MALE RATS VERSUS DOSE OF NEONATALLY INJECTED o,p'-DDT
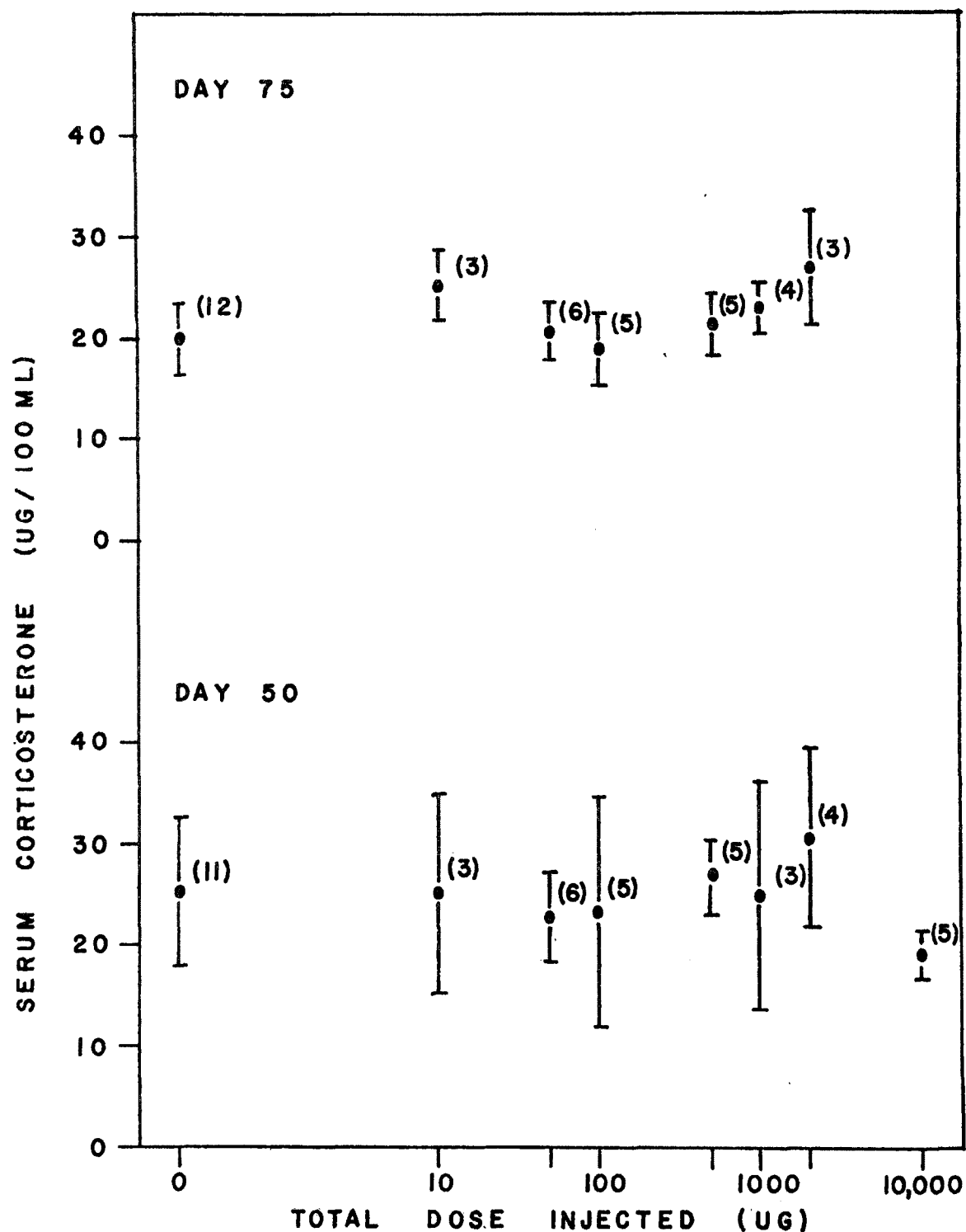
ANOVA led to the conclusion that no alteration from control levels existed at either 50 or 75 days of age. However, if low and very high dose levels were eliminated from the analysis at 75 days of age a weak correlation with dose appeared, R = 0.637. This regression line allowed extrapolation of the o,p'-DDT results to the serum corticosterone levels shown by treatment with 200 ug of EV and, subsequently, to an estimation of estrogenic potency. The calculations showed that 1 ug of EV was equivalent to approximately 2,500 ug of o,p'-DDT on day 75. This estimate was well within an order of magnitude of the 1/10,000 ratio calculated by Cecil et al. (147) using a uterine glycogen-response bioassay.

The interpretation, that no dose-response effect existed, was probably true, particularly on day 50, in view of the large standard deviations which existed in what was a slightly immature and presumably unstable system. But the possibility that a multiphasic dose-response existed and was manifested by a weak dose-response relationship only in the intermediate dose range should not be ignored. DDT generates what may be antagonistic responses in the liver (microsomal induction (11-15)) and in estrogen-sensitive tissues (estrogen mimetic (22,24,26,27)). The thresholds for expression of these two effects are different (15,22) so the possibility that low levels of o,p'-DDT (0-50 ug) stimulated

corticosterone breakdown and led to a decrease in serum corticosterone levels appears reasonable. Because the balance exists, it also seems reasonable that further increments (100 ug - 2 mg) of o,p'-DDT dosage could have favored increased serum corticosterone levels by an estrogen-like stimulation of the adrenals and/or an increased catabolism of circulating androgens. Even further increments (10 mg) of DDT dosage may have caused breakdown of the active form(s) of o,p'-DDT by further increasing microsomal metabolism and/or upset the balance between adrenal output and hepatic corticosterone catabolism; either mechanism would result in a drop in serum corticosterone levels. It is therefore neither impossible nor unrealistic to think that the weak correlation between dose and serum corticosterone which held for intermediate dosages of o,p'-DDT at 75 days of age might indicate an action of o,p'-DDT. Obviously, only further investigations concerning these possibilities will clarify the meaning of these results.

D. Serum LH:

Earlier studies by Rybakova (28,29) on adult rats chronically fed low levels of technical DDT uncovered a stimulatory action on the pituitary gonadotrophs, which were examined histologically, and on the pituitary content of LH, which was measured by bioassay. Gellert et al., working on the female rat, had also shown that serum LH could be suppressed by o,p'-DDT in adult rats (150) and

that the LH rise in response to adult ovariectomy could
be blunted (54). Since the precedents existed for an
effect of o,p'-DDT on hypothalamo-hypophysial function,
determinations of serum LH were conducted concurrently
with my examinations of tissue histology and serum corti-
costerone. A summary of these measurements is given in
Figure 25.

The highly variable LH titers reported for immature
male rats (77,82,83) were evident in control animals on
day 25 and, to a somewhat lesser extent, on day 50. By
day 75 the mean control level had stabilized near the
lower reliable limit of the RIA, 10 ng B-640/ml.
DDT-treated rats also showed highly variable levels on
days 25 and 50. But, in contrast to the controls, only
group VI, the lowest dosage group, had approached stability
on day 50. On days 75 and 110 the levels of all the groups
were statistically indistinguishable from the controls.
On day 110 the high value for group III was probably again
a result of illness (35,36).

Estrogen treatment caused a uniform suppression of
serum LH to essentially nonmeasurable levels until day 75.
A slight rise was evident by day 110. This rise may be
what was reflected in the movement of several of the
normalized organ weights toward control values at this
time. Testosterone treatment similarly suppressed LH
levels on day 25 but it apparently had lost its influence
on tonic LH secretion after day 25 since an essentially

Figure 25. Serum LH in Neonatally Injected Male Rats

Levels of serum LH, in ng B-640 per ml, are shown as functions of age and dose of o,p'-DDT or steroid. Serum samples are those obtained from trunk-blood at decapitation of the animals shown in Figures 13-20. (They are the same samples used to determine serum corticosterone - Figures 23 and 24). Radioimmunoassays, using the method of Niswender et al. (169), were done at least in duplicate for each serum sample. The mean of the duplicates forms the basis for the group means (+1 standard deviation) shown; pooled samples were used for groups II-VI on day 25 of age, otherwise, n = 3-6 for groups I-VI and 7-12 for groups C, EV and TP (B-640 = 0.03 x NIH-LH-S1 by OAAD).

The large group variances at any given age mask any statistically significant differences in group means. However, two general trends are evident: 1) the decrease from generally high levels during immaturity toward low levels in maturity (75 and 110 days) and 2) the uniformly low levels of serum LH in the estradiol-treated animals.

FIGURE 25
SERUM LH IN NEONATALLY INJECTED MALE RATS

SERUM LH (NG B-640/ML)

TREATMENT GROUP

AGE (DAYS)

25

50

75

110
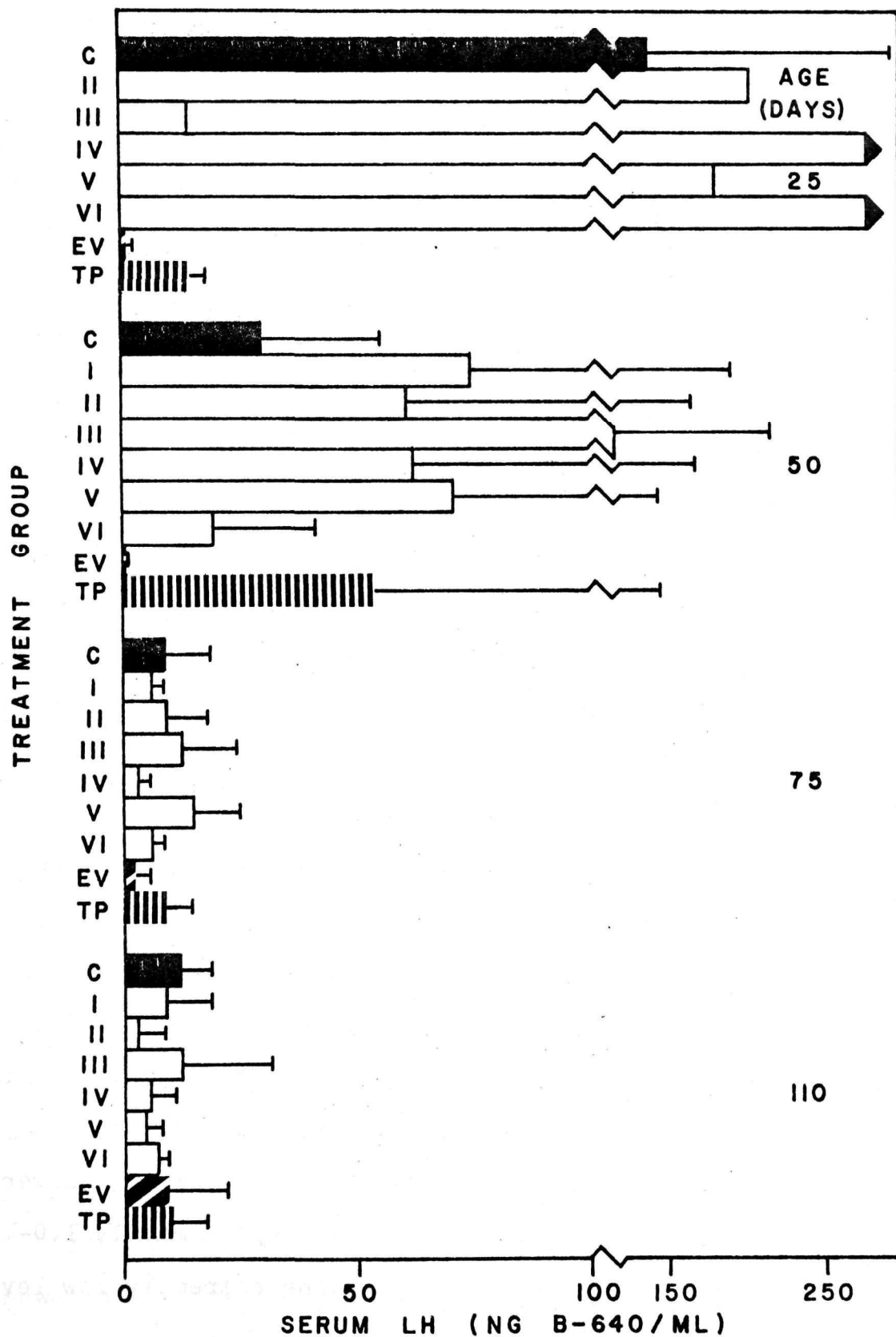
normal pattern ensued. Again, as was the case with group EV, organ weights such as testes, adrenals, seminal vesicles and ventral prostate seem coordinated with, and perhaps by, the changes in serum LH.

Although the pituitaries for this study had been committed to histology, other subsequent small experiments did yield some limited data on hypophysial content of LH:

Pituitary LH (ug B-640/mg wet tissue)

	Day 25	Day 50	Day 75
Control	35 ± 2 (3)*	77 ± 10 (3)	36 ± 8 (3)
2 mg DDT	---	---	37 ± 11 (3)
10 mg DDT	27 ± 8 (3)	69 ± 5 (5)	---

* Number of animals measured is indicated in parentheses.

The control values agree with those of Yamamoto et al. (180) and do not differ significantly from those of the DDT-treated rats.

The pituitary LH values for 81-110 day old neonatally castrated rats receiving only sesame oil (5 animals) or a total of 2 mg o,p'-DDT (7 animals) were 61 ± 9 and 52 ± 9, respectively, which were similar to the content in 50 day old males. Neonatal castrates injected with a total of 200 ug EV (6 animals), however, yielded only 6 ± 3 ug B-640/mg wet tissue; their serum levels were only 50 ± 20 ng/ml in contrast to approximately 1.0-1.3 ug B-640/ml for the other groups. The extremely low level of

pituitary LH in the EV group easily explains the serum level. If deductive reasoning can be applied to the absence of serum LH in intact estrogenized animals from group EV of experiment 2, the result in castrates implies a similar low pituitary content in the intact rats, a situation actually found in females by Arai (211) using OAAD assays. Either of these situations can be explained by the continued presence of EV until at least 80-100 days of age. Although such a conclusion is possible on the basis of Gorski's findings with TP (61), it would not be in accord with the findings of Schiavi (207) in the pituitary. Schiavi found levels only slightly suppressed in 60 day old intact rats treated on day 5 with 100 ug of 17β-estradiol-17-benzoate and a two-fold elevation of pituitary LH content above controls at 180 days of age. Since the same vehicle was used in both Schiavi's experiments and my own the only simple explanations for the differences reside in the timing, size and form of the dose and the total volume in which it was dissolved. Each of these factors would, from what is known about imprinting (57-61) tend to favor a less drastic and prolonged effect in the experiments of Schiavi. Whether the reason is prolonged presence or imprinting, neonatal estrogen markedly affected LH in both the serum and pituitary of neonatally treated male rats for extremely long periods of time.

In summary, this second experiment affirmatively answered the initial experimental query; o,p'-DDT did

indeed demonstrate an effect on the steroidogenic
endocrines - it altered the early histological appearance
of the adrenal cortex and possibly its function as well.
Hints of other possible changes involving spermatogenesis
and the production and/or release of LH at 50 days of age
were found. The effects of testosterone propionate and
estradiol valerinate were reexamined in a coordinated
series of measurements not used before. Results stemming
mostly from the estrogen-treated animals shed some doubt
on the idea that estrogen esters given in oil act directly
for only a short period of time after which their effects
are manifestations of steroidal imprinting. This does not
imply, however, that imprinting may, or does, not occur
since too many experiments using other more soluble forms
of the steroid or more easily absorbed vehicles substanti-
ate the initial findings of Pfeiffer (164). Rather, the
question posed is whether or not sesame oil may be a
problematic vehicle in endocrinologic or screening studies.

III. <u>Serum LH Response to Adult Castration</u>:

While some data had been generated on the existence
of an effect on the immature adrenal gland, the total
absence of an effect on the hypothalamo-hypophysial-gonadal
system of male rats did not seem reasonable in view of the
observations of Rybakova (28,29), Gellert <u>et al</u>. (53,54,
150,151) and Krause <u>et al</u>. (208). To test the possibility
that such an effect did exist but was only apparent under

stressed or competitive situations, a measurement of serum LH response to adult castration was undertaken.

Six litters of male pups were divided among 4 treatment groups. Two litters received injections of sesame oil, 0.05 ml s.c. daily, on days 1-5 of age; 4 litters received 5 daily doses of 2 mg o,p'-DDT in 0.05 ml sesame oil, a total dose of 10 mg. Two of the DDT-treated litters also received other drugs; one litter received 10 equal i.p. doses of 100 ug sodium phenobarbital (PB) in 0.05 ml saline spaced 12 hours apart over the first 5 days of life while the other received 5 daily i.p. injections of 125 ug SKF-525A in 0.05 ml saline. The latter two groups were included to determine if hepatic activation played any role in mediating the effects of neonatally administered o,p'-DDT.

After allowing the animals to mature until 57 days of age 4 animals from each of the 4 groups were chosen for the experiment. Two sample bleedings (TVBE) were done on days 57 and 59 to establish a baseline. Castration was performed on day 60 and serum samples were taken at intervals until 14 days after castration, by which time serum LH levels should have begun to plateau (180). On day 14 an s.c. injection of 0.75 mg/kg of testosterone was given 6 hours prior to bleeding. The dose was small enough not to drive LH levels to baseline but large enough to ascertain if the negative feedback loop of the animal was functional (170). On day 15 after castration the

animals were anesthesized lightly with ether and decapi-
tated; trunk-blood samples were collected for LH measure-
ment to determine if steroid clearance of the injected
testosterone and the rebound of serum LH were normal.
After the RIA was completed the group means were calcu-
lated for each day. They were plotted versus time after
castration and submitted to 2-way ANOVA (212,213).

Results for the control and DDT-treated groups are
plotted in Figure 26, those for the PB + DDT and
SKF-525A + DDT groups are plotted in Figure 27. The
analysis of variance, which covers days 1-11 after castra-
tion (the period after castration but before steroid
treatment), is summarized in Table 8.

The expected hyperbolic rise in serum LH was seen in
all four groups as was a dip of 25-40% subsequent to
testosterone injection and a rise back to pre-testosterone
levels. It was concluded that all groups possessed a
functional feedback system. However, the plateau of the
serum LH levels seen in the control and DDT-treated groups
were distinctly, and statistically, different. The DDT
animals reached a plateau somewhat earlier at a level
fully 30% below the controls. This corresponded to a p
value of < 0.005 for an ANOVA which included only the
control and DDT groups (this ANOVA is not shown).

Both drug treated groups, SKF-525A + DDT and PB + DDT,
had patterns which fell between the controls and the
DDT-treated group. The pattern of the hepatically induced

Figure 26. Response of Serum LH to Adult Castration in

Neonatally Injected Male Rats

Serum levels of LH, given in ng B-640 per ml, are
plotted versus time after adult castration for groups of
rats which were injected daily, s.c., on days 1-5 after
birth with either 0.05 ml sesame oil (Control, o-o) or
2 mg o,p'-DDT in 0.05 ml sesame oil (DDT, x-x). Bleedings
were done at 15.00-18.00 (EST) by tail-vein under light
ether anesthesia, the same animals being used throughout
the study. Castration was performed on day 60 of age after
2 baseline bleedings. No treatment other than bleeding
occurred until 14 days after castration when hypothalamic
feedback control was tested by injecting 0.75 mg/kg
testosterone (T) s.c. in sesame oil 6 hours prior to
bleeding. Bleeding on day 15 was without further treat-
ment. The serum collected was assayed, at least in dupli-
cate, by the radioimmunoassay procedure of Niswender et al.
(169). The results shown are means, ± 1 standard deviation,
for groups of 4 animals, except for the controls on days
14 and 15 in which cases n = 3 and 2, respectively.
Analysis of variance for all of the values indicate a 95%
probability that the control group values were, in fact,
higher throughout the experiment; a similar analysis indi-
cates that, for the days after castration and before treat-
ment with testosterone, the chance occurrence of the differ-
ences seen has a probability of < 0.005. Table 8 shows the
2-way ANOVA for these values and those of Figure 27.

FIGURE 26

RESPONSE OF SERUM LH TO ADULT CASTRA-
TION IN NEONATALLY INJECTED MALE RATS
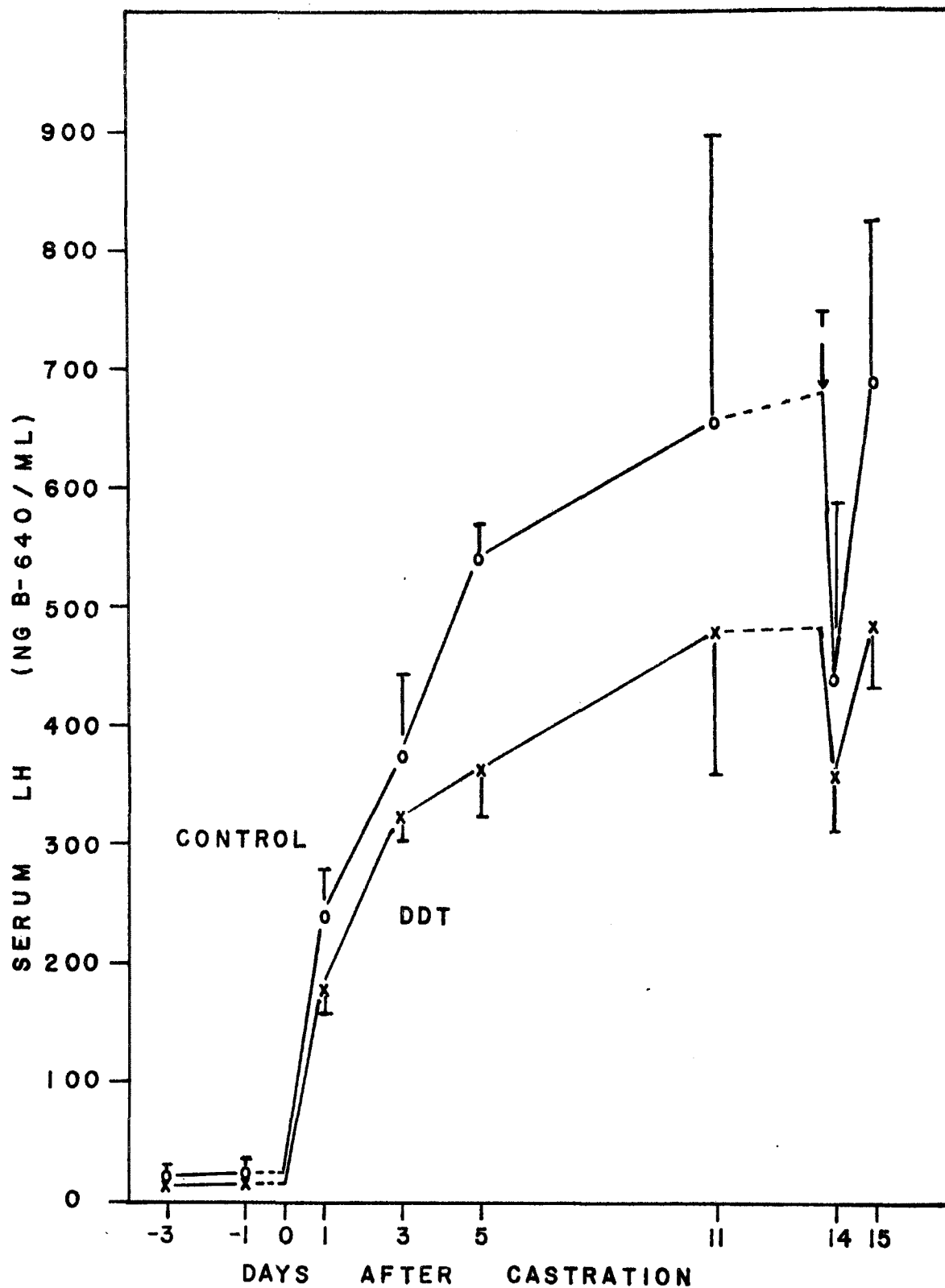
Figure 27. Response of Serum LH to Adult Castration in
Neonatally Injected Male Rats

Serum levels of LH, given in ng B-460 per ml, are
plotted versus time after adult castration for groups of
rats which were treated on days 1-5 of age as follows:
group SKF-525A + DDT (▼-▼) was injected daily with 2 mg
o,p'-DDT in 0.05 ml sesame oil, s.c., and 125 ug (25 mg/kg)
of SKF-525A in 0.05 ml neutral saline, i.p.; group pheno-
barbital + DDT (●-●) was injected daily with 2 mg o,p'-DDT
in 0.05 ml sesame oil, s.c., and 2 doses - 12 hours apart -
of 100 ug (40 mg/kg) sodium phenobarbital in neutral
saline, i.p. Bleedings, castration, testosterone treatment
and sample assays were the same as described in Figure 26.

The results shown are means, ± 1 standard deviation
for groups of 4 animals. All values were examined by
analysis of variance simultaneously with the groups shown
in Figure 26. Comparisons of the SKF-525A + DDT and/or
phenobarbital + DDT groups with the control group (Figure
26) over the entire test period or over the period after
castration and prior to treatment with testosterone indi-
cate no significant differences between these groups (as
determined by Tukey's test for multiple comparisons (214)).

FIGURE 27

RESPONSE OF SERUM LH TO ADULT CASTRA-
TION IN NEONATALLY INJECTED MALE RATS

SKF-525A + DDT

PHENOBARBITAL+DDT

SERUM LH (NG B-640/ML)

DAYS AFTER CASTRATION
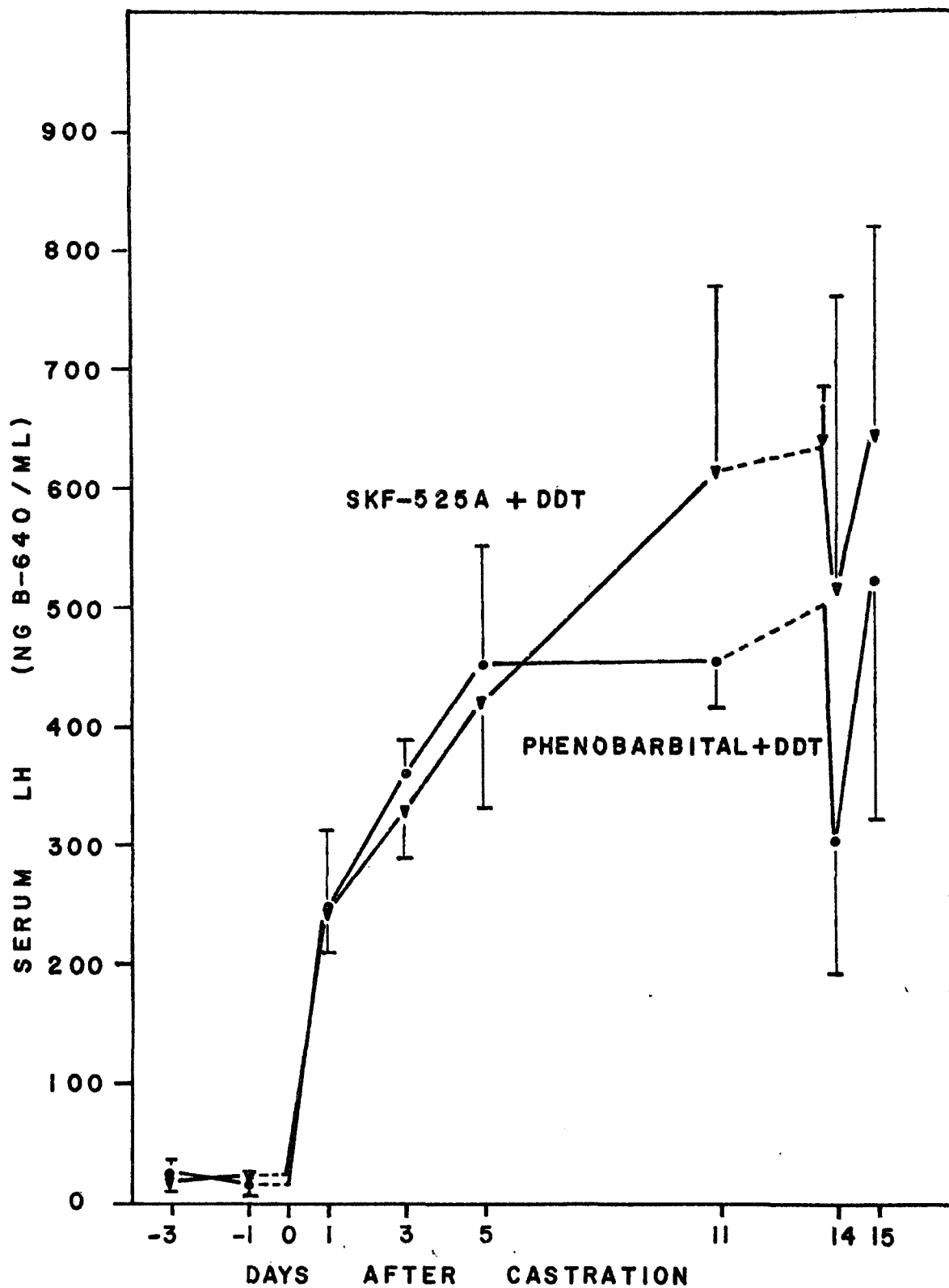
Table 8

Two-Way Analysis of Variance for Castration Response Data from Adult, Neonatally

Injected Male Rats: Analysis of Days After Castration and

Before Testosterone Administration

Treatment	Raw Data from Figures 26 and 27 (LH in ng B-640/ml Serum)				$\sum x_i$	$\sum x_i^2$
	Day					
	1	3	5	11		
Control	180.72	382.09	543.76	702.58		
	240.74	323.72	518.11	435.10		
	233.54	315.40	579.29	972.29	7,190.72	3,833,909.77
	285.25	470.43	511.26	496.14		
o,p'-DDT	210.98	343.78	394.95	373.95		
	155.18	295.99	311.08	425.95		
	172.65	336.16	349.40	465.07	5,382.56	2,052,224.82
	173.00	315.30	402.25	656.87		
o,p'-DDT + SKF-525A	250.17	289.34	312.60	696.10		
	226.26	330.84	441.90	484.66		
	270.06	367.22	510.74	788.93	6,341.33	2,919,215.24
	209.88	201.95	390.58	470.10		
o,p'-DDT + PB	260.49	331.72	575.83	489.70		
	278.75	392.79	409.13	412.20		
	139.33	381.01	327.74	464.79	6,063.25	2,472,126.38
	292.38	358.00	499.39	450.00		
$\sum x_i$	3,579.38	5,535.74	7,078.31	8,784.43	24,977.86	

Table 8 (Cont.)

$\sum x_i^2$ 836,070.53   1,946,633.19   3,260,199.02   5,234,573.47   11,277,476.20

$(\sum x_i)^2 = (24,977.86)^2 = 623,893,490.1$

$\sum_1^{N_b} (\sum_1^4 x_i)^2 = (940.25)^2 + (1491.64)^2 + (2152.72)^2 + (2606.11)^2 + (711.81)^2 + (1291.23)^2 +$
$(1457.68)^2 + (1921.84)^2 + (956.37)^2 + (1289.35)^2 + (1655.82)^2 + (2439.79)^2 +$
$(970.95)^2 + (1463.52)^2 + (1812.09)^2 + (1816.69)^2 = 43,467,370.57$

$\sum^{Day} (\sum^{R_x} X)^2 = (7190.72)^2 + (5382.56)^2 + (6341.33)^2 + (6063.25)^2 = 157,653,873.0$

$\sum^{R_x} (\sum^{Day} X)^2 = (3579.38)^2 + (5535.74)^2 + (7078.31)^2 + (8784.43)^2 = 170,725,061.4$

Summary Computations

Source of Variation	Amount (SS)	D.F.	Mean Variance (MS)	F versus Error	Occurrence by Chance
Treatment (R_x)	105,031.28	3	35,010.43	4.0925	< 0.025
Day after Castration	921,980.55	3	307,326.85	35.9242	< 0.0005
Interaction	91,495.03	9	10,166.11	1.1883	> 0.05
Error	410,633.56	48	8,554.87	---	---
Total	1,529,140.42	63			

$N_b = 16 =$ Number of boxes, i.e. groups of measurements with the same treatment on the same day.

group, PB + DDT, tended to resemble the DDT hyperbola more while the SKF-525A + DDT, or hepatically inhibited, group favored the control pattern. Neither of these groups differed significantly from the control group or from each other. A set of pairwise analyses (ANOVA) did show that the SKF-525A + DDT group was different from the DDT group ($p < 0.05$) and that the PB + DDT group approached statistical difference from both control and DDT-treated groups ($0.05 < p < 0.10$).

The analysis of variance including all four treatment groups, Table 8, demonstrated the anticipated effect of time after castration by giving it a p value of < 0.0005, i.e., time after castration contributed significantly to the overall variance of the results from the overall (grand) mean of the results. Treatment also showed a significant effect, $p < 0.025$. Time after castration and treatment were shown to be independent when they exhibited no interaction, $p > 0.05$. Tukey's (honestly significant difference) tests (214) were performed for each of the possible data contrasts within the data set but only one reached significance at the 5% level (Control - DDT). The value of the (Control - (PB + DDT)) contrast approached, but did not reach significance. A simple estimation of F for the maximum and minimum cell variances indicated that the data had fulfilled the requirement for homoscedasticity and that the use of ANOVA was fully justified.

The observed similarity of the SKF-525A + DDT group and the controls implied that SKF-525A had antagonized whatever action of o,p'-DDT had resulted in making the DDT-treated group different from the controls. Because SKF-525A acts as an inhibitor of microsomal function and can presumably block DDT breakdown in mammals as it does in insects (107,215), the amelioration of the DDT effect on the postcastration rise in LH implied that an active metabolite of o,p'-DDT was involved in producing the LH suppression seen in the DDT-treated group. Further support for this conclusion was provided by the position of the PB + DDT curve. Phenobarbital is a known inducer of DDT metabolism in the rat (175) as is DDT itself (115). The net result of injection of both PB and DDT could be either a more rapid formation of an active DDT metabolite than occurs with DDT treatment alone or a more rapid inactivation of DDT or its metabolite(s) than occurs with DDT alone. The first alternative should potentiate any observed actions of DDT while the second should alleviate them. Since the PB + DDT curve fell between that of DDT and SKF-525A + DDT, a balance between the two alternatives appeared to have occurred.

Other measurements on these animals indicated their similarities. Variables such as organ weights and pituitary LH content (144 ± 12 ug B-640/mg wet weight) did not differ significantly between the control, DDT and drug

treated groups nor did they differ from published values
(180,207).

To summarize, a second experiment has indicated the
existence of an effect of o,p'-DDT on the steroidogenic
endocrine system of the male rat. This experiment has
shown the occurrence in male rats neonatally treated with
o,p'-DDT of the same blunting of the LH response to adult
castration which Gellert et al. (54) demonstrated in the
neonatally injected female rat. At the o,p'-DDT dose used
the decrease of 30%, observed at 11 days postcastration,
agrees well with the 25% diminution seen in the female at
3 weeks postovariectomy. In either sex the difference
between controls and the DDT-treated animals may be an
hypothalamic imprinting (as proposed by Gellert et al.),
a peripherally mediated change of gonadotrophin or steroid
metabolism, or an alteration of hypothalamic, pituitary or
gonadal hormone sensitivity caused by occupation of
steroid receptors by o,p'-DDT or one of its metabolites.
The indication that a metabolite might be involved is seen
in the present results in the similarity of the responses
of the hepatically-induced, DDT-treated group (PB + DDT)
and the DDT-treated group. It is also seen in the similar-
ity of the responses of the hepatically inhibited,
DDT-treated group (SKF-525A + DDT) and the control group.
These results reinforce the hypotheses of an active
o,p'-DDT metabolite put forward by several authors (22,146,
147) to explain such phenomena as the inhibition of the

uterotropic action of o,p'-DDT by pretreatment with the

microsomal poison carbon tetrachloride (22).

CHAPTER 4

EXPERIMENTS ON THE EFFECTS OF ADMINISTERING o,p'-DDT TO

NEONATAL MALE RATS VIA THEIR MOTHER'S MILK

The last chapter dealt with experiments in animals
directly injected with doses of o,p'-DDT. This chapter is
a description of the dosage of neonatal rats with
o,p'-DDT via their dams and some of the results of such
dosage. Since most of these experiments were conducted
concurrently with those described in Chapter 3 and some
used similar designs and methodology, they do not repre-
sent advanced experiments but describe results obtained
by using a different route of exposure.

I. Administration of o,p'-DDT to Neonatal Rats Via the
    Dam:

Since the natural route of exposure to o,p'-DDT, or
other synthetic chemicals, during the perinatal period is
via the placenta and/or mother's milk, and since many
materials, including DDT, have been shown to be passed via
those routes (55,56,153,187), it appeared expedient to
ascertain the effects of o,p'-DDT when administered to the
pup via such a natural route. Therefore, after delivery
and assignment of pups to litters of the needed sizes (6
to 12 per dam) the dams were given daily i.p. injections,
until weaning, of either 0.1 ml of DMSO or the same volume
containing 50 mg of o,p'-DDT dissolved in DMSO. The daily
dose of o,p'-DDT chosen was 1/10 of an acute LD_{50}.

Body weights for both control and DDT-injected dams were monitored for one group of dams; growth of the pups was also measured. Control and DDT-treated dams showed similar weight profiles through weaning as did their pups. Growth of the litters was similar to the sesame oil injected controls used in other experiments and to the untreated litters of similar size described by Oštádalová et al. (163,216). Other than local discomfort immediately (0-15 minutes) following injection, the dams did not show any noticeable untoward effects.

The transfer of o,p'-DDT from dams fed diets containing 20 or 200 ppm of technical DDT to their pups was implied in a study (187) of the concentration of DDT metabolites in the milk curds found in the stomachs of suckling pups. In that study Ottoboni and Ferguson demonstrated a DDT concentration in milk lipid ranging from 3 to 10 times the concentration in the dams' feed; the concentration ratio (milk/feed) decreased with increased DDT concentration in the feed. The study did not, however, indicate the amount of the DDT dose absorbed by the pups. Because that study was based on data generated by long term feeding, because it did not measure DDT uptake by the pups and because it included feeding during pregnancy it could only serve as a general model for an investigation specifically designed to determine what dose of o,p'-DDT and/or its metabolites were absorbed by pups suckling an injected dam. The present experiment measured both the

secretion of o,p'-DDT and its neutral metabolites and their absorption by suckling pups.

On the day of parturition pups were sorted into litters of 6 male pups each and the daily injections of DMSO or DDT into the dams were begun. The next day, day 1, one pup from each of 3 DDT-treated and 4 control litters were removed at random, killed and the stomachs dissected out for separate storage. The pups were immediately replaced in the litters by females of similar age. The same procedure was repeated on days 3, 5, 10, 15 and 20 after birth (only 1-3 samples were collected from each treatment group after day 10). Collected samples were processed and measured by the methods described in Figures 9 and 10 (see Chapter 2, Section IV.E.). Only the curded milk in samples taken at 15 and 20 days was processed to avoid spuriously low values due to the presence of uncontaminated rat chow in the contents of the stomach. No o,p'-DDE was found in either milk curd or whole body extracts.

Group means were generated for each compound measured for each sampling day and these were plotted against time after birth (which closely approximated time after initial o,p'-DDT administration). The observed differences in analog secretion (Figure 28) and uptake (Figure 29) between control and DDT-treated groups were dramatic.

Concentration of o,p'-DDT in milk (Figure 28) rose gradually from 1.3 ppm on day 1 to 4 ppm on day 20. The

Figure 28. Secretion of DDT Analogs in Rat Milk

The concentration, in ug per g of milk curd, of various DDT analogs extracted from the curded milk removed from rat pups of various ages is shown. The pups were reared in litters of 6 pups each by dams which were injected daily, i.p., from day 0 (= birth) onward with either 0.1 ml DMSO (x-x) or 0.1 ml DMSO containing 50 mg o,p'-DDT (•-•). The curds were extracted according to the procedure shown in Figure 9 and subjected to gas chromatography under the conditions listed in Figure 10. The data shown are means, ± 1 standard error, for both treatment groups; error bars do not appear for those points where the symbol covers the error interval. Each point represents 2-4 milk curds except for days 10-20 of the controls where single values are shown.

FIGURE 28

SECRETION OF DDT ANALOGS IN RAT MILK
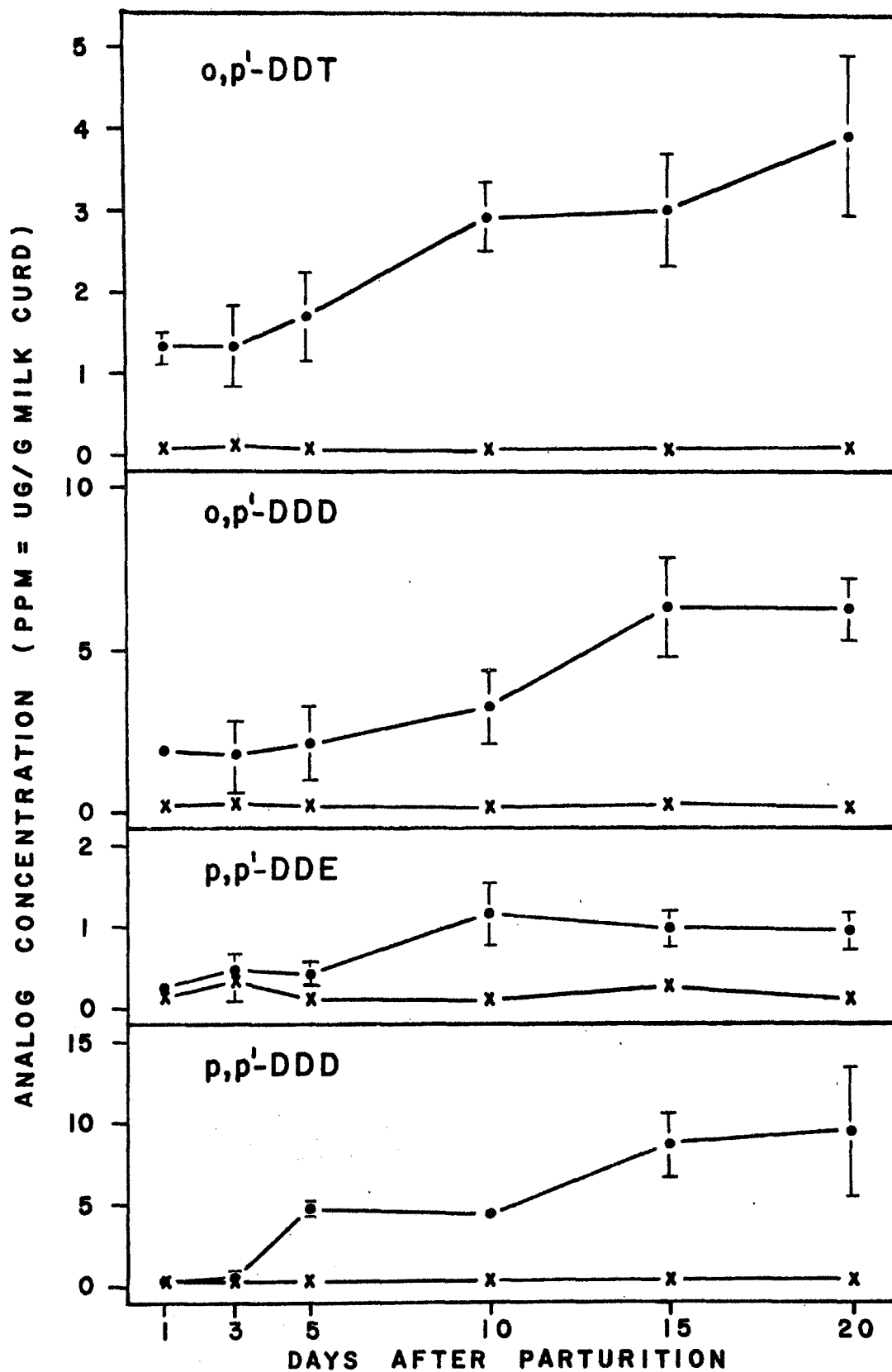
Figure 29. Uptake of DDT Analogs by Suckling Rats

The tissue concentrations of various DDT analogs, in ug per g of body weight, is shown for rat pups of various ages. The carcasses of the pups described in Figure 28 were processed according to the protocol of Figure 9 and quantitated by gas chromatography under the conditions described in Figure 10. The data from litters suckling vehicle-injected dams (x-x) or o,p'-DDT injected dams (●-●) are shown as means ± 1 standard error. The number of pups per point is 2-4 except for days 1 and 20 for the controls where a single animal is shown. Error bars are omitted where symbols cover the error intervals.

FIGURE 29

UPTAKE OF DDT ANALOGS BY SUCKLING RATS
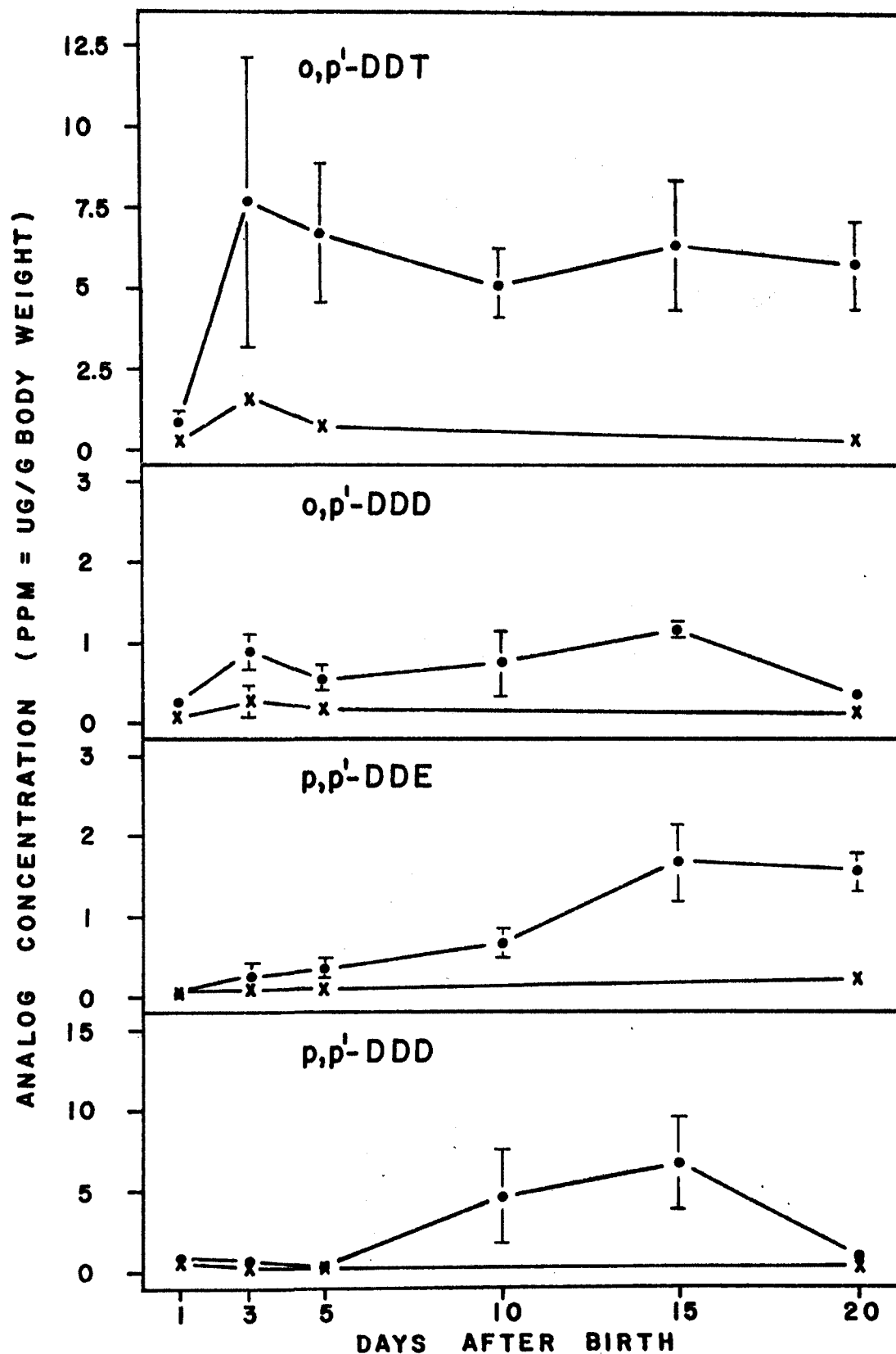
concentration of o,p'-DDD, the major neutral metabolite
of o,p'-DDT, rose in a fashion parallel to o,p'-DDT from
2 ppm on day 1 to 6 ppm on days 15 and 20. Evidence of
the contamination of the o,p'-DDT, which was injected into
the dams, with the p,p'-isomer, in addition to that pro-
vided in the chemical analysis described in Chapter 2,
Section II, was provided by the appearance of p,p'-DDE and
p,p'-DDD residues. Their concentrations rose somewhat
more slowly above control levels than the o,p'-isomers due
to the smaller amount of p,p'-DDT injected and probably to
the presence of a lag phase of 3-5 days (113,217) for the
hepatic induction by DDT which promoted their formation
in the dam. The metabolites reached amounts of 1 ppm
(p,p'-DDE) and 9 ppm (p,p'-DDD) by days 15 and 20. The
more rapid metabolism of the o,p'-isomers, causing their
loss in the urine and feces, undoubtedly contributed sub-
stantially to the implied unequal retention of the
p,p'-DDT isomer by the dam and to her subsequent secretion
in milk of disproportionately large amounts of p,p'-DDE
and p,p'-DDD.

Whole body o,p'-DDT concentrations in the pups
(Figure 29) rose abruptly from control levels on day 1 to
a plateau of 6-7.5 ppm by day 3. This clearly demonstrated
the absorption of o,p'-DDT. If an estimation of 70% water
was made for total body weight, and a similar estimation
of 75-80% water was made for stomach curd weight (218),
the concentrations of o,p'-DDT based on solid weights were

18 and 12-15 ppm for the whole body and curd, respectively.
Thus, accumulation was at least an equilibration of the
concentrations in the pups' body tissues with those in the
milk.

Though the body concentrations of o,p'-DDD followed
the temporal pattern of o,p'-DDT accumulation they never
reached the levels found in milk, on either a total or
solid weight basis. A partial answer to this discrepancy
with the results found for o,p'-DDT may lie in the occur-
rence of on-column breakdown of o,p'-DDT to o,p'-DDD.
This breakdown was encountered in an analysis of pure
o,p'-DDT conducted after analysis of the body residues and
the milk curd samples had been completed. A mass of
10-20% of an injection of o,p'-DDT appeared as o,p'-DDD,
probably as the result of interaction with lipid residues
deposited on the column near the injection port. The same
problem has been described for p,p'-DDT by Burke (219).
It constitutes a needed correction (increase) of less than
20% for the o,p'-DDT values in milk curd samples (with an
equivalent decrease in measured o,p'-DDD levels).
Analyses of pure pesticides conducted between the body
residue and milk curd analyses indicated no breakdown
problem. Therefore, any adjustments would apply to the
milk curd results only. Possibilities which are more
capable of fully explaining the difference seen in the
milk and body residues of o,p'-DDD in comparison to those
seen with o,p'-DDT may be the occurrences of a more active

metabolism or excretion of ingested o,p'-DDD by the pups
or a selective exclusion of o,p'-DDD by the gut.

Body uptake of p,p'-DDE approximates a sigmoidal curve
less steep than the uptake of the o,p'-compounds. The rise
to 1.5 ppm between day 10 and day 15 follows both attain-
ment of the plateau of secretion in milk and the critical
hypothalamic imprinting period. On a solid weight basis
the concentrations of both tissue and milk would be near
4-5 ppm on day 15, a situation adequately described as a
passive accumulation driven by a concentration gradient;
just as might have been expected for a nonmetabolizable,
hydrophobic compound such as p,p'-DDE.

As with body residues of o,p'-DDD, body residues of
p,p'-DDD never approach the levels theoretically possible
on the basis of the residues seen in milk, levels which
might cause neural or reproductive toxicity (see p. 30-31)
or levels approaching those of o,p'-DDT during the critical
imprinting period. A possible on-column breakdown of the
0.1 ng p,p'-DDT per ul, originally added as an internal
standard in milk curd analyses could only account for 1-2%
of the p,p'-DDD mass found in milk and therefore is incon-
sequential in explaining the difference between concentra-
tions seen in milk curds and total body extracts. Time
courses for either p,p'-DDD or o,p'-DDD uptake in pups do,
however, indicate that breakdown after absorption is a very
active process. The abrupt fall to control levels after 15
days is not accompanied by similar falls in o,p'-DDT or

p,p'-DDE as would be the case if only decreased uptake, due to introduction of solid food (163,216), was the cause. Therefore, active metabolism by pups in the presence of decreased intake of all forms of DDT must have caused the observed fall in body residues after day 15 and was probably responsible at earlier times for maintaining DDD levels below those expected by passive accumulation. Hepatic induction in pups (112,153) was not clearly indicated but may have been reflected in the dip in o,p'-DDD and o,p'-DDT values which occurred between days 3 and 10 since secretion in milk continued to rise throughout that period.

Total dosage for the period of birth through weaning was calculated by integrating a set of curves, extrapolated to 25 days of age, over time. Total administered dose (cumulative dose) was obtained from milk curd concentrations while total effective or absorbed dose (body burden) was calculated from body concentrations. Milk production was assumed to be 24.7 g/day - the mean value obtained by Hanwell and Linzell (220) for dams suckling litters of 6 pups. Mean body weights, exclusive of stomach, were used in computation.

When body burden of o,p'-DDT was plotted against cumulative dose of o,p'-DDT (Figure 30) a curve intermediate between a straight line and a hyperbola was generated. Since any curve fitted to the results lay above a line with a slope of unity and thus forced the conclusion that more o,p'-DDT was absorbed than was supplied, one of two conditions must have occurred. Either the assumed

Figure 30. Uptake of o,p'-DDT by Suckling Rat Pups: Total

Body Burden Versus Cumulative Dose

Total body burden, calculated as the product of

o,p'-DDT tissue concentration and average body weight at

the time of sacrifice, is plotted versus cumulative

ingested dose of o,p'-DDT, calculated as the product of

daily milk weight and milk curd concentration integrated

over the age of the animals in days. Milk weight was

taken as 24.7 g/day based on the findings of Hanwell and

Linzell (220) for litters of 6 pups. The empirical curve

was generated from the data for the DDT exposed pups

described in Figures 28 and 29.

The inset shows a graph of the inverse functions with

a comparison to a line $\Delta x/\Delta y = 1$, describing complete

storage of ingested residues. It should be noted that the

empirical lines describe systems which are storing more

residues than are being ingested. If the errors for both

uptake and storage measurements (see Figures 28 and 29)

are considered, however, the data may be found to include

the approximation of a line with a slope of 1. Thus the

data appear to describe a nonsaturable system, i.e., a

residue sink.

FIGURE 30

UPTAKE OF o,p'-DDT BY SUCKLING RAT PUPS:
TOTAL BODY BURDEN VERSUS CUMULATIVE DOSE
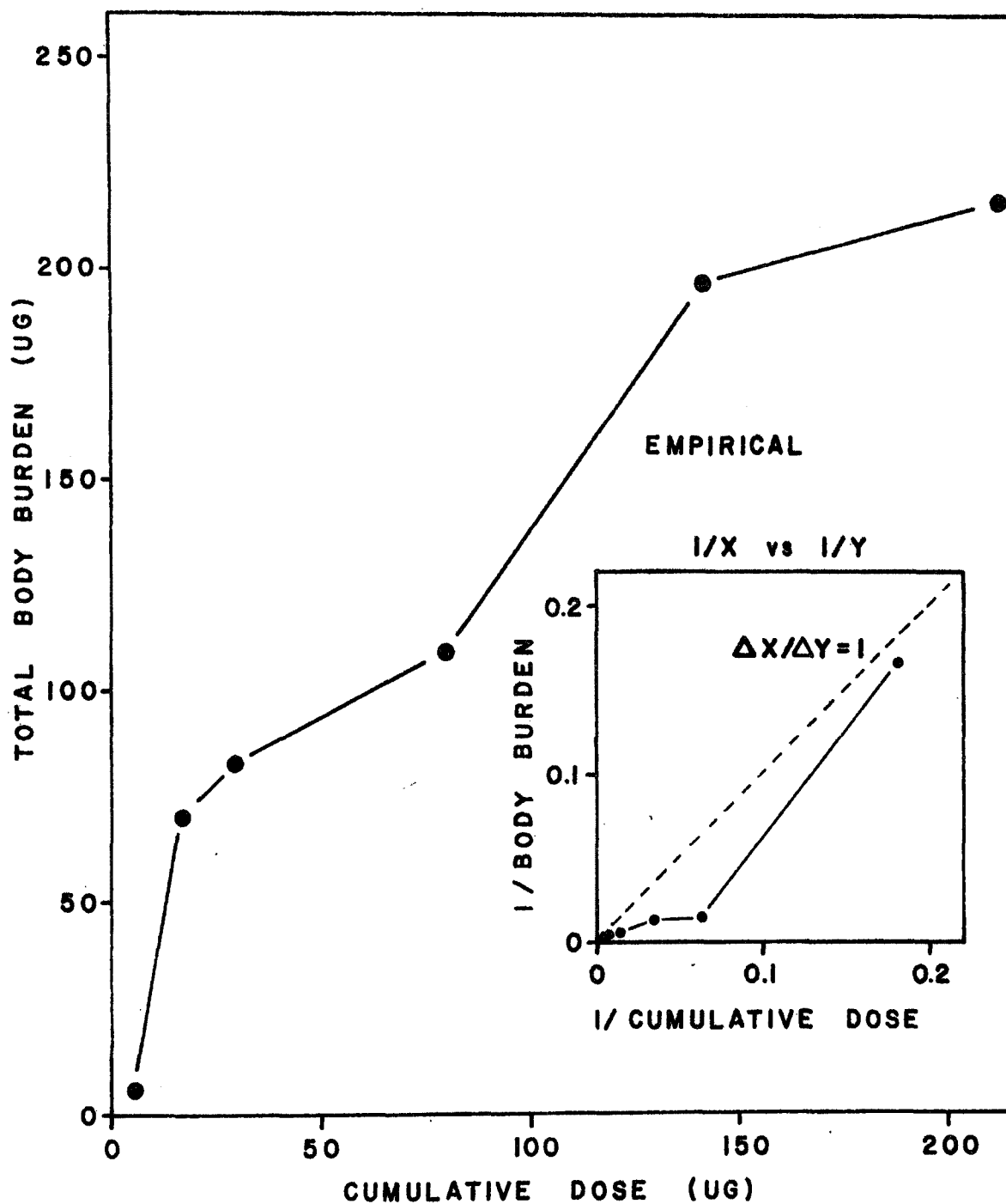
production of milk was too low or the estimates of milk
residues were too low. If allowances were made for a
10-20% underestimate of o,p'-DDT, caused by on-column
breakdown, and for the variances of both milk curd and
body residues, the results adequately approximate a
straight line with a slope of unity. This line (slope
equal to 1.0) indicated that the pups were extremely
efficient in absorbing, and storing, o,p'-DDT administered
in milk; they acted as sinks for o,p'-DDT. Furthermore,
it implied that the total dose absorbed by rats treated in
this manner was between 50 and 100 ug by day 5 or 200-300
ug by day 25. If similar analysis was done for o,p'-DDD,
total doses were 10 and 50 ug for days 5 and 25, respec-
tively. Comparison to cumulative administered dose of
o,p'-DDD indicated that only 10-12% of that supplied in
the milk was stored in these animals.

Other computations indicated that only 0.35-0.40% of
the total dose administered to the dam was eliminated in
her milk and that a total of only 0.19% of the total dose
administered to the dam was absorbed by the pups. The
absorption rate appeared to be fairly uniform until
self-feeding began at 15 days of age; total retention by
25 days of age was only 0.12% of the total dose admin-
istered to the dam. It should be noted that the absorption
and retention values given above are probably higher than
the actual exposure experienced by the larger litters used
in other experiments described later since milk production

per pup falls somewhat as the litter size increases (220).

Conversely, because total milk production increases with

larger litters, total dose secreted increases. Therefore,

this experiment only describes an upper limit of exposure

per pup and a lower limit of total secretion by the female.

In summary, this experiment was designed to determine

the dosage of o,p'-DDT administered to rat pups suckled by

DDT-injected dams; it showed that the doses administered

in the milk and absorbed by the pups were well within the

dose-range examined in directly injected rats. It gener-

ated some information on the dynamics of this transfer

from mother to offspring in terms of the forms and

quantities involved. And, it inferred the role of hepatic

induction in transformations in both the dam and pup.

Finally, the experiment indicated that if a "DDT-saturated"

female were used as a foster dam for a portion of the neo-

natal or preweaning period the efficient absorption of

o,p'-DDT would allow dose response experiments to be con-

ducted by using the natural route of exposure.

II. Organ Weights in Adult Rats Neonatally Treated with

o,p'-DDT Via Their Dams:

A brief experiment similar to the initial experiment

on directly injected rats was performed using the injected

dam as the means of exposure. Litter size averaged 8 pups.

Autopsies were done at 102-138 days of age; both intact

and neonatally castrated animals were examined. Growth

was only examined sporatically but indicated general agreement among the groups prior to 40-50 days of age.

Final body weights (Table 9) were similar in both castrate groups but the DDT-treated intact animals were about 10-12% lighter than their vehicle-exposed controls. All body weights were indistinguishable from the corresponding groups shown in Table 6 and Figure 13.

Organ weights, normalized to body weight, demonstrated the effects of castration but did not reflect any overt changes due to treatment with o,p'-DDT. Only the testes showed a slight change in normalized weight due to the presence of DDT, but this was explained by the difference in body weights between the control and DDT-treated group; raw organ weights did not differ. The other normalized organ weights, with the exception of the pituitary, were similar to those seen in the directly injected rats described in Table 6 and in Figures 14-20.

The weight differences which exist between these findings and those obtained in the experiments done on directly injected animals (Table 6 and Figure 14), in regard to the pituitaries of both the intact and castrate groups, were not explicable on the basis of differing body weights. The difference was also not reflected in a change in pituitary LH content or serum LH levels in the castrate groups; pituitary LH content was 55 ± 7 and 55 ± 6 ug B-640/mg wet tissue for control (3 animals) and DDT-treated (3 animals) groups, respectively; serum LH levels were

184

Table 9

Effects of Suckling an o,p'-DDT Injected Dam[1] on Body[2] and Organ Weights of

Intact and Neonatally Castrated Male Rats

Percent of Body Weight[3]	Intact		Castrated	
	Control (6)	DDT (6)	Control (9)	DDT (14)
	(455 ± 47)	(401 ± 29)**	(333 ± 21)	(353 ± 35)
Pituitary x 10^3	3.37 ± 0.19	3.51 ± 0.38	4.77 ± 0.68	4.27 ± 1.34
Adrenals x 10^2	1.36 ± 0.09	1.30 ± 0.06	2.05 ± 0.28	2.09 ± 0.43
Testes	0.74 ± 0.07	0.80 ± 0.05*	-----	-----
Seminal } x 1	0.33 ± 0.09	0.31 ± 0.09	-----	----- .
Vesicles } x 10^3	-----	-----	8.36 ± 3.36	11.37 ± 7.06**
Ventral Prostate	0.09 ± 0.02	0.11 ± 0.02	-----	-----
Liver	-----	-----	3.92 ± 0.68	3.54 ± 0.50
Kidneys	-----	-----	0.76 ± 0.15	0.71 ± 0.13

[1] All pups were suckled prior to weaning at 25 days of age by dams who received daily i.p. injections of 50 mg o,p'-DDT in 0.1 ml DMSO or 0.1 ml DMSO alone. Litter size averaged 8 pups per litter and sacrifice was at 102 to 138 days of age.

[2] Body weights are given in grams in parentheses under each heading.

185

Table 9 (Cont.)

---

[3] All data are given as mean weights or mean % of body weight ± 1 standard deviation; numbers in parentheses after the treatment designation are the numbers of animals examined; comparisons are all versus the appropriate control groups and are made by unpaired Student's t-tests, * $p < 0.05$, ** $p < 0.025$.

1.04 ± 0.32 and 0.86 ± 0.39 ug B-640/ml for control and DDT-treated groups, respectively. Nor did a different group of pituitaries (Section V, this Chapter), taken from animals treated via their dams, demonstrate weights differing from those found in directly injected animals for either control or DDT-exposed groups. Possible explanations of the present results include variability in litter sizes during the experiment, possible problems with malnutrition due to the presence of primiparous dams and/or inexperience or inconsistency in dissection and handling of the pituitary. Whatever the cause, the later determinations, on intact rats, similarly dosed in litters of 6 and 12 pups (Section V, this Chapter), gave values for normalized pituitary weights of 2.59 ± 0.24 for control animals (21 animals) and 2.69 ± 0.25 for DDT-treated animals (20 animals); both values agree with those found in directly injected groups. Similarly, a later determination of the normalized hypophysial weights of neonatally castrated, suckled rats raised in 8 pup litters showed values of 3.74 ± 0.30 and 3.37 ± 0.14 mg/100 g body weight for control (2 animals) and DDT-treated (3 animals) groups, respectively; values again in accord with those determined for directly injected animals.

The above results yielded no clue as to any possible effect of o,p'-DDT on the male rat. They again, as was the case with directly injected rats, implied the need for the use of more sensitive measurements. To that end, and

with the data of Rybakova (29,29) and Gellert et al. (53,

54,150,151) in mind, several experiments were done to

probe aspects of pituitary release of LH.

III. Periodicity of LH Release in Adult Rats Neonatally

Exposed to DDT:

Adult castration superimposed on neonatal DDT-treat-

ment was shown to expose a measurable difference between

the responses of sesame oil and DDT-treated LH control

systems (Chapter 3, Section III). Ether had analogously

been shown to alter the 24-hour periodicity of serum LH

levels in untreated animals (Chapter 2, Section IV.D.2.)

(183-186). The following experiment, though originally

performed to measure the direct effect of o,p'-DDT expo-

sure on the 24-hour periodicity of serum LH in both

intact and neonatally castrated rats, is best discussed

in light of the effect of superimposing a second stressor,

ether, on what may be an LH control system which has

already been perturbed by neonatal exposure to o,p'-DDT.

Differences in ether response throughout the day are, in

fact, what was measured.

Two groups of rats were subdivided into 2 subgroups

each, 2 of which were treated with o,p'-DDT via their

dams (DDT-treated) and 2 of which were suckled by vehicle

injected dams (Controls). One subgroup under each treat-

ment was castrated on the day of birth. The pups were

reared in 8 pup litters and were used for the experiment

at 76-89 days of age. After weaning, a pair of subgroups,

one DDT-treated and one control, were each split into 3 sets of 2-5 animals each, i.e., 3 sets of 2-5 control animals were formed and 3 sets of 2-5 DDT-treated animals were formed. Beginning at 09.00 or 11.00 (EST) individual animals from a pair of the sets (one DDT-treated set and one control set) were alternately bled under light ether anesthesia (TVBE). Animals were returned to their cages and to the normal light/dark cycle when fully conscious after bleeding; total elapsed time between removal of an animal from the cage to its return was about 6-8 minutes. The serum was separated from the collected blood and stored at $-20^{O}C$ until analysis by the RIA procedure of Niswender et al. (169). The bleeding procedure was repeated at 2 hour intervals, using the sets of animals pairwise and in rotation so each animal was bled once every 6 hours, i.e., a total of 4 times over the course of the test period.

Means for each treatment group and each time of day were calculated from the results obtained from the RIA. The means were plotted versus time of day and visually examined for the existence of any discernable circadian rhythm(s). Corresponding means for DDT-treated and control rats were tested for difference by Student's t-tests. Both the DDT-treated and the control group results for intact animals were also divided into halves, before 20.00 and after 20.00 (EST), and examined in a 2-way ANOVA for the effects of both time, day and night, and exposure to DDT.

The LH patterns obtained for both neonatally
castrated and intact rats are shown in Figure 31. No
significant differences between DDT-treated and control
groups were found at any single time of day for either neo-
natally castrated or intact animals. No rhythm was shown
in either treatment group of castrated rats. The levels
of LH seen in castrates were similar to those reported by
Lawton and Smith (186). The intact animals generated a
picture similar to that seen in Figure 8 which describes
intact, normal, adult males exposed to ether. In intact
males the LH values found during the day were more variable
and higher ($p < 0.005$) than those obtained at night. A
superficial rise near 24.00 (EST) may reflect the LH peak
which coincides with peak activity (see Chapter 2, Section
IV.D.2. and Figure 8), however, the values are too near
the reliable limit of the assay to distinguish that point
from any others between 18.00 and 06.00 (EST). Though
there was no significant difference seen in LH values
between the entire set of results from the control and
DDT-treated intact groups ($0.05 < p < 0.10$), the com-
bined values for the results between 15.00 and 19.00 (EST)
did differ for the two groups ($t_{15} = 2.4625$, $p < 0.05$),
the controls being higher than the test group.

The possibility that the castrates demonstrated a
larger ether effect than the intact animals was not sup-
ported by the similarity of the present results to the
values of serum LH in long-term castrates bled by

Figure 31. 24-Hour Periodicity of Serum LH in Adult Male

Rats Treated Neonatally Via Their Dams

Serum LH in ng B-640 equivalents per ml is plotted
versus time of day for groups of adult male rats. Both
neonatally castrated and intact rats are shown. The ani-
mals were further grouped by treatment, being suckled in
8 pup litters by dams injected daily, i.p., from day 0 to
25 after parturition, with 0.1 ml DMSO (control, ●-●) or
with 0.1 ml DMSO containing 50 mg o,p'-DDT (DDT, x-x).
Bleedings were done by tail-vein under light ether (see
Figure 8) on groups of 2-5 rats. All animals in a given
experiment and treatment group were placed in 3 subgroups
which were bled in rotation every 2 hours; each individ-
ual subgroup being bled every 6 hours. Animals were
allowed free access to food and water throughout the
experiment. Sera were analyzed for LH by the method of
Niswender et al. (169) using B-640 (= 0.03 x NIH-LH-S1 by
OAAD) as the primary standard. Values shown are means ± 1
standard error; n = 1-5 for each point. No statistically
significant differences between the DDT and control groups
were found.

FIGURE 31

24-HOUR PERIODICITY OF SERUM LH IN ADULT MALE
RATS TREATED NEONATALLY VIA THEIR DAMS
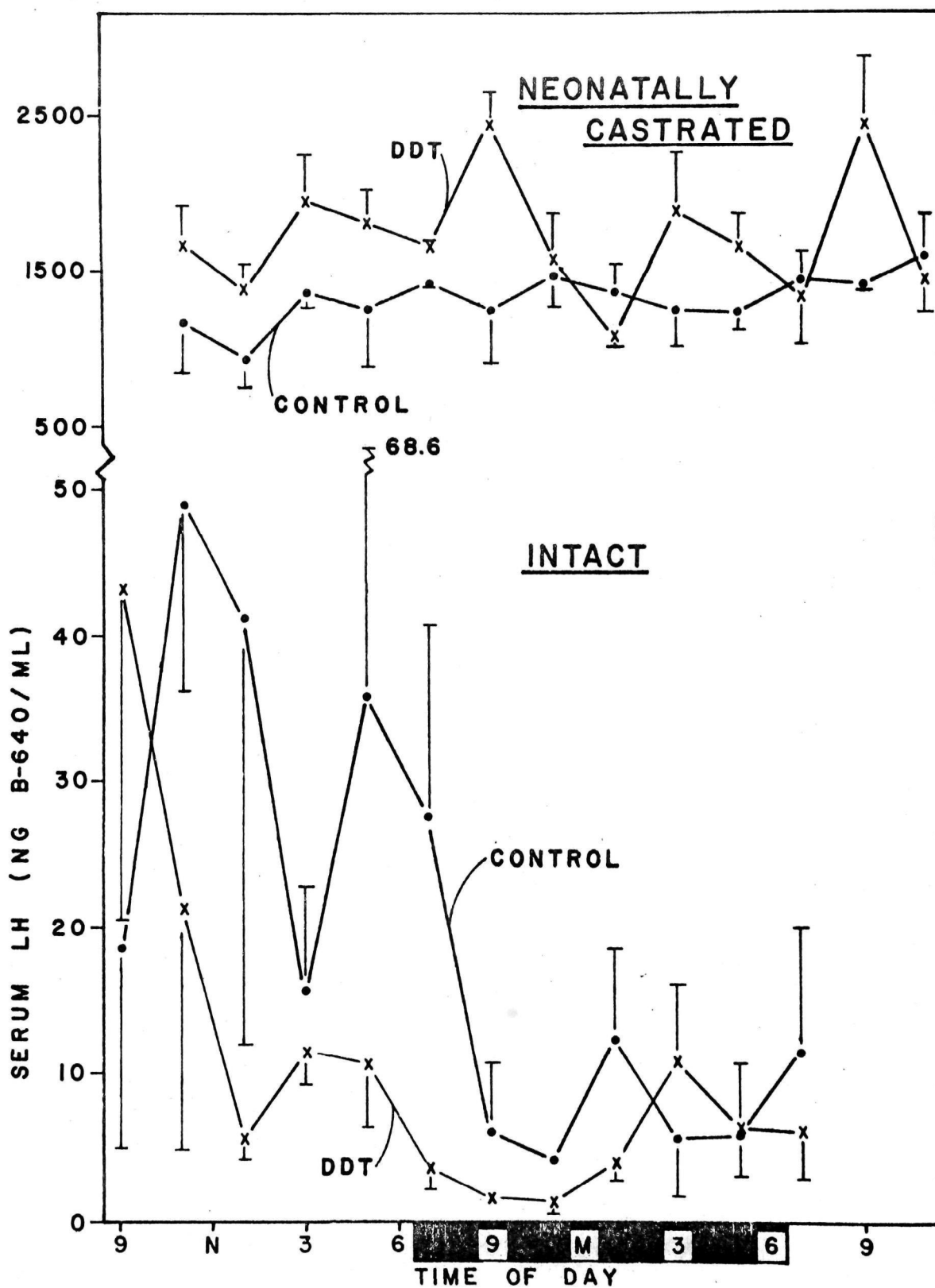
decapitation as published by Yamamoto et al. (180). The possibility that DDT-treated rats showed an ether response which compensated for a depression in LH caused by o,p'-DDT cannot be ruled out. However, the observation that a response to ether did not eliminate the difference between DDT-treated and control animals castrated as adults (Chapter 3, Section III) argues against such compensation. The present finding that no difference was seen due to o,p'-DDT exposure when a difference did occur after adult castration in directly injected animals may have been due either to the longer period which had elapsed after castration - serum LH continued to rise for at least a month after castration (180) - or to the difference in the absorbed dosage (pups treated via the dam absorbed less than 0.5 mg over a period of 25 days while injected pups received 10 mg over 5 days).

The conclusion reached from the experimental results was that neonatal exposure to o,p'-DDT does not significantly alter the 24-hour periodicity of the response of serum LH to tail-vein bleeding under ether in either neonatally castrated or intact rats. There does appear, however, to be a tendency toward lower values in intact DDT-treated animals at least during the late afternoon.

IV. Serum LH Response to Adult Castration:

Since no significant alteration of the circadian pattern of LH response to ether was generated by neonatal DDT exposure in the preceding experiment, an experiment

was undertaken to measure the effect of neonatal DDT-exposure on the LH response to adult castration. The experiment was later repeated in directly injected animals (the experiment discussed in Chapter 3, Section III). As was the case in the other experiments dealing with LH release the empirical bases for the experiment were the observations of Rybakova (28,29), showing increased pituitary LH levels in male and female rats chronically fed technical DDT, and the observations of Gellert et al. (54), showing the blunting of the response to adult castration in female rats neonatally injected with 0.001-1.0 mg of o,p'-DDT.

The experiment was initiated with two litters of 8 neonatal pups each. One litter received o,p'-DDT via its treated dam (DDT-treated) while the dam of the other litter received vehicle only (control). Attrition due to undetermined causes during development and prior to the beginning of the experiment on day 57 of age, limited the number of animals to 3 in the control group and 4 in the DDT-treated group.

To establish a response baseline serum samples were taken between 15.00 and 18.00 (EST) by TVBE on days 57, 58 and 59; the sera were stored for later assay. On day 60 the animals were castrated but no blood samples were taken. On days 1, 3, 6 and 10 following castration serum was obtained from all the rats by TVBE at 15.00-18.00 (EST). Two rats succumbed to ether and a preexisting respiratory infection leaving 2 control rats and 3 test animals. An

injection of testosterone (0.75 mg/kg) was given 6 hours prior to bleeding on day 14 to ascertain if the gonadal-hypothalamo-hypophysial feedback loop was functioning differently in the DDT-treated and control animals (170). Finally, on day 15 postcastration, the animals were anesthesized with ether and decapitated; trunk-blood was collected to determine if LH had rebounded normally from the testosterone injection.

Sera were analyzed by RIA using the procedure of Niswender et al. (169). Means were calculated for each experimental day and each treatment; a plot of these results is presented as Figure 32. Two-way ANOVA was also done on the values obtained between the time of castration and the time of testosterone administration, i.e., on the results from days 1-10.

Due to the small number of animals involved the standard deviations were quite large. At the 5% significance level no difference was apparent between the control and DDT groups. If the acceptance level for significance was relaxed to 10%, however, the control showed significantly higher values than the DDT-treated animals during the period of 1-10 days postcastration; values for DDT-treated animals were 15-20% lower than those for the controls. The measured LH values for the period following day 6 were quite similar for both control and DDT-treated groups to those encountered in the better balanced experiment done on directly injected animals. Controls

Figure 32. Response of Serum LH to Adult Castration in
Male Rats Treated Via Their Dams

Serum LH as ng B-640 (= 0.03 x NIH-LH-S1 by OAAD)
equivalents per ml is plotted versus time after adult
castration for two groups of male rats. The control rats
(●-●) were suckled prior to weaning in a litter of 8 pups
by a dam which received daily i.p. injections of 0.1 ml
DMSO while the DDT-treated rats (x-x) were reared in a
litter of similar size by a dam which received daily i.p.
injections of 0.1 ml DMSO containing 50 mg o,p'-DDT. The
animals were reared with free access to food and water and
were castrated at 60 days of age. Blood samples were taken
from the same animals throughout the experiment by TVBE at
15.00-18.00 (EST) on the days indicated. On day 14 an
injection of 0.75 mg/kg testosterone (T) was given s.c. in
sesame oil 6 hours prior to bleeding. Sera were assayed
by the method of Niswender et al. (169). Values shown are
means $\pm$1 standard deviation for groups of either 2 control
or 3 DDT animals. Two-way ANOVA of the results between
castration and testosterone injection (days 1-10) yielded
a p value between 5 and 10% for the difference between
control and DDT-treated groups.

FIGURE 32

RESPONSE OF SERUM LH TO ADULT CASTRATION IN MALE RATS TREATED VIA THEIR DAMS
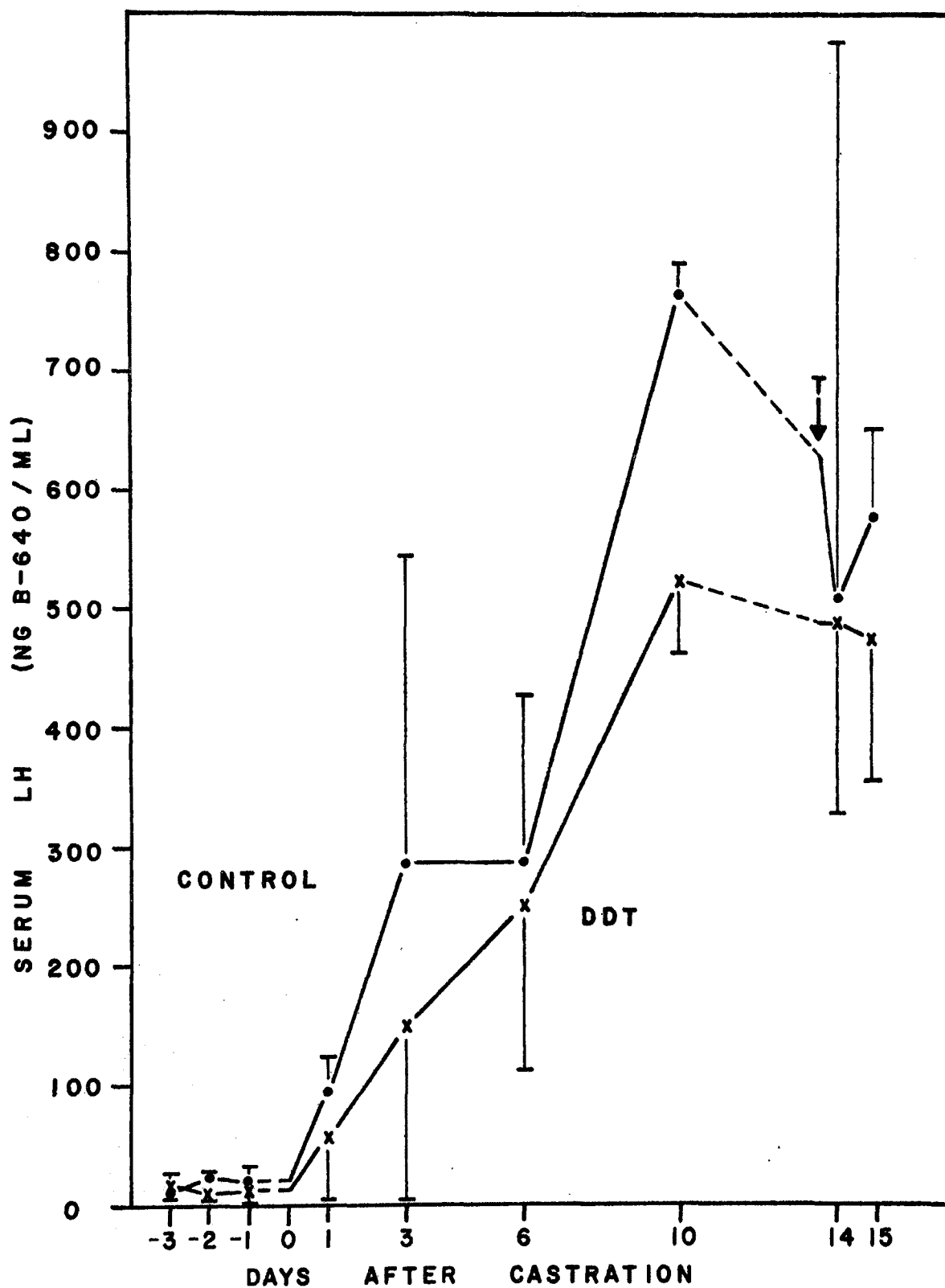
demonstrated a response to testosterone administration which appeared similar to that observed in directly injected animals. DDT-exposed animals, on the other hand, did not demonstrate this response clearly. However, since the error for the mean on day 14 included the range of response seen in directly injected rats a total lack of feedback inhibition could not be inferred.

If the relaxation of the acceptance level for significance is allowed, the agreement of these results with those observed in directly injected animals is striking. Their similarity to the data given by Gellert et al. (54) for female rats is equally good. Such correspondence is particularly important in view of the differences in effective administered dose. The directly treated rats in my studies received 10 mg o,p'-DDT while Gellert et al. observed effects with 0.1-1.0 mg; the calculated total absorbed dose of all forms of DDT in the suckled animals was less than 0.5 mg over the full 25 day preweaning period. If only the first 5 days, the critical period for hypothalamic imprinting in the male, is considered, the suckled animals absorbed less than 100 ug of all forms of DDT and only 80-85 ug of o,p'-DDT. Still, in view of the small numbers of animals used and the resulting large standard deviations, a final assessment of these experimental results and their importance must await the confirmation of future studies.

V. Serum LH Response to Exogenous LHRH:

Studies by Rybakova (28,29) indicated a change in the pituitary content of LH in rats chronically fed low levels of technical DDT. Other studies by Arai (211) had shown changes in pituitary stores of LH and hypothalamic stores of LHRH in response to neonatal estrogen treatment. Several of the experiments already discussed also implied a change in the function of the overall feedback system controlling gonadal steroidogenesis. On the basis of these observations, and in order to begin answering the question of the locus of action of neonatal o,p'-DDT, a measurement of the response of this feedback system to exogenously administered LHRH was undertaken.

The design of the experiment included the use of two different litter sizes and two periods of injection of the dam in order to probe the results of different absorbed dosages. A 3-level factorial design was generated for 8 litters; a control and a DDT-treated group were constructed in each of 2 litter sizes (6 and 12 pups per litter) and at each of 2 durations of injection of the dam (5 and 25 days). Growth was monitored by regularly checking body weights. Beginning at 149-154 days of age 4-6 animals from each litter were subjected to a test of their serum LH response to injection of synthetic LHRH. Rats derived from dams injected for 5 days were tested separately from those reared by dams injected for 25 days.

Treatment Groups

Litter Size	Treatment of the Dam	
	Injected Chemical	Number of Injections
6	DMSO	5
6	DMSO	25
6	o,p'-DDT	5
6	o,p'-DDT	25
12	DMSO	5
12	DMSO	25
12	o,p'-DDT	5
12	o,p'-DDT	25

On the third day of each test[1] simple TVBE was done at
17.00-19.00 (EST) to generate a basal level of LH for
reference. The animals from the 6-pup litters in both the
control and DDT-exposed groups were bled in rotation (to
prevent temporal bias) followed by those from the 12-pup
litters. On the fourth day the same rotation was followed
except that, before bleeding each group, each animal
received a 1.0 ug i.p. injection of synthetic LHRH in 0.1
ml of saline. The bleedings were begun 20 minutes after
injection and were completed on each group less than 60
minutes after injection. The same procedure, including
LHRH injection, was repeated on the fifth day of the test.

---

[1] The test results for the present experiment were plotted
beginning on day 3 to allow comparison of LH levels to
the range of LH values seen in a group of rats which
were neonatally injected with sesame oil and bled as
adults on 8 consecutive days during which they were
injected with saline on days 3-6.

On the sixth day no bleeding or injections were performed.
On the seventh day of the test, animals were weighed,
stunned, decapitated, bled and autopsied. Weights and
organ weights were measured as previously described and
assays for LH were done on both sera and pituitary
extracts. Results for the LH assays were plotted versus
day of the test and analyzed for differences by a 3-way
ANOVA as well as by individual t-tests between the means
of each DDT-treated group and its paired control (paired
both in litter size and length of dam injection) on each
day of the experiment. Student's t-tests were likewise
used to compare values for body weights, etc., from each
paired control and DDT-exposed litter.

Growth for litter sizes of 6 and 12 pups diverged as
expected (163,216) between 5 and 10 days of age; o,p'-DDT
injection into the dams for either 5 or 25 days did not
alter weight changes. Pups from 6-pup litters grew
slightly more rapidly than those from 8-pup litters while
pups from 12-pup litters grew somewhat more slowly than
those from 8-pup litters. Final body weights for rats
from 6-pup litters were fully 10% above those of rats from
12-pup litters (significant at $p < 0.001$). Normalized
organ weights, however, did not differ because of differ-
ence in litter size, DDT treatment or length of DDT-injec-
tion into the dam.

A representative plot of the experimental results
obtained for serum LH is shown in Figure 33 - values

Figure 33.   Response of Serum LH to Injection of Large

Doses of LHRH in Adult Male Rats Treated Via

Their Dams

The results of a test of the pituitary response to
repeated injection of LHRH in vivo is shown.   Serum LH is
plotted versus time for 2 groups of rats raised in 12-pup
litters: Controls (x-x) which were suckled prior to
weaning by a dam which received daily i.p. injections of
0.1 ml DMSO , and DDT treated animals (o-o) which suckled
a dam which received daily i.p. injections of 0.1 ml DMSO
containing 50 mg o,p'-DDT.   The 149-154 day old animals,
which had free access to food and water throughout the
experiment, were bled at 17.00-19.00 (EST) on the days
shown by TVBE.   Simple bleedings were performed on days 3
and 7 but 20-60 minutes (unbiased between groups) prior to
the bleedings on days 4 and 5 the animals were injected
i.p. with 1.0 ug of synthetic LHRH in physiological saline.
Sera were assayed by the method of Niswender et al. (169)
using B-640 (= 0.03 x NIH-LH-S1) as standard.   Values
shown are the means $^+_-$1 standard deviation for groups of 4-6
rats.   A Student's t-test for day 5 of the experiment shows
a large (p < 0.005) dependence on DDT treatment for the
observed response to the second daily injection of LHRH.

Simple bleedings were also done on a group of male
rats which received neonatal injections of sesame oil
(S.O.);   the limits of the range of their LH levels (===)
is shown for comparison with the LHRH treated animals.

FIGURE 33

RESPONSE OF SERUM LH TO INJECTION OF LARGE DOSES OF LHRH IN ADULT MALE RATS TREATED VIA THEIR DAMS
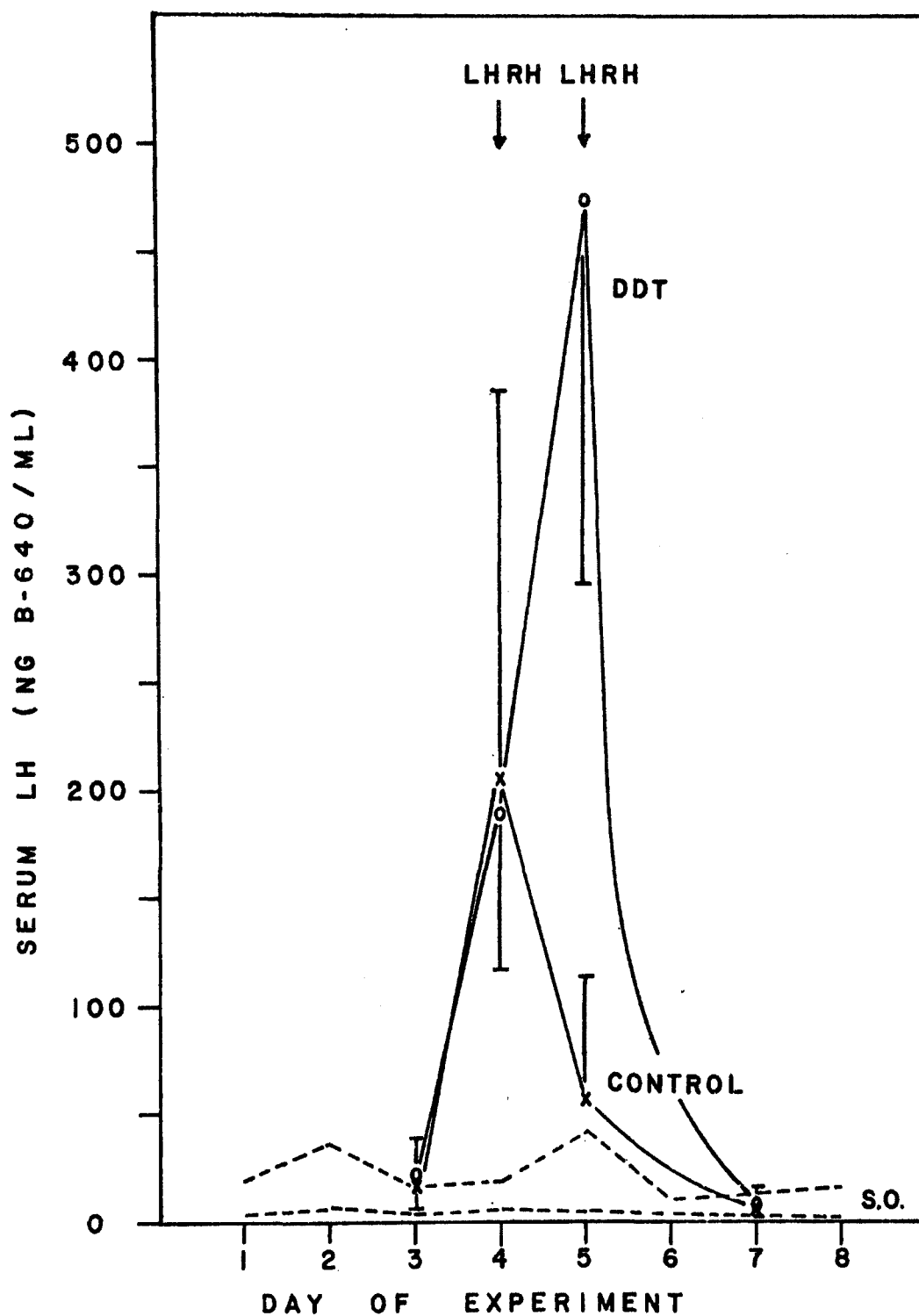
obtained for rats reared in 12-pup litters and suckled by
dams injected for 25 days. Plots for the other litter
sizes and for the other length of dam injection have not
been included.

Serum LH levels did not differ on the third day of the
test due to treatment or length of treatment of the dam.
However, 12-pup litters appeared ($p < 0.05$) to have higher
basal serum LH values than did 6-pup litters. The result
was probably only apparent because many of the values were
at or below the reliable limit of the RIA.

On the first day of LHRH injection, DDT-treatment,
length of dam injection or litter size did not, themselves,
cause a difference in response to LHRH. Some interaction
between litter size and either the presence of DDT-exposure
or the length of DDT injection into the female was
indicated ($p < 0.05$), implying some dependency of the
response to injected LHRH on the size of the total
absorbed dose of o,p'-DDT. The absence of any effect of
the major treatment conditions, however, does not allow
clear interpretation of the results.

The second day of LHRH injection yielded a highly
visible difference in the response of those rats in litters
of 6 or 12 pups neonatally exposed to o,p'-DDT by a dam
injected for 25 days and the response of rats exposed only
to DMSO under similar conditions (Figure 33). The
DDT-treated rats gave serum LH responses to injected LHRH
2.5-10 times those given by control rats ($p < 0.001$).

Interaction between treatment and both length of treatment alone ($p < 0.005$) and length of treatment and litter size ($p < 0.05$) imply that total dosage of o,p'-DDT may have been the deciding factor in the observed results. Rats neonatally suckled by females injected for only 5 days did not differ from their controls.

No differences among LH values from any of the groups or treatments was evident in the sera collected at the termination of the experiment. The LH values of the pituitary extracts (not presented) implied a dependence of pituitary LH content on the length of treatment of the dam ($p < 0.005$), i.e., on the number of injections given the dam or, possibly, on slightly different amounts of injected LHRH for the animals in one half of the experiment (reared by dams injected for 25 days) versus those in the other half (reared by dams injected for 5 days).

Interpretation of the results of this experiment is definitely complex. First of all, unintentional experimental differences between the animals derived from dams injected for 25 days and those derived from dams injected for 5 days may have arisen because the former group was tested for LHRH response 5-10 days before the latter. This may have generated artifactual differences which would be reflected in significance of the experimental factor associated with the length of the injection period for the dam regardless of the presence or absence of DDT-treatment. If, for example, LHRH degraded measurably

during storage in solution, the responses to LHRH for the
animals reared by dams injected for 5 days and injected
with stored LHRH would be less marked than those in animals
reared by dams injected for 25 days and injected with
freshly diluted LHRH. Such a difference would explain
the overall lower serum and pituitary LH values seen in
the second group tested and would also explain the absence
of a measurable potentiation of the response to the second
LHRH injection in rats suckled by dams injected for only 5
days. If, on the other hand, no such problems occurred,
the difference in the results for the two dam injection
periods might be explained on the basis of lower doses
having been received by those animals suckled by dams
injected for only 5 days. Or they might be explained on
the basis of nutritional changes, which were not reflected
in body weight and which potentiated the effect(s) of DDT,
having occurred in rats suckled by dams injected for 25
days.

Whatever the cause of the difference in outcome
related to the 2 different treatment periods, the experi-
ment dealing with animals suckled by dams injected for 25
days with o,p'-DDT did demonstrate a marked increase of
the serum LH response to the second injection of LHRH. In
rats from both litter sizes the potentiation in DDT-exposed
rats was coupled with a decrease in response in control
animals. This may indicate that the two groups differ in
either their rates of LHRH degradation or their rate of

LH synthesis. Though the amount of LHRH injected was large, calculation of the effective concentration following injection, based on the half-life of 6.5 minutes given by Schally et al. (9), indicated that the injected dose would be cleared in less than 5.3 hours. Even slowing this down 2-3 fold would not explain the results on the basis of residual LHRH. Potentiation based on the priming effect of closely spaced injections of LHRH (172) would similarly not be favored due to the length of time between the doses of LHRH.

The remaining possibilities for explaining the differences seen between the control and DDT exposed groups may involve altered sensitivities of target organs in the hypothalamo-hypophysial-gonadal system to circulating hormones as would be caused by neonatal imprinting or they may involve the continued presence of residual DDT which interferes with the feedback suppression of pituitary LH release and/or synthesis. The second mechanism is highly unlikely since measurements of DDT residues in 120 day old females injected neonatally with 3 mg of o,p'-DDT demonstrated no more residues than control rats (53). Since the animals in the present experiment were even older male animals, which should have metabolized DDT even faster than females (105,107), residues should not act directly during the period used to measure the responses to LHRH. The alternative mechanism, i.e., imprinted sensitivity changes which are expressed in the presence of high levels of

circulating LHRH, is in concert with my earlier findings
of probable alterations in the gonadal steroidogenic
control loop. The opposite direction of the changes in
serum LH seen in the DDT-treated rats in the response to
adult castration and the response to repeated LHRH injec-
tions may be manifestations of different DDT effects or
merely the expression of a different facet of the same
effect.

The smaller response of serum LH observed with the
second injection of LHRH, relative to the first, in con-
trol animals may be produced by a depletion of releasable
pituitary LH stores. Initially, this would be due to the
presence of massive amounts of LHRH. Subsequently it
might be due to direct suppression of LH synthesis within
the depleted pituitary by the large quantities of testos-
terone generated as a result of the LH release brought
about by the first injection of LHRH. The precise
mechanism is not clear at this time.

In summary, treatment of neonatal animals with
o,p'-DDT via an injected dam appears quite effective.
Nearly all of the o,p'-DDT which enters the gut in the
milk was absorbed by suckling pups. Such treatment was not
effective in altering body or organ weights, as might have
been anticipated on the basis of both the calculated
absorbed dose and the previous findings in the present
studies. DDT-treatment via the dam was not effective in
altering overall serum LH periodicity. It was, however,

effective in modifying the serum LH responses to adult

castration and to repeated large injections of LHRH.

CHAPTER 5

DISCUSSION OF ANIMAL STUDIES

This study began, experimentally, as an attempt to measure direct interaction between the metabolism of o,p'-DDT and the androgenic steroids in the testes of adult male rats chronically exposed to o,p'-DDT, i.e., it began as a study of interactions _in vitro_. During the course of initial experiments, however, technical problems due to a variety of causes limited the results obtained to a few very imprecise observations which indicated little or no effect of DDT exposure. At that juncture (1971) a growing awareness of the literature made it apparent that very few effects of DDT or its analogs had been observed - or tested for - _in vivo_, much less _in vitro_ - in the steroidogenic tissues of any mammalian species; the effect of o,p'-DDD on the adult adrenal in several species was the only real exception. Since the available literature indicated only that o,p'-DDT was a weak estrogen (22, 24,146,148,152) the best approach to the problem appeared to be to conduct a brief but very broad study _in vivo_ which would indicate which portions of the endocrine system would be most profitably studied by subsequent studies run _in vitro_. Body and organ weights seemed to be the most appropriate measurements for such a screening experiment since they should indicate changes which might occur in the the adrenals, the gonads, the liver and the

hypothalamic-pituitary complex. Before the study was begun, however, a report by Heinrichs et al. (53) became available. It indicated that some marked changes occurred in the steroidogenic system of the female rat following neonatal injection with moderate doses of o,p'-DDT. The approach of using neonatal injection appeared profitable both because it yielded quite demonstrable changes and because it allowed the use of a large body of literature on neonatal steroid imprinting in the rat (57-63,69-71) as a background for comparisons. After several short experiments using the neonatally injected male had failed to demonstrate any changes in adult organ weights, it was decided that a longer study incorporating several ages, several dose levels and examinations of organ histologies, serum corticosterone and serum LH might prove more illuminating. This study, described in Chapter 3, Section II, did yield an indication that development of adrenal morphology, and possibly function, was effected by neonatal injection with a range of concentrations of o,p'-DDT. The experiment, however, also indicated that if any changes in parameters dealing directly with gonadal steroidogenesis had occurred they were rather subtle. Subsequent experiments (Chapter 3, Section III) based on the work of Rybakova (28,29) and Gellert et al. (54) concentrated on measurements of serum LH under conditions which stressed part of the hypothalamo-hypophysial-gonadal axis. They were designed to determine "reserve" capacity, i.e., the

capacity to respond to stress. Adult castration was found in such experiments to generate a picture of altered "reserve" or response capacity in male rats neonatally injected with o,p'-DDT.

While these experiments on injected rats were being conducted a parallel series was initiated using the natural source of environmental dietary contaminants for the pup, i.e., the mother's milk, as the vehicle for neonatal exposure to o,p'-DDT. Again, as with directly treated animals, the results indicated that some changes in the capacity of the gonad-brain-pituitary axis had occurred due to DDT exposure but that the changes were exposed only by adult castration or exogenous LHRH. However, the changes occurred at moderately low levels of exposure; 100 ug of DDT in a 5 day old, 10 g pup is a body burden of 10 ppm which is near the levels found in some environmental exposures. Taken together the results from the two sets of experiments, in directly injected and suckled rats, indicated that at least two loci of o,p'-DDT effects were demonstrable within the realm of the steroidogenic systems: 1) the immature adrenal cortex, and 2) the hypothalamic-pituitary complex.

I. The Change in the Immature Adrenal Cortex:

The adrenal has been implicated in the onset of puberty in the female rat (98) and in the maturation of the testicular response to hCG (78,79). If the process of maturation of the adrenal is interfered with it is

conceivable that the maturation of other tissues influenced by the adrenal will be affected. The coordination of the rise and fall of several hormone levels can have marked influence on the normal maturation of tissues, e.g., only the proper coordination of LH, FSH and E_2 will allow a developing follicle within the ovary to develop to the stage of ovulation; other conditions result in atresia (89). Because of this, either adrenal degeneration, or a precocious adrenal development, as was found in these studies, may result in pathologic states which could effect the development of any of a myriad of other tissues which are known to be effected by the function of the adrenal cortex (206).

If precocious development is viewed as accelerated aging, then the precocious development of the adrenal cortex seen in animals treated neonatally with estradiol or o,p'-DDT may result in eventual degeneration similar to that seen after treatment of the adult adrenal of several species with o,p'-DDD. Since the adrenals of o,p'-DDT-treated rats did not exhibit the specific type of involuted morphology seen in other species treated with o,p'-DDD (37-49) the DDT effects seen may not be the result of this metabolite. Furthermore, since the adult rat exhibits a refractoriness to the action of o,p'-DDD it is improbable, though not impossible, that o,p'-DDD also would be effective in the neonate.

A more likely explanation for the results is that the estrogenicity of o,p'-DDT may be the cause of its action on the adrenal. The adrenal is known to be positively effected both morphologically and functionally by the presence of circulating estrogen (4). This is true functionally from at least 25 days of age onward and morphologically (normallized weight) from 50 days onward (see Chapter 3, Section II). Since steroids influence tissues by way of intracellular mechanisms subsequent to binding a steroid receptor, it may be assumed that o,p'-DDT may function by binding to estradiol receptors. DDT has been shown to bind to uterine estradiol receptors with affinities of the order of $1\text{-}10 \times 10^{-6}$ M (K_d) - affinity for estradiol is 4×10^{-10} M (26). It has also been shown to bind to hepatic P-450 (221) and adrenal P-450 (222,223) in such a manner that the substrate difference spectra of P-450-steroid and P-450-DDT are similar (Type I). Furthermore, the estimate of estrogenic potency found (Chapter 3, Section II.C) for intermediate dosages of o,p'-DDT on the basis of corticosterone secretion agrees with that found using a totally different assay of estrogen potency, the rat uterine-glycogen bioassay (147).

Still, there is the element of the time lag between injection and the observation of an effect which must be explained before an adequate hypothesis based on estrogenicity is possible. The potential for the sesame oil vehicle to act as a depot has already been discussed in

regard to the steroids; the same arguments apply to
o,p'-DDT. Another possible mechanism of estradiol and
o,p'-DDT action would be via a peripheral imprinting of
the adrenal cortex analogous to those encountered in the
gonads and/or the liver. Finally, the adrenal precosity
found in animals neonatally injected with estradiol or
o,p'-DDT may be an indirect effect of hepatic microsomal
induction. In this instance an early compensatory hyper-
trophy of the neonatal adrenal cortex would take place to
maintain serum corticosteroid levels and would not have
fully regressed by 25 days of age. This explanation is,
however, unsatisfactory on two bases: 1) estradiol in
adult rats inhibits microsomal metabolism and induction
(5), and 2) the serum levels of corticosterone are higher
than normal. Still, unless the levels of free corticos-
terone in serum are measured rather than total corticos-
terone (which includes corticosterone bound to corticos-
teroid-binding-globulin (CBG)) the actual effective level
of the hormone in serum is unknown and the second argument
is weakened.

Obviously, a great deal of research remains to be
done to clarify the meaning and significance of the
observed early development of the adrenal cortex in
treated animals. If the present studies were to be carried
forward a repeat of the first 20-30 days of the direct
injection study using several doses of o,p'-DDT dissolved
in DMSO or ethanol and similar doses dissolved in sesame

oil could be used to verify the initial observations while
eliminating the question of long-term, depot-injection
effects. Such studies would also provide tissues for some
of the following measurements:

1. Measurements of cholesterol conversion into gluco-
corticoids in quartered adrenals in the presence or
absence of ACTH and/or estradiol or o,p'-DDT would deter-
mine the level of functionality of the adrenal at the time
the altered histological appearance was in evidence and
would also test for the altered adrenal's sensitivity to
ACTH, estradiol and o,p'-DDT.

2. Measurements of serum corticosterone and CBG would
establish the amount of free serum corticosterone and could
be used to help differentiate direct effects on the adrenal
from those mediated by the liver.

3. Measurements of hepatic microsomal breakdown of
corticosteroids would also differentiate direct effects on
the adrenal from those mediated by the liver.

4. Measurements of serum ACTH would help to deter-
mine whether the current observation is due to the con-
tinued presence of estradiol or o,p'-DDT or to an imprinted
change in pituitary output of ACTH or hypothalamic produc-
tion and release of CRH.

Other studies involving surgical manipulations, e.g.,
hypophysectomy, of the dosed animals are, of course,
possible. However, their interpretation would probably be
less precise than the more direct measurements listed above.

II. The Change in the Hypothalamic-Hypophysial Complex:

Because LH (and FSH) are involved in controlling steroidogenesis and spermatogenesis the observed changes in the hypothalamic-pituitary axis probably have the potential for a more direct effect on reproductive capacity and/or development than those described for the adrenal. However, the apparent normality of the development of the DDT-treated animals argues against such an effect. On the other hand, it is doubtful whether any such change would manifest itself in altered reproductive performance in laboratory rats for whom the stresses of obtaining and maintaining a mate are in most situations considerably less than in animals in the wild state. Therefore, situations in which a somewhat abnormal LH response capacity might play a role in depressing reproductive success such as courtship or repulsion of rivals may not occur in tests of reproductive capacity with such laboratory rats.

The subjective appearance of at least transient oligospermia in DDT-injected males could result directly in reduced reproductive success. The observations of Krause et al. (208) might be used at this point to support the argument for oligospermia and reduced fecundity, however, the   quantities of DDT which were administered in that study could well have caused hepatic induction and testosterone breakdown long after their injection. For that reason that study is potentially misleading and

cannot be used as clear support for an argument favoring a
hypothalamic or pituitary alteration resulting in impaired
spermatogenesis in DDT-treated rats.

Even if the observed changes in LH response capacity
does not have any effect on reproductive success it is
still of interest from the viewpoint of understanding the
mechanisms controlling LH synthesis and release, i.e.,
endocrine control. To my knowledge it is the first
observation in the male rat of long term effects caused
by a chemical agent on hypothalamic-pituitary responses to
adult castration or LHRH injection. Therefore, it leads
to some interesting speculations concerning the mechanism
of the response and provides a base for future experimenta-
tion. The apparent differences in the directions of the
two effects (LHRH-response and adult castration response)
may be based on preservation of the cyclic center of the
hypothalamus in DDT-treated male rats but not in the
controls (Chapter 1, Section II). Such a preservation
might occur by occupation of the center's estrogen recep-
tors by the very weak estrogen, o,p'-DDT, during the
critical phase of hypothalamic development. After the
critical imprinting period had passed and the dosage had
been discontinued the o,p'-DDT (or the actual active
metabolite) would be gradually eliminated by catabolism.
Since endogenous testosterone would thus be unable to act
neonatally, the cyclic center would remain intact. Adult
castration, which removes the source of negative feedback

onto the tonic center (57-61), would not reveal the presence of such an intact cyclic center. But large doses of exogenous LHRH, LH, testosterone or, possibly, estrogen might stimulate an LH surge of the type seen in the cycling female rat near ovulation. These agents could cause the appearance of an LH response superimposed on an LH surge on the second day of LHRH injection in DDT rats which would not be possible in control rats. This would be the case because the endogenous source(s) of LHRH, which would be necessary for replenishment of pituitary LH stores after the first injection with LHRH, would be suppressed by the testosterone produced as a result of the first LHRH injection in the control but not in the DDT-treated rats. This mechanism would not explain the lower plateau of the DDT-treated rats after the adult castration unless some change in the tonic center had also occurred during its period of exposure to o,p'-DDT. Still, simultaneous changes in both cyclic and tonic centers have been described for neonatal treatment with androgens and estrogens (61) and thus would not be implausible for o,p'-DDT.

A second more inclusive explanation would involve either a continued presence of o,p'-DDT which could bind to hypothalamic steroid (estrogen?) receptors or an imprinting which caused a modification in the number or affinity of the testosterone (estrogen?) sensitive receptors within the brain. Either of these conditions would bring about a state in which a fraction of the total

steroid receptors were, in effect, inactive or occupied by a weak steroid analog. This fraction would be unavailable to act in totally suppressing LHRH release but would constantly function at a low level to suppress unstressed LH levels. This situation would result in an inability of testosterone, generated perhaps by a burst of LH released in response to a single LHRH injection, to fully suppress LHRH release and therefore to prevent stimulation of the replenishment of releasable LH within the pituitary. It would also result in an effectively slower fall of serum testosterone relative to receptor concentration after castration in DDT-treated as opposed to control animals. This, in turn, would cause a slower rise in serum LH following castration. Thus, altered numbers of steroid receptors could explain both the lower serum LH plateau after adult castration in the DDT-treated rat and the lack of a difference between serum LH levels in treated and untreated animals which were neonatally castrated and examined as adults (Chapter 4, Section III).

Again, as was the case with the adrenal, additional postulates for mechanisms cannot be excluded. Only further studies will fully illuminate the picture. The initial observations may help guide research on the neonatally dosed male rat by eliminating several approaches from further consideration and by indicating several research directions which may be productive :

1. Measurements of the adult castration response for longer periods of time after castration should determine if a final equilibrium state similar to that of controls does exist.

2. Measurements of LHRH response should be repeated using more than two injections of LHRH to further define the magnitude of the response. Combined injections of LHRH and testosterone or o,p'-DDT might also give clues as to the functional state of the tonic hypothalamic center.

3. Measurements of pituitary release of LH and FSH _in vitro_ in response to LHRH, testosterone and o,p'-DDT should be done to provide data on the comparative sensitivities of the pituitaries from DDT-treated and untreated animals. Such studies could also measure sensitivity to corticosterone and determine basal ACTH secretion.

4. Histochemical or RIA measurements of brain LHRH levels could also be done and would probably reveal much concerning the relative sensitivities of the brains from DDT-treated and untreated animals to stimuli such as exogenous LHRH, testosterone, o,p'-DDT, ether, light, etc.

5. Further studies involving testicular sensitivity to LH or hCG could also be conducted. They must, however, utilize cholesterol as substrate (224). An initial measurement using tritiated acetate of very high specific activity, conducted during these studies, failed to demonstrate any incorporation of label into the isolated

testosterone in either the control tissue, as could have been predicted (224), or the tissue from rats neonatally injected with 10 mg of o,p'-DDT. This result could only imply that the large cholesterol pool (or whatever other mechanism acts to prevent rats from incorporating labelled acetate into testosterone) had not been disturbed sufficiently in DDT-treated rats to allow incorporation of label into testosterone even in the presence of 10 ug/ml hCG.

6. Studies of specific steroidogenic pathways in testes taken from DDT-treated and control groups could be studied. This approach formed the basis for a few in vitro experiments during this study which will be discussed in Chapter 6. Briefly stated, they have shown no conclusive effect of neonatal treatment with o,p'-DDT on adult testicular steroid production. In view of the known ability of o,p'-DDT to bind to receptors and the P-450 forms found in the adrenal (223) more data should be collected to test for direct interference of o,p'-DDT (or its metabolites) with steroidogenesis.

If the male rat continues to be an object of study, other types of measurements, including assays for serum androgens, FSH and prolactin, could well be used to monitor hormonal status in much the same manner as has been attempted in this research.

III. <u>Summary</u>:

Further investigation of the parameters studied or proposed in this project may well allow more rapid toxicological screening of materials for effects on the functional status of the steroidogenic endocrine systems. This should also provide support for, if not an alternative means to, predictions of effects on reproductive capacity and viability. Use of the neonatally treated animal is to be recommended when possible because its susceptibility to permanent damage is apparent in these and other studies (53,54,57-61,150,151).

The studies demonstrate the type of data which may be generated in a broad investigation. As a consequence of emphasizing scope and of the time consumed in examining it, depth of study of the positive results has been sacrificed. The projected experiments were designed to measure the systems and tissues which have been demonstrated in this study to be effected by neonatal treatment with o,p'-DDT in more detail.

The whole-animal experiments described demonstrate that neonatal treatment with o,p'-DDT does effect the steroidogenic system of the male rat. It causes change in

at least two loci, the immature adrenal cortex and the adult hypothalamo-pituitary axis; such treatment does not, however, grossly affect development. At least part of the effects of the treatment may be due to a metabolite of o,p'-DDT rather than the parent compound. And, the parent compound and at least some of its metabolites are effectively transferred to suckling pups who are, in turn, affected by such naturally administered material.

Though these results seem modest and many other experiments building on them are possible, let it suffice that these experiments serve as a basis for more specifically focused studies which may yield mechanistic information on the mode of o,p'-DDT action, more insight into the control and development of the steroidogenic endocrines and, lastly, more specific rapid and simple testing procedures which may be of use in clinical and environmental monitoring situations.

CHAPTER 6

INVESTIGATION OF Δ^5-PREGNENOLONE CONVERSION IN VITRO

The original conception of this project
involved an examination of the mutual interactions of the
metabolism of o,p'-DDT and the metabolism of steroids
within the testes of animals which had been treated with
o,p'-DDT or a vehicle. Since o,p'-DDT had been shown to
act as an estrogen (22,24,53,146-148) and to bind to the
hepatic P-450 involved in steroid hydroxylations (5,12-15,
18,128,129), and since the closely related metabolite,
o,p'-DDD, was known to interfere with steroidogenesis in
the adrenal (37,40,42-47), it appeared reasonable to study
the interactive metabolisms of DDT and steroids within the
testes. That is, it seemed reasonable to attempt to
measure the effects of o,p'-DDT and the metabolites which
it formed in vitro on the metabolism of Δ^5-pregnenolone
to androgens in vitro and to concurrently measure the
effects of Δ^5-pregnenolone and the metabolites, e.g.,
androgens, which it formed in vitro on the metabolism of
o,p'-DDT to its metabolites in vitro. To that end incuba-
tion procedures, extraction protocols and metabolite
assay methods were designed to allow measurements to be
done on both sets of metabolites when they were formed in
a single incubation mixture. The attractiveness of this
combined approach lies in its efficiency in terms of the
time, materials and tissues used to generate a set of

results which reflect the multiple forms of interaction which might occur <u>in vivo</u>. The procedures used in these studies were developed and improved throughout much of the study; some of them still need further improvements (present versions were described in Chapter 2, Section IV.F.1).

The examination of the metabolisms of two rather different sets of compounds required that some adjustments of the incubation conditions commonly used with each be made to accomodate incubations of both simultaneously. The discrepant time courses for metabolic incubations previously done on the steroids (192,225-227) and DDT analogs (104,113,133) indicated the necessity of measuring steroid and DDT metabolites in aliquots of incubations taken over the course of a reasonable time period (1 hour). Because the potential for DDT interactions with the mitochondrial P-450 was unknown, this subcellular fraction was not discarded before incubations were conducted although its presence for conversions of Δ^5-pregnenolone to androgens was unnecessary (Chapter 1, Section I) (6). The similarity of cofactor requirements for steroid conversions in the testis (6) and for DDT conversions in the liver (113), along with the length of the incubation time, dictated the addition of reduced pyridine nucleotides or systems capable of generating them. Finally, the protein concentrations used appeared to be an adequate compromise

between those used previously in steroidal studies (192, 226) and in DDT catabolism studies (104).

The possibility that o,p'-DDT might act at any of several metabolic steps on the pathways from Δ^5-pregnenolone to testosterone brought up the question of the value of measuring intermediates. Since time course data was already to be generated it was decided that several of the known intermediates (progesterone, 17α-hydroxyprogesterone, dehydroepiandrosterone and Δ^4-androstenedione, i.e., products 4 or D, 5 or E, 1 or A and 2 or B in the computer program printout of Appendix I) should be examined at each time point in addition to the substrate and end-product in order to provide a good picture of the metabolic flux from the substrate, Δ^5-pregnenolone (substrate and product 3 or C in the computer printout of Appendix I), to the end-product, testosterone (product 2 or B in the computer printout of Appendix I). Since the metabolism of o,p'-DDT in the testis was unknown the potential production of fairly polar metabolites could not be excluded. The procedures adopted for extraction, however, limited examination of the metabolites of DDT to those which were neutral. Because of this limitation on the number of DDT metabolites which could be examined, because of technical limitations on time available for the entire proposed analysis and because alterations of steroid metabolism due to treatment with DDT were more critical to the focus of the project on altered

reproductive capacity, emphasis was placed on examining steroidal flux within the incubation systems given in Table 3 (Chapter 2, Section IV.F.1).

Steroid flux of this type appeared to be best studied by using radioactive substrate (195,228,229). Use of a second label added after stopping active metabolism could provide added information by accounting for losses which occurred during extraction and purification. Similar information, or information additional to it, could also be generated by using a sensitive mass detection system. Since gas chromatography had already been used extensively for measuring steroids (195,228,229), and because it was a method of choice for measuring DDT metabolites, it was chosen as the mass determination system. The GC was also capable of separating a number of steroids simultaneously and, thus, could serve as a separation, as well as a quantitation instrument. Therefore, it seemed reasonable that if radioactivity could be successfully monitored for individual chromatographic peaks it should be possible to generate a series of doubly labelled intermediates and analyze them simultaneously by combined GC-double label scintillation techniques. Since the main object of the overall studies was to analyze the differences between DDT-treated and untreated animals some decreased precision in separations and peak identifications was allowable if samples from incubations of tissues from both types of animals were analyzed similarly.

With this experimental conception in mind a model for
the analysis of a single compartment metabolic system
emerged.

$$Y \rightleftharpoons S_Y + \overset{\overset{S}{\downarrow^{3}H}}{} + S_E \rightleftharpoons P_Y + P_E + P_{3_H} + P_O + P_Z \rightleftharpoons Z$$

$$\downarrow + S_C = Cl \qquad\qquad \downarrow + P_C = Cl'$$

$$A_S = \left\{ S_Y + S_E + S_{3_H} + S_C \right\} \quad A_P = \left\{ P_Y + P_E + P_{3_H} + P_O + P_Z + P_C \right\}$$

$$\Downarrow \quad \Downarrow \quad \Downarrow \qquad\qquad \Downarrow \quad \quad \Downarrow$$

$$C2 \quad S_{NE} \quad C3 \qquad\qquad C2' \quad P_{NE} \quad C3'$$
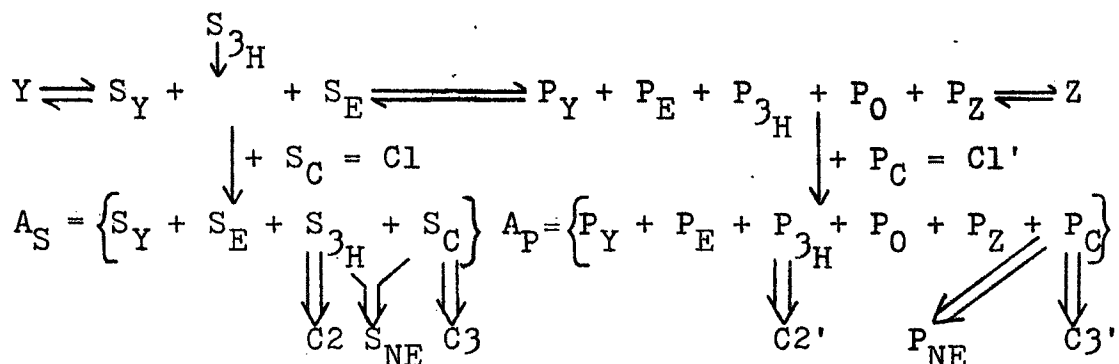
In this model Y is the endogenous precursor for formation
of substrate S, and subsequently for the formation of P
and Z from S. During the incubation, Y produces amounts
of S and P (S_Y and P_Y) which are in addition to the
amounts of endogenous S and P (S_E and P_O) which exist prior
to the incubation. S_E also forms an amount of product,
P_E, during incubation as does Z, any alternate source of
product, P_Z. If tritiated substrate S_{3_H} is added prior to
incubation it also forms product, P_{3_H}, during the incuba-
tion. If a sample of an incubation is stopped at time
zero and amounts of carbon-14 substrate and product, S_C
and P_C, are added, the total concentration of substrate is
due to S_E, S_{3_H} and S_C (two of which are known) (Equation
III, Appendix I). Similarly, the product contained in the
sample would be made up of P_O and P_C (one of which is
known) (Equation IV, Appendix I). Because all but one
portion of the mass is known a priori a zero time point
should allow determination of the mass of endogenous

substrates (S_E) (Equation X, Appendix I) and endogenous products, P_O (Equation XI, Appendix I). At times greater than zero substrate is made up of S_Y, S_E, S_{3_H} and S_C (Equation V, Appendix I) while product is made up of P_Y, P_{3_H}, P_O, P_Z and P_C (Equation VI, Appendix I). In both cases if mass is corrected for any nonenzymatic breakdown by measuring an incubated system containing only S_{3_H}, which results in substrate and product masses of S_{3_H} + $S_C (= S_{NE})$ (Equation I, Appendix I) and P_C $(=P_{NE})$ (Equation II, Appendix I), the conversion from Y to S_Y and P_Y (Equation XV, Appendix I) and from S_{3_H} and S_E to P_{3_H} and P_E (Equation 2, Appendix I) can be computed. This is done by recalculating the specific activity of the tritiated substrate to include the initial mass of the cold endogenous substrate S_E at time zero or the entire measured mass of the substrate other than S_C at times greater than zero.

Chromatographic data, A_S and A_P, or other measurements of total mass can be combined with the levels of radioactivity (C1, C2, C3, C1', C2', C3') to generate values for mass of the various subsets of total mass of substrate or product. By measuring mass and radioactivity at time zero S_E and P_O are determined as stated above. Radioactivity based on the initial specific activity of S_{3_H} allows computation of the quantities of S_{3_H} and P_{3_H} at any time beyond zero (Equation 2, Appendix I). If the specific activity of S_{3_H} is altered to include S_E then P_E can also be determined (Equation 3, Appendix I). If the specific

activity is adjusted to include S_Y as well, P_Y is also
determined (Equation 4, Appendix I) and by default, P_Z is
generated (Equation XVI, Appendix I). Total net conver-
sions for all forms of substrate or for all forms of
product could be measured at any time of incubation by
correcting the measured masses for added S_{Cl} (Equation XII,
Appendix I) or $P_{Cl'}$ (Equation XIII, Appendix I).

The computer program METFLX, presented in Appendix I,
was generated from this model and was subsequently updated
to conform to empirical observations. The program pre-
supposes nothing and generates negative concentration
values for a product if conversions to later products
exceed its formation from endogenous product and all forms
of substrate. It also reflects analytical error in the
radiochemical assays or mass determinations, especially if
too large an amount of cold carrier (added to increase
recovery) is used.

The program uses recovery corrections made possible by
the added ^{14}C-steroids which were used as internal recovery
standards. It bases chromatographic determination of mass
on the detector response of known products relative to a
chromatographic internal standard and on calibration plots
of $\log_{10}$ (relative response) versus $\log_{10}$ (concentration)
which were generated from mixtures of all of the compounds
of interest and the internal standard. The use of relative
peak areas minimizes errors due to misinjection of samples
or to slight changes in the sensitivity of the mass

detector from injection to injection. It corrects results for individual chromatographic peaks for variations in sample volume injected, in sample size, in efficiency of capillary tube capture, in differences in mass flow to the detector and capture tube, in differences between internal standard concentration in the samples and calibration mixtures, etc. Finally, it bases radioactivity determinations on the specific activity of the added substrate and the computed ^{3}H-dpm and ^{14}C-dpm for captured effluent.

Proof of this program was delayed due to problems involving identification of appropriate GC conditions which would optimize steroid separations and give the cleanest results for the proposed approach. Over thirty single and combined column phases were examined for their ability to separate the steroids which were chosen for study. Parent compounds, silyl-methyl-oxime derivatives, silyl-butyl-oxime derivatives, silyl-benzyl-oxime derivatives, acetate derivatives and benzoate derivatives were examined on one or several of the phases studied. Choice of cholesterol-3-propyl ether as the chromatographic internal standard was made after examining its chromatographic properties with respect to those of both the steroids of interest and 5α-androstane, 5α-pregnane, estradiol, estriol and cholesterol. The standard had the desirable characteristics of being stable, fairly similar in structure to the compounds of interest and of

chromatographing in a fashion quite similar to the
steroids of interest. A series of modifications of the
effluent capture procedure led to the final choice of a
simple chilled capillary capture tube. The choice of the
GC column conditions and steroid form were a compromise
of obtaining workable separations and capture efficiencies
without requiring extensive preliminary treatment of the
samples to be measured - the parent compounds were
separated on a 350 cm x 2 mm column containing Gas Chrom
Q coated with 1.5% each of SE-54 and OV-7 by using a
temperature gradient of 1°/minute from 175° to 275^oC and
a flow rate of 25 ml/minute for the carrier gas, nitrogen
(Chapter 2, Section IV.F.2). Separations were done using
relatively crude extracts containing amounts of carrier
steroid, sufficient to yield good recoveries of radio-
active steroids throughout isolation and analysis.

By the time these technical matters had been resolved,
the whole animal studies were well under way. The gen-
erality of the program which had been written made it
quite desirable to test it on experimental results even if
part of the initial strong impetus to look at direct
interactions of DDT and steroids within the testes had
somewhat waned. Since the testicular steroid conversions
in DDT-treated and untreated animals could still fit into
the scope of the overall project and still might generate
some insight into either any imprinted changes in steroid
metabolism within treated testes or any changes in

steroid-DDT interactions within treated tissues, it was decided to test the program and do the originally proposed measurements on the testes from DDT-treated and control animals.

Testes derived from rats exposed neonatally not only to DDT, but also to EV and TP were incubated under the protocol given in Table 3. The incubation mixture aliquots were extracted and stored. Extracts from incubations Type 2 (Δ^5-pregnenolone only) and Type 5 (non-enzymatic incubation) were measured under the conditions listed in Figures 11 and 12 (Chapter 2, Section IV.F.1 and 2). Counting results were normalized to the values which would have been expected for 1 ul injection volumes to allow more rapid pre-program comparisons. Relative peak areas were computed both by means of the fourth revision of an automatic integration program which linked the GC to an electronic calculator and by manual triangulation. Examination of the raw data indicated adequate capture of ^{3}H- and ^{14}C-counts (minimally 50-100 dpm/ul above chromatographic background counts for each radioactive label) and good agreement between automatic and manual integration for relative areas for all but the 17α-hydroxyprogesterone peak, which only manual integration measured. Plots of the ^{3}H/^{14}C ratio (not shown) for each of the steroid peaks in the chromatograms from the testicular incubations of control animals (neonatally injected with sesame oil) indicated the predicted pattern of ^{3}H-labelling.

Tritiated substrate declined to essentially zero levels by
30 minutes of incubation. The products of both the Δ^4-
and Δ^5-pathways, testosterone and Δ^4-androstenedione,
rose beyond 5-10 minutes of incubation until essentially
all the ^{3}H-label was incorporated into them by 30-60
minutes. Progesterone and 17α-hydroxyprogesterone rose
to a peak by 10 minutes of incubation and declined to base-
line by 30 minutes. Dehydroepiandrosterone (or the label-
led compound associated with that chromatographic peak -
Chapter 2, Section IV.F.2) rose slowly after 10-30 minutes
of incubation to a level of about 1/5 that seen for
testosterone plus Δ^4-androstenedione. (This agreement of
the radioactivity pattern for dehydroepiandrosterone with
the predicted pattern tends to support the identity of the
label in this chromatographic effluent with the carrier
steroid.) Analogous plots for testicular incubations done
on tissues from rats neonatally injected with EV (200 ug
total), TP (1000 ug total) or o,p'-DDT (500 ug total)
indicated somewhat similar profiles.

METFLX was run using the results from the incubation
of all the treatment groups. The resultant data are pre-
sented in Appendix I, as an example of the program
printout.

The plots shown at the end of the printout illustrate
conversion of the ^{3}H-label and demonstrate patterns
similar to the plots obtained from ^{3}H-/^{14}C- ratios, as
expected. It can be seen in the second plot (Treatment II)

that steroid metabolism in EV-treated animals appears more
rapid than in the controls; this may, however, be due to
a larger number of Leydig cells per gram of testicular
weight than in the control (62) as may also be the case
in the third plot (Treatment III, TP treatment). Both
sets of results, however, agree with the findings of
Joseph and Kincl (230) in similarly treated rats that
metabolic capability is preserved in the testis of such
animals even though spermatogenesis is largely halted.
The fourth plot (Treatment IV) which describes the situa-
tion for DDT-treated animals appears different in that the
metabolic conversions appear much slower. This may,
however, be an artifact due to inadequate aeration of the
incubation since a larger incubation volume was used for
this tissue; it was incubated somewhat earlier than the
steroid and control tissues. Still, it may also indicate
a reduced rate of steroid synthesis in treated animals
and with the results from the whole animal studies imply
an overall sluggishness of response within the hypo-
thalamo-pituitary-testicular axis of DDT-treated rats.

Other data given in the program printout show masses
which reflect the amount of carrier steroids (about 300
nmoles each) which had been added during the isolation
procedure. It is obvious that this mass is too large to
allow good estimation of many of the parameters the program
was designed to calculate. An attempt to measure the
steroids in this system by using a very large incubation

but no carrier steroid demonstrated that GC-measurement using the FID detection system was possible, but that the extract would have to be more rigorously purified prior to injection onto the column in order to allow the accurate manual capture of the desired effluents.

The approach at this stage appears usable if a few modifications are made; the program seems to function very well, generating accurate results for the desired parameters and being limited only by the precision of the values on which it must act. Modifications of this approach which could make it quite useful in the area of steroid metabolism and/or comparative steroid metabolism (as here) would be: 1) to move to the use of electron capture detection of appropriate derivatives to allow precise measurements of very small amounts of steroids; 2) to use minimal amounts of carrier, e.g. 1-10 ng per steroid, to decrease artifactual change due to deviations in carrier concentration, 3) to interpose TLC, lipophilic Sephadex chromatography or some other purification and/or steroid-group separation between extraction and GC analysis to assist in decreasing both chemical and radio-activity background, and 4) to use capillaries filled with terphenyl crystals to optimize capture efficiencies so that smaller amounts of ^{14}C-carrier and/or ^{3}H- could be accurately detected in the GC effluents (195,228,229). Potential major modifications of the methodology used in the present study have evolved elsewhere during the course

of these studies so that now it may be feasible to use GC-mass spectral equipment in combination with Fourier transform techniques to measure mass and isotope ratios simultaneously. Or, RIA might be used for determination of the masses of most of the steroids involved.

The attractiveness of using an approach such as the one used in these studies is that when fully developed it can yield data not only on conversion of labelled substrate but also on conversion of other forms of substrate and on sizes of metabolic pools. Presently, such data are generated by doing a series of several experiments, many times a separate set for each individual product or intermediate. Obviously, for such a limited approach to be useful in clinical or environmental monitoring the tests must be quite rapid. The approach which was proposed and tested in these studies would be perhaps more rapid by generating all the data for all the compounds of interest in a somewhat longer single procedure.

The attractiveness of the program lies in its generality. It may be used to generate solely mass or radioactivity data or a combination of both; it may be used to calculate data from any combination of four incubations - 2 test groups and 2 controls, 4 different buffers, etc.; and it may be used to compute metabolic flux through a single compartment system for any of a large variety of compounds, e.g., amino acids, sugars, drugs, etc. In drug studies as an example, it can even incorporate measurements

of chemically unidentified intermediates as long as some sort of standard curve can be generated from which masses (or absorbance units, etc.) can be computed.

Measurements on the steroids and DDT metabolites from incubation Types 1, 3 and 4 (Table 3) can still be run on the samples which were collected during this investigation though their results must be viewed as preliminary until they can be repeated using one or more of the suggested modifications in the isolation, purification and measurement protocols. Measurements of other types of incubation, e.g., incorporating hCG and ^{3}H-cholesterol in DDT-treated and control testicular incubations could well extend the results and spirit of the present investigation.

Contributions of these methodological studies were twofold: 1) a method of measuring doubly-labelled steroids in GC effluents, which was proposed earlier as a desirable objective by Eik-Nes (195) for samples containing single compounds, and 2) a FORTRAN program, METFLX, which can analyze results of the type generated in this study but can also be used in other studies of rather varied forms.

LIST OF REFERENCES

Note: For brevity when the full chemical names of DDT analogs appear in article titles they have been replaced by appropriate abbreviations, e.g., 2-(2-chloro-phenyl)-2-(4-chlorophenyl)-1,1,1-trichloroethane appears as [o,p'-DDT] .

1. Tomkins, G. M., Gelehrter, T. D., Granner, D., Martin, D., Jr., Sammuels, H. H. and Thompson, E. B. Control of specific gene expression in higher organisms. Science 166 (1969) 1474-1480.

2. O'Malley, B. W., Sherman, M. R. and Toff, D. O. Progesterone "Receptors" in the cytoplasm and nucleus of chick oviduct target tissue. Proc. Nat. Acad. Sci. 67 (1970) 501-508.

3. Schally, A. V., Kastin, A. J. and Arimura, A. The hypothalamus and reproduction. Am. J. Obstet. Gynecol. 114 (1972) 423-442.

4. Schriefers, H. Factors regulating the metabolism of steroids in the liver. Adv. in the Biosciences 2 (1969) 69-90.

5. Conney, A. H. Pharmacological implications of microsomal enzyme induction. Pharm. Rev. 19 (1967) 317-366.

6. Tamaoki, B.-I. Steroidogenesis and cell structure. J. Steroid Biochem. 4 (1973) 89-118.

7. Rasmussen, H. Ions as 'Second Messangers', Chapter 20. In Cell Membranes, 203-212. Ed. G. Weissmann and R. Claiborne. New York: H. P. Publishing Co., Inc., 1975.

8. Cooke, B. A., vander Molen, H. J. and Setchell, B. P. The testis. Research in Reproduction 5 (1973).

9. Schally, A. V., Arimura, A. and Kastin, A. J. Hypothalamic regulatory hormones. Science 179 (1973) 341-350.

10. Frieden, E. and Lipner, H. Biochemical endocrinology of the vertebrates. Englewood Cliffs, New Jersey: Prentice-Hall, Inc., 1971.

11. Conney, A. H., Schneidman, K., Jacobson, M. and Kuntzman, R. Drug-induced changes in steroid metabolism. Ana. N.Y. Acad. Sci. 123 (1965) 98-109.

12. Kuntzman, R., Sansur, M. and Conney, A. H. Effect of drugs and insecticides on the anesthetic action of steroids. _Endocrinology_ 77 (1965) 952-954.

13. Conney, A. H., Jacobson, M., Levin, W., Schneidman, K. and Kuntzman, R. Decreased central depressant effect of progesterone and other steroids in rats pretreated with drugs and insecticides. _J. Pharmacol. Exp. Therap._ 154 (1966) 310-318.

14. Welch, R. M., Levin, W. and Conney, A. H. Insecticide inhibition and stimulation of steroid hydroxylases in rat liver. _J. Pharmacol. Exp. Therap._ 155 (1967) 167-173.

15. Conney, A. H., Welch, R. M., Kuntzman, R. and Burns, J. J. Effects of pesticides on drug and steroid metabolism. _Clin. Pharmacol. Therap._ 8 (1967) 2-10.

16. Welch, R. M., Levin, W. and Conney, A. H. Stimulatory effect of phenobarbital on the metabolism _in vivo_ of estradiol-17β and estrone in the rat. _J. Pharmacol. Therap._ 160 (1968) 171-178.

17. Levin, W., Welch, R. M. and Conney, A. H. Effect of carbon tetrachloride and other inhibitors of drug metabolism on the metabolism and action of estradiol-17β and estrone in the rat. _J. Pharmacol. Therap._ 173 (1970) 247-255.

18. Welch, R. M., Levin, W., Kuntzman, R., Jacobson, M. and Conney, A. H. Effect of halogenated hydrocarbon insecticides on the metabolism and uterotropic action of estrogens in rats and mice. _Toxicol. Appl. Pharmacol._ 19 (1971) 234-246.

19. Levin, W., Welch, R. M. and Conney, A. H. Effect of chronic phenobarbital treatment on the liver microsomal metabolism and uterotropic action of 17β -estradiol. _Endocrinology_ 80 (1967) 135-140.

20. Levin, W., Welch, R. M. and Conney, A. H. Effect of phenobarbital and other drugs on the metabolism and uterotropic action of estradiol-17β and estrone. _J. Pharmacol. Exp. Therap._ 159 (1968) 362-371.

21. Levin, W., Welch, R. M. and Conney, A. H. Decreased uterotropic potency of oral contraceptives in rats pretreated with phenobarbital. _Endocrinology_ 83 (1968) 149-156.

22. Welch, R. M., Levin, W. and Conney, A. H. Estrogenic action of DDT and its analogs. *Toxicol. Appl. Pharmacol.* 14 (1969) 358-367.

23. Levin, W., Welch, R. M. and Conney, A. H. Inhibitory effect of phenobarbital or chlordane pretreatment on the androgen-induced increase in seminal vesicle weight in the rat. *Steroids* 13 (1969) 155-161.

24. Bitman, J. and Cecil, H. C. Estrogenic activity of DDT analogs and polychlorinated biphenyls. *J. Agr. Food Chem.* 18 (1970) 1108-1112.

25. Smith, M. T., Thomas, J. A., Smith, C. G., Mawhinney, M. G. and Lloyd, J. W. Effects of DDT on radioactive uptake from testosterone-1,2-^{3}H by mouse prostate glands. *Toxicol. Appl. Pharmacol.* 23 (1972) 159-164.

26. Nelson, J. A. Effects of dichlorodiphenyltrichloro-ethane (DDT) analogs and polychlorinated biphenyl (PCB) mixtures on 17β - ^{3}H - estradiol binding to rat uterine receptor. *Biochem. Pharmac.* 23 (1974) 447-451

27. Foster, M. S., Gellert, R. J. and Heinrichs, W. L. The estrogenic capacity of organochlorine pesticides. *Soc. Gyn. Invest.* (Abs.) 5 (1974) 35-36.

28. Rybakova, M. N. Comparative toxic effects of Sevin and DDT used in the treatment of food crops. *Voprosy Pitaniia* 26 (1967) 9-15. Translated by A. Nichols.

29. Rybakova, M. N. About several pesticide effects on the hypophysis and its gonadotrophic function. *Gigiena I Sanitariia* 33 (1969) 27-31. Translated by L. Ratermanis and M. Mizens.

30. Shtenberg, A. I. and Rybakova, M. N. Effect of carbaryl on the neuroendocrine system of rats. *Fd. Cosmet. Toxicol.* 6 (1968) 461-467.

31. Ottoboni, A. Effect of DDT on the reproductive life-span in the female rat. *Toxicol. Appl. Pharmacol.* 22 (1972) 497-502.

32. Ware, G. W. and Good, E. E. Effects of insecticides on reproduction in the laboratory mouse. II. Mirex, telodrin and DDT. *Toxicol. Appl. Pharmacol.* 10 (1967) 54-61.

33. Harr, J. R., Claeys, R. R., Bone, J. F. and McCorcle, T. W. Dieldrin toxicosis: rat reproduction. *Am. J. Vet. Res.* 31 (1970) 181-189.

34. Beattie, C. W., Campbell, C. S., Nequin, L. G., Soyka, L. F. and Schwartz, N. B. Barbiturate blockade of tonic LH secretion in the male and female rat. _Endocrinology_ 92 (1973) 1634-1638.

35. Ajika, K., Kalra, S. P., Fawcett, C. P., Krulich, L. and McCann, S. M. The effect of stress and nembutal on plasma levels of gonadotropins and prolactin in ovariectomized rats. _Endocrinology_ 90 (1972) 707-715.

36. Howland, B. E., Beaton, D. B. and Jack, M. I. Changes in serum levels of gonadotropins and testosterone in the male rat in response to fasting, surgery and ether. _Experimentia_ 30 (1974) 1223-1225.

37. Gallagher, T. F., Fukushima, D. K. and Hellman, L. The effect of ortho,para'DDD on steroid hormone metabolites in adrenocortical carcinoma. _Metabolism_ 11 (1962) 1155-1161.

38. Bradlow, H. L., Fukushima, D. K., Zumoff, B., Hellman, L. and Gallagher, T. F. A peripheral action of o,p'-DDD on steroid biotransformation. _J. Clin. Endocr._ 23 (1963) 918-922.

39. Bledsoe, T., Island, D. P., Ney, R. L. and Liddle, G. W. An effect of o,p'-DDD on the extra-adrenal metabolism of cortisol in man. _J. Clin. Endocr._ 24 (1964) 1303-1311.

40. Southern, A. L., Tochimoto, S., Strom, L., Ratuschni, A., Ross, H. and Gordon, G. Remission in Cushing's syndrome with o,p'-DDD. _J. Clin. Endocr._ 26 (1966) 268-278.

41. Southern, A. L., Tochimoto, S., Isurugi, K., Gordon, G., Krikun, E. and Stypulkowski, W. The effect of [o,p'-DDD] on the metabolism of infused cortisol-7-^{3}H. _Steroids_ 7 (1966) 11-29.

42. Nelson, A. A. and Woodard, G. Severe adrenal atrophy (cytotoxic) and hepatic damage produced in dogs by feeding [p,p'-DDD]. _Arch. Pathol._ 48 (1949) 387-394.

43. Cobey, F., Taliaferro, I. and Haag, H. B. Effect of DDD and some of its derivatives on plasma 17-OH-corticosteroids in the dog. _Science_ 123 (1956) 140-141.

44. Nichols, J. and Hennigar, G. Studies on [DDD]. _Exp. Med. Surg._ 15 (1957) 310-316.

45. Cueto, C., Brown, J. H. U. and Richardson, A. P. Biological studies on an adrenocorticolytic agent and the isolation of the active components. <u>Endocrinology</u> <u>62</u> (1958) 334-339.

46. Vilar, O. and Tullner, W. W. Effects of o,p'-DDD on histology and 17-hydroxycortico-steroid output of the dog adrenal cortex. <u>Endocrinology</u> <u>65</u> (1959) 80-86.

47. Kaminsky, N., Luse, S. and Hartroft, P. Ultrastructure of adrenal cortex of the dog during treatment with DDD. <u>J. Nat. Cancer Inst.</u> <u>29</u> (1962) 127-159.

48. Kupfer, D., Balazs, T. and Buyske, D. A. Stimulation by o,p'-DDD of cortisol metabolism in the guinea pig. <u>Life Sciences</u> <u>3</u> (1964) 959-964.

49. Balazs, T. and Kupfer, D. Effect of DDT on the metabolism and production rate of cortisol in the guinea pig. <u>Toxicol. Appl. Pharmacol.</u> <u>9</u> (1966) 40-43.

50. Foster, T. S. Effect of some pesticides on the adrenal glands in the rat. <u>Can. J. Biochem.</u> <u>46</u> (1968) 1115-1120.

51. Reif, V. D. and Sinsheimer, J. E. Metabolism of [o,p'-DDD] in rats. <u>Drug Metab. Disp.</u> <u>3</u> (1975) 15-25.

52. Hiddemen, J. W. III. "The effects of dieldrin and tolbutamide on exogenous and endogenous glucose in the rat." Ph.D. Dissertation, The University of Michigan, 1970.

53. Heinrichs, W. L., Gellert, R. J., Bakke, J. L. and Lawrence, N. L. DDT administered to neonatal rats induces persistent estrus syndrome. <u>Science</u> <u>173</u> (1971) 642-643.

54. Gellert, R. J., Heinrichs, W. L. and Swerdloff, R. Effects of neonatally-administered DDT homologs on reproductive function in male and female rats. <u>Neuroendocrinology</u> <u>16</u> (1974) 84-94.

55. Moffenson, H. C., Greensher, J. and Horowitz, R. Hazards of maternally administered drugs. <u>Clin. Toxicol.</u> <u>7</u> (1974) 59-68.

56. Knowles, J. A. Breast milk: a source of more than nutrition for the neonate. <u>Clin. Toxicol.</u> <u>7</u> (1974) 69-82.

57. Harris, G. W. Sex hormones, brain development and brain function. <u>Endocrinology</u> <u>75</u> (1964) 627-648.

58. Barraclough, C. A. Modifications in the CNS regulation of reproduction after exposure of prepubertal rats to steroid hormones. Rec. Prog. Hormone Res. 22 (1966) 503-539.

59. Barraclough, C. A. Modifications in reproductive function after exposure to hormones during the prenatal and early postnatal period, Chapter 19. In Neuroendocrinology, Vol. II, 62-99. Ed. L. Martini and W. F. Ganong. New York and London: Academic Press, 1967.

60. Barraclough, C. A. Alterations in reproductive function following prenatal and early postnatal exposure to hormones. Adv. Reprod. Physiol. 3 (1968) 81-112.

61. Gorski, R. A. Gonadal hormones and the perinatal development of neuroendocrine function, Chapter 9. In Frontiers in Neuroendocrinology, 1971, 237-290. Ed. W. F. Ganong and L. Martini. New York: Oxford Univ. Press, 1971.

62. Kincl, F. A., Folch Pi, A. and Lasso, L. H. Effect of estradiol benzoate treatment in the newborn male rat. Endocrinology 7 (1963) 966-968.

63. Dörner, G., Döcke, F. and Hinz, G. Paradoxical effects of estrogen on brain differentiation. Neoroendocrinology 7 (1971) 146-166.

64. Einarsson, K., Gustafsson, J.-Å. and Stenberg, Å. Neonatal imprinting of liver microsomal hydroxylation and reduction of steroids. J. Biol. Chem. 248 (1973) 4987-4997.

65. Berg, A. and Gustafsson, J.-Å. Regulation of hydroxylation of 5α-androstane-3α,17β-diol in liver microsomes from male and female rats. J. Biol. Chem. 248 (1973) 6559-6567.

66. Gustafsson, J.-Å. and Ingelman-Sundberg, M. Regulation and properties of a sex-specific hydroxylase system in female rat liver microsomes active on steroid sulfates. I. General characteristics. J. Biol. Chem. 249 (1974) 1940-1945.

67. Gustafsson, J.-Å. and Ingelman-Sundberg, M. Age-dependent induction and repression of rat liver microsomal hydroxylase systems by estradiol. Biochim. Biophys. Acta 354 (1974) 172-181.

68. Gustafsson, J.-Å. and Stenberg, Å. Specificity of neonatal, androgen-induced imprinting of hepatic steroid metabolism in rats. Science 191 (1976) 203-204.

69. Flerkó, B., Mess, B. and Illei-Donhoffer, A. On the mechanism of androgen sterilization. Neuroendocrinology 4 (1969) 164-169.

70. McGuire, J. L. and Lisk, R. D. Oestrogen receptor in androgen or oestrogen sterilized female rats. Nature 221 (1969) 1068-1069.

71. Simmons, J. E. Uptake of $[1,2-^3H]$-testosterone in oestrogenized male rats. Acta Endocrinologica 67 (1971) 535-543.

72. Bullock, L. P. and Bardin, C. W. In vivo and in vitro testosterone metabolism by the androgen insensitive rat. J. Steroid. Biochem. 4 (1973) 139-151.

73. Mennin, S. P., Kubo, K. and Gorski, R. A. Pituitary responsiveness to luteinizing hormone-releasing factor in normal and androgenized female rats. Endocrinology 95 (1974) 412-416.

74. Niemi, M. and Ikonen, M. Histochemistry of the Leydig cells in the postnatal prepubertal testis of the rat. Endocrinology 72 (1963) 443-448.

75. Clermont, Y. and Perey, B. Quantitative study of the cell population of the seminiferous tubules in immature rats. Am. J. Anat. 100 (1957) 241-260.

76. Warren, D. W., Haltmeyer, G. C. and Eik-Nes, K. B. The effect of gonadotrophins on the fetal and neonatal rat testis. Endocrinology 96 (1975) 1226-1229.

77. Döhler, K. D. and Wuttke, W. Changes with age in levels of serum gonadotropins, prolactin, and gonadal steroids in prepubertal male and female rats. Endocrinology 97 (1975) 898-907.

78. Engel, W. and Frowein, J. Glucocorticoids and hCG sensitivity of rat testicular Leydig cell. Nature 251 (1974) 146-148.

79. Frowein, J. and Engel, W. Constitutivity of the HCG-receptor protein in the testis of rat and man. Nature 249 (1974) 377-379.

80. Resko, J. A., Feder, H. H. and Goy, R. W. Androgen concentrations in plasma and testis of developing rats. <u>J. Endocr.</u> <u>40</u> (1968) 485-491.

81. Knorr, D. W., Vanha-Perttula, T. and Lipsett, M. B. Structure and function of rat testis through pubescence. <u>Endocrinology</u> <u>86</u> (1970) 1298-1304.

82. Miyachi, Y., Nieschlag, E. and Lipsett, M. B. The secretion of gonadotropins and testosterone by the neonatal male rat. <u>Endocrinology</u> <u>92</u> (1973) 1-5.

83. Payne, A. H., unpublished results.

84. Araki, S., Toran-Allerand, C. D., Ferin, M. and Vande Wiele, R. L. Immunoreactive gonadotropin-releasing hormone (Gn-RH) during maturation in the rat: ontogeny of regional hypothalamic differences. <u>Endocrinology</u> <u>97</u> (1975) 693-697.

85. Goldman, B. D., Grazia, Y. R., Kamberi, I. A. and Porter, J. C. Serum gonadotropin concentrations in intact and castrated neonatal rats. <u>Endocrinology</u> <u>88</u> (1971) 771-776.

86. Eldridge, J. C., Dmowski, W. P. and Mahesh, V. B. Effects of castration of immature rats on serum FSH and LH, and of various steroid treatments after castration. <u>Biol. Reprod.</u> <u>10</u> (1974) 438-446.

87. Korenbrot, C. C., Paup, D. C. and Gorski, R. A. Effects of testosterone propionate and dihydrotestosterone propionate on plasma FSH and LH levels in neonatal rats and on sexual differentiation of the brain. <u>Endocrinology</u> <u>97</u> (1975) 709-717.

88. Peters, H. The development of the mouse ovary from birth to maturity. <u>Acta Endocrinologia</u> <u>62</u> (1969) 98-116.

89. Midgley, A. R., Jr., Zeleznik, A. J., Richards, J. S. and Han, S. S. Gonadotropin and estradiol receptors and follicular development. In <u>Advances in Fertility Regulation Through Basic Research</u>. Ed. W. A. Sadler and S. Segal. New York: Plenum Press, 1974. Preprint.

90. Baker, F. D. and Kragt, C. L. Maturation of the hypothalamic-pituitary-gonadal negative feedback system. <u>Endocrinology</u> <u>85</u> (1969) 522-527.

91. Ojeda, S. R., Wheaton, J. E., Jameson, H. E. and McCann, S. M. The onset of puberty in the female rat: changes in plasma prolactin, gonadotropins, luteinizing hormone-releasing hormone (LHRH), and hypothalamic LHRH content. Endocrinology 98 (1976) 630-638.

92. Swerdloff, R. S., Walsh, P. C., Jacobs, H. S. and Odell, W. D. Serum LH and FSH sexual maturation in the male rat: effect of castration and cryptorchidism. Endorcinology 99 (1971) 120-128.

93. Odell, W. D., Swerdloff, R. S., Jacobs, H. S. and Hescox, M. A. FSH induction of sensitivity to LH: one cause of sexual maturation in the male rat. Endocrinology 92 (1973) 160-165.

94. Howard, E. The representation of the adrenal x zone in rats, in the light of observations on x zone variability in mice. Am. J. Anat. 62 (1938) 351-373.

95. Milkovic, K. and Milkovic, S. Functioning of the pituitary-adrenocortical axis in rats at and after birth. Endocrinology 73 (1963) 535-539.

96. Haltmeyer, G. C., Denenberg, V. H., Thatcher, J. and Zarrow, M. X. Response of the adrenal cortex of the neonatal rat after subjection to stress. Nature 212 (1966) 1371-1373.

97. Eguchi, Y., Hirai, O., Morikawa, Y. and Hashimoto, Y. Critical time in hypothalamic control of the pituitary-adrenal system in fetal rats: observations in fetuses subjected to hypervitaminosis A and hypothalamic destruction. Endocrinology 93 (1973) 1-11.

98. Gorski, M. E. and Lawton, I. E. Adrenal involvement in determining the time of onset of puberty in the rat. Endocrinology 93 (1973) 1232-1234.

99. Haller, H. L., Bartlett, P. D., Drake, N. L., Newman, M. S., Cristol, S. J., Eaker, C. M., Hayes, R. A., Kilmer, G. W., Magerlein, B., Mueller, G. P., Schneider, H. and Wheatley, W. The chemical composition of technical DDT. J. Am. Chem. Soc. 67 (1945) 1591-1602.

100. Peterson, J. E. and Robison, W. H. Metabolic products of p,p'-DDT in the rat. Toxicol. Appl. Pharmacol. 6 (1964) 321-327.

101. Pinto, J. D., Camien, M. N. and Dunn, M. S.
Metabolic fate of [p,p'-DDT] in rats. _J. Biol._
Chem. _240_ (1965) 2148-2154.

102. Miskus, R. P., Blair, D. P. and Casida, J. E.
Conversion of DDT and DDD by bovine rumen fluid,
lake water, and reduced porphyrins. _J. Agr. Food_
Chem. _13_ (1965) 481-483.

103. Glass, B. L. Relation between the degradation of
DDT and the iron redox system in soils. _J. Agr. Food_
Chem. _20_ (1972) 324-327.

104. Hassall, K. A. and Forrest, T. J. Reductive
dechlorination of DDT by heated liver. _Nature New_
Biology _236_ (1972) 214-216.

105. Hayes, W. J., Jr. Review of the metabolism of
chlorinated hydrocarbon insecticides especially in
mammals. _Ann. Rev. Pharmacol._ _5_ (1965) 27-52.

106. Durham, W. F. The influence of other factors on the
toxicity of pesticides, Chapter 23. In _Chemical_
Fallout, 433-446. Ed. M. W. Miller and G. G. Berg.
Springfield, Ill.: Charles C. Thomas, 1969.

107. Durham, W. F. The interaction of pesticides with
other factors. _Residue Reviews_ _18_ (1967) 21-103.

108. Dale, W. E., Gaines, T. B. and Hayes, W. J., Jr.
Storage and excretion of DDT in starved rats.
Toxicol. Appl. Pharmacol. _4_ (1962) 89-106.

109. Gillett, J. W. Microsomal epoxidation: effect of
age and duration of exposure to dietary DDT on induc-
tion. _Bull. Environ. Contam. Toxicol._ _4_ (1969)
159-168.

110. Boyd, E. M. and Krijnen, C. J. Dietary protein and
DDT toxicity. _Bull. Environ. Contam. Toxicol._ _4_
(1969) 256-261.

111. Wagstaff, D. J. and Street, J. C. Ascorbic acid
deficiency and induction of hepatic microsomal
hydroxylative enzymes by organochlorine pesticides.
Toxicol. Appl. Pharmacol. _19_ (1971) 10-19.

112. Chadwick, R. W., Linko, R. S., Freal, J. J. and
Robbins, A. L. The effect of age and long-term
low-level DDT exposure on the response to enzyme
induction in the rat. _Toxicol. Appl. Pharmacol._ _31_
(1975) 469-480.

113. Morello, A. Induction of DDT-metabolizing enzymes in microsomes of rat liver after administration of DDT. Can. J. Biochem. 43 (1965) 1289-1293.

114. Sánchez, E. DDT-induced metabolic changes in rat liver. Can. J. Biochem. 45 (1967) 1809-1817.

115. Hart, L. G. and Fouts, J. R. Effects of acute and chronic DDT administration on hepatic microsomal drug metabolism in the rat. Proc. Soc. Exp. Biol. Med. 114 (1963) 388-392.

116. Hart, L. G. and Fouts, J. R. Further studies on the stimulation of hepatic microsomal drug metabolizing enzymes by DDT and its analogs. Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak. 249 (1965) 486-500.

117. Straw, J. A., Waters, I. W. and Fregly, M. J. Effect of o,p'-DDD on hepatic metabolism of phenobarbital in rats. Proc. Soc. Exp. Biol. Med. 118 (1965) 391-394.

118. Kupfer, D. and Peets, L. The effect of o,p'-DDD on cortisol and hexobarbital metabolism. Biochem. Pharmacol. 15 (1966) 573-581.

119. Azarnoff, D. L., Grady, H. J. and Svoboda, D. J. The effect of DDD on barbiturate and steroid-induced hypnosis in the dog and rat. Biochem. Pharmacol. 15 (1966) 1985-1993.

120. Kuntzmann, R., Jacobson, M., Schneidman, K. and Conney, A. H. Similarities between oxidative drug-metabolizing enzymes and steroid hydroxylases in liver microsomes. J. Pharmacol. Exp. Therap. 146 (1964) 280-285.

121. Platt, O. S. and Cockrill, B. L. Biochemical changes in rat liver in response to treatment with drugs and other agents - II. Effects of Halothane, DDT, other chlorinated hydrocarbons, thioacetamide, dimethyl-nitrosamine and ethionine. Biochem. Pharmacol. 18 (1969) 445-457.

122. Nowicki, H. G. and Norman, A. W. Enhanced hepatic metabolism of testosterone, 4-androstene-3,17-dione, and estradiol-17β in chickens pretreated with DDT and PCB. Steroids 19 (1972) 85-99.

123. Alary, J. G., Brodeur, J., Côté, M. G., Panisset, J. C., Lamothe, P. and Guay, P. The effect of a pre-treatment with phenobarbital on the metabolism of DDT in dairy cows and young bulls. Rev. Can. Biol. 27 (1968) 269-271.

124. Street, J. C. DDT antagonism to dieldrin storage in adipose tissue of rats. _Science_ <u>146</u> (1964) 1580-1581.

125. Nowicki, H. G., Wong, R. G., Myrtle, J. F. and Norman, A. W. Inhibition of biological activity of cholecalciferol...by o,p'-DDT or p,p'-DDT in rachitic cockerel. _J. Agr. Food Chem._ <u>20</u> (1972) 376-380.

126. Novicki, H. G., Myrtle, J. F. and Norman, A. W. Effects of organochlorine insecticides on metabolism of cholecalciferol (vitamin D_3) in rachitic cockerel. _J. Agr. Food Chem._ <u>20</u> (1972) 380-384.

127. Conney, A. H. and Kuntzman, R. Metabolism of normal body constituents by drug-metabolizing enzymes in liver microsomes. In _Handbook of Experimental Pharmacology_ 28/2, 401-421. Ed. B. B. Brodie and J. Gillette. New York: Springer-Verlag, 1971.

128. Conney, A. H., Welch, R., Kuntzman, R., Chang, R., Jacobson, M., Munro-Faure, A. D., Peck, A. W., Bye, A., Poland, A., Poppers, P. J., Finster, M. and Wolff, J. A. Effects of environmental chemicals on the metabolism of drugs, carinogens, and normal body constituents in man. _Ann. N.Y. Acad. Sci._ <u>179</u> (1971) 155-172.

129. Conney, A. H. and Burns, J. J. Metabolic interactions among environmental chemicals and drugs. _Science_ <u>178</u> (1972) 576-586.

130. McCully, K. A., McKinley, W. P. and Phillips, W. E. J. The effect of the route of administration of single doses of p,p'-DDT on residues in adipose and liver tissues. _J.A.O.A.C._ <u>51</u> (1968) 1050-1057.

131. McCully, K. A., Villeneuve, D. C., McKinley, W. P., Phillips, W. E. J. and Hidiroglou, M. Metabolism and storage of DDT in beef cattle. _J.A.O.A.C._ <u>49</u> (1966) 966-973.

132. Plimmer, J. R., Kearney, P. C. and Von Endt, D. W. Mechanism of conversion of DDT to DDD by _Aerobacter aerogenes_. _J. Agr. Food Chem._ <u>16</u> (1968) 594-597.

133. Sink, J. D., Varela-Alvarez, H. and Hess, C. Metabolism of ^{14}C-DDT by ovine rumen fluid _in vitro_. _J. Agr. Food Chem._ <u>20</u> (1972) 7-9.

134. Laug, E. P., Nelson, A. A., Fitzhugh, O. G. and Kunze, F. M. Liver cell alteration and DDT storage in the fat of the rat induced by dietary levels of 1 to 50 p.p.m. DDT. J. Pharmacol. Exp. Therap. 98 (1950) 268-273.

135. Bailey, S., Bunyan, P. J., Rennison, B. D. and Taylor, A. The metabolism of [p,p'-DDT] and [p,p'-DDD] in the pigeon. Toxicol. Appl. Pharmacol. 14 (1969) 13-22.

136. Bailey, S., Bunyan, P. J., Rennison, B. D. and Taylor, A. The metabolism of [p,p'-DDE] and [p,p'-DDMU] in the pigeon. Toxicol. Appl. Pharmacol. 14 (1969) 23-32.

137. Feil, V. J., Lamoureux, C. H., Styrovoky, E., Zaylskie, R. G., Thacker, E. J., and Holman, G. M. Metabolism of o,p'-DDT in rats. J. Agr. Food Chem. 21 (1973) 1072-1078.

138. Reif, V. D., Sinsheimer, J. E., Ward, J. C. and Schteingart, D. E. Aromatic hydroxylation and alkyl oxidation in metabolism of Mitotane (o,p'-DDD) in humans. J. Pharmaceutical Sciences 63 (1974) 1730-1736.

139. Cranmer, M. F. Absence of conversion of o,p'-DDT to p,p'-DDT in the rat. Bull. Environ. Contam. Toxicol. 8 (1972) 121-124.

140. Gaines, T. B. Acute toxicity of pesticides. Toxicol. Appl. Pharmacol. 14 (1969) 515-534.

141. Metcalf, R. L. Organic Insecticides. New York: Interscience Pub. Inc., 1955.

142. Hrdina, P. D., Singhal, R. L., Peters, D. A. V. and Ling, G. M. Role of brain acetylcholine and dopamine in acute neurotoxic effects of DDT. Eur. J. Pharmacol. 15 (1971) 379-382.

143. Hrdina, P. D., Singhal, R. L. and Ling, G. M. DDT and related chlorinated hydrocarbon insecticides: pharmacological basis of their toxicity in mammals. Adv. Pharmacol. Chemotherapy 12 (1975) 31-88.

144. Burlington, H. and Lindeman, V. F. Effect of DDT on testes and secondary sex characters of white Leghorn cockerels. Proc. Soc. Exp. Biol. Med. 74 (1950) 48-57.

145. Fischer, A. L., Keasling, H. H. and Schueler, F. W. Estrogenic action of some DDT analogues. **Proc. Soc. Exp. Biol. Med.** 81 (1952) 439-441.

146. Bitman, J., Cecil, H. C., Harris, S. J. and Fries, G. F. Estrogenic activity of o,p'-DDT in the mammalian uterus and avian oviduct. **Science** 162 (1968) 371-372.

147. Cecil, H., Bitman, J. and Harris, S. J. Estrogenicity of o,p'-DDT in rats. **J. Agr. Food Chem.** 19 (1971) 61-65.

148. Singhal, R. L., Valadares, J. R. E. and Schwark, W. S. Metabolic control mechanism in mammalian systems-IX. Estrogen-like stimulation of uterine enzymes by [o,p'-DDT]. **Biochem. Pharmacol.** 19 (1970) 2145-2155.

149. Quinby, G. E., Hayes, W. J., Armstrong, J. F. and Durham, W. F. DDT storage in the U.S. population. **J. Am. Med. Assoc.** 191 (1965) 109-113.

150. Gellert, R. J., Heinrichs, W. L. and Swerdloff, R. S. DDT homologues: estrogen-like effects on the vagina, uterus and pituitary of the rat. **Endocrinology** 91 (1972) 1095-1100.

151. Gellert, R. J. and Heinrichs, W. L. Effects of DDT homologs administered to female rats during the perinatal period. **Biol. Neonate** 26 (1975) 283-290.

152. Peakall, D. B. p,p'-DDT: effect on calcium metabolism and concentration of estradiol in the blood. **Science** 168 (1970) 592-594.

153. Alvares, A. P. and Kappas, A. Induction of aryl hydrocarbon hydroxylase by polychlorinated biphenyls in the foeto-placental unit and neonatal livers during lactation. **FEBS Letters** 50 (1975) 172-174.

154. Holan, G. New halocyclopropane insecticides and the mode of action of DDT. **Nature** 221 (1969) 1025-1029.

155. Kagan, Y. S., Fudel-Ossipova, S. I., Khaikina, B. J., Kuzminskaya, U. A. and Kouton, S. D. On the problem of the harmful effect of DDT and its mechanism of action. **Residue Reviews** 27 (1969) 43-79.

156. Carson, R. **Silent Spring.** Greenwich, Conn.: Fawcett Publications, Inc. 1962.

157. Hickey, J. J. (Ed.) Peregrine Falcon Populations, Their Biology and Decline. Madison, Wisc.: Univ. of Wisc. Press, 1969.

158. U.S. The Executive Office of the President. Office of Science and Technology. Ecological Effects of Pesticides on Nontarget Species by D. Pimentel. Washington, D. C.: Government Printing Office, 1971.

159. Robinson, J. Dynamics of organochlorine insecticides in vertebrates and ecosystems. Nature 215 (1967) 33-35.

160. Risenbrough, R. W., Rieche, P., Herman, S. G., Peakall, D. B., Kirven M. N. Polychlorinated biphenyls in the global ecosystem. Nature 220 (1968) 1098-1102.

161. Ottoboni, A. Effect of DDT on reproduction in the rat. Toxicol. Appl. Pharmacol. 14 (1969) 74-81.

162. Duby, R. T., Travis, H. F. and Terrill, C. E. Uterotropic activity of DDT in rats and mink and its influence on reproduction in the rat. Toxicol. Appl. Pharmacol. 18 (1971) 348-355.

163. Babický, A., Parízek, J., Oštádalová, I. and Kolár, J. Initial solid food intake and growth of young rats in nests of different sizes. Physiologica Bohemoslovaca 22 (1973) 557-566.

164. Pfeiffer, C. A. Sexual differences of the hypophyses and their determination by the gonads. Am. J. Anat. 58 (1936) 195-225.

165. Preece, A. A Manual for Histologic Technicians. Boston, Mass.: Little, Brown and Co., 1972.

166. Bitman, J., Cecil, H. and Fries, G. F. Nonconversion of o,p'-DDT to p,p'-DDT in rats, sheep, chickens and quail. Science 174 (1971) 64-66.

167. Alltech Associates. Gas Chromatography Supplies, Catalog No. 15, 1974-1975.

168. Bush, E. T. and Hansen, D. L. Improvement of liquid scintillation counting efficiencies by optimization of scintillator composition. Relative efficiencies for three new fluors. In Radioisotope Sample Measurement Techniques in Medicine and Biology, 395-408. Vienna: International Atomic Energy Agency, 1965.

169. Niswender, G. D., Midgley, A. R., Jr., Monroe, S. E. and Reichert, L. E., Jr. Radioimmunoassay for rat luteinizing hormone with antiovine LH serum and ovine-LH-^{131}I. Proc. Soc. Exp. Biol. Med. 128 (1968) 395-408.

170. Hutchinson, J. S. and Goldman, B. D. The relationship between the rate of testosterone infusion and gonadotropin secretion. Endocrinology 97 (1975) 725-730.

171. Schally, A. V., Arimura, A., Baba, Y., Nair, R. M. G., Matsuo, H., Redding, T. W. and Debeljuk, L. Isolation and properties of the FSH and LH-releasing hormone. Biochem. Biophys. Res. Commun. 43 (1971) 393-399.

172. Aiyer, M. S., Chiappa, S. A. and Fink, G. A priming effect of luteinizing hormone releasing factor on the anterior pituitary gland in the female rat. J. Endocr. 62 (1974) 573-588.

173. Greeley, G. H., Jr., Allen, M. B. and Mahesh, V. B. FSH and LH response in the rat after intravenous, intracarotid or subcutaneous administration of LH-RH. Proc. Soc. Exp. Biol. Med. 147 (1974) 859-862.

174. Mortimer, C. H., Besser, G. M., Hook, J. and McNeilly, A. S. Intravenous, intramuscular, subcutaneous and intranasal administration of LH/FSH-RH: the duration of effect and occurrence of asynchronous pulsatile release of LH and FSH. Clinical Endocrinology 3 (1974) 19-25.

175. Harbison, R. D. Comparative toxicity of some selected pesticides in neonatal and adult rats. Toxicol. Appl. Pharmacol. 32 (1975) 443-446.

176. Mattingly, D. A simple fluorimetric method for the estimation of free 11-hydroxycorticoids in human plasma. J. Clin. Pathol. 15 (1962) 374-379.

177. Silber, R. H., Busch, R. D. and Oslapas, R. Practical procedure for estimation of corticosterone or hydrocortisone. Clin. Chem. 4 (1958) 278-285.

178. Discussion, Chapter 20. In Statistics in Endocrinology, 279-392. Ed. J. W. McArthur and T. Colton. Cambridge, Mass.: The M.I.T. Press, 1970.

179. Rodbard, D., Raylord, P. L. and Ross, G. T. Statistical quality control of radioimmunoassays, Chapter 23. In <u>Statistics in Endocrinology</u>, 411-429. Ed. J. W. McArthur and T. Colton. Cambridge, Mass.: The M.I.T. Press, 1970.

180. Yamamoto, M., Diebel, N. D., Bogdanove, E. M. Analysis of initial and delayed effects of orchidectomy and ovariectomy on pituitary and serum LH levels in adult and immature rats. <u>Endocrinology</u> <u>86</u> (1970) 1102-1111.

181. Midgley, A. R., Jr., Niswender, G. D., Gay, V. L. and Reickert, L. E., Jr. Use of antibodies for characterization of gonadotropins and steroids. <u>Rec. Prog. Hormone Res</u>. <u>27</u> (1971) 235-301.

182. Karsch, F. J. personal communication.

183. Dunn, T. D., Arimura, A. and Scheving, L. E. Effect of stress on circadian periodicity in serum LH and prolactin concentration. <u>Endocrinology</u> <u>90</u> (1972) 29-33.

184. Hefco, E. and Lackey, R. L. Circadian variations of plasma levels of LH, FSH, prolactin and GH in normal male rats. <u>Fed. Proc</u>. <u>32</u> (1973) 281.

185. Krulich, L. and Illner, P. Effect of stress on plasma LH, FSH, prolactin, TSH and GH in normal male rats. <u>Fed. Proc</u>. <u>32</u> (1973) 281.

186. Lawton, I. E. and Smith, S. W. LH secretory patterns in intact and gonadectomized male and female rats. <u>Am. J. Physiol</u>. <u>219</u> (1970) 1019-1022.

187. Ottoboni, A. and Ferguson, J. I. Excretion of DDT compounds in rat milk. <u>Toxicol. Appl. Pharmacol</u>. <u>15</u> (1969) 56-61.

188. Widmark, G., Institute of Analytical Chemistry, Univ. of Stockholm, Sweden. In a letter to J. B. Moore, Vice President and Director of Research, McLaughlin Gormley King Co., Minneapolis, Minnesota. October 26, 1967.

189. McCully, K. A. and McKinley, W. P. Determination of chlorinated pesticide residues in fat by electron capture gas chromatography. <u>J.A.O.A.C</u>. <u>47</u> (1964) 652-658.

190. Abou-Donia, M. B. and Menzel, D. B. DDT-type compounds: separation and identification by a system combining thin layer chromatography, gas chromatography, and infrared spectroscopy. J.A.O.A.C. 51 (1968) 1247-1260.

191. Moldeus, P. W., Young-nam, C., Cinti, D. L. and Schenkman, J. B. Hepatic organelle interaction-III. Mitochondrial modification of microsomal drug metabolism. J. Biol. Chem. 248 (1973) 8574-8584.

192. Coffey, J. C., French, F. S. and Nayfeh, S. N. Metabolism of progesterone by rat testicular homogenates. IV. Further studies of testosterone formation in immature testis in vitro. Endocrinology 89 (1971) 865-872.

193. Sowell, J. G., Folman, Y. and Eik-Nes, K. B. Androgen metabolism in rat testicular tissue. Endocrinology 94 (1974) 346-354.

194. Layne, E. Spectrophotometric and turbidimetric methods for measuring proteins. In Methods in Enzymology, Volume III, 450-451. Ed. S. P. Colowick and N. O. Kaplan. New York: Academic Press, Inc., 1957.

195. Eik-Nes, K. B. Gas Phase Chromatography of Steroids. Monograph on Endocrinology Volume 2. Ed. K. B. Eik-Nes and E. C. Horning. Berlin: Springer-Verlag, 1968.

196. Okita, G. T., Kabara, J. J., Richardson, F. and LeRoy, G. V. Assaying compounds containing ^{3}H and ^{14}C. Nucleonics 15(6) (1957) 111-114.

197. Oštádalová, I. and Pařízek, J. Delayed retardation of somatic growth of rats injected with oestrogens during the first days of postnatal life. Physiologia Bohemoslovaca 17 (1968) 217-228.

198. Dorfman, R. I. and Shipley, R. A. Androgens. New York: John Wiley and Sons, Inc., 1956.

199. Selye, H. The effect of testosterone on the kidney. J. Urology 42 (1939) 637-641.

200. Levine, S. and Mullins, R., Jr., Estrogen administered neonatally affects adult sexual behavior in male and female rats. Science 144 (1964) 185-187.

201. Kitay, J. I.   Effects of estradiol on pituitary-adrenal function in male and female rats.   *Endocrinology* 72 (1963) 947-954.

202. Simmons, J. E., Di Clementi, D. and Maxted, G. Aspects of adrenal function in neonatally estrogenized male mice.   *J. Exp. Zool.* 186 (1973) 123-126.

203. Freud, J.   Conditions of hypertrophy of the seminal vesicles in rats.   *Biochem. J.* 27 (1933) 1438-1450.

204. Yates, F. E., Herbst, A. L. and Urguhart, J.   Sex difference in rate of ring A reduction of Δ^4-3-keto-steroids *in vitro* by rat liver. *Endocrinology* 63 (1958) 887-902.

205. Sufrin, G. and Coffey, D. S.   A comparison of the hormone responsiveness of the prostate and seminal vesicles.   *Investigative Urology* 11 (1974) 386-391.

206. Potter, V. R., Baril, E. F., Watanabe, M. and Whittle, E. D.   Systematic oscillations in metabolic functions in liver from rats adapted to controlled feeding schedules.   *Fed. Proc.* 27 (1968) 1238-1245.

207. Schiavi, R. C.   Adenohypophyseal and serum gonadotrophins in male rats treated neonatally with estradiol benzoate.   *Endocrinology* 82 (1968) 983-988.

208. Krause, W., Hamm, K. and Weissmuller, J.   The effect of DDT on spermatogenesis of the juvenile rat.   *Bull. Environ. Contam. Toxicol.* 14 (1975) 171-179.

209. Maqueo, M. and Kincl, F. A.   Testicular histo-morphology of young rats treated with oestradiol-17β-benzoate.   *Acta Endocrinologica* 46 (1964) 25-30.

210. Holmquist, D. L., Retiene, K. and Lipscomb, H. S. Circadian rhythms in rats: effects of random lighting.   *Science* 152 (1966) 662-664.

211. Arai, Y.   The content of luteinizing hormone of the anterior pituitary and of luteinizing hormone releasing factor of the hypothalamus in estrogen-induced persistent-diestrus rats.   *Proc. Japan Acad.* 39 (1963) 605-609.

212. Crow, E. L., Davis, F. A. and Maxfield, M. W. *Statistics Manual*. New York: Dover Publications, Inc., 1960.

213. Croxton, F. E. Elementary Statistics: with Applications in Medicine and the Biological Sciences. New York: Dover Publications, Inc., 1959.

214. Steel, R. G. D. and Torrie, J. H. Principles and Procedures of Statistics, 110-114. New York: McGraw-Hill Book Co., Inc., 1960.

215. Morello, A. Role of DDT-hydroxylation in resistance. Nature 203 (1964) 785-786.

216. Babický, A., Oštádalová, I., Pařízek, J., Kolář, J. and Bíbr, B. Onset and duration of the physiological weaning period for infant rats reared in nests of different sizes. Physiologia Bohemoslovaca 22 (1973) 449-456.

217. Hart, L. G., Schutice, R. W. and Fouts, J. R. Stimulatory effects of chlordane on hepatic microsomal drug metabolism in the rat. Toxicol. Appl. Pharmacol. 5 (1963) 371-386.

218. Cox, W. M., Jr. and Mueller, A. J. The composition of milk from stock rats and an apparatus for milking small laboratory animals. J. Nutrition 13 (1937) 249-261.

219. Burke, J. A. Gas chromatography for pesticide residue analysis; some practical aspects. J.A.O.A.C. 48 (1965) 1037-1058.

220. Hanwell, A. and Linzell, J. L. A simple technique for measuring the rate of milk secretion in the rat. Comp. Biochem. Physiol. 43A (1972) 259-270.

221. Schenkman, J. B., Remmer, H. and Estabrook, R. W. Spectral studies of drug interaction with hepatic microsomal cytochrome. Mol. Pharmacol. 3 (1967) 113-123.

222. Narasimhulu, S., Cooper, D. Y. and Rosenthal, O. Spectrophotometric properties of a Triton-clarified steroid 21-hydroxylase system of adrenocortical microsomes. Life Sciences 4 (1965) 2101-2107.

223. Young, R. B., Bryson, M. J., Sweat, M. L. and Street, J. C. Complexing of DDT and o,p'-DDD with adrenal cytochrome P-450 hydroxylating systems. J. Steroid Biochem. 4 (1973) 585-591.

224. Sandler, R. and Hall, P. F. The response of rat testis to interstitial cell-stimulating hormone in vitro. Comp. Biochem. Physiol. 19 (1966) 833-843.

225. Nayfeh, S. N., Barefoot, S. W., Jr. and Baggett, B. Metabolism of progesterone by rat testicular homogenates. II. Changes with age. Endocrinology 78 (1966) 1041-1048.

226. Inano, H. and Tamaoki, B.-I. Bioconversion of steroids in immature rat testes in vitro. Endocrinology 79 (1966) 579-590.

227. Matsumoto, K. and Yamada, M. 5α -Reduction of testosterone in vitro by rat seminiferous tubules and whole testes at different stages of development. Endocrinology 93 (1973) 253-255.

228. Eik-Nes, K. B. The Androgens of the Testis. New York: Marcel Dekker, Inc., 1970.

229. Sommerville, I. F. Determination of Steroid Hormones. Radiochemical review booklet. Amersham, England: Amersham/Searle Corp., about 1970 (no date given).

230. Joseph, A. A. and Kincl, F. A. Neonatal sterilization of rodents with steroid hormones: 5. A note on the influence of neonatal treatment with estradiol benzoate or testosterone propionate on steroid metabolism in the brain and testes of adult male rats. J. Steroid Biochem. 5 (1974) 227-231.

231. Ettre, L. S. and Slatkis, A. (Ed.) The Practice of Gas Chromatography. New York: Interscience, 1967.

APPENDIX I

THE PROGRAM METFLX

The program METFLX was designed to reduce data gener-
ated in a comparative metabolism problem. Its purpose is
to work data into a form usable in drawing correlations and
conclusions concerning the metabolic flux of a substrate
through a tissue when incubated in vitro under a variety of
conditions. Specifically, it was written to evaluate a
combination of ^{3}H-^{14}C count data and gas chromatographic
results which were both to be generated during the course
of experiments on androgen production in rat testes under
the influence of DDT.

The problem was one of taking recovery data, gener-
ated by a ^{14}C internal standard, and combining it with
total mass data, generated by gas chromatography, and
conversion data, generated by ^{3}H counts derived from
labelled substrate, and converting these to: 1) the values
which actually occurred within the incubation in terms of
total steroid present at given times; 2) the contributions
of the ^{3}H substrate, the endogenous substrate and all other
substrate sources to the total amount of a particular
steroid present; 3) the amount of endogenous steroid
present before the start of the experiment; 4) the net
converions to and from the substrate and products; and
5) the amounts of a particular steroid which are formed
from unlabelled substrates, including the one added,

precursors of the one added and all others which generate the product by alternate pathways. Graphs of net conversions at given sampling times and under four different test conditions were plotted to allow graphic comparisons of flux and kinetics.

The calculations for the program were based on those generally accepted for either gas chromatographic or double-label quantitation experiments (195,196,229,231). They were modified to accommodate the simultaneous use of both methods in order to measure flux within a system which contained labelled and unlabelled substrate, endogenous substrate, endogenous substrate precursors, endogenous product, alternate product precursors and an amount of recovery (internal) standard containing a second label.

The flux model, described in Chapter 5, and some initial empirical observations dictated the following equations which yielded the desired pieces of data. Equation 1) calculates the nanomoles of products or substrate found per milligram of protein at any given time under any given condition. This equation was calculated by the function XMOLE.

1) nmoles/mg protein = (C1/C3) x (10 Exp((Log$_{10}$(A1)-SL-AP)/B)) x (VF/VI) x FF x CE x V x 0.001 /GP

C1 = number of ^{14}C counts added

C3 = number of ^{14}C counts recovered

Al = chromatographic area of the compound of interest relative to that of the internal standard (chromatographic) in the same injection of sample

SL = the Log_{10} of the ratio of the concentration of the internal standard (chromatographic) in the calibration samples to the concentration of the internal standard in the sample

AP = the intercept of the calibration plot of Log_{10} (relative chromatographic area) versus Log_{10} (compound concentration)

VF = final total volume of the sample

VI = initial total volume of the sample

FF = fraction of total gas flow passing through the capture capillary

CE = efficiency of capillary capture for the compound of interest

V = volume injected into the chromatograph in ul

GP = concentration of protein in the sample incubation in mg/ml

This equation was used to generate the following additional equations and data:

Equation	Data
I	nmoles/mg-protein of substrate in the nonenzymatic incubation
II	nmoles/mg-protein of product in the nonenzymatic incubation

Equation	Data
III	nmoles/mg-protein of substrate at time 0 in a test incubation
IV	nmoles/mg-protein of product at time 0 in a test incubation
V	nmoles/mg-protein of substrate at times greater than 0 in a test incubation
VI	nmoles/mg-protein of product at times greater than 0 in a test incubation

Equation 2) calculated the tritiated product formed during a test incubation. This equation was calculated by function H3 and was referred to as Equation 1 in the output.

2) nmoles/mg protein = $((C_2 \times (C_1/C_3))/S_0)/GP$

C_2 = number of tritium counts recovered

S_0 = the initial specific activity of the added tritiated substrate

Equation 3) calculated the product formed from tritiated and initially present, endogenous substrate during a test incubation. This equation was calculated by function H3E and was referred to as Equation 2 in the output.

3) nmoles/mg protein = $((C_2 \times C_1)/C_3)/(C_0/((C_0/S_0) +$
$(III \times GP) - (I))/GP$

C_0 = initial number of ^3H counts added

Equation 4) calculated product formed from all forms of substrate during a test incubation. This equation was calculated by the function ALL and was referred to as Equation 3 in the output.

4) nmoles/mg protein = ((C2 x C1)/C3/(C0/((C0/S0) +

    (V x GP) - (I))/GP

Finally, from the above equations the following equations and data were obtained:

Equation	Data
X = III-I	nmoles/mg-protein of endogenous substrate
XI = IV-II	nmoles/mg-protein of endogenous product
XII = III-V	the net number of nmoles of substrate converted
XIII = VI-IV	the net number of nmoles of product formed
XIV = VIII-VII	nmoles/mg-protein of product formed from endogenous substrate
XV = IX-VIII	nmoles/mg-protein of product formed from substrate precursors
XVI = VI-IV-IX	nmoles/mg-protein of product formed from sources other than the substrate under study.

Negative values could be obtained from the above calculations. Such values conveyed the information that endogenous product or alternative product precursors had been converted to subsequent products in amounts exceeding the amount of product produced from the specific substrate under study.

Mnemonics for use in METFLX are summarized in Table
10, while the logical sequence of program steps is out-
lined in the flowchart given in Figure 34.

A printout of the program and its output were gener-
ated for exemplary purposes from results collected in a
series of control incubations. These incubations were
conducted under the conditions described for incubations
type 2 and 5 in Table 3 (Chapter 2, Section IV.F.1),i.e.,
they were incubations of $[7 \text{ n-}^3\text{H}]$ Δ^5-pregnenolone in the
presence or absence of the 500 x g supernatants of 6:1
testicular homogenates from adult rats. The homogenates
were derived from 4 groups of rats neonatally injected
with 5 consecutive daily s.c. injections on days 1-5 of
age. The injections were each of 0.05 ml of sesame oil
containing: nothing (treatment 1), 40 ug EV (treatment
2), 200 ug TP (treatment 3) or 500 ug o,p'-DDT (treatment
4). After the incubations the steroids were extracted
under the protocol listed in Figure 11 (Chapter 2, Section
IV.F.1) and were analyzed under the conditions given in
Figure 12. The steroids were numbered in order of elution
from the GC column: 1 = dehydroepiandrosterone, 2 =
Δ^4-androstenedione and testosterone, 3 = Δ^5-pregnenolone,
4 = progesterone and 5 = 17α-hydroxyprogesterone. All
the steroids were computed as products in the present
program run in order to allow a check on the rate of sub-
strate (product 3) disappearance with time of incubation
as calculated by function H3.

Table 10

Mnemonics of METFLX

All Data and calculations were stored in arrays according to incubation type and time point (0, 5, 10, 30 and 60 minutes). Product data and calculations had the added dimension of product number.

Symbol	Definition
ESDATA	Array in which substrate data from the non-enzymatic incubation were stored
ESUB	Vector in which the calculated nmoles/mg-protein of substrate from the nonenzymatic incubation were stored
SDATA	Array in which the substrate data from the enzymatic incubation were stored
SUB	Array in which the calculated nmoles/mg-protein of substrate in the enzymatic incubation were stored
BP	The slope of the standard - calibration - curve of $Log_{10}(Al)$ versus Log_{10}(concentration)
AP	The intercept of the standard - calibration - curve of $Log_{10}(Al)$ versus Log_{10}(concentration)
PDATA	Array in which the product data from the non-enzymatic incubation were stored
EPRO	Array in which the calculated nmoles/mg-protein of product in the nonenzymatic incubation were stored
PRO	Array in which the data for the product from the enzymatic incubation were stored
PROD	Array in which the calculated nmoles/mg-protein of product in the enzymatic incubation were stored
XPROD	Array where the calculated values for H3 were stored
YPROD	Array where the calculated values for H3E were stored

Table 10 (Cont.)

Symbol   Definition

ZPROD   Array where the calculated values for ALL were stored

X to XVI   Arrays in which the calculated values for equations X to XVI were stored

T   Vector in which the sampling times were stored

NSCALE   Vector which held scaling information for use in PLOT1

CHAR   Vector which held the character labels for use in PLOT 4

POINT   The value of the ordinate for points plotted by PLOT1-4

GP   Vector which held the values of mg-protein per ml for all the incubation conditions

VI   Vector which held the values of the initial sample volumes for all the incubation conditions

CE   Vector which held the values of the capture efficiency of capillary capture for each of the chromatographic peaks examined

FF   The value which defined the flow fraction of gas which passed through the capillary capture tube

SL   The value which defined the Log_{10} of the ratio of the concentration of the internal chromatographic standard in the calibration mixture over the concentration of the internal chromatographic standard in the measured sample

VF   The value which defined the final total volume of the sample

N   The value which defined the number of products measured

B   BP for the substrate

SO   Initial specific activity of the added tritiated substrate in dpm/nmole

CO   Initial number of tritium counts added in dpm

Table 10 (Cont.)

Symbol	Definition
XMOLE	The function which calculated the total number of nmoles/mg-protein in the sample
PROCAL	The subroutine which calculated PROD, XPROD, YPROD, ZPROD, XI, XIII, XIV, XV and XVI
C1	The initial number of carbon-14 dpm added
C2	The number of tritium dpm recovered
C3	The number of carbon-14 dpm recovered
A1	The area of the chromatographic peak which corresponded to a given compound relative to the area of the internal standard peak in the same injection
V	The volume of the sample injected for measurement
YMIN	The minimum value for the ordinate for a given data plot, used in PLOT1-4
YMAX	The maximum value for the ordinate of a given data plot, used in PLOT1-4
PLOT1-4	A series of computer printer plot subroutines located within the *LIBRARY of the MTS computer system of The University of Michigan
H3	The function which calculated the conversion of tritium dpm from substrate to product for only tritiated substrate
H3E	The function which calculated the conversion of endogenous plus tritiated substrate to product
ALL	The function which calculated the formation of product from all sources of substrate
ALOG10	The single precision estimate of the logarithm to the base 10
SUB0	SUB at time zero

Figure 34

Flowchart for METFLX

Figure 34 (Cont.)

Write: EPRO(M,I),
PROD(M,I,J),
XPROD(M,I,J),
YPROD(M,I,J),
ZPROD(M,I,J),
XI(M,I,),
XIII(M,I,J),
XIV(M,I,J),
XV(M,I,J),
XVI(M,I,J)

M > N — no

yes

Determine YMIN and YMAX

Do 300 I = 1,4

Call Plot1

Call Plot2

Do 601 M = 1,N

Do 602 K = 1,5

XPROD(M,I,K) = POINT(K)

K > 5 — no

yes

Call Plot3

M > N — no

yes

Call Plot4

I > 4 — no

yes

End

XMOLE

Calculate Function XMOLE

Return

PROCAL

XMOLE

Store in PROD(M,I,J)

H3

Store in XPROD(M,I,J)

H3E

Store in YPROD(M,I,J)

ALL

Store in ZPROD(M,I,J)

Calculate:
XI and Store in XI(M,I)
XIII and Store in XIII(M,I,J)
XIV and Store in XIV(M,I,J)
XV and Store in XV(M,I,J)
XVI and Store in SVI(M,I)

Return

H3

Calculate Equation H3

Return

H3E

Calculate Equation H3E

Return

ALL

Calculate Equation ALL

Return

271

Chromatographic area data and radioactivity levels (^{14}C-dpm and ^{3}H dpm) were normalized to 1 ul of GC injection volume prior to the computer run in order to facilitate comparisons of the raw data. Values·for samples were entered in order of incubation number, 1-4, and time of incubation - 0, 5, 10, 30 or 60 minutes. These normalized results were entered according to the format shown in Table 11 along with the calculated values for such items as the initial specific activity of the substrate (SO), slopes and intercepts for the GC calibration curves for the various steroid peaks (BP and AP), capillary tube capture efficiencies for each of the steroid chromatographic peaks (CE), etc.

The program output is given in 5 sections. First, a program listing of the steps used in METFLX is given on pages 277-282. Second, a printout echoing the values entered on cards is given on pages 283-285; this serves as a visual check on the correctness and order of the values entered. Third, the same data is printed in organized form according to substrate and product number, according to variable and according to the presence or absence of tissue within the steroid incubation (pages 286-291). The order of values, proceeding from top to bottom in each list, is by time of incubation sample and treatment number, i.e., values for treatment 1, zero minutes, treatment 1, five minutes, ... treatment 4, 60 minutes. Fourth, the data output of the computations is

Table 11

Description of the Input Data Format for METFLX

There are 10 types of data cards which were entered
in the following order:

1. One card
   Constants CE(1), CE(2), ... , CE(6)

2. One card
   Constants FF, SL, VF

3. One card
   Constants N, GP(1), GP(2), GP(3), GP(4)

4. One card
   Constants VI(1), VI(2), VI(3) and VI(4)

5. One card
   Constants B, SO, CO, AP(3), CE(6)

6. Five cards
   Data cards for the substrate from the
   enzymatic incubation
   Each card contained 4 values: C1, C3, A1 and V
   Cards were read according to increasing time:
   0, 5, 10, 30, 60 minutes

7. Twenty cards
   Data cards for the substrate from the enzymatic
   incubations
   Each card contained values for: C1, C3, A1
   and V
   Each card represented one time point; they were
   read in the same order as in 6.
   Each set of 5 cards represented one incubation;
   there were a total of 4 incubations

8. One card
   Constants BP and AP

9. Five cards
   Data cards for the product from the nonenzymatic
   incubation
   Each card contained 4 values: C1, C3, A1 and V
   Cards were read according to increasing time:
   0, 5, 10, 30 and 60 minutes

Table 11 (Cont.)

10. Twenty cards
    Data cards for the product from the
    enzymatic incubation
    Each card contained 5 values: C_1, C_2, C_3,
    A_1 and V
    Each card represented one time point; they
    were read in the same order as in 9.
    Each set of 5 cards represented one incuba-
    tion; there were a total of 4 incubations

Formats 8., 9. and 10. were repeated as many times as

there were products.

printed (pages 292 to 302). The substrate is listed first followed by each of the products (product 3 = substrate). The values for the various incubation conditions, times and equations are printed in clearly labelled order. Fifth, plots of the content of ^{3}H-labelled substrate and products versus time of incubation are given for each animal treatment group. These allow easy comparison of rates and levels of conversion[1].

The results generated demonstrate the need for several modifications in the experimental procedures. First, the total nanomoles of substrate and products closely resemble the amounts added as carrier. This destroys the utility of the program for computing endogenous levels of substrate and/or product or quantities based on those levels. Therefore, large reductions, or elimination of carrier is suggested. This might necessitate the use of electron capture detection of appropriate steroid derivatives but that possibility is presently used in a number of laboratories (195;229). Second, the need to use uniform sample sizes is illustrated by the differences seen between treatment groups 1-3, which used 1 ml sampling aliquots and those in group 4 which used 5 ml sampling aliquots.

---

[1] In the program printout the following numbers and letters are used to identify specific steroid chromatographic peaks: 1=A=dehydroepiandrosterone, 2=B=Δ ^{4}-androstenedione + testosterone, 3=C=Δ ^{5}-pregnenolone, 4=D=progesterone, 5=E=17α-hydroxyprogesterone.

Modifications of the program are indeed possible. Extensions of printout to include plots of all the equation results and inclusion of the protein concentrations in each incubation could easily be made. Similarly, additional correction or dilution factors may be included as well as extensions to different numbers of sampling times or incubation or treatment conditions.

Finally, utility of the program is not limited to the coupled GC-scintillation counting system described here. Any mass determination which does not interfere with the simultaneous quantitation of ^{3}H- and ^{14}C- may just as easily be used in the context of this program. Similarly, the program is not limited to use with steroids but is easily extended to numerous other metabolic problems. It is hoped that the generality of its inception may serve to make the program useful in many studies both in and out of the area of steroid biochemistry.

```
C METFLX CALCULATES THE METABOLIC FLUX OF COMPOUNDS IN A TISSUE
C INCUBATION BASED ON DATA GENERATED BY GC AND DOUBLE-LABEL SCINTILLA-
C TION COUNTING.  INITIALLY APPLIED TO ANDROGEN METABOLISM.
      DIMENSION ESDATA(5,5),ESUB(5),SDATA(4,5,5),SUB(4,5),BP(6),AP(6)
      DIMENSION PDATA(6,5,5),EPRO(6,5),PRO(6,4,5,6)
      DIMENSION PROD(6,4,5),XPROD(6,4,5),YPROD(6,4,5),ZPROD(6,4,5)
      DIMENSION X(4),XI(6,4),XII(4,5),XIII(6,4,5),XIV(6,4,5),
     1XV(6,4,5),XVI(6,4,5)
      DIMENSION T(5),NSCALE(5),CHAR(7)
      DIMENSION POINT(5),GP(4),VI(4)
      DIMENSION CE(6)
  410 FORMAT(6F10.8)
      READ(5,410)(CE(I),I=1,6)
      WRITE(6,411)(CE(I),I=1,6)
  411 FORMAT(' ',6F10.8)
      READ(5,420)FF,SL,VF
      WRITE(6,421)FF,SL,VF
  420 FORMAT(3F10.8)
  421 FORMAT(' ',2F10.8,F10.3)
      READ(5,201)N,(GP(I),I=1,4)
      WRITE(6,202)N,(GP(I),I=1,4)
  201 FORMAT(I1,4F10.8)
  202 FORMAT(' ',I3,4F10.4)
      READ(5,207)(VI(I),I=1,4)
  207 FORMAT(4F10.3)
      WRITE(6,208)(VI(I),I=1,4)
  208 FORMAT(' ',4F10.3)
      READ(5,1)B,SO,CO,AP(3),CE(6)
      WRITE(6,203)B,SO,CO,AP(3),CE(6)
  1   FORMAT(5F10.8)
  203 FORMAT(' ',F10.6,2F10.2,F10.6,F10.8)
      READ(5,2)((ESDATA(I,J),J=1,4),I=1,5)
      WRITE(6,204)((ESDATA(K,L),L=1,4),K=1,5)
  2 FORMAT(F10.4,10X,3F10.4)
  204 FORMAT(' ',F10.2,10X,F10.2,F10.4,F10.2)
      DO 100 I=1,5
  100 ESUB(I)=XMOLE(ESDATA(I,1),ESDATA(I,2),ESDATA(I,3),ESDATA(I,4),
     1B,1.0,AP(3),FF,SL,CE(6),VI(1),VF)
      READ(5,2)(((SDATA(I,J,K),K=1,4),J=1,5),I=1,4)
      WRITE(6,204)(((SDATA(L,M,K),K=1,4),M=1,5),L=1,4)
      DO 101 I=1,4
      DO 101 J=1,5
  101 SUB(I,J)=XMOLE(SDATA(I,J,1),SDATA(I,J,2),SDATA(I,J,3),
     3SDATA(I,J,4),B,GP(I),AP(3),FF,SL,CE(6),VI(I),VF)
      DO 105 I=1,4
  105 X(I)=SUB(I,1)-ESUB(I)/GP(I)
      DO 106 I=1,4
      DO 106 J=1,5
  106 XII(I,J)=SUB(I,1)-SUB(I,J)
      DO 200 M=1,N
      READ(5,23)BP(M),AP(M)
      WRITE(6,205)BP(M),AP(M)
   23 FORMAT(2F10.6)
  205 FORMAT(' ',2F10.6)
```

```
      READ(5,2)((PDATA(M,I,J),J=1,4),I=1,5)
      WRITE(6,204)((PDATA(M,K,L),L=1,4),K=1,5)
      DO 102 I=1,5
  102 EPRO(M,I)=XMOLE(PDATA(M,I,1),PDATA(M,I,2),PDATA(M,I,3),
     5PDATA(M,I,4),BP(M),1.0,AP(M),FF,SL,CE(M),VI(1),VF)
      READ(5,3)(((PRO(M,I,J,K),K=1,5),J=1,5),I=1,4)
      WRITE(6,206)(((PRO(M,I,J,K),K=1,5),J=1,5),I=1,4)
    3 FORMAT(3F10.2,F10.4,F10.5)
  206 FORMAT(3F10.2,F10.4,F10.2)
      CALL PROCAL(PRO,SUB,ESUB,EPRO,BP,SO,CO,PROD,XPROD,YPROD,ZPROD,
     4XI,XIII,XIV,XV,XVI,GP,M,AP,FF,SL,CE,VI,VF)
  200 CONTINUE
      WRITE(6,660)
  660 FORMAT('1','DEFINITION OF VARIABLES:')
      WRITE(6,670)
  670 FORMAT('0',4X,'B(P) - SLOPE OF STANDARD PLOT OF RELATIVE AREA VERS
     1US CONCENTRATION OF X')
      WRITE(6,881)
  881 FORMAT(' ',4X,'AP - INTERCEPT OF STANDARD PLOT OF RELATIVE AREA VE
     3RSUS CONCENTRATION OF X')
      WRITE(6,680)
  680 FORMAT(' ',4X,'SO - INITIAL SPECIFIC ACTIVITY, DPM/NM, OF 3H SUBST
     1RATE ADDED')
      WRITE(6,690)
  690 FORMAT(' ',4X,'CO - INITIAL NUMBER OF 3H-SUBSTRATE COUNTS ADDED TO
     1 SAMPLE')
      WRITE(6,700)
  700 FORMAT(' ',4X,'C1 - INITIAL NUMBER OF 14C DPM ADDED TO SAMPLE AS X
     1')
      WRITE(6,710)
  710 FORMAT(' ',4X,'C2 - NUMBER OF 3H DPM RECOVERED FROM SAMPLE AS X')
      WRITE(6,720)
  720 FORMAT(' ',4X,'C3 - NUMBER OF 14C DPM RECOVERED FROM SAMPLE AS X')
      WRITE(6,730)
  730 FORMAT(' ',4X,'A1 - RELATIVE AREA OF PEAK FROM COMPONENT X IN ALIQ
     1UOT OF SAMPLE')
      WRITE(6,750)
  750 FORMAT(' ',4X,'V - VOLUME INJECTED FOR MEASUREMENT')
      WRITE(6,880)
  880 FORMAT(' ',4X,'CE - EFFICIENCY OF CAPILLARY CAPTURE FOR THIS COMPONENT
      WRITE(6,61)B,SO,CO,AP(3),CE(6)
   61 FORMAT('-','B=',F10.6,3X,'SO=',F10.2,3X,'CO=',F10.2,3X,'AP=',F10.6 'CE
     3=',F8.6)
      WRITE(6,62)
   62 FORMAT('-','NONENZYMATIC SUBSTRATE DATA')
      WRITE(6,63)
   63 FORMAT('-',4X,'C1',10X,'C3',9X,'A1',9X,'V')
      WRITE(6,64)((ESDATA(I,J),J=1,4),I=1,5)
   64 FORMAT(' ',F10.2,F10.2,F10.4,F10.2)
      WRITE(6,65)
   65 FORMAT('-','ENZYMATIC SUBSTRATE DATA')
      WRITE(6,63)
      WRITE(6,64)(((SDATA(I,J,K),K=1,4),J=1,5),I=1,4)
      DO 400 M=1,N
```

```
      WRITE(6,69)BP(M),AP(M),CE(M)
   69 FORMAT('-','BP=',F10.6,5X,'AP=',F10.6,5X,'CE=',F10.6)
      L=M
      WRITE(6,66)L
   66 FORMAT('-','NONENZYMATIC PRODUCT',1X,I1,1X,'DATA')
      WRITE(6,63)
      WRITE(6,64)((PDATA(M,I,J),J=1,4),I=1,5)
      WRITE(6,67)L
   67 FORMAT('-','ENZYMATIC PRODUCT',1X,I1,1X,'DATA')
      WRITE(6,68)
   68 FORMAT(4X,'C1',10X,'C2',8X,'C3',8X,'A1',9X,'V')
      WRITE(6,70)(((PRO(M,I,J,K),K=1,5),J=1,5),I=1,4)
   70 FORMAT(' ',3F10.2,F10.4,F10.2)
  400 CONTINUE
      WRITE(6,40)
   40 FORMAT('1','EQUATIONS:')
      WRITE(6,41)
   41 FORMAT('0',4X,'1 - 3H PRODUCT FORMED')
      WRITE(6,42)
   42 FORMAT(' ',4X,'2 - PRODUCT FORMED FROM 3H AND ENDOGENOUS SUBSTRATE')
      WRITE(6,43)
   43 FORMAT(' ',4X,'3 - PRODUCT FORMED FROM ALL SOURCES')
      WRITE(6,44)
   44 FORMAT(' ',4X,'X - ENDOGENOUS SUBSTRATE')
      WRITE(6,45)
   45 FORMAT(' ',4X,'XI - ENDOGENOUS PRODUCT')
      WRITE(6,46)
   46 FORMAT(' ',4X,'XII - NET CONVERSION OF SUBSTRATE')
      WRITE(6,47)
   47 FORMAT(' ',4X,'XIII - NET FORMATION OF PRODUCT')
      WRITE(6,48)
   48 FORMAT(' ',4X,'XIV - PRODUCT FORMED FROM ENDOGENOUS SUBSTRATE')
      WRITE(6,49)
   49 FORMAT (' ',4X,'XV - PRODUCT FORMED FROM SUBSTRATE PRECURSORS')
      WRITE(6,50)
   50 FORMAT(' ',4X,'XVI - PRODUCT FORMED FROM OTHER SUBSTRATES')
      WRITE(6,800)
  800 FORMAT('-','ANIMAL TREATMENTS:')
      WRITE(6,810)
  810 FORMAT('0',4X,'1(I) - CONTROL, SESAME OIL INJECTED')
      WRITE(6,820)
  820 FORMAT(' ',4X,'2(II) - ESTRADIOL INJECTED')
      WRITE (6,830)
  830 FORMAT(' ',4X,'3(III) - TESTOSTERONE INJECTED')
      WRITE(6,840)
  840 FORMAT(' ',4X,'4(IV) - DDT INJECTED')
      WRITE(6,4)
    4 FORMAT('-','NMOLES SUBSTRATE: NONENZYMATIC INCUBATION SAMPLE')
      WRITE(6,5)
    5 FORMAT('0',8X,'0 MIN',5X,'5 MIN',4X,'10 MIN',4X,'30 MIN',4X,
     2'60 MIN')
      WRITE(6,6)(ESUB(I),I=1,5)
    6 FORMAT(' ',4X,5(F10.4))
      WRITE(6,7)
```

```
  7 FORMAT('-','NMOLES/MG-PROTEIN, SUBSTRATE: ENZYMATIC INCUBATION')
    WRITE(6,5)
    WRITE(6,8)(I,(SUB(I,J),J=1,5),I=1,4)
  8 FORMAT(' ',I1,3X,5F10.4)
    WRITE(6,15)
 15 FORMAT('-','EQUATION X')
    WRITE(6,16)(I,X(I),I=1,4)
 16 FORMAT(' ',I1,3X,F10.4)
    WRITE(6,18)
 18 FORMAT('-','EQUATION XII')
    WRITE(6,5)
    WRITE(6,8)(I,(XII(I,J),J=1,5),I=1,4)
    DO 500 M=1,N
    L=M
    WRITE(6,31)L
 31 FORMAT('1',21X,'PRODUCT',1X,I1)
    WRITE(6,9)
  9 FORMAT('-','NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE')
    WRITE(6,5)
    WRITE(6,6)(EPRO(M,I),I=1,5)
    WRITE(6,10)
 10 FORMAT('-','NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION')
    WRITE(6,5)
    WRITE(6,8)(I,(PROD(M,I,J),J=1,5),I=1,4)
    WRITE(6,12)
 12 FORMAT('-','EQUATION 1')
    WRITE(6,5)
    WRITE(6,8)(I,(XPROD(M,I,J),J=1,5),I=1,4)
    WRITE(6,13)
 13 FORMAT('-','EQUATION 2')
    WRITE(6,5)
    WRITE(6,8)(I,(YPROD(M,I,J),J=1,5),I=1,4)
    WRITE(6,14)
 14 FORMAT('-','EQUATION 3')
    WRITE(6,5)
    WRITE(6,8)(I,(ZPROD(M,I,J),J=1,5),I=1,4)
    WRITE(6,17)
 17 FORMAT('-','EQUATION XI')
    WRITE(6,16)(I,XI(M,I),I=1,4)
    WRITE(6,19)
 19 FORMAT('-','EQUATION XIII')
    WRITE(6,5)
    WRITE(6,8)(I,(XIII(M,I,J),J=1,5),I=1,4)
    WRITE(6,20)
 20 FORMAT('-','EQUATION XIV')
    WRITE(6,5)
    WRITE(6,8)(I,(XIV(M,I,J),J=1,5),I=1,4)
    WRITE(6,21)
 21 FORMAT('-','EQUATION XV')
    WRITE(6,5)
    WRITE(6,8)(I,(XV(M,I,J),J=1,5),I=1,4)
    WRITE(6,22)
 22 FORMAT('-','EQUATION XVI')
    WRITE(6,5)
```

```
      WRITE(6,8)(I,(XVI(M,I,J),J=1,5),I=1,4)
  500 CONTINUE
      T(1)=0.
      T(2)=5.
      T(3)=10.
      T(4)=30.
      T(5)=60.
      DATA CHAR(1),CHAR(2),CHAR(3),CHAR(4),CHAR(5),CHAR(6)/'A','B',
     4'C','D','E','F'/
      YMAX=0
      YMIN=0
      DO 900 M=1,N
      DO 900 I=1,4
      DO 900 J=1,5
      IF(XPROD(M,I,J).GT.YMAX) GO TO 901
      IF(XPROD(M,I,J).LT.YMIN) GO TO 902
      GO TO 903
  902 YMIN=XPROD(M,I,J)
      GO TO 903
  901 YMAX=XPROD(M,I,J)
  903 CONTINUE
  900 CONTINUE
      DO 300 I=1,4
      CALL PLOT1(0,6,9,7,9)
      CALL PLOT2(0,T(5),T(1),YMAX,YMIN)
      DO 601 M=1,N
      DO 602 K=1,5
  602 POINT(K)=XPROD(M,I,K)
  601 CALL PLOT3(CHAR(M),T,POINT,5,4)
      GO TO(81,82,83,84),I
   81 WRITE(6,85)
   85 FORMAT('1',26X,'CONVERSION OF 3H: TREATMENT I')
      GO TO 89
   82 WRITE(6,86)
   86 FORMAT('1',26X,'CONVERSION OF 3H: TREATMENT II')
      GO TO 89
   83 WRITE(6,87)
   87 FORMAT('1',26X,'CONVERSION OF 3H: TREATMENT III')
      GO TO 89
   84 WRITE(6,88)
   88 FORMAT('1',26X,'CONVERSION OF 3H: TREATMENT IV')
   89 CALL PLOT4(17,'NMOLES/MG PROTEIN')
  300 WRITE(6,303)
  303 FORMAT(' ',34X,'TIME IN MINUTES')
      STOP
      END
      SUBROUTINE PROCAL(PRO,SUB,ESUB,EPRO,BP,SO,CO,PROD,XPROD,YPROD,
     5ZPROD,XI,XIII,XIV,XV,XVI,GP,M,AP,FF,SL,CE,VI,VF)
      DIMENSION PRO(6,4,5,6),SUB(4,5),ESUB(5),EPRO(6,5)
      DIMENSION PROD(6,4,5),XPROD(6,4,5),YPROD(6,4,5),ZPROD(6,4,5)
      DIMENSION XI(6,4),XIII(6,4,5),XIV(6,4,5),BP(6)
      DIMENSION XV(6,4,5),XVI(6,4,5),GP(4),VI(4)
      DIMENSION AP(6),CE(6)
      DO 99 I=1,4
```

```
      DO 99 J=1,5
      PROD(M,I,J)=XMOLE(PRO(M,I,J,1),PRO(M,I,J,3),PRO(M,I,J,4),
     7PRO(M,I,J,5),BP(M),GP(I),AP(M),FF,SL,CE(M),VI(I),VF)
      XPROD(M,I,J)=H3(PRO(M,I,J,1),PRO(M,I,J,2),PRO(M,I,J,3),SO,GP(I))
      YPROD(M,I,J)=H3E(CO,PRO(M,I,J,1),PRO(M,I,J,2),PRO(M,I,J,3),SO,
     8SUB(I,1),ESUB(J),GP(I))
   99 ZPROD(M,I,J)=ALL(CO,PRO(M,I,J,1),PRO(M,I,J,2),PRO(M,I,J,3),SO,
     9SUB(I,J),ESUB(J),GP(I))
      DO 98 I=1,4
   98 XI(M,I)=PROD(M,I,1)-EPRO(M,1)/GP(I)
      DO 97 I=1,4
      DO 97 J=1,5
   97 XIII(M,I,J)=PROD(M,I,J)-PROD(M,I,1)
      DO 96 I=1,4
      DO 96 J=1,5
      XIV(M,I,J)=YPROD(M,I,J)-PROD(M,I,1)
      XV(M,I,J)=ZPROD(M,I,J)-YPROD(M,I,J)
   96 XVI(M,I,J)=XIII(M,I,J)-ZPROD(M,I,J)
      RETURN
      END
      FUNCTION XMOLE(C1,C3,A1,V,B,GP,AP,FF,SL,CE,VI,VF)
      XMOLE=((C1/C3)*(10**(((ALOG10(A1)-SL-AP)/B))*(VF/VI)*FF*CE*V*.001)/GP
      RETURN
      END
      FUNCTION H3(C1,C2,C3,SO,GP)
      H3=((C2*(C1/C3))/SO)/GP
      RETURN
      END
      FUNCTION H3E(CO,C1,C2,C3,SO,SUBO,ESUB,GP)
      H3E=((C2*(C1/C3))/(CO/((CO/SO)+(SUBO*GP)-(ESUB))))/GP
      RETURN
      END
      FUNCTION ALL(CO,C1,C2,C3,SO,SUB,ESUB,GP)
      ALL=((C2*(C1/C3))/(CO/((CO/SO)+(SUB*GP)-(ESUB))))/GP
      RETURN
      END
```

```
0.685827970.589727340.884790600.780579860.491454540.88479060
0.74367738-.25467902      0.200
   5     9.1730      7.7820      7.7030     29.4550
      1.000      1.000      1.000      5.000
   1.0469241898380.001846948.00  -3.5120810.88479060
   62016.00                    90.88     0.8440      1.00
   62016.00                    90.88     0.8440      1.00
   62016.00                    90.88     0.8440      1.00
   62016.00                    93.60     0.7870      1.00
   62016.00          .         93.60     0.7870      1.00
   62016.00                    33.93     0.4520      1.00
   62016.00                    80.09     0.5480      1.00
   62016.00                    74.55     0.5210      1.00
   62016.00                    76.88     0.8005      1.00
   62016.00                    39.62     0.6965      1.00
   62016.00                    51.21     0.7480      1.00
   62016.00                    90.99     0.7880      1.00
   62016.00                    92.45     0.7345      1.00
   62016.00                    92.75     0.7635      1.00
   62016.00                    45.64     0.8580      1.00
   62016.00                    54.05     0.8295      1.00
   62016.00                    95.06     0.9090      1.00
   62016.00                    83.07     0.7530      1.00
   62016.00                    91.89     0.8105      1.00
   62016.00                    49.49     0.6805      1.00
   62016.00                    53.78     0.5190      1.00
   62016.00                    50.78     0.3375      1.00
   62016.00                    61.51     0.3970      1.00
   62016.00                    48.10     0.3240      1.00
   62016.00                    37.27     0.3840      1.00
   1.005030  -3.375531
   61734.00                    76.27     0.8280      1.00
   61734.00                    76.27     0.8280      1.00
   61734.00                    76.27     0.8280      1.00
   61734.00                    79.78     0.7940      1.00
   61734.00                    79.78     0.7940      1.00
   61734.00     14.52          22.86     0.4105      1.00
   61734.00     73.67          75.35     0.4670      1.00
   61734.00     94.48          64.60     0.4235      1.00
   61734.00    151.77          80.27     0.7795      1.00
   61734.00    144.60          26.40     0.5770      1.00
   61734.00     18.45          32.73     0.6320      1.00
   61734.00    495.79          87.27     0.7210      1.00
   61734.00    224.79          72.11     0.6375      1.00
   61734.00    435.25          83.53     0.7075      1.00
   61734.00    236.25          29.53     0.7395      1.00
   61734.00     41.51          50.51     0.7130      1.00
   61734.00    155.66          96.96     0.7325      1.00
   61734.00     83.03          78.33     0.7210      1.00
   61734.00    270.20          88.96     0.8045      1.00
   61734.00    249.77          42.66     0.5830      1.00
   61734.00    153.70          37.56     0.4960      1.00
   61734.00    194.15          50.26     0.3860      1.00
   61734.00    361.49          53.23     0.4340      1.00
```

61734.00	631.14	43.67	0.3850	1.00
61734.00	531.94	30.51	0.3315	1.00
0.988246	-3.357901			
128091.00		163.41	1.8700	1.00
128091.00		163.41	1.8700	1.00
128091.00		163.41	1.8700	1.00
128091.00		163.38	1.6890	1.00
128091.00		163.38	1.6890	1.00
128091.00	35.54	54.86	0.9385	1.00
128091.00	58.90	150.90	1.2230	1.00
128091.00	158.72	127.57	1.0495	1.00
128091.00	1396.45	160.67	1.5425	1.00
128091.00	635.75	60.34	1.2985	1.00
128091.00	42.07	65.77	1.3160	1.00
128091.00	274.97	173.57	1.4615	1.00
128091.00	1445.56	184.33	1.6740	1.00
128091.00	1334.55	169.50	1.5400	1.00
128091.00	655.33	70.31	1.5250	1.00
128091.00	48.09	93.40	1.2285	1.00
128091.00	131.50	182.33	1.5965	1.00
128091.00	453.02	169.02	1.6350	1.00
128091.00	1508.75	183.81	1.6060	1.00
128091.00	876.23	95.52	1.3680	1.00
128091.00	268.61	99.22	1.1020	1.00
128091.00	208.53	109.81	0.8220	1.00
128091.00	273.08	117.28	0.8500	1.00
128091.00	2432.41	98.68	0.8140	1.00
128091.00	3920.06	79.98	0.7620	1.00
1.046924	-3.512081			
62016.00		90.88	0.8440	1.00
62016.00		90.88	0.8440	1.00
62016.00		90.88	0.8440	1.00
62016.00		93.60	0.7870	1.00
62016.00		93.60	0.7870	1.00
62016.00	588.77	33.93	0.4520	1.00
62016.00	1790.69	80.09	0.5480	1.00
62016.00	814.44	74.55	0.5210	1.00
62016.00	111.15	76.88	0.8005	1.00
62016.00	182.80	39.62	0.6965	1.00
62016.00	1141.55	51.21	0.7480	1.00
62016.00	193.14	90.99	0.7880	1.00
62016.00	117.34	92.45	0.7345	1.00
62016.00	122.13	92.75	0.7635	1.00
62016.00	151.03	45.64	0.8580	1.00
62016.00	1461.38	54.05	0.8295	1.00
62016.00	1176.97	95.06	0.9090	1.00
62016.00	319.97	83.07	0.7530	1.00
62016.00	121.10	91.89	0.8105	1.00
62016.00	171.91	49.49	0.6805	1.00
62016.00	6207.46	53.78	0.5190	1.00
62016.00	6568.69	50.78	0.3375	1.00
62016.00	5871.14	61.51	0.3970	1.00
62016.00	1172.33	48.10	0.3240	1.00
62016.00	582.00	37.27	0.3840	1.00

1.003495	-3.329786			
63686.00		97.01	0.8530	1.00
63686.00		97.01	0.8530	1.00
63686.00		97.01	0.8530	1.00
63686.00		100.92	0.7730	1.00
63686.00		100.92	0.7730	1.00
63686.00	64.46	33.20	0.4570	1.00
63686.00	191.31	89.29	0.6615	1.00
63686.00	444.83	84.81	0.5275	1.00
63686.00	193.24	93.80	0.7690	1.00
63686.00	95.74	42.22	0.6670	1.00
63686.00	130.17	51.89	0.7690	1.00
63686.00	840.37	104.41	0.7395	1.00
63686.00	281.70	121.07	0.7785	1.00
63686.00	93.57	102.12	0.8100	1.00
63686.00	101.39	44.25	0.7435	1.00
63686.00	166.70	64.40	0.7065	1.00
63686.00	690.68	112.74	0.8665	1.00
63686.00	521.33	104.72	0.8155	1.00
63686.00	146.58	109.41	0.8020	1.00
63686.00	109.77	56.01	0.6695	1.00
63686.00	553.61	50.75	0.4220	1.00
63686.00	584.10	54.73	0.3345	1.00
63686.00	932.11	59.06	0.3405	1.00
63686.00	1230.05	50.47	0.2980	1.00
63686.00	526.88	41.36	0.3170	1.00
0.993589	-3.482027			
62367.00		68.38	0.4530	1.00
62367.00		68.38	0.4530	1.00
62367.00		68.38	0.4530	1.00
62367.00		73.06	0.3220	1.00
62367.00		73.06	0.3220	1.00
62367.00	36.54	23.31	0.2780	1.00
62367.00	79.51	75.36	0.4565	1.00
62367.00	281.96	65.62	0.3520	1.00
62367.00	167.03	78.07	0.4650	1.00
62367.00	60.93	25.41	0.3970	1.00
62367.00	42.98	35.75	0.3710	1.00
62367.00	284.47	89.69	0.4545	1.00
62367.00	189.74	92.73	0.3825	1.00
62367.00	92.43	86.71	0.4805	1.00
62367.00	64.06	31.37	0.4645	1.00
62367.00	80.54	43.37	0.4365	1.00
62367.00	261.73	97.92	0.5200	1.00
62367.00	542.09	83.00	0.4160	1.00
62367.00	127.47	93.92	0.4790	1.00
62367.00	96.72	40.79	0.3870	1.00
62367.00	237.39	49.90	0.4550	1.00
62367.00	257.80	57.97	0.3310	1.00
62367.00	413.96	56.57	0.2205	1.00
62367.00	1042.03	50.18	0.1615	1.00
62367.00	391.59	35.03	0.1650	1.00

DEFINITION OF VARIABLES:

    B(P) - SLOPE OF STANDARD PLOT OF RELATIVE AREA VERSUS CONCENTRATION
    AP - INTERCEPT OF STANDARD PLOT OF RELATIVE AREA VERSUS CONCENTRATION
    SO - INITIAL SPECIFIC ACTIVITY, DPM/NM, OF 3H SUBSTRATE ADDED
    CO - INITIAL NUMBER OF 3H-SUBSTRATE COUNTS ADDED TO SAMPLE
    C1 - INITIAL NUMBER OF 14C DPM ADDED TO SAMPLE AS X
    C2 - NUMBER OF 3H DPM RECOVERED FROM SAMPLE AS X
    C3 - NUMBER OF 14C DPM RECOVERED FROM SAMPLE AS X
    A1 - RELATIVE AREA OF PEAK FROM COMPONENT X IN ALIQUOT OF SAMPLE
    V - VOLUME INJECTED FOR MEASUREMENT
    CE - EFFICIENCY OF CAPILLARY CAPTURE FOR THIS COMPONENT

B= 1.046924   SO=1898380.00   CO=1846948.00   AP= -3.512081   CE=0.884790

NONENZYMATIC SUBSTRATE DATA

C1	C3	A1	V
62016.00	90.88	0.8440	1.00
62016.00	90.88	0.8440	1.00
62016.00	90.88	0.8440	1.00
62016.00	93.60	0.7870	1.00
62016.00	93.60	0.7870	1.00

ENZYMATIC SUBSTRATE DATA

C1	C3	A1	V
62016.00	33.93	0.4520	1.00
62016.00	80.09	0.5480	1.00
62016.00	74.55	0.5210	1.00
62016.00	76.88	0.8005	1.00
62016.00	39.62	0.6965	1.00
62016.00	51.21	0.7480	1.00
62016.00	90.99	0.7880	1.00
62016.00	92.45	0.7345	1.00
62016.00	92.75	0.7635	1.00
62016.00	45.64	0.8580	1.00
62016.00	54.05	0.8295	1.00
62016.00	95.06	0.9090	1.00
62016.00	83.07	0.7530	1.00
62016.00	91.89	0.8105	1.00
62016.00	49.49	0.6805	1.00
62016.00	53.78	0.5190	1.00
62016.00	50.78	0.3375	1.00
62016.00	61.51	0.3970	1.00
62016.00	48.10	0.3240	1.00
62016.00	37.27	0.3840	1.00

BP= 1.005030   AP= -3.375531   CE= 0.685828

NONENZYMATIC PRODUCT 1 DATA

C1	C3	A1	V
61734.00	76.27	0.8280	1.00
61734.00	76.27	0.8280	1.00
61734.00	76.27	0.8280	1.00
61734.00	79.78	0.7940	1.00
61734.00	79.78	0.7940	1.00

ENZYMATIC PRODUCT 1 DATA

C1	C2	C3	A1	V
61734.00	14.52	22.86	0.4105	1.00
61734.00	73.67	75.35	0.4670	1.00
61734.00	94.48	64.60	0.4235	1.00
61734.00	151.77	80.27	0.7795	1.00
61734.00	144.60	26.40	0.5770	1.00
61734.00	18.45	32.73	0.6320	1.00
61734.00	495.79	87.27	0.7210	1.00
61734.00	224.79	72.11	0.6375	1.00
61734.00	435.25	83.53	0.7075	1.00
61734.00	236.25	29.53	0.7395	1.00
61734.00	41.51	50.51	0.7130	1.00
61734.00	155.66	96.96	0.7325	1.00
61734.00	83.03	78.33	0.7210	1.00
61734.00	270.20	88.96	0.8045	1.00
61734.00	249.77	42.66	0.5830	1.00
61734.00	153.70	37.56	0.4960	1.00
61734.00	194.15	50.26	0.3860	1.00
61734.00	361.49	53.23	0.4340	1.00
61734.00	631.14	43.67	0.3850	1.00
61734.00	531.94	30.51	0.3315	1.00

287

BP= 0.988246   AP= -3.357901   CE= 0.589727

NONENZYMATIC PRODUCT 2 DATA

C1	C3	A1	V
128091.00	163.41	1.8700	1.00
128091.00	163.41	1.8700	1.00
128091.00	163.41	1.8700	1.00
128091.00	163.38	1.6890	1.00
128091.00	163.38	1.6890	1.00

ENZYMATIC PRODUCT 2 DATA

C1	C2	C3	A1	V
128091.00	35.54	54.86	0.9385	1.00
128091.00	58.90	150.90	1.2230	1.00
128091.00	158.72	127.57	1.0495	1.00
128091.00	1396.45	160.67	1.5425	1.00
128091.00	635.75	60.34	1.2985	1.00
128091.00	42.07	65.77	1.3160	1.00
128091.00	274.97	173.57	1.4615	1.00
128091.00	1445.56	184.33	1.6740	1.00
128091.00	1334.55	169.50	1.5400	1.00
128091.00	655.33	70.31	1.5250	1.00
128091.00	48.09	93.40	1.2285	1.00
128091.00	131.50	182.33	1.5965	1.00
128091.00	453.02	169.02	1.6350	1.00
128091.00	1508.75	183.81	1.6060	1.00
128091.00	876.23	95.52	1.3680	1.00
128091.00	268.61	99.22	1.1020	1.00
128091.00	208.53	109.81	0.8220	1.00
128091.00	273.08	117.28	0.8500	1.00
128091.00	2432.41	98.68	0.8140	1.00
128091.00	3920.06	79.98	0.7620	1.00

BP= 1.046924   AP= -3.512081   CE= 0.884791

NONENZYMATIC PRODUCT 3 DATA

C1	C3	A1	V
62016.00	90.88	0.8440	1.00
62016.00	90.88	0.8440	1.00
62016.00	90.88	0.8440	1.00
62016.00	93.60	0.7870	1.00
62016.00	93.60	0.7870	1.00

ENZYMATIC PRODUCT 3 DATA

C1	C2	C3	A1	V
62016.00	588.77	33.93	0.4520	1.00
62016.00	1790.69	80.09	0.5480	1.00
62016.00	814.44	74.55	0.5210	1.00
62016.00	111.15	76.88	0.8005	1.00
62016.00	182.80	39.62	0.6965	1.00
62016.00	1141.55	51.21	0.7480	1.00
62016.00	193.14	90.99	0.7880	1.00
62016.00	117.34	92.45	0.7345	1.00
62016.00	122.13	92.75	0.7635	1.00
62016.00	151.03	45.64	0.8580	1.00
62016.00	1461.38	54.05	0.8295	1.00
62016.00	1176.97	95.06	0.9090	1.00
62016.00	319.97	83.07	0.7530	1.00
62016.00	121.10	91.89	0.8105	1.00
62016.00	171.91	49.49	0.6805	1.00
62016.00	6207.46	53.78	0.5190	1.00
62016.00	6568.69	50.78	0.3375	1.00
62016.00	5871.14	61.51	0.3970	1.00
62016.00	1172.33	48.10	0.3240	1.00
62016.00	582.00	37.27	0.3840	1.00

BP= 1.003495   AP= -3.329786   CE= 0.780580

NONENZYMATIC PRODUCT 4 DATA

C1	C3	A1	V
63686.00	97.01	0.8530	1.00
63686.00	97.01	0.8530	1.00
63686.00	97.01	0.8530	1.00
63686.00	100.92	0.7730	1.00
63686.00	100.92	0.7730	1.00

ENZYMATIC PRODUCT 4 DATA

C1	C2	C3	A1	V
63686.00	64.46	33.20	0.4570	1.00
63686.00	191.31	89.29	0.6615	1.00
63686.00	444.83	84.81	0.5275	1.00
63686.00	193.24	93.80	0.7690	1.00
63686.00	95.74	42.22	0.6670	1.00
63686.00	130.17	51.89	0.7690	1.00
63686.00	840.37	104.41	0.7395	1.00
63686.00	281.70	121.07	0.7785	1.00
63686.00	93.57	102.12	0.8100	1.00
63686.00	101.39	44.25	0.7435	1.00
63686.00	166.70	64.40	0.7065	1.00
63686.00	690.68	112.74	0.8665	1.00
63686.00	521.33	104.72	0.8155	1.00
63686.00	146.58	109.41	0.8020	1.00
63686.00	109.77	56.01	0.6695	1.00
63686.00	553.61	50.75	0.4220	1.00
63686.00	584.10	54.73	0.3345	1.00
63686.00	932.11	59.06	0.3405	1.00
63686.00	1230.05	50.47	0.2980	1.00
63686.00	526.88	41.36	0.3170	1.00

BP= 0.993589   AP= -3.482027   CE= 0.491455

NONENZYMATIC PRODUCT 5 DATA

C1	C3	A1	V
62367.00	68.38	0.4530	1.00
62367.00	68.38	0.4530	1.00
62367.00	68.38	0.4530	1.00
62367.00	73.06	0.3220	1.00
62367.00	73.06	0.3220	1.00

ENZYMATIC PRODUCT 5 DATA

C1	C2	C3	A1	V
62367.00	36.54	23.31	0.2780	1.00
62367.00	79.51	75.36	0.4565	1.00
62367.00	281.96	65.62	0.3520	1.00
62367.00	167.03	78.07	0.4650	1.00
62367.00	60.93	25.41	0.3970	1.00
62367.00	42.98	35.75	0.3710	1.00
62367.00	284.47	89.69	0.4545	1.00
62367.00	189.74	92.73	0.3825	1.00
62367.00	92.43	86.71	0.4805	1.00
62367.00	64.06	31.37	0.4645	1.00
62367.00	80.54	43.37	0.4365	1.00
62367.00	261.73	97.92	0.5200	1.00
62367.00	542.09	83.00	0.4160	1.00
62367.00	127.47	93.92	0.4790	1.00
62367.00	96.72	40.79	0.3870	1.00
62367.00	237.39	49.90	0.4550	1.00
62367.00	257.80	57.97	0.3310	1.00
62367.00	413.96	56.57	0.2205	1.00
62367.00	1042.03	50.18	0.1615	1.00
62367.00	391.59	35.03	0.1650	1.00

EQUATIONS:

    1 - 3H PRODUCT FORMED
    2 - PRODUCT FORMED FROM 3H AND ENDOGENOUS SUBSTRATE
    3 - PRODUCT FORMED FROM ALL SOURCES
    X - ENDOGENOUS SUBSTRATE
    XI - ENDOGENOUS PRODUCT
    XII - NET CONVERSION OF SUBSTRATE
    XIII - NET FORMATION OF PRODUCT
    XIV - PRODUCT FORMED FROM ENDOGENOUS SUBSTRATE
    XV - PRODUCT FORMED FROM SUBSTRATE PRECURSORS
    XVI - PRODUCT FORMED FROM OTHER SUBSTRATES

ANIMAL TREATMENTS:

    1(I) - CONTROL, SESAME OIL INJECTED
    2(II) - ESTRADIOL INJECTED
    3(III) - TESTOSTERONE INJECTED
    4(IV) - DDT INJECTED

NMOLES SUBSTRATE: NONENZYMATIC INCUBATION SAMPLE

0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
302.5964	302.5964	302.5964	274.8206	274.8206

NMOLES/MG-PROTEIN, SUBSTRATE: ENZYMATIC INCUBATION

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	48.6618	24.7792	25.3664	37.0729	62.9832
2	61.4888	36.3720	33.4725	34.6214	78.6538
3	64.9663	40.3137	38.5391	37.3770	58.7261
4	2.1821	1.5321	1.4770	1.5556	2.3614

EQUATION X

1	15.6741
2	22.6047
3	25.6834
4	-7.1481

EQUATION XII

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	23.8826	23.2954	11.5889	-14.3214
2	0.0	25.1168	28.0163	26.8674	-17.1650
3	0.0	24.6527	26.4272	27.5894	6.2402
4	0.0	0.6500	0.7051	0.6265	-0.1793

292

PRODUCT 1

NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE

0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
280.0796	280.0796	280.0796	256.8162	256.8162

NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	50.6822	17.4812	18.5000	27.3204	61.5816
2	64.1021	27.4085	29.3472	28.1022	83.0680
3	47.3132	25.3178	30.8498	30.2928	45.8517
4	2.3192	1.3505	1.4329	1.5503	1.9121

EQUATION 1

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0023	0.0035	0.0052	0.0067	0.0194
2	0.0024	0.0237	0.0130	0.0218	0.0334
3	0.0035	0.0068	0.0045	0.0128	0.0247
4	0.0045	0.0043	0.0075	0.0160	0.0192

EQUATION 2

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.3350	0.5157	0.7714	1.1886	3.4433
2	0.4283	4.3161	2.3683	4.5804	7.0326
3	0.7090	1.3850	0.9144	2.9863	5.7566
4	-1.1022	-1.0404	-1.8291	-3.4371	-4.1464

EQUATION 3

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.3350	-0.2648	-0.3674	0.4562	6.0653
2	0.4283	-0.4533	-0.5508	-0.0990	11.6227
3	0.7090	0.0621	-0.0219	0.1854	4.5354
4	-1.1022	-1.1244	-1.9892	-3.7397	-4.0419

EQUATION XI

1	20.1492
2	28.1114
3	10.9534
4	-7.1895

EQUATION XIII

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-33.2010	-32.1822	-23.3618	10.8994
2	0.0	-36.6936	-34.7549	-35.9999	18.9660
3	0.0	-21.9954	-16.4634	-17.0205	-1.4615
4	0.0	-0.9687	-0.8863	-0.7689	-0.4072

EQUATION XIV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-50.3472	-50.1665	-49.9108	-49.4936	-47.2389
2	-63.6738	-59.7859	-61.7337	-59.5217	-57.0695
3	-46.6043	-45.9283	-46.3988	-44.3269	-41.5566
4	-3.4214	-3.3597	-4.1483	-5.7563	-6.4656

EQUATION XV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-0.7805	-1.1388	-0.7324	2.6219
2	0.0	-4.7694	-2.9192	-4.6794	4.5901
3	0.0	-1.3229	-0.9363	-2.8009	-1.2212
4	0.0	-0.0839	-0.1600	-0.3026	0.1045

EQUATION XVI

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-0.3350	-32.9362	-31.8148	-23.8180	4.8341
2	-0.4283	-36.2403	-34.2040	-35.9009	7.3433
3	-0.7090	-22.0575	-16.4415	-17.2059	-5.9969
4	1.1022	0.1556	1.1028	2.9708	3.6348

PRODUCT 2

NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE

0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
586.0391	586.0391	586.0391	528.7720	528.7720

NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	94.7259	45.0190	45.6145	53.4746	119.6202
2	131.1253	55.2489	59.6842	59.6514	142.3873
3	87.0086	58.1029	64.2080	57.9821	94.8595
4	3.8378	2.5776	2.4966	2.8401	3.2777

EQUATION 1

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0048	0.0029	0.0092	0.0639	0.0775
2	0.0055	0.0137	0.0680	0.0683	0.0808
3	0.0045	0.0063	0.0235	0.0719	0.0804
4	0.0062	0.0044	0.0053	0.0565	0.1123

EQUATION 2

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.7090	0.4272	1.3616	11.3371	13.7433
2	1.0083	2.4973	12.3622	14.3604	16.9998
3	0.9216	1.2910	4.7976	16.7451	18.7139
4	-1.5129	-1.0613	-1.3012	-12.1633	-24.1854

EQUATION 3

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.7090	-0.2193	-0.6485	4.3515	24.2081
2	1.0083	-0.2623	-2.8753	-0.3104	28.0954
3	0.9216	0.0579	-0.1148	1.0396	14.7439
4	-1.5129	-1.1469	-1.4151	-13.2343	-23.5761

EQUATION XI

1	30.8385
2	55.8183
3	10.9293
4	-16.0582

EQUATION XIII

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-49.7069	-49.1115	-41.2513	24.8942
2	0.0	-75.8764	-71.4410	-71.4739	11.2620
3	0.0	-28.9057	-22.8006	-29.0265	7.8509
4	0.0	-1.2602	-1.3412	-0.9977	-0.5601

EQUATION XIV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-94.0170	-94.2988	-93.3643	-83.3889	-80.9826
2	-130.1169	-128.6280	-118.7630	-116.7649	-114.1255
3	-86.0870	-85.7176	-82.2110	-70.2635	-68.2947
4	-5.3508	-4.8991	-5.1391	-16.0011	-28.0233

EQUATION XV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-0.6465	-2.0101	-6.9855	10.4648
2	0.0	-2.7595	-15.2375	-14.6708	11.0956
3	0.0	-1.2331	-4.9124	-15.7056	-3.9700
4	0.0	-0.0856	-0.1139	-1.0710	0.6094

EQUATION XVI

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-0.7090	-49.4876	-48.4630	-45.6028	0.6862
2	-1.0083	-75.6141	-68.5658	-71.1635	-16.8334
3	-0.9216	-28.9636	-22.6858	-30.0660	-6.8930
4	1.5129	-0.1133	0.0739	12.2365	23.0159

PRODUCT 3

NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE

0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
302.5964	302.5964	302.5964	274.8206	274.8206

NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	48.6618	24.7792	25.3664	37.0729	62.9832
2	61.4888	36.3720	33.4725	34.6214	78.6538
3	64.9663	40.3137	38.5391	37.3770	58.7261
4	2.1821	1.5321	1.4770	1.5556	2.3614

EQUATION 1

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0618	0.0796	0.0389	0.0051	0.0164
2	0.0936	0.0089	0.0053	0.0055	0.0139
3	0.1147	0.0525	0.0163	0.0056	0.0147
4	0.1280	0.1435	0.1059	0.0270	0.0173

EQUATION 2

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	9.1944	11.8468	5.7886	0.9130	2.9138
2	17.0130	1.6200	0.9687	1.1628	2.9222
3	23.4314	10.7300	3.3381	1.3017	3.4309
4	-31.2300	-34.9998	-25.8259	-5.8228	-3.7307

EQUATION 3

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	9.1944	-6.0829	-2.7568	0.3505	5.1325
2	17.0130	-0.1701	-0.2253	-0.0251	4.8294
3	23.4314	0.4810	-0.0799	0.0808	2.7031
4	-31.2300	-37.8230	-28.0857	-6.3355	-3.6367

EQUATION XI

1	15.6741
2	22.6047
3	25.6834
4	-8.0911

EQUATION XIII

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-23.8826	-23.2954	-11.5889	14.3214
2	0.0	-25.1168	-28.0163	-26.8674	17.1650
3	0.0	-24.6527	-26.4272	-27.5894	-6.2402
4	0.0	-0.6500	-0.7051	-0.6265	0.1793

EQUATION XIV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-39.4675	-36.8150	-42.8732	-47.7488	-45.7480
2	-44.4758	-59.8688	-60.5201	-60.3260	-58.5666
3	-41.5349	-54.2363	-61.6283	-63.6647	-61.5354
4	-33.4121	-37.1819	-28.0080	-8.0049	-5.9128

EQUATION XV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-17.9297	-8.5454	-0.5626	2.2187
2	0.0	-1.7902	-1.1940	-1.1879	1.9073
3	0.0	-10.2490	-3.4180	-1.2209	-0.7278
4	0.0	-2.8232	-2.2597	-0.5127	0.0940

EQUATION XVI

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-9.1944	-17.7998	-20.5386	-11.9394	9.1889
2	-17.0130	-24.9466	-27.7909	-26.8423	12.3356
3	-23.4314	-25.1337	-26.3474	-27.6702	-8.9433
4	31.2300	37.1730	27.3806	5.7090	3.8160

PRODUCT 4

NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE

0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
242.7879	242.7879	242.7879	211.5665	211.5665

NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	41.5246	22.3201	18.7537	24.6867	47.5952
2	52.6021	25.1429	22.8225	28.1485	59.6456
3	39.3501	27.5486	27.9188	26.2812	42.8831
4	1.5628	1.1496	1.0844	1.1111	1.4419

EQUATION 1

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0071	0.0078	0.0192	0.0075	0.0083
2	0.0108	0.0347	0.0100	0.0039	0.0099
3	0.0113	0.0267	0.0217	0.0058	0.0085
4	0.0124	0.0122	0.0180	0.0278	0.0145

EQUATION 2

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	1.0565	1.1658	2.8540	1.3361	1.4707
2	1.9661	6.3083	1.8236	0.8309	2.0778
3	2.3037	5.4522	4.4305	1.3589	1.9879
4	-3.0310	-2.9654	-4.3852	-5.9794	-3.1254

EQUATION 3

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	1.0565	-0.5986	-1.3592	0.5128	2.5905
2	1.9661	-0.6625	-0.4241	-0.0180	3.4340
3	2.3037	0.2444	-0.1060	0.0844	1.5661
4	-3.0310	-3.2046	-4.7689	-6.5059	-3.0466

EQUATION XI

1	15.0569
2	21.4034
3	7.8314
4	-6.6799

EQUATION XIII

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-19.2045	-22.7709	-16.8379	6.0706
2	0.0	-27.4592	-29.7796	-24.4536	7.0435
3	0.0	-11.8014	-11.4312	-13.0689	3.5331
4	0.0	-0.4132	-0.4784	-0.4517	-0.1209

EQUATION XIV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-40.4681	-40.3587	-38.6706	-40.1885	-40.0539
2	-50.6360	-46.2938	-50.7785	-51.7712	-50.5243
3	-37.0464	-33.8979	-34.9195	-37.9912	-37.3622
4	-4.5938	-4.5282	-5.9481	-7.5422	-4.6882

EQUATION XV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0	-1.7644	-4.2131	-0.8232	1.1198
2	0.0	-6.9708	-2.2478	-0.8489	1.3562
3	0.0	-5.2078	-4.5365	-1.2745	-0.4217
4	0.0	-0.2392	-0.3837	-0.5265	0.0787

EQUATION XVI

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-1.0565	-18.6059	-21.4117	-17.3507	3.4801
2	-1.9661	-26.7966	-29.3555	-24.4356	3.6095
3	-2.3037	-12.0458	-11.3252	-13.1532	1.9669
4	3.0310	2.7914	4.2905	6.0541	2.9257

PRODUCT 5

NMOLES PRODUCT: NONENZYMATIC INCUBATION SAMPLE

0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
173.2276	173.2276	173.2276	114.9919	114.9919

NMOLES/MG-PROTEIN, PRODUCT: ENZYMATIC INCUBATION

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	33.8900	17.2686	15.2664	16.9816	44.4992
2	34.8253	17.0276	13.8450	18.6271	49.7619
3	34.1568	18.0429	17.0045	17.3189	32.1739
4	1.6190	1.0117	0.6888	0.5676	0.8309

EQUATION 1

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.0056	0.0038	0.0154	0.0077	0.0086
2	0.0051	0.0134	0.0086	0.0045	0.0086
3	0.0079	0.0114	0.0279	0.0058	0.0101
4	0.0053	0.0050	0.0082	0.0232	0.0125

EQUATION 2

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.8353	0.5622	2.2896	1.3588	1.5229
2	0.9227	2.4344	1.5705	0.9466	1.8135
3	1.6185	2.3295	5.6921	1.3481	2.3553
4	-1.2945	-1.2101	-1.9911	-4.9892	-2.6858

EQUATION 3

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0.8353	-0.2887	-1.0904	0.5216	2.6825
2	0.9227	-0.2557	-0.3653	-0.0205	2.9971
3	1.6185	0.1044	-0.1362	0.0837	1.8556
4	-1.2945	-1.3077	-2.1654	-5.4285	-2.6181

EQUATION XI

1	15.0054
2	12.5653
3	11.6685
4	-4.2621

EQUATION XIII

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0·0	-16·6214	-18·6236	-16·9084	10·6092
2	0·0	-17·7977	-20·9803	-16·1982	14·9366
3	0·0	-16·1139	-17·1524	-16·8380	-1·9829
4	0·0	-0·6073	-0·9301	-1·0513	-0·7881

EQUATION XIV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-33·0547	-33·3277	-31·6003	-32·5311	-32·3671
2	-33·9025	-32·3909	-33·2548	-33·8787	-33·0118
3	-32·5383	-31·8273	-28·4647	-32·8087	-31·8016
4	-2·9134	-2·8290	-3·6101	-6·6082	-4·3048

EQUATION XV

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	0·0	-0·8509	-3·3800	-0·8372	1·1596
2	0·0	-2·6900	-1·9357	-0·9671	1·1836
3	0·0	-2·2251	-5·8283	-1·2644	-0·4996
4	0·0	-0·0976	-0·1742	-0·4393	0·0677

EQUATION XVI

	0 MIN	5 MIN	10 MIN	30 MIN	60 MIN
1	-0·8353	-16·3327	-17·5331	-17·4299	7·9267
2	-0·9227	-17·5420	-20·6150	-16·1777	11·9395
3	-1·6185	-16·2184	-17·0162	-16·9216	-3·8386
4	1·2945	0·7004	1·2352	4·3771	1·8300

CONVERSION OF 3H; TREATMENT 1

CONVERSION OF 3H; TREATMENT II

CONVERSION OF 3H; TREATMENT III

Y-axis: N M O L E S / M G   P R O T E I N

Y-axis values: 0.143, 0.115, 0.086, 0.057, 0.029, -0.000

X-axis: TIME IN MINUTES

X-axis values: 0.0, 10.000, 20.000, 30.000, 40.000, 50.000, 60.000

305

CONVERSION OF 3H: TREATMENT IV
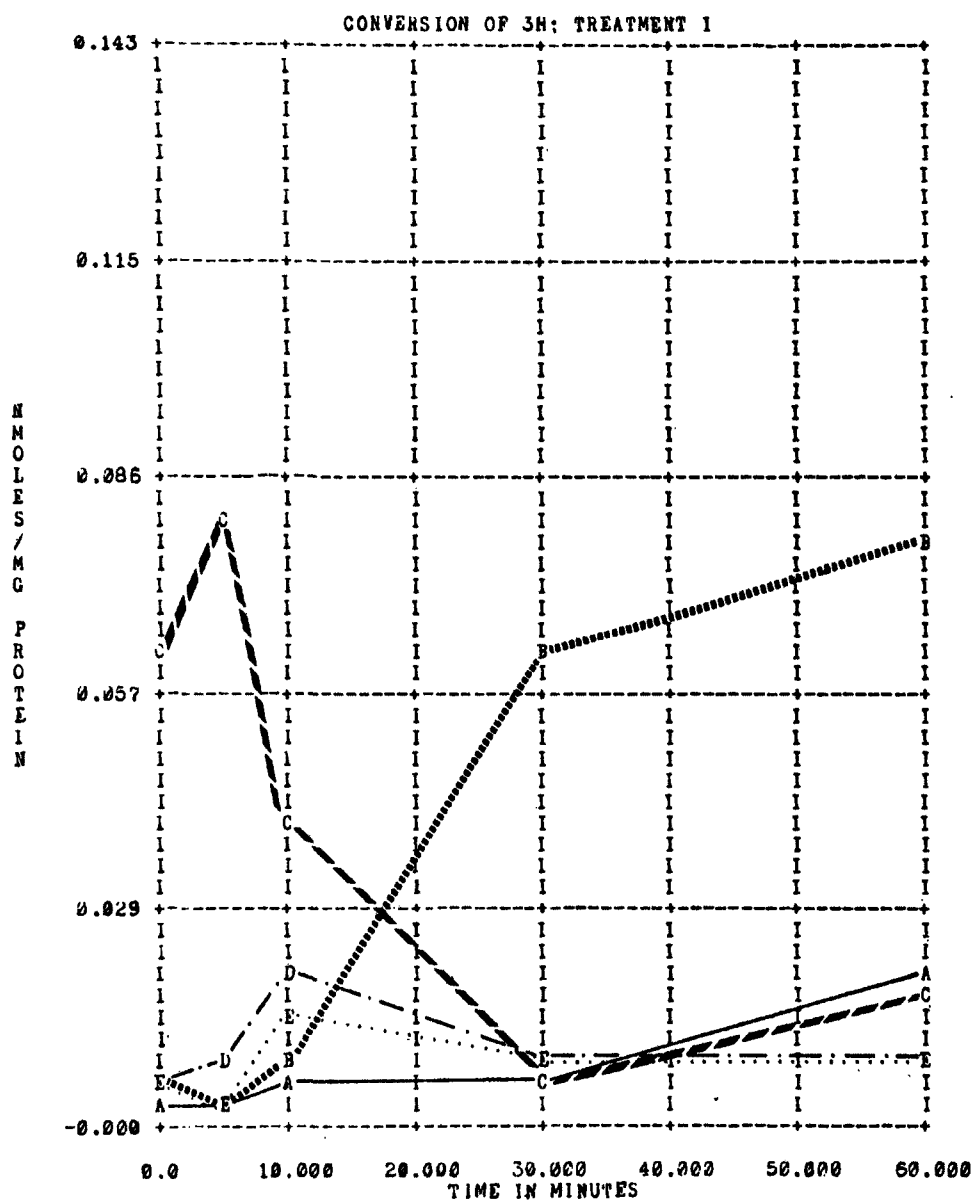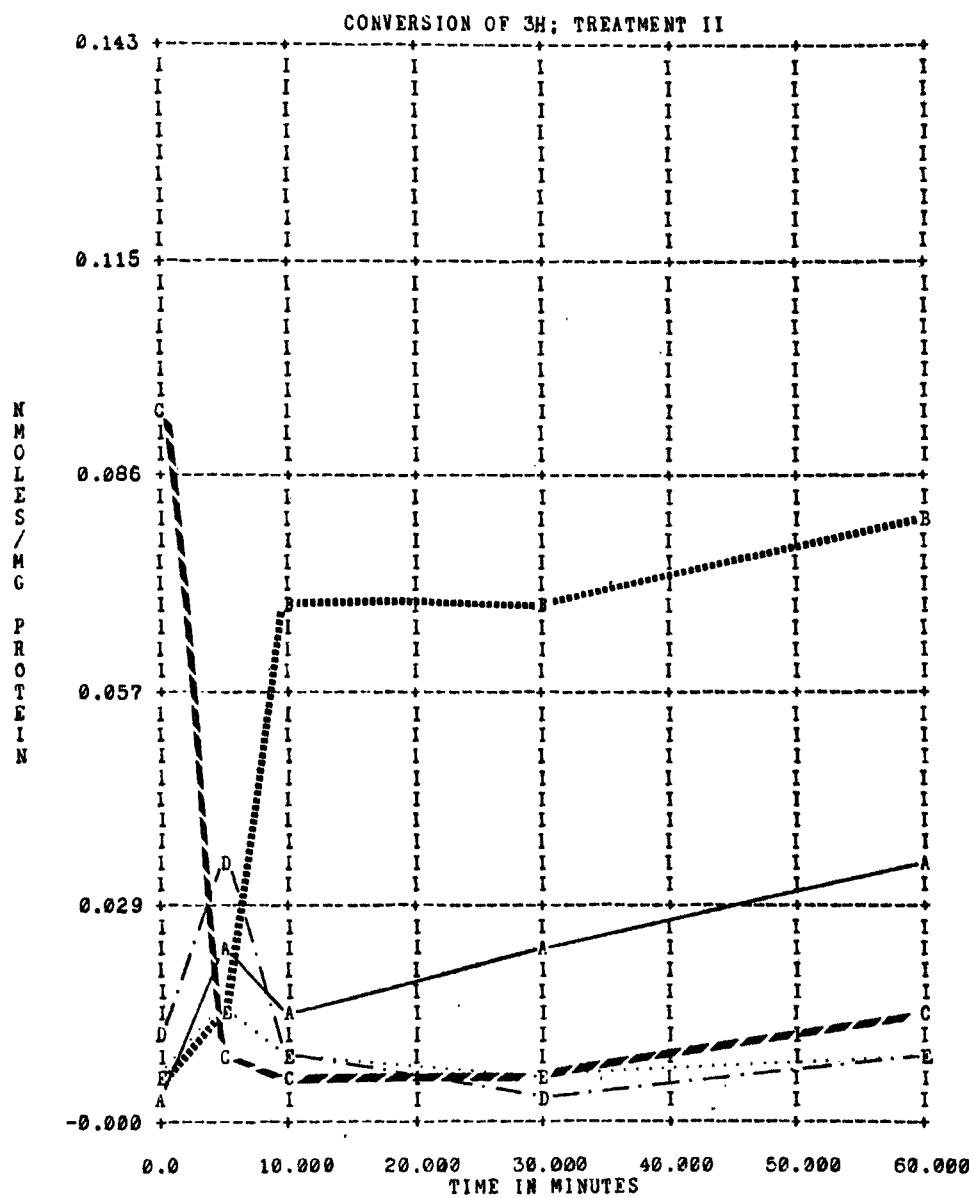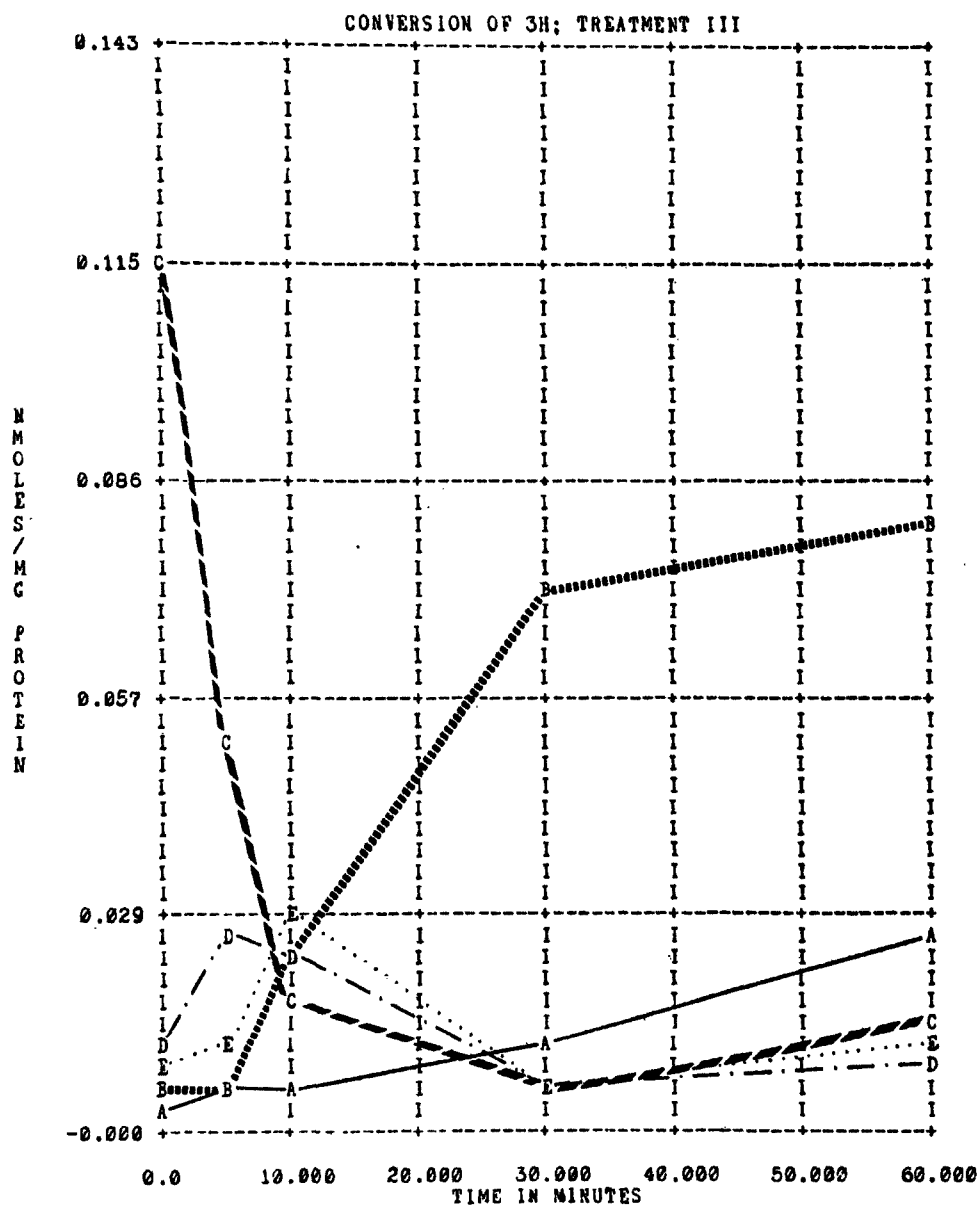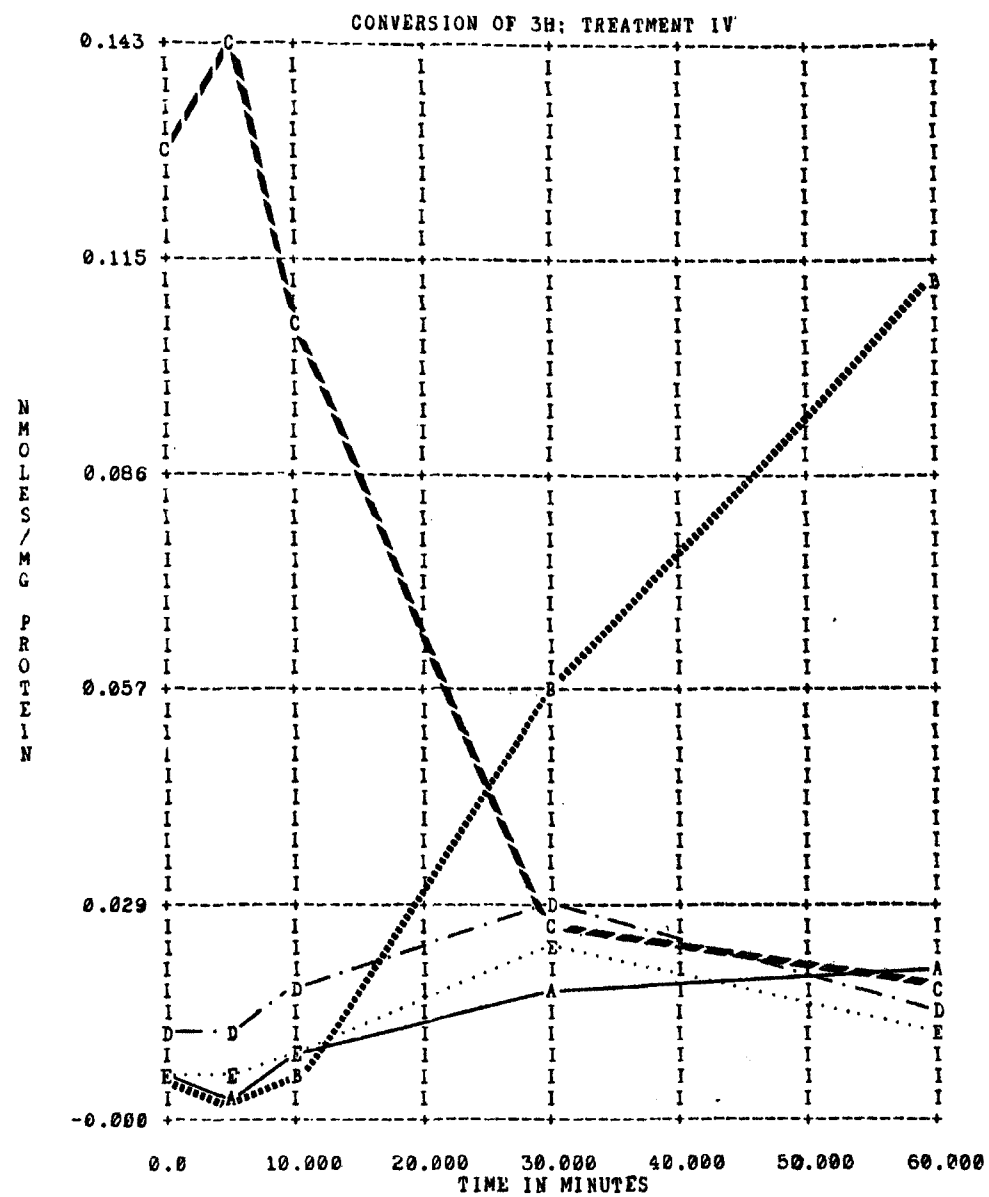
TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/1-77-042	2.	3. RECIPIENT'S ACCESSION NO.

4. TITLE AND SUBTITLE METABOLIC INTERACTIONS OF HORMONAL STEROIDS AND CHLORINATED HYDROCARBONS - Effects of Neonatal Treatment with o,p'-DDT on the Development of the Steroidogenic Endocrine System of the Male Rat	5. REPORT DATE September 1977
	6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S) Kenneth Lyle Campbell	8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS The University of Michigan Medical School Ann Arbor, Michigan 48104	10. PROGRAM ELEMENT NO. 1EA615
	11. CONTRACT/GRANT NO. R-800637

12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory RTP,NC Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, N.C. 27711	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES

16. ABSTRACT

This study was an attempt to generate approaches to the measurements of the potential of xenobiotics to alter reproductive capacity and viability which may allow improvement of those currently in use. More specifically, it was an attempt to identify the effects of neonatal exposure to the model compound, o,p'-DDT (1,1,-trichloro-2-(2-chlorophenyl) -2-(4-chlorophenyl)-ethane), on the developing steroidogenic endocrine system of the model animal, the male rat. The effect of exposures by direct injections before day 5 of age and by indirect treatment via an o,p'-DDT injected dam were examined initially by measurement of growth, organ weights, organ histology, and of serum corticosterone and luteinizing hormone (LH). Later experiments included determinations of the effects of o,p'-DDT on the serum LH responses to challenges of the hypothalamo-pituitary axis with adult castration and repeated injections of luteinizing hormone releasing hormone (LHRH). The observed effects indicated a precocious development of the adrenal cortex in immature animals treated neonatally with o,p'-DDT similar to that seen in rats neo-natally injected with 17 -estradiol-17-valerinate. They also demonstrated altered serum LH responses in treated rats to challenges with adult castration or repeated injections of LHRH. These results are discussed in relation to previous work on steroids and o,p'-DDT in several animal systems, to possible mechanisms, to prospective experiments and to the original goal of the research.

17.	KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b.IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group	
Chlorohydrocarbons Steroids Endocrine glands Metabolism Biochemistry Rats DDT		06 A, F	

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS *(This Report)* UNCLASSIFIED	21. NO. OF PAGES 322
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (9-73)