"Secondary Effects of Oral Contraceptives"

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ABBREVIATIONS USED

ALA	amino laevulinic acid
ALAS	amino laevulinic acid synthetase
ADP	adenosine diphosphate
BMR	basal metabolic rate
^{o}C	degrees Centigrade
CHA	chlormadinone acetate
CNS	central nervous system
DEAE	diethylaminoethyl
DES	diethylstilboestrol
DNA	deoxyribonucleic acid
EE	ethinyl estradiol
EDDA	ethynodiol diacetate
e-r	estrogen receptor
FSH	follicle stimulating hormone
FSH-RH	follicle stimulating hormone releasing factor
g	gram
g x 1 000	gravity
g value	electron paramagnetic resonance signal
GNRH	gonadotropin releasing hormone
h	hours
HGH	human growth hormone
HDL	high density lipids
ip	intraperitoneally
IUD	intra uterine device
Kd	dissociation constant
kg	kilogram
K_M	Michaelis constant

K_s	Spectral dissociation constant
LH	luteinizing hormone
LH-RH	luteinizing hormone releasing hormone
m	molar
max	maximum
MEE	mestranol
min	minute
mm	millimetre
mRNA	messenger ribonucleic acid
NEFA	non-esterified fatty acid
NHP	non-histone protein
nm	nanometer
nmol	nanomole
OC	oral contraceptive
OCs	oral contraceptive steroids
p	probability
%	per cent
p-r	progesterone-receptor
r	free receptor
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RI	reverse type I spectrum
S	sedimentation values
SDS	sodium dodecyl sulphate
SER	smooth endoplasmic reticulum
-SH	sulphydryl group
Sh-Rc	steroid hormone receptor complex
S-Rc	steroid cytoplasmic receptor
S-Rn	steroid nuclear receptor

tRNA	transfer ribonucleic acid
μg	microgram
μl	microlitre
UK	United Kingdom
USA	United States of America
XA	xanthanuric acid
<	less than

KEY TO ELECTRON MICROGRAPHS

A	Artifact
BC	Bile canaliculus
c	Site of continuity between RER and SER
CM	Cell membrane
EDB	Electron dense body
ER	Endoplasmic reticulum
G	Glycogen
L	Lipid
LSO	Light staining organelle
M	Mitochondrion
MF	Myelin figure
N	Nucleus
Nu	Nucleolus
NP	Nuclear pore
RER	Rough endoplasmic reticulum
SER	Smooth endoplasmic reticulum

All material was fixed in glutaraldehyde and osmium tetroxide.

Embedded in Epon 812. Stained in uranyl acetate and lead citrate.

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ABSTRACT

Norethynodrel, a common progestin in oral contraceptives, produces in female rats several significant physiological, cytological and biochemical changes at dose levels of 1 mg and 20 mg per kg:

1) a relative increase in liver mass
2) modification of appearance and extent of the endoplasmic reticulum
3) augmentation of the protein content of the liver
4) increase of the level of cytochrome P-450 in the liver as determined by:
    a) difference spectroscopy
    b) increases in biotransformation of aniline and aminopyrine *in vitro* and
    c) reduction of sleeping times of rats dosed with phenobarbital

The significance of these findings becomes evident when it is realized that norethynodrel affects and is affected by the same enzyme system which oxidizes medicaments in general in the body: induction of cytochrome P-450 by administration of norethynodrel may interfere with the action of other drugs.

Ethinyl estradiol alone showed none of the inductive effects. At high dose levels (20 mg per kg) both norethynodrel and ethinyl estradiol caused a marked inhibition of growth of the animals, producing a net loss of body mass over the 30-day experimental period.

Electron micrographic evidence implies that there is also a lowering of glycogen content and a chemical change in the lipids of adrenocortical and liver cells accompanying the use of these agents.

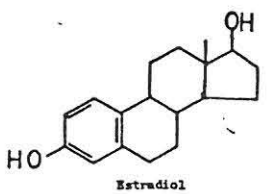
LITERATURE REVIEW

SECTION 1

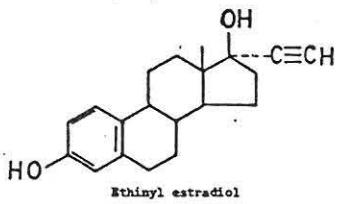
REVIEW OF THE BIOLOGICAL EFFECTS OF ORAL CONTRACEPTIVE STEROIDS

The ingestion of steroids brings about alterations not only in the reproductive system but in nearly all physiological processes. The oral contraceptives are a group of synthetic steroids which are structurally dissimiliar from the natural hormones and from each other yet, it can be expected that they may be subject to the same metabolism or display the same activity as the natural steroids.

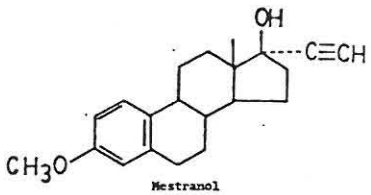
Many orally active synthetic steroids are available on prescription today. Each may vary in its relative estrogenic or anti-estrogenic, progestational or anti-progestational activities on the reproductive tract or in its degree of induction of hepatic microsomal enzymes. The structures of the compounds most commonly used as contraceptive drugs are illustrated below.



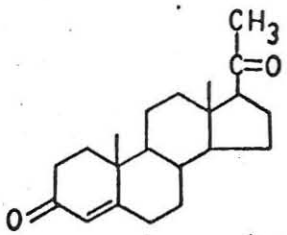
Estradiol



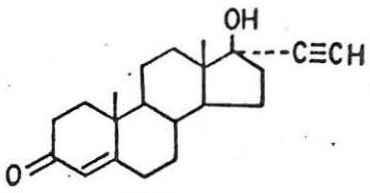
Ethinyl estradiol



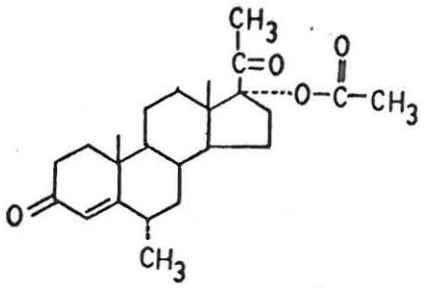
Mestranol



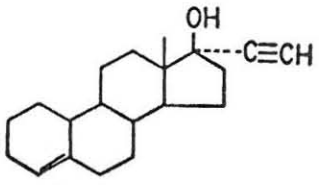
Progesterone



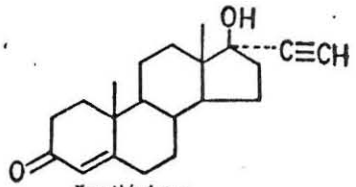
Ethisterone



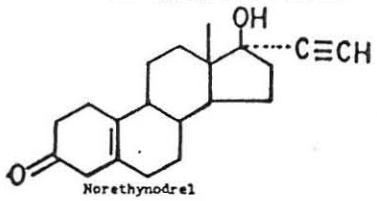
Medroxyprogesterone acetate



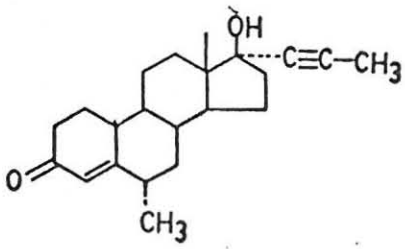
Lynestrenol



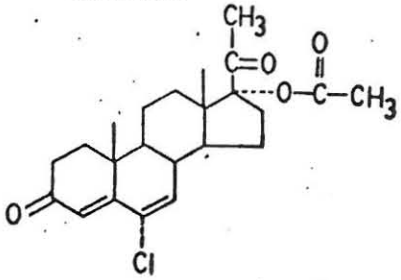
Norethindrone



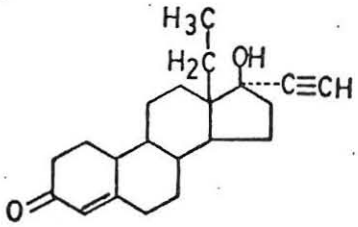
Norethynodrel



Dimethisterone



Chlormadinone acetate



Norgestrel

The oral contraceptive drugs available in South Africa are listed in Table 1 by their product names with the dose of the individual progestin and estrogen in each tablet.

TABLE 1. List of Oral Contraceptives Available in South Africa

Product	Tablet Composition		
	Progestin (mg)	Estrogen (mg)	
		Mestranol	Ethinylestradiol
	Norethynodrel		
Conovid E	2,50	0,10	
Enovid	4,925	0,075	
	Norethindrone		
Micro-novum	0,35	-	-
Noriday	0,35	-	-
Norinyl-1	1,0	0,05	-
Ortho-novum 1/80	1,0	0,08	-
Ortho-novin	2,0	0,10	-
	Norethisterone acetate		
Anovlar	4	-	0,05
Gynovlar	3	-	0,05
Minovlar-ED	1	-	0,05
Norlestrin -FE	1	-	0,05
	Lynestrol		
Exluton	0,5	-	-
Lyndiol	2,5	-	0,05
Ovostat - 28	1		0,05

Table 1 continued

Product	Tablet Composition		
	Progestin (mg)	Estrogen (mg)	
		Mestranol	Ethinylestradiol
	Ethynodiol Diacetate		
Demulen	1	-	,05
Metrulen	2	-	0,1
Ovulen-21	1	0,1	-
Fumulen	0,5	-	-
	Megestrol Acetate		
	4	-	0,05
	Norgestrel		
Nordette - 28	0,15	-	0,03
Nordiol	2,5	-	0,05
Ovral	0,5	-	0,05

1.1 The Role of OCs in the Control of Fertility

When consideration is given to more modern methods of contraception, two emerge as highly effective: oral contraception and the use of intrauterine devices (IUD).

John Rock and the late Gregory Pincus were pioneers in the field of oral contraceptives (OCs) and it was due largely to their efforts that the first OCs were made available in 1960.

Enovid appeared in that year and became known as "the pill". With the advent of OCs arose the possibility of virtually 100% effectiveness in the prevention of pregnancy. Very few drugs used in medicine have such a high degree of effectiveness. Thus, it is not surprising that in 1965 the number of users reached about 3,8 million in the U.S.A. alone.

Today, approximately 50 million women throughout the world are practising oral contraception.

Dosages and Regimen

OCs in use today are prescribed as combined tablets or sequential tablets.

1.1.1 Combined Tablets

These are more popular than sequential tablets, and one course usually comprises 20 or 21 identical tablets containing one of several synthetic progestins as well as an estrogen. They are taken from day 5 to day 24 or 25 of the cycle.

1.1.2 Normocyclic Tablets

These are also combined progestin and estrogen tablets. The normocyclic tablets take into account physiological conditions existing during the normal menstrual cycle. In the first half of the cycle, considerably less progestin (0,05 mg/day) is ingested than in the second half of the cycle (0,125 mg/day). The estrogen dose remains the same throughout the entire cycle.

These doses are administered according to a proven 11/10 day regimen, which conforms with phases of the normal cycle.

1.1.3 Sequential OCs

Under this regimen, 15 or 16 tablets containing estrogen are ingested,

followed by 5 tablets containing estrogen and progestin. One tablet is ingested daily. In view of the serious side effects caused by the estrogens, sequential OCs have become unpopular and most of them have been withdrawn.

1.1.4 Interference with Fertility by OCs

The female reproductive cycle is a complicated, delicately balanced system controlled by the subtle interplay of hormones.

Shown in Figure 1 are the numerous target organs and feedback connections that may be upset by oral contraceptives.

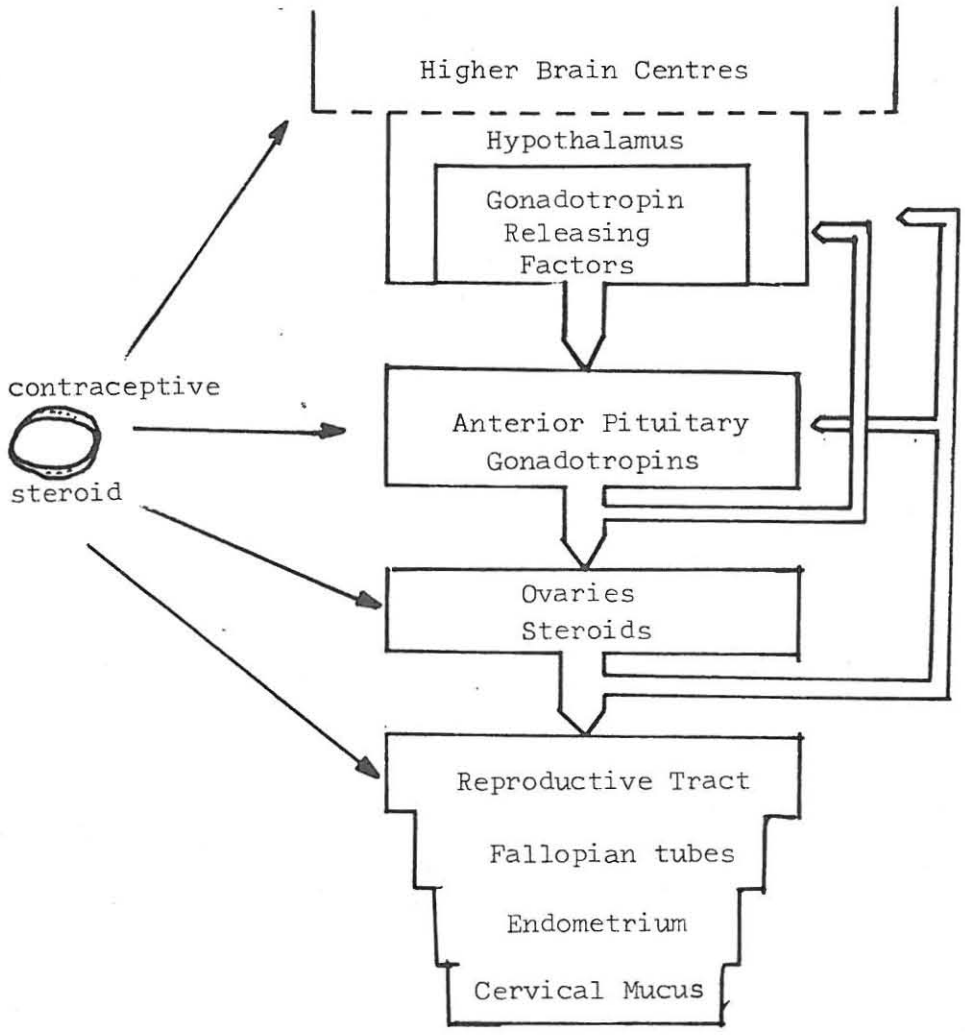


Figure 1.

Schematic Representation of Inter-relationship and Feedback Mechanisms of Female Reproductive System and Possible Areas of Interference by OCs.

The anti-fertility effect of the OCs may arise from the alteration of one or several of these vulnerable areas.

To understand the mechanism of OC contraception, it would be useful to consider hormonal interactions during the normal cycle and during pregnancy. The activity of the ovaries is controlled by the pituitary-hypothalamic system. Under the influence of gonadotropin releasing hormones (GNRH) produced by the hypothalamus, the pituitary secretes the gonadotrophic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (McCaan and Ramriz 1964; Zanatu et al.1974; and Schally and Kastin 1970). FSH and LH control maturation of the follicle, ovulation, the formation of the corpus luteum, and the production of oestrogens and progesterone (Keys et al.1973; and Crossland 1970). In their turn, the steroid hormones produced by the ovary act on the hypothalamus to regulate the temporal and quantitative release of the gonadotrophic hormones from the anterior lobe of the pituitary.

It can be seen from this explanation that the normal regular cycle is the result of an intricately balanced regulating mechanism, which is referred to as a feedback system. These steroids may exert either a positive or a negative feedback on gonadotrophin release which acts directly on the pituitary or indirectly on the hypothalamus.

Evidence suggests that a negative feedback (Bogandove et al.1975; and Everette 1969) between the various steroids exists as exemplified by Figures 2 and 3.

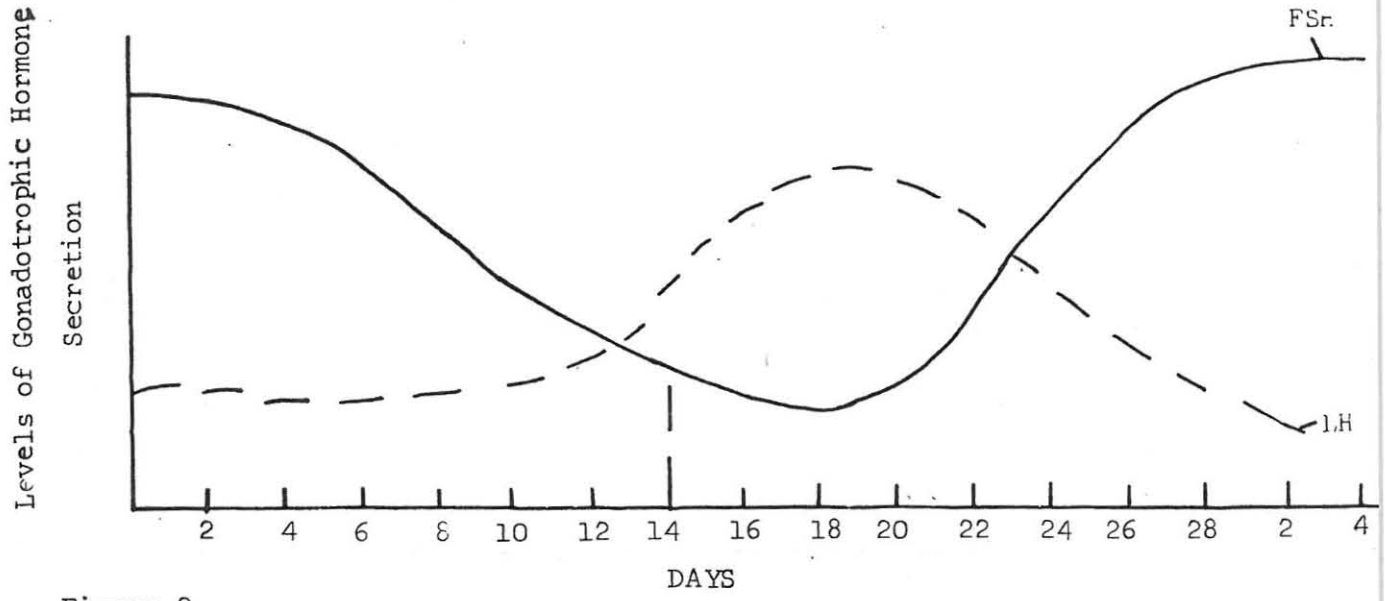


Figure 2.

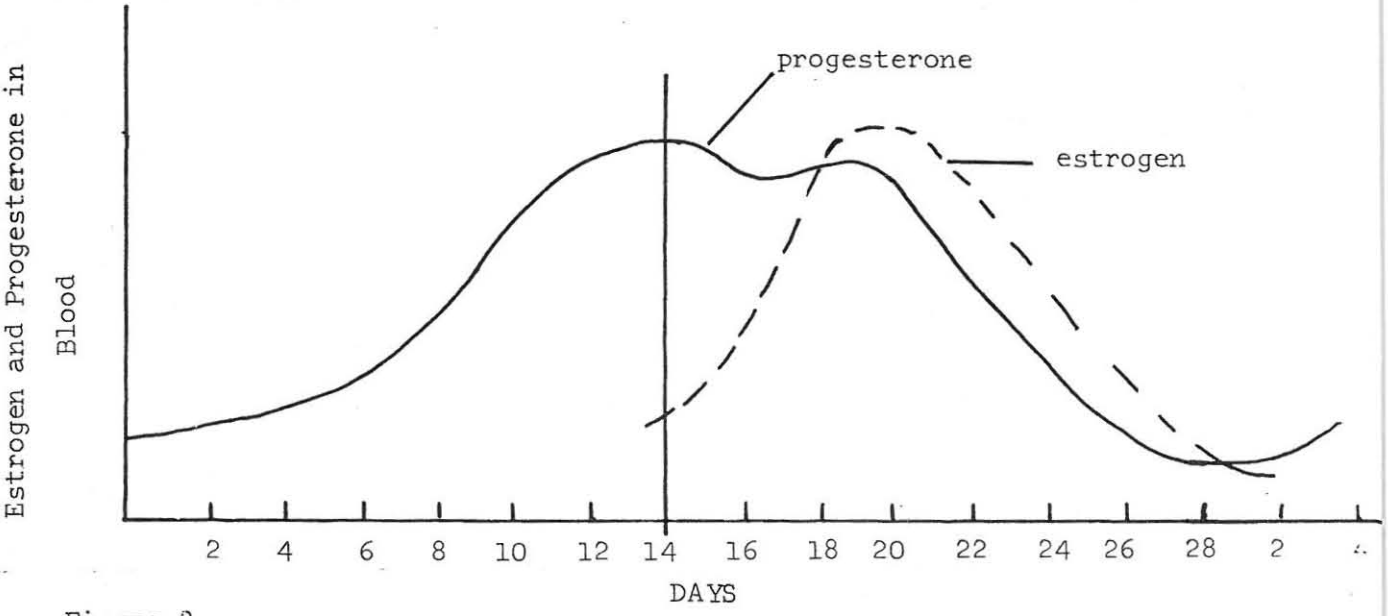


Figure 3.

Other evidence suggesting a negative feedback system is that the secretion of gonadotropins is inversely proportional to levels of gonadal steroids and that, on administration of estrogen to ovariectomized rats, post-menopausal women and premenopausal women in the follicular phase of the cycle, circulating gonadotropins decreased (Everett 1969; Saunders 1964; and Scharwz 1969).

Schally and Kastin (1970) cite evidence for the "auto-feedback", in addition to the two mechanisms for control of LH and FSH (hypothalamic

releasing hormones and sex steroids). In the "auto-feedback", the inhibitory impulse is provided by LH and FSH themselves.

The median eminence of the hypothalamus is thought to contain receptors sensitive to high plasma levels of FSH and LH. High plasma levels of these hormones reduce the secretion of further amounts of FSH and LH through inhibition of release of FSH-RH and LH-RH respectively.

Sensitive radio-immunoassay techniques have made it possible to determine the blood levels of gonadotropins, estrogens and progesterone which are characteristic of the ovulatory cycle.

Prior to ovulation, a distinct rise in serum estrogens occur, which is then followed by an LH peak.

The sharp elevation of luteinizing hormone is apparently a prerequisite for the induction of ovulation.

Following ovulation, a corpus luteum develops from the ruptured follicle and produces appreciable amounts of progesterone (Figures 2 and 3.) This process can be recognized from a distinct rise in serum progesterone, measured as urinary pregnanediol, during the second half of the cycle (Figures 2 and 3).

Progesterone secretion from the ovary only occurs after ovulation and after a functional corpus luteum has been formed. It is important to note that a large amount of pregnanediol is excreted during the first half of the cycle, the progesterone being secreted from the adrenal and possibly from the corpus luteum of the previous cycle (Drills 1966). Thus, high serum level of pregnanediol should not be taken as absolute proof that ovulation has occurred.

During pregnancy, the corpus luteum of pregnancy, and soon afterwards the placenta, produce increased amounts of steroid hormones. These inhibit the secretion of gonadotropins by the pituitary via the hypothalamus, preventing the maturation of further follicles - this is the normal cycle.

1.2 Mechanism of Action of OCs

Extensive studies have been carried out with OCs and their activities have been reviewed (Pincus 1966; Saunders 1964; Drills 1966; and Diczfalusy 1968).

OCs Mechanism of Action can be ascribed to

(1) effect on the CNS-hypothalamus pituitary axis

(2) inhibition of nidation

(3) changes in viscosity of cervical mucus, making it inhospitable to sperm motility and transport.

It can be concluded that most, if not all, of the OCs exert some effect on the female reproductive tract.

A discussion of all the possible modes of action of OCs is beyond the scope of this work. Only the effect on CNS-hypothalamus pituitary axis will be reviewed since this seems to represent the principle action of OCs and the other two may be a secondary result of the effect of OCs on this system.

1.3 Effect of OCs on CNS-hypothalamus-pituitary Axis

Animal Studies

Drills (1966) and Saunders (1964) demonstrated that norethynodrel decreases the gonadotropin content of the pituitary gland of ovariectomized rats. Saunders (1964) has shown that, in addition, norethindrone and

ethynodiol diacetate also have this effect of inhibiting the secretion of pituitary gonadotropins in the rat.

Pincus (1965) provided indirect evidence that OCs depress pituitary gonadotropin function, inhibit spermatogenesis, reduce testes mass and cause atrophy in Leydig cells of the male rat.

It has been generally accepted that steroid ovulation inbitors inhibit gonadotropin release, particularly LH release. This can be accomplished in numerous ways - by decreasing the sensitivity of certain area in the hypothalamus or pituitary to LH-RH and FSH-RH, or by decreasing the synthesis and release of FSH-RH and LH-RH.

Hillard et al. (1966) and Spies (1969) seem to belong to the first school of thought. Hillard et al. (1966) reported that norethisterone (norethindrone) could block ovulation in rabbits by intrapituitary infusion of crude hypothalamic extract. They also established that pretreatment with estrogen partially prevented this blockage of ovulation - thus they concluded that the suppression of release of LH by norethisterone is mediated through its ability to block estrogen receptors in the pituitary and median eminence. Spies (1969) using chlormadinone acetate, arrived at the same conclusion. It must be borne in mind that Hillard et al. (1966) and Spies (1969) employed crude preparations of hypothalamic extracts in their experiments so that the effect of interfering substances (in addition to LH-RH) cannot be entirely excluded.

Schally and Kastin (1970) cite experimental work performed by Döck et al., work which is in agreement with the results obtained by Spies (1969). This group found that intrapituitary implants of chlormadinone blocked ovulation in rats. They explained their results by claiming that progestogens, in the absence of estrogen, block the pituitary receptor

sites and depress their sensitivity to LH-RH.

At variance with these claims are Ramirez and McCaan (1963) and Schalley et al. (1967 and 1968), who showed that concomitant administration of progesterone and estrogen does not block the responsiveness to LH-RH.

Schally (1968) also showed that an excellent response to LH-RH is elicited in ovariectomized rats pretreated with large doses of various progestins in the complete absence of estrogen.

Other factors, such as a decrease in the responsiveness of the ovary to exogenous (or endogenous) LH after treatment with the progestin (chlormadinone), could have contributed to the results of Spies (1969). However, this seems unlikely as Drills (1966) showed that norethynodrel, another progestin, does not inhibit the increase in ovarian mass induced by injections of gonadotropin. Eckstein et al. (1961) using immature rats, also showed, that norethindrone does not affect ovarian mass response. Therefore, it seems unlikely that the OCs could decrease the responsiveness of the ovary to gonadotropins.

A great deal of effort has been devoted to gaining insight into the effect of ethinylestradiol and mestranol (estrogens) and various progestins on the plasma FSH and LH levels and response to LH-RH in ovariectomized rats (Pincus 1966 and Drills 1966).

It can be concluded from these studies that:

(1) large doses of various progestins and their combination with estrogens exert inhibitory effects on LH secretion, since plasma levels of LH in ovariectomized rats are low (Schally et al. 1968);

(2) combination of progestins and estrogens also decrease FSH levels in the same animals. The extent of suppression of FSH is however less than

LH. Not much work has been done however on the effect of OCs on FSH levels (Pincus 1966);

(3) none of these steroids blocked the responsiveness of LH-RH;

(4) only small doses of OCs are required to block LH release.

In conclusion then, in animals it seems that inhibition of ovulation by steroidal agents basically appears to involve interference with hypothalamic processes governing pituitary gonadotropin synthesis and release, rather than a decrease in sensitivity, to LH-RH and FSH. The hypothalamus rather than the pituitary is the principal site of action. Pincus (1966) cited evidence that hypothalamic implants of OCs are more effective in preventing ovulation than are pituitary implants.

1.4 Effects of OCs on Gonadotropin, Progestins and Estrogens in Humans

1.4.1 Gonadotropins

Not much can be added to the previous conclusions that sequential OCs and combined OCs suppress the midcycle surge of FSH and LH (Diczfalusy 1968; Swerdloff and Odell 1969).

Many reviews have been published on the effects of OCs on gonadotropins (Saunders 1964; Pincus 1966; Drills 1966; Diczfalusy 1968), and the following conclusions can be drawn:

(1) In most women on OC regimen, total gonadotropin is reduced in the urine, however the degree of suppression is variable. Diczfalusy (1968) and Cargille et al. (1973) provided evidence for a variable effect of ethinyl estradiol on plasma FSH and LH. This group indicated that the effect of estrogens on the circulatory levels of gonadotropins is determined by the "timing of estrogen administration during the menstrual cycle". This provides evidence that it is the estrogen component which

is responsible for suppression of FSH (Vory et al. 1965 and Weisz et al. 1973).

Combined OCs, but not progestin-only OCs, depress FSH, however by and large, there is a fairly consistent elimination of midcycle FSH and LH peaks by all preparations except that, with many of the new "mini-pills", (progestin-only), the LH peak may be reduced or occasionally unchanged.

(2) Urinary preganediol is highly reduced in the late cycle in most OC users, but ovulation occurs in a number of women on the mini-pill and chlormadinone acetate. Martinez-Manautou (1967) estimated that about 60% of those receiving low dose of chlormadinone acetate will ovulate. Weisz et al. (1973) report one case of a patient on chlormadinone acetate who ovulated). In these women, plasma progesterone and urinary pregnanediol seem to approach levels seen in untreated cycles. Whether the anti-fertility action of these "mini-pills" and chlormadinone acetate is tied up with cervical mucus that inhibits sperm transport (Martinez-Manautou 1967), with its interference with progesterone metabolism (Diczfalusy 1968), or with its allowing luteinization of the follicle without release of ovum is not clear.

(3) Since about 5-10% of the active estrogen produced daily is excreted in the urine, determination of estrogenic activity furnishes a useful index of estrogen production. That OCs reduce urinary estrogens has been indicated by histological examination of ovarian biopsies and by studies on urinary excretion (Pincus 1965 and Diczfalusy 1968). This is to be expected since the principle action of the OCs is to inhibit ovulation. This inhibition should decrease ovarian estrogen production since no graafian follicle forms in the absence of ovulation.

Weisz et al. (1973) found a similiar decrease in estrogens in ovarian and peripheral venous plasma in women receiving OCs. Previous evidence leads to the conclusion that the OCs must either prevent the pituitary gonadotropins from stimulatory functions or they must act to inhibit the release of gonadotropins from the anterior pituitary.

Modern research is directed towards the effect of the newer mini-pills on gonadotropins. Saunders (1971) measured plasma LH and FSH at intervals through a control cycle (pretreatment cycle) and the first cycle following dosing with 0,5 mg chlormadinone acetate. These workers found that, during the control cycle, eight of the nine women used in the test ovulated, while during the treatment cycle, four out of nine ovulated. Delvoye et al. (1973) reported studies with d-norgestrel (0,25 and 2,5 mg) and 10 mg norethisterone acetate administered from day 5 to day 26 of the first treatment cycle. d-Norgestrel at 0,25 mg had no consistent effect on basal levels of LH or FSH, one woman showing a sharp LH peak. d-Norgestrel at 2,5 mg daily definitely inhibits LH secretion, but increases FSH. Norethisterone acetate at 10 mg daily has no effect on FSH but causes a period of LH surge followed by a decrease in basal levels.

Moghissi and Marks (1971) also studied serum FSH and LH in six women receiving 0,075 mg dl-norgestrel daily. They found a fairly constant inhibition of the two gonadotropins and also noted that basal secretion was unchanged. The results concerning FSH are at variance with the results of Delvoye et al. (1973), who studied the effects of 2,5 mg d-norgestrel. They found FSH increased. These results could possibly be explained on the basis of dose related effects in that, at lower doses, the effects seem to be variable. However, Briggs et al. (1970) cited evidence that progestins given alone do not depress levels of FSH. Mishell and Odell (1971)

worked on dose related effects of progestin - only products on serum LH. They administered doses ranging from 0,1 to 2 mg of ethynodiol diacetate. At doses of 1 and 2 mg daily, the mid-cycle surge of LH was eliminated, while lower doses had a very variable effect. Garmendia et al. (1973) also reported a study on 0,03 mg d-norgestrel administered daily. Of 10 treated women, 9 showed significant LH peaks, but these peaks occurred earlier in the cycle than in the controls.

Achtari et al. (1971), using radio-immunoassay, determined separate FSH and LH concentrations in women treated with OCs throughout the cycle, then again during the first post-treatment cycle after 8-13 months of exposure. They found that mid-cycle peaks were absent and that only about one-third of their patients returned to a normal LH, FSH secretion pattern during the first post-treatment cycle.

In summary, it appears that estrogens inhibit FSH, progestins the mid-cycle LH peak, and combined preparations result in the suppression of both peaks. The newer mini-pills seem to have complex effects on gonadotropin secretion. They appear to cause much less disruption of pituitary function than the combination or sequential OCs and seem to allow patterns of gonadotropin release associated with ovulation.

1.4.2 Estrogens

Direct examination of the ovary during estrogen-progestin therapy shows the absence of newly formed copora lutea and a lack of follicular development. This indicates that the ovary is inactive and resembles the ovary during the menopause. This state of inactivity of the ovary is confirmed by the reduced excretion of estrone, estradiol and estriol, three estrogens. Normally, the ovary secretes estradiol primarily at mid-

cycle with large amounts of estrogens also released in response to the "mid-cycle surge" of gonadotropins (this estimated feedback on pituitary decreased gonadotropin secretion). A second peak of estrogen is seen toward the end of the cycle; this is secreted by the corpus luteum.

The results of Weisz et al.(1973) indicated that steroid OCs that block ovulation decrease the production of estradiol and estrone.

Brown et al.(1962) provided evidence indicating that norethisterone and its acetate administered in sufficient dosages early in the cycle will suppress follicular activity.

Nygren and Johannsen (1971) reported that relatively large doses of progestins are required to inhibit steroid hormone secretion by the corpus luteum. From their results, it seems unlikely that such inhibition is possible with newer mini-pills which provide low levels of progestin.

Moghissi and Marks (1971) determined urinary estrogens in women receiving 0.075 mg dl-norgestrel (mini-pill) daily. They observed irregular peaks at various times of the cycle in three out of six women. In these three cases, total urinary estrogen was higher than for untreated cycles, while in the other three women, total estrogen was decreased. The elevation of levels appears rather strange since most workers seem to agree that OCs lower or abolish mid-cycle estrogen. Also, only progestin was administered so that it cannot be argued that some exogenous estrogen, e.g. ethinylestradiol, could have entered into the estradiol and estrone fractions and thus have been included in total estrogens. The only comment that can be made for these results at this time is that, at low dosages, the OCs have a variable effect.

Larsson-Cohn et al.(1971) determined urinary estrogen excretion in patients

receiving d-norgestrel 0,03 mg/day. They observed mid-cycle peaks in all, together with late cycle rises in most - i.e. these patients had more or less a normal excretion pattern for estrogens. These results are in agreement with the results of earlier workers (Martinez-Manautou 1967), who found that the mini-pills cause less disruption of pituitary function than combined or sequential OCs, so that the mini-pills allow almost normal levels of gonadotropin release. Since one of the basic physiological functions of the estrogens is to reduce FSH levels and stimulate LH secretion, the observation of "normal levels" of estrogens should lead to normal levels of gonadotropin. Since estrogen is implicated in the stimulation of LH release during the normal cycle, the suppression of estrogen during OC therapy, a suppression indicated by lower levels of plasma estradiol, could be a basic mechanism for the prevention of LH release.

Other functions of estrogens are that they induce the proliferative phase of the uterine cycle. They also produce an effect on the cervical mucus, increasing its amounts, lowering its viscosity and raising its pH. These changes favour the transport, motility and longevity of spermatozoids. Alteration of the estrogen levels during OC treatment could alter these factors and account for some of their anti-fertility effects. Estrogens play a minor role in metabolism, contributing to small increases in B.M.R. and protein anabolism, and accelerating the processes of bone formation.

1.4.3 Progesterone

During the normal cycle, the corpus luteum is responsible for the secretion of progesterone. Thus, a rise in plasma progesterone or urinary pregnanediol signifies that ovulation has occurred.

Low plasma progesterone levels seem to be associated with OC therapy -

this could be due to:

(1) OCs allowing luteinization of the follicle without release of ovum;

(2) inhibition of ovulation, which would mean that no corpus luteum would form. This would account directly for a decrease in plasma progesterone levels or urinary pregnanediol;

(3) ovulation. The low plasma levels of progesterone could be due to inhibition of progesterone secretion by the corpus luteum, a possibility which was investigated by Nygren and Johannsen (1971). They found that large doses of progestins are required to inhibit the corpus luteum. From their results it seems that the dose contained in most mini-pills is insufficient to have any action on the corpus luteum.

The dosages they used are summarized in the Table 2 below:

TABLE 2. Dosages of Progestin Employed by Nygren and Johannsen(1971)

norgestrel	12 mg
norethisterone	30 mg
chlormadinone acetate	300 mg
medroxyprogesterone acetate	360 mg

Saunders et al. (1971) determined plasma progesterone levels at various intervals in women on 0,5 mg daily of chlormadinone acetate. In three out of nine women, they found a significant progesterone peak during late cycle. These three women also showed mid-cycle peaks of FSH and LH which is indicative of ovulation. These results are in harmony with those of Martinez-Manautou (1967).

Larsson-Cohn (1971) examined five women on 0,03 mg daily of d-norgestrel. All five women showed a rise of plasma progesterone during the late cycle, characteristic of a normal ovulatory cycle.

These workers followed the effects of the dl-norgestrel on these same patients for several successive cycles and found a decrease in plasma progesterone. The finding prompted them to suggest that these OCs cause a progressive reduction in corpus luteum function. Since only low doses were employed, this conclusion seems unlikely, as Nygren and Johannsen (1971) showed that high doses were required to inhibit the corpus luteum. A possible explanation for the elevated levels of progesterone in OC users during the late cycle could be the contribution of progesterone from ovarian and non-ovarian sources as Aftergood and Aflin-Slater indicate that steroidogenesis is stimulated by OCs in the adrenal gland.

1.5 Effect of OCs on Levels of Other Hormones

1.5.1 Corticosteroids

Billiar et al. (1975) measured the levels of plasma corticosteroid-binding globulin (transcortin) in ovariectomized and hysterectomized women maintained on 0,2 mg of ethinyl estradiol daily over a period of three weeks, in trying to find the effects of estrogens on the metabolism of progesterone in vivo.

Their findings, in agreement with those of other workers, are that estrogen increases the plasma transcortin concentration.

This group found that, after the third day of estrogen administration, the transcortin levels increased significantly. Withdrawal of estrogen therapy for three weeks caused transcortin levels to return to normal. Briggs et al. (1970) in their review cite a large number of studies on the effect of OCs on plasma corticosteroids. There is general agreement that corticosteroid levels are raised in women receiving most combined OCs.

Burke(1970)showed that low doses of estrogens, combined OCs, or progestin-only OCs, have less effect in increasing corticosteroid levels in the blood.

This group also determined unconjugated (urinary) control and found an increase in OC users.

The increase in transcortin levels in OC users is a result of estrogen administration, which results in a decrease in the metabolic clearance rate of cortisol (Billar· et al. 1975).

The significance of high corticosteroid production and excretion seen in patients on OC therapy is presently unknown.

1.5.2 Aldosterone

Aldosterone acts specifically on the renal tubule to increase the absorption and retention of sodium (and thus of water, chloride and bicarbonate).

Aldosterone is also secreted by the adrenal gland and, since large increases in plasma cortisol in OC users have been observed, it is reasonable to expect OC therapy will increase aldosterone levels.

Beckerhoff et al. (1973) studied the effect of OCs on aldosterone in ten women and found that, within the first week of OC therapy, aldosterone levels increased significantly, and this increase became progressively greater as treatment continued.

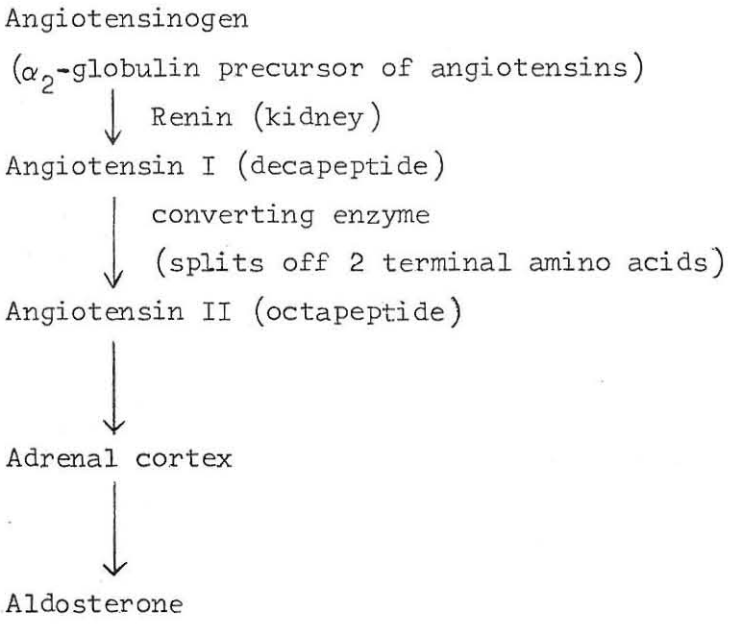
Higher levels of aldosterone in OC users may be associated with hypertension since, at physiological levels, the function of aldosterone is to retain sodium and maintain normal blood pressure, larger quantities increasing sodium retention and consequently elevating blood pressure. The mechanism may be related to the effects of the OCs on the renin-angiotensin system.

1.5.3 Renin-angiotensin System

A number of cases of hypertension associated with the use of OCs have been reported (Newton et al. 1968 and Tyson 1968). More recently, Fregly (1973) observed increases in blood pressure in rats on long term administration of Enovid.

Investigations on the relationship between hypertension and OCs has been focussed on the renin-angiotensin system (Newton 1968; Fregly 1973; and Lowenstein 1972) - see Scheme 1. The renin-angiotensin system operates through an interaction between renin, angiotensin and aldosterone. Normal blood plasma contains an α_2-globulin known as angiotensinogen. Angiotensinogen is acted upon by renin, a proteolytic enzyme released by the kidney. The first result of the renin-angiotensinogen interaction is angiotensin I (a decapeptide), which has no hypertensive action. A converting enzyme in the blood removes the two terminal amino acids of angiotensin I to yield angiotensin II, an octapeptide.

Angiotensin II is the most potent pressor agent known. It also stimulates the adrenal cortex to release aldosterone.



Scheme 1. Renin-angiotensin System.

The earlier above workers found increased levels of angiotensin in the plasma of OCs users, and this increase could explain the increase in aldosterone levels (see Scheme 1).

The angiotensin II produced constricts arterioles and, consequently, raises blood pressure, and also acts on the adrenal cortex to stimulate aldosterone secretion. Both effects increase renal arterial pressure and blood flow and, through negative feedback, shut off the secretion of renin.

Sarutu et al. (1970) and Weir et al. (1970) maintain that women who develop hypertension during OC treatment could have a diminished feedback suppression of renin. This view is supported by the findings of most workers that, in patients who have OC-induced hypertension, increased renin activity and angiotensinogen concentrations have been observed.

Though OCs elevate levels of angiotensin in the blood, one should be wary of concluding that this is the mechanism by which OCs induce hypertension. Crane et al. (1966) cite evidence that, during pregnancy and following estrogen regimen, plasma renin levels may be elevated without an accompanying rise in blood pressure. It appears that the disruption to the renin-angiotensin system brought about by the OCs is an increased secretion of renin-substrate concentration, the other changes to renin and aldosterone follow.

1.5.4 Insulin and Carbohydrate Metabolism

There seems to be general agreement that OCs cause an increase in plasma fasting level of insulin (Briggs 1976). Briggs (1970) showed that earlier studies on OCs and insulin levels appear controversial and results varied markedly from place to place. For this reason, only the results of the Spellacy group are summarized in Table 3.

TABLE 3. Results of Plasma Insulin Levels from the Spellacy Group

GROUP	NO.	PLASMA INSULIN μm/ml mean ± SD	REFERENCE
Pretreatment	91	10,10 ± 10,9	Spellacy et al. 1971b
CHA-sequential (6 months)	91	13,2 ± 8,4	
Pretreatment	53	8,8 ± 5,5	Spellacy et al. 1971c
CHA-sequential (12 months)	53	10,8 ± 6,7	
Pretreatment	31	11,2 ± 1,4	Spellacy et al. 1973
0,25 mg EDDA	31	13,9 ± 1,7	
Pretreatment	130	11,0 ± 1,2	Spellacy et al. 1973
1,0 mg EDDA + 100 μg MEE	130	12,6 ± 1,0	
Pretreatment	36	9,3 ± 1,4	Spellacy et al. 1973
1,0 mg EDDA + 50 μg EE	36	16,3 ± 2,5	
Pretreatment	26	18,1 ± 2,9	Spellacy et al. 1973
50 μg EE	26	15,9 ± 3,4	
Pretreatment	28	11,9 ± 2,2	Spellacy et al. 1973
80 μg MEE	28	10,5 ± 2,0	

Similar results were obtained by Starup et al.(1968). This group found no significant change in plasma-insulin levels, before or after twelve months of treatment with a OCs preparation containing 0,1 mg mestranol and 5 mg megestrol acetate.

The results obtained by the Spellacy group show that only in two instances, 50 μg of ethinyl estradiol and 80 μg of MEE, are values for the treated group less than those for controls. More recently, Dhall et al.(1977) observed that there were no significant differences in insulin levels before

and after treatment with OCs.

1.5.4.1 Glucose Tolerance Tests

General agreement exists in so far as fasting blood glucose is concerned. Fasting blood glucose concentration apparently is unaffected by OC treatment (Spellacy 1971b, 1971c and Briggs 1976). Yet, this is not uniformly the case. In some women, an alteration in glucose tolerance is induced by the estrogenic OCs. Individual responses are extremely variable in these cases and are likely to be determined by a diabetic predisposition.

According to Spellacy (1969), the disruption of carbohydrate metabolism is at least partially determined by the characteristics of the individual on hormone therapy. Older women with a high parity, with a history of familial diabetes mellitus, and who have delivered large infants are more susceptible to abnormal response to glucose tolerance tests.

Pyruvate, a major component of carbohydrate metabolism, has also been shown to be elevated following oral and intravenous glucose tests in subjects taking OCs according to Doar et al.(1969); Wynn et al.(1969); Doar and Wynn (1969) and Mason et al.(1973).

Results of these studies show that obesity increases blood pyruvate levels, while the highest values are seen in hypertensive women on OCs.

Other factors such as age and the chemical nature of the estrogen may be important.

Beck (1969) postulated that there is a relationship between the chemical structure of the OCs and their diabetogenic effect. Apparently, the presence or absence of the C_{19} methyl group, or of an hydroxyl, acetyl,

ethinyl or acetoxy substituents at the C_{17} position however does not alter glucose or insulin metabolism. According to Beck (1969), a prerequisite for such alterations is the presence of a positive charge at C_5 of the steroid. This positive charge at C_5 is common to mestranol and all the pregnene and nortestosterone derivatives which are known to give rise to increased insulin production after glucose stimulation.

In summary, it seems that the disruption of glucose tolerance induced in some users of OCs is due to individual response, which may be enhanced by a diabetic predisposition; other factors, such as high parity, age and the chemical nature of the OCs, may also be of importance.

1.5.5 Thyroid Hormone

Investigation of a large number of OCs has shown that they interfere with the interpretation of thyroid function tests. This interference is thought to be due to the estrogen component (Briggs et al.1970). Winikoff (1968) has confirmed this and has also shown that the progestins, with the exception of norethynodrel, do not have this effect.

Briggs et al.(1976) indicate that thyroid diseases can be "diagnosed biochemically" without having to suspend OC therapy, providing that thyroxine binding globulin levels are not disrupted above the expected limits reached in OC therapy.

1.5.6 Prolactin

Synthetic estrogens suppress estrogen synthesis but seem to stimulate prolactin release. This phenomenon might be due to a direct action on the anterior pituitary.

Delvoye (1973) determined serum prolactin levels in women receiving progestogens only and found that norethisterone acetate, 10 mg/day, and d-norgestrel, 0,25 or 2,5 mg/day, have no effect on prolactin levels, while ethinyl estradiol (an estrogen), 50 µg/day, stimulates prolactin release throughout the cycle. The rise in prolactin level, coupled with a decrease in lactation, seems to indicate that antagonism exists at the mammary level.

1.5.7 Growth Hormone (HGH)

OC therapy affects growth hormone secretion in that the OCs stimulate the secretion of this hormone. Significantly, increased serum growth hormone levels were reached when subjects taking OCs were given hypoglycemia tests (Spellacy 1969).

Spellacy (1969, 1970) found these elevated serum levels of growth hormone with combination type pills as well as sequential OCs.

The increased secretion of HGH appears to be associated with carbohydrate metabolism, but the actual role played by these increased levels with regard to altering the glucose or insulin levels is still obscure.

1.6 Vitamins

Although earlier work on the effects of OCs on vitamin levels have been confined to folic acid, recently there has been an increase in interest in the vitamin metabolism of a number of vitamins in women on an OC regimen. Results of this work suggest that the use of OCs does, in fact, affect the metabolism of vitamins.

1.6.1 Vitamin A

Gal et al. (1971) and Ahmed et al. (1976) have observed a significant

increase in vitamin A levels in subjects on OCs as compared with controls. In a later study, Gal et al. (1973) determined vitamin A levels in pregnant and non-pregnant women on an OC regimen and reported increased serum levels. Although these levels were high in some cases, none approached levels indicative of hypervitaminosis. These workers also noted that, in certain cases, the increased vitamin A levels may have had a beneficial effect, since hair, skin and nail conditions improved in women on OCs. However, the beneficial or harmful effects of raised levels of vitamin A have not been adequately evaluated. Studies by Gal (1972) and Crombie et al. (1970) have however indicated that use of synthetic sex hormones, taken either before, or immediately after conception, may have teratogenic effects due to the effect they have on vitamin A metabolism.

Gal et al. (1971) determined the concentration of plasma retinol and β-carotene in non-OC users and in OC users. In non-OC users, plasma retinol was only increased during the late cycle rather than during the follicular phase. The OC group had high values of plasma retinol at all stages.

Briggs et al. (1972) confirmed the elevation of plasma retinol. Retinol is transported in human plasma highly bound to transretinol (a specific carrier protein) and a likely explanation of the above results is that OCs increase plasma transretinol levels. However, β-carotene is only loosely bound to various non-specific plasma proteins and thus, is unaffected by hormone administration.

1.6.2 Folic Acid

Conflicting reports have been published with respect to serum folate levels following OC use, but the balance of evidence seems to favour a

decrease of serum folate levels (Shojania et al. 1968, 1969; Shojania 1971; Gafaar et al. 1973; and Shojania 1975).

Red blood cell folate levels, a more reliable index of tissue stores than serum levels, have been investigated by Shojania et al. (1971) and Gafaar et al. (1973) and were found to be lower in contraceptive users than in controls. Spray (1968), on the other hand, found no change in red cell folate levels in OC users.

A deficiency of folates could arise as a result of a number of causes. The most likely causes of folate deficiency are; defective intestinal absorption, abnormally increased requirements, impaired utilization in tissues, insufficient dietary intake, increased plasma clearance, and increased urinary excretion.

Shojania et al. (1971) and Gafaar et al. (1973) determined urinary formiminoglutamate levels and found significantly increased levels of excretion in patients on OCs therapy. Shojania (1975) reports that in women on OCs as well as in control subjects an association exists between urinary folate excretion and serum or red cell folate levels. The higher the serum or cell folate levels, the greater the urinary folate excretion. Shojania (1975) found however that women on OCs excreted more folate in their urine for any given level of serum or red cell folate than did control subjects.

Stephens et al. (1972) found no reduction in absorption of pteroylpoly-glutamate, the major form of dietary folate, after presaturation of the body's stores with folic acid, in OC users. These workers maintain that OCs do not affect the absorption of folic acid but instead may increase the clearance rate from plasma. Shojania and Hornady (1973) also reported

that OCs do not impair the absorption of folates. They measured the absorption of monoglutamate and polyglutamate forms of folate in OC users and found no consistent effect. Thus, it would appear that malabsorption of folates cannot explain reduced serum and red cell folate levels in OC users.

Shojania and Hornady (1973) maintain that a minority of patients do exhibit a low polyglutamate absorption, probably due to a genetic predisposition. Since these people exhibit a "mild and clinically unrecognizable form of malabsorption" of folates, they will have low serum and red cell folate levels. Thus, Shojania and Hornady (1973) conclude that in most cases of low folate levels, these levels cannot be associated directly with OC regimen, but may be due to an alteration of folate metabolism caused indirectly by OCs. Although OCs do appear to decrease serum folate and red cell folate levels, disturbances in folate levels are hardly serious enough to cause symptoms of megaloblastic anaemia. Rare cases of megaloblastic anaemia due to OC intake have been documented (Paton 1969 ; Lindebaum et al. 1975 ; Johnson et al. 1973); however, Johnson et al. (1973) indicate that two cases cited developed a severe folate deficiency anaemia while on OCs due to an intestinal disease rather than to the pill.

In experimental animals, estrogen administered orally seems to accelerate the metabolism of folic acid and its metabolites by intestinal enzymes. This observation suggests that a similar acceleration of metabolism of folic acid by the tissues might be the cause of lowered levels of the vitamin. According to Beck et al. (1971), the folic acid requirements increase six-fold during pregnancy, and thus, it seems possible that women who discontinue OC ingestion in order to conceive may run the risk of developing folic acid deficiency during pregnancy.

Systemic folic acid deficiency may affect cell systems, other than blood cells, which have a high turnover rate. Van Niekerk (1962) reported that cytologic changes in patients (not OC users) were associated with folate deficiency. These cytological changes included multi-nucleation, nuclear enlargement and cytoplasmic vacoulation.

More recently, Lindebaum et al. (1975) demonstrated that about 20% of patients on OCs show mild changes in the cervical epithelium of the vagina. This condition was corrected by folic acid supplementation. In conclusion, it appears that OC hormone ingestion frequently leads to reduced folate levels (which generally cannot be explained by malabsorption of folate) in serum and red blood cells. Only in the rare patient with some underlying disorder of folate absorption, metabolism or intake, does this contribute to the development of folate depletion serious enough to cause megaloblastic anaemia.

The available evidence also suggests that no benefit would be derived from routine folate supplementation in women on OC therapy.

1.6.3 Vitamin B_6

OC administration appears to induce a state of vitamin B_6 deficiency (Salkeld et al. (1973)), as evidenced by erythrocyte glutamate oxaloacetate transaminase activities. It was found that 48% of the subjects studied were deficient or were marginally deficient in vitamin B_6. An anonymous report (1973) claims that approximately 25% of women show clear evidence of vitamin B_6 deficiency.

Davis and Smith (1973) determined the relationship between serum levels of pyridoxal phosphate and age in OC users and non-users and found that there was a reduction of this co-enzyme in OC users.

Vitamin B_6 deficiency gives rise to abnormal metabolites of tryptophan, so that OC users who show a deficiency of vitamin B_6 also show an increase in urinary excretion of tryptophan metabolites (see Section 1.7.1.).

Rose (1969), Salkeld et al. (1973), and Sauberlick (1975) cite evidence that it is the estrogenic component of the pill that is responsible for the alteration of tryptophan metabolism due to the induction of tryptophan oxygenase. This enzyme catalyses the initial step of the tryptophan-nicotinic acid ribonucleotide pathway.

Salkeld et al. (1973) found that the effect on vitamin B_6 levels by the estrogen was not dose dependent since low dose estrogen (0,05 mg) and high dose (0,075 mg - 0,1 mg) both gave rise to lowered vitamin B_6 levels.

Briggs (1976), Rose (1969), Salkeld (1973) and anonymous (1973) indicated that abnormal tryptophan metabolism can be corrected by administration of supplementary vitamin B_6. Briggs (1976), Salkeld (1973) and anonymous (1973) are of the opinion that a minimum of 20 mg of vitamin B_6 should be administered daily to correct tryptophan metabolism. None of the above studies mention any of the clinical implications of vitamin B_6 deficiency caused by OCs, nor do the authors indicate the nutritional state of their experimental subjects who were deficient in vitamin B_6.

In more affluent countries, the diet may supply an intake of vitamin B_6 adequate to counteract the deficiency caused by the "pill", but what of those people in parts of the world where laboratory and clinical evidence of vitamin B_6 deficiency exists? The use of OC agents could only worsen the deficiency state and work should be done to investigate if vitamin B_6 supplementation would be useful in these cases.

1.6.4 Ascorbic Acid

Ascorbic acid concentration in leucocytes is generally considered the most useful index of ascorbic acid intake. Consequently, most work on the effect of OCs on ascorbic acid has involved investigations of leucocyte ascorbate levels. There is general agreement that OCs depress leucocyte ascorbate levels (McElroy et al. and Briggs et al. 1976). Briggs and Briggs (1972) report a decrease of leucocyte and platelet ascorbic acid levels. Plasma ascorbate levels also appear to be depressed, yet Rivers and Devine (1972) and Prasad et al. (1975) found no change in plasma ascorbate levels.

Metabolism and blood levels of ascorbic acid seem to be sex and age dependent (Dodds 1969 and Loh 1972), males appearing to metabolise ascorbic acid faster than females. This difference appears at adolescence. Dodds (1969) mentions that on an equivalent intake of ascorbic acid females have higher blood ascorbate levels, suggesting that there are hormonal interrelationships in ascorbic acid metabolism.

Cyclic variation in ascorbate levels in blood seems to occur. McElroy et al. (1973) and Rivers and Devine (1972) reported that the maximum concentration of ascorbic acid in the plasma occurs at ovulation and that the OCs prevented the cyclic changes in ascorbic acid in plasma and total ascorbic acid concentration.

The decreased levels of ascorbic acid in plasma and leucocytes could be due to a number of factors; oral ingestion of OCs could impair absorption of ascorbic acid; the OCs could increase metabolism and utilisation of ascorbic acid; or they could increase excretion or deposition of ascorbic acid into tissues. However, the results obtained by Rivers and Devine (1972) point to an increased excretion of ascorbic acid. McElroy et al.

(1973) suggest that, on the basis of their results, supplements of ascorbic acid for OC users will not aid in maintaining normal levels in tissues of these women.

More recently, investigators have shown that ascorbic acid was implicated in cholesterol metabolism. Anonymous (1973) showed that, in ascorbic acid deficient guinea pigs, the conversion of cholesterol to bile acids was retarded. This group is of the opinion that ascorbic acid is required for hydroxylation of cholesterol and, perhaps, low ascorbate levels in OC users may be a contributory factor to high cholesterol levels in the same subjects.

Kritchevsky et al. (1973) found that ascorbic acid did not stimulate cholesterol oxidation and that 7α-hydroxylation of cholesterol by hepatic microsomes of rats were unaffected. This group also found that 7α-hydroxylation of cholesterol was increased in guinea pig liver by ascorbic acid but not significantly so.

1.6.5 Vitamin E

OC utilization by women is throught to lower α-tocopherol levels (Briggs 1976; Aftergood and Aflin-Slater 1973). This condition also arises in female rats treated with OCs (Aftergood and Aflin-Slater 1973).

It is possible that α-tocopherol levels are associated with lipid metabolism since vitamin E is concerned with the maintenance of lipids.

Aftergood and Aflin-Slater (1973) propose that depressed α-tocopherol levels in treated patients may be due to a primary effect of OCs on the lipoprotein distribution, which imposes a shortage of protein carrier.

Briggs (1976) indicates that vitamin E plasma levels may be depressed in

OC users. This fact might be explained by the observation that lipid levels increased in plasma and these lipids might increase the solubility of this vitamin. Vitamin E also has a hormonal activity since it is involved in haem synthesis. Thus in vitamin E deficiency, a reduced capacity to synthesize haem may exist. Presently, no clear cut evidence for the exact effects of OCs on vitamin E deficiency exists.

The effects of OC administration on vitamins are summarized in Table 4.

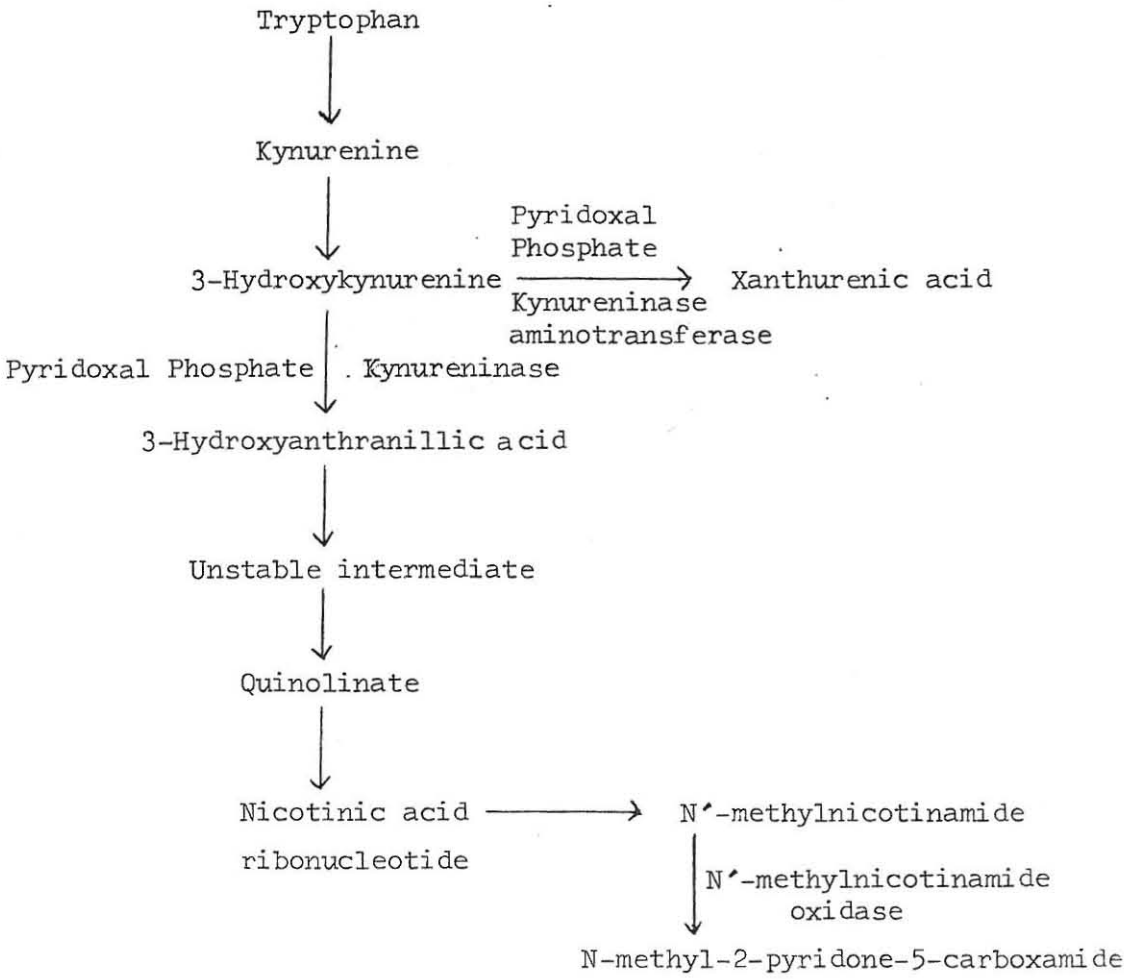
TABLE 4. Influence of OCs on Vitamins

Vitamin	Effect	Reference
A	Increased plasma levels	Gal et al. 1971, 1973.
B_6	Increased requirement, altered tryptophan metabolism	Rose 1969; Salkeld et al. 1973; and Sauberlich 1975.
C	Reduced plasma ascorbate levels	McElroy et al. 1973; and Prasad et al. 1975.
Folic acid	Reduced serum and erythrocyte levels, megaloblastic changes of epithelium of vagina	Shojania et al. 1971; and Lindebaum 1975.
Niacin	Increased urinary excretion of N'-methylnicotinamide	Rose 1969; and Sackeld 1973.
E	Reduced α-tocopherol levels in blood	Briggs 1976; and Aftergood et al. 1974.
B_{12}	Reduced serum levels	Shojania 1971 and Briggs 1976.

1.7 Amino Acids and Proteins

1.7.1 Tryptophan

The metabolic pathway of tryptophan to yield nicotinic acid ribonucleotide is shown in Scheme 2.



SCHEME 2. Metabolic Pathway of Tryptophan

Rose (1969), Briggs (1976) and Anonymous (1973) indicated that vitamin B_6 deficiency causes kynurenine, 3-hydroxykynurenine and xanthurenic acid to be excreted in unusually large amounts following a tryptophan load.

It appears that a lack of pyridoxal 5-phosphate coenzyme affects the enzyme kynureninase more severely than it does kynurenine aminotransferase, causing a reduction in the conversion of 3-hydroxykynurenine to 3-hydroxy-anthranillic acid with a corresponding increase in xanthurenic acid, which

is used as an index of vitamin B_6 status following a loading dose of tryptophan (see section on vitamin B_6 - 1.6.3).

As mentioned in a previous section, supplementation of pyridoxine reversed the abnormal excretion of these substances and returned the pattern to normal. Thus, the abnormal excretion of tryptophan metabolites was interpreted as being due to a vitamin B_6 deficiency.

A parallel situation exists during late pregnancy when a disturbed tryptophan metabolism also exists, pregnant women excreting larger amounts of xanthurenic acid after a loading dose of tryptophan than do non-pregnant women. This result was difficult to interpret since it was uncertain whether this vitamin B_6 deficiency was due to the hormonal changes associated with pregnancy or due to the demands of the foetus. This metabolic difference was also corrected by pyridoxine administration.

Rose (1969) indicates that the effects of estrogens and progestogens on tryptophan metabolism are similar to those of pregnancy.

Further, Salkeld (1973) and Rose (1969) cite evidence that there is a sex difference in the urinary metabolites, higher levels occurring in women than in men; yet, there is no difference in excretion of these metabolites in post-menopausal women and in men. Increased levels of these metabolites occur at ovulation when the estrogen levels are at their highest, a situation leading to the conclusion that, in OC users, it is the estrogen component that is responsible for the dysfunction of tryptophan metabolism.

There seems to be general agreement on the possible mechanism by which estrogens disrupt tryptophan metabolism (Salkeld et al. 1973 and Briggs 1976). Estrogens may cause induction of tryptophan oxygenase, thus increasing the amounts of tryptophan passing down the pathway, or they may inhibit pyridoxal phosphate dependent enzymes such as kynureninase, which will lead to an increase in the metabolites prior to this step.

Davis and Smith (1973) however, measured pyridoxal phosphate levels in OC users and non-users and found no significant difference, a result which is difficult to correlate with disruption in tryptophan metabolism from lack of kynureninase. Yet, increased levels of tryptophan pyrolase are not the sole cause of the changed pattern of excretion.

Rose et al.(1968) reported an increase in excretion of N'-methylnicotinamide, the precursor of 2-pyridone, in women receiving Enovid E, without an increase in the excretion of 2-pyridone. The enhanced excretion of this metabolite occurred with or without a tryptophan load. This group explained their results by postulating that inhibition of N'-methylnicotinamide oxidase activity occurs. Such a situation could account for the rise in N'-methylnicotinamide excretion without a corresponding increase in 2-pyridone. Gluecksohn-Waelsch et al.(1967) report such an effect of estrogens on this enzyme in mice.

More recently, Brown et al.(1975) determined the effect of OC usage on the nutritional requirement for vitamin B_6. Control women and OC users were depleted of vitamin B_6 for one month. This procedure was followed by a month of repletion in these subjects with various doses of pyridoxine hydrochloride per day. At weekly intervals, various indices of vitamin B_6 nutrition (assay of urinary tryptophan metabolites after 20 g oral load of tryptophan was applied, urinary crystathionine, urinary 4-pyridoxic acid, plasma pyridoxal phosphate) were carried out. Their results indicate that usage of OCs does not generally change the requirement for vitamin B_6 but rather alters the specific activity of enzymes beyond kynurenine in the pathway of tryptophan metabolism.

Adams et al.(1976) pointed out that a possible relationship between abnormal tryptophan metabolism and the disturbance of glucose tolerance of treated

women may exist. However, these workers indicated (although they observed increased xanthenuric acid (X.A.) excretion in OC users) that carbohydrate intolerance in women on OCs is unlikely to be mediated by the formation of X.A. with insulin.

The biological effects of this change in tryptophan metabolism is uncertain but may be related to the high incidence of depression in women on OCs (Madsen 1974). Since serotonin is formed from tryptophan, excessive liver metabolism of this amino acid could reduce plasma and brain tryptophan and serotonin.

Adams et al. (1973) reported a good response to vitamin B_6 in depressed women on OCs.

The studies of Leklem and Brown (1976) indicate that abnormal tryptophan metabolism may be a predisposing factor in bladder carcinogenesis.

1.7.2 Binding Proteins

1.7.2.1 Hormone Binding Proteins

The changes induced by OCs in plasma hormone levels have been discussed elsewhere. The most significant changes occurred in the case of corticosteroids, sex hormones and thyroxine. In general, the hormones are known to be present in the plasma largely in the protein bound state.

1.7.2.2 Metalloproteins

The metalloproteins most widely investigated in relation to OCs have been haptoglobin (haemoglobin binding globulin), transferrin (an iron containing protein) and ceruloplasmin (a copper containing protein).

Early investigations have shown that mainly the high dose combined OCs had a

significant effect on these proteins and, as a result of a report by Inman et al.(1970), they have now been withdrawn. Prasad et al.(1975) reported an increase in ceruloplasmin and in copper levels in women using OCs, the increase in copper being due, it is thought, to an increase in ceruloplasmin.

It appears that the estrogen component of the OCs is responsible for the increase in ceruloplasmin since this increase is also noted during pregnancy. Progestogens (e.g. norethisterone acetate) with estrogenic properties, when given alone, also produce small changes in levels of ceruloplasmin.

Transferrin levels are also found to increase in conjunction with total iron binding capacity in OC users (Prasad et al. 1975). This finding is in agreement with that of Briggs (1976).

Since ceruloplasmin and transferrin, a β-globulin, are synthesized in the liver, it seems reasonable to assume that increases in these levels, due to OC usage, can be ascribed to secondary effects on liver metabolism and may be due to an increased biosynthesis or release of these proteins, or both. Serum haptoglobin levels seem to decrease, while haemoglobin levels seem largely unaffected (Prasad et al. 1975). In this same study, a reduction of serum albumin was observed in the patients, and this observation led the authors to speculate that, since albumin is a zinc carrier, it is its decrease which leads to decreased zinc levels in OC users. Generally, OCs are found to have no effect on haemoglobin levels but cause increased levels of copper ceruloplasmin, transferrin and iron binding capacity. The biochemical mechanisms are still obscure; however, increased transferrin and iron binding also occur in hepatitis. Ceruloplasmin possesses ascorbate oxidase activity and could be implicated in low blood ascorbate levels in women taking OCs (Briggs and Briggs 1972).

1.8 Clotting Mechanisms

Since blood platelets act as the precipitating factor in blood clotting, possible changes in platelet number and function as a result of OC ingestion are important.

Investigative procedures include bleeding time (*in vivo* measure of effectiveness of platelet adhesiveness) and aggregatability (an *in vitro* test to measure platelet adhesion to foreign surface such as glass and aggregation in response to chemicals such as ADP) (Zahavi *et al.* 1973; Sanderson *et al.* 1973).

Zahavi *et al.* (1973) and Sanderson *et al.* (1973) report an increased platelet adhesion response to ADP on OC administration. This increase in sensitivity to ADP may be used as a crude index of the degree to which erythrocyte and platelets form clumps. This increased tendency to form clumps may be related to an increased incidence of thromboembolic disorder in OC users.

OCs have also been implicated in alteration of clotting mechanism of blood. Lecompte and Ranaud (1973) report hypercoagulability and reduction in plasma clotting time in pregnant rats, pregnant women and women on OCs. In women on OCs, plasma clotting time was reduced by 22% compared with controls. In all three groups, this hypercoagulability was attributed to an increased activity of platelet III factor. These workers did not observe an increased susceptibility of platelets to thrombin and ADP aggregation in pregnant women or women on OCs but they did detect increases in pregnant rats.

Renaud *et al.* (1973) report an increase in platelet count and suggest that this phenomenon might be the reason for reduction in clotting time of blood from pregnant rats and OC users.

Sanderson et al. (1973) noted however that platelet counts actually fall during the first month, the period in which platelet aggregates most commonly appear (Zahavi et al. 1973).

The difficulty in correlating the studies on platelet activity may be explained in terms of technical details which seem to affect some of the results obtained. Renaud et al. (1973) showed for instance that discrepancies in results were obtained from blood drawn from a common source depending on whether or not blood was collected in siliconized glassware and whether the final determinations were performed in plastic or glass.

The fibrinolytic system has also been investigated for changes during OC usage.

Sobrero et al. (1971) and Asbeck et al. (1973) reported an increase in plasminogen in treated women. Asbeck et al. (1973) also found an increased plasmin and activator activity which existed predominatly in the first month and decreased during the second and third months of treatment. The increased plasmin activity and activator activity reflect stimulation of the fibrinolytic system during OC administration.

Bergsjo et al. (1973) investigated the relationship between chlormadinone acetate and anti-thrombin III levels since changes in anti-thrombin are important as this protein gives protection against intravascular clots by inactivation of thrombin. Bergsjo et al. (1973) found increased levels while other workers (Kaulla et al. 1971) found reduced levels.

Asbeck et al. (1973) are of the opinion that the fibrinolytic system is not impaired during OC therapy.

1.9 Thromboembolic Diseases

Oral contraceptives have been shown to enhance the risk of thromboembolism, and a positive correlation with the amount of estrogen in the pill was noted (Inman et al. 1970). After 1970, the regulatory committees of several countries suggested that no more than 50 μg of estrogen should be included in each tablet. Vessey and Inman (1973) cite evidence that, as the amount of estrogen was decreased, in accordance with the recommendation of the Committee on Safety of Drugs in Britain, so did the number of deaths. These two workers studied the mortality trends from thromboembolic diseases associated with OC usage in England and Wales from 1953 to 1971 and showed clearly that the mortality statistics correlate with the incidence of thromboembolic effect and the OCs. In summary then, the incidence of thromboembolic disease appears to be higher in women using OCs than in non-users. Vessey et al. (1969) calculated that the risk is many times greater in OC users than non-users. Moreover, Jick et al. (1969) indicated in a co-operative study in U.S.A., the U.K. and Sweden on women on OC regimens that those with blood types A, B and AB are more susceptible than those with O blood type.

Kubik et al. (1973) report three cases of myocardial infarction associated with OC use. Their results however appear difficult to evaluate since all three patients had a family history of myocardial infarction and the number of patients (four) studied was small.

1.10 Lipids

Previous investigations indicate that OCs give rise to a fasting hyperlipemia. This manifestation seems to be due in part to an increase in triglycerides.

Some investigators report an increase in plasma lipoprotein concentrations

and some changes in other fractions of plasma lipids have been documented. There are major groups of lipoproteins which are important; the fraction that contains most of the plasma cholesterol is the "beta" or low density lipoprotein fraction. In addition, most of the plasma phospholipids occurs in the "alpha" or high density lipoprotein fraction. The third important fraction is the triglyceride-rich, very low density "pre-beta" lipoprotein fraction.

Alvarez et al. (1973) investigated serum total lipids and found an increase.

1.10.1 Triglycerides

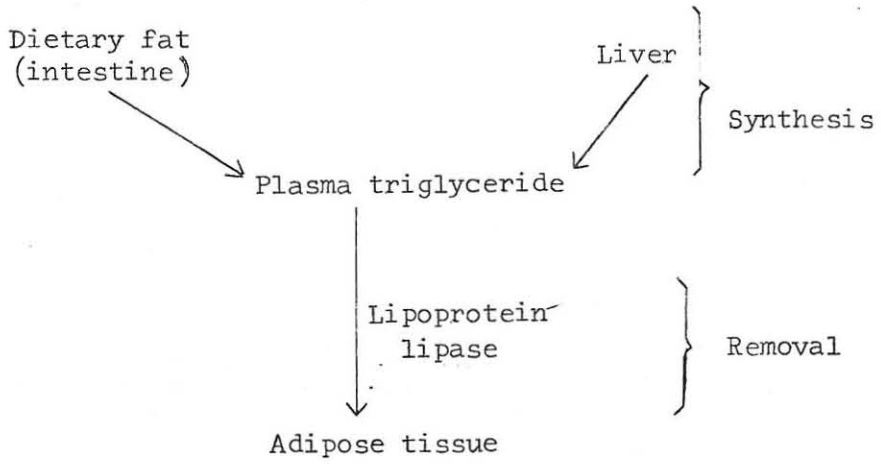
The triglyceride fraction has been widely investigated and there seems to be general agreement that OCs cause an increase in the fasting levels of triglycerides in women (Bierman 1969; Wynn and Doar 1969; Hazzard et al. 1969 and Mason et al. 1973). Spellacy et al. (1973) disagree with these results. They report a decrease of serum triglyceride levels over 12 months in OC users on OCs containing 1,25 mg/dose ethynodiol diacetate. Schillinger and Gerards (1973) report hypertriglyceridemia in rats treated with ethinyl estradiol for 14 days. Singh and Swarthout (1972) report an increase in triglyceride in cervical mucus of OCs users. Using liver perfusates, Weinstein et al. (1973) studied the effect of short-term (4 day treatment) and long-term treatment (1 year) of Enovid on the release of triglyceride and cholesterol from the livers of rats. These workers attempted to find some correlation between these observations and the concentration of cholesterol and triglycerides in the serum of donor animals prior to the removal of the liver. Their findings indicate a reduced rate of release of cholesterol and triglycerides from the liver, while serum levels of those two types of lipids remained unchanged for the long-term study. They also found no effect on the concentration of cholesterol or triglyceride in the liver after the experiment. This

finding seems to be at variance with the results of Aftergood and Alfin-Slater (1969), who also used Enovid and found that liver cholesterol of rats increased after four days in one study. The results of Weinstein et al. (1975) are also in contrast to the many observations of increased serum triglycerides in women ingesting OCs. The authors attempt to explain their results by pointing out that the rats in the experimental group may have been in the fasted state as compared to controls, since the treated rats consumed less food and weighed less.

A fasted state is thought to give rise to a reduced rate of release of triglycerides in liver perfusates.

The increase in triglyceride levels in OC users could be due to increased plasma triglyceride, in part from exogenous sources (diet) or endogenous sources (liver), impaired triglyceride removal, or both. The removal of triglycerides is mediated through an enzyme, lipo-protein lipase.

Two tissues in the body, the liver and the intestine, are responsible for the synthesis and release of triglyceride into the blood stream. One major removal process exists in the adipose tissue. This can be represented schematically (Scheme 3).



SCHEME 3. The Synthesis and Removal of Triglyceride

Bierman (1969) indicated that hypertriglyceridemia due to OC use might be associated with an effect of these agents on triglyceride transport. It is thought that OCs may increase triglyceride-rich lipoprotein synthesis or impair its removal. Most workers are of the opinion that it is the estrogen component of the OCs that is responsible for the hypertriglyceridemia, for those effects can be produced by administration of estrogen alone, but not by the progestin. This view is consistent with the historical evidence that hypertriglyceridemia is common during pregnancy in humans and animals (Pincus 1966), when estrogen levels are high.

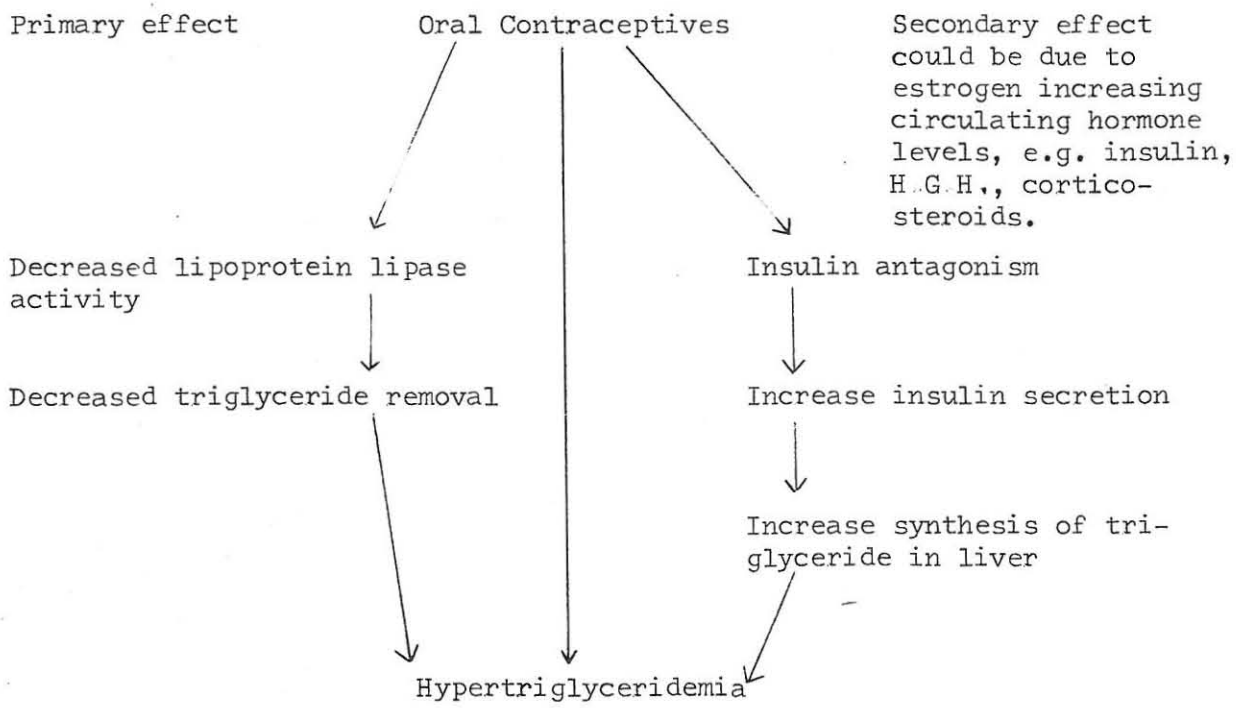
It is improbable that the uptake of fat from the diet via intestinal wall synthesis and secretion of chylomicrons into the lymph with subsequent transport from lymph into the blood stream is affected, since Bierman (1969) reported an increase in triglyceride levels of one subject taking 0,625 mg Premarin/day on a fat free diet. This suggests that OCs may aggrevate existing hypertriglyceridemia by a similar mechanism as the estrogen induced elevation of triglyceride levels in all subjects.

If OCs effect either triglyceride or lipoprotein synthesis, the action is likely to be localized in the liver. Furthermore, lipogenesis requires insulin, and thus, the degree of exposure of the liver to insulin can affect the rate of triglyceride synthesis. It is known that OCs raise insulin levels (Spellacy et al. 1971b, 1971c, 1973). This known effect of OCs on insulin suggests a role in the alterations of triglyceride transport as well as the reported changes of carbohydrate tolerance.

The enzyme, lipoprotein lipase, is responsible for the removal of triglyceride. Studies on animals have revealed a decreased tissue level of lipoprotein lipase activity during late pregnancy (Otway 1968) and this depressed activity of lipolytic activity indicates an impairment of tri-

glyceride removal (Hazzard et al. 1969; Hazzard et al. 1972). The activity of this enzyme can be determined since it is released into the blood stream by the administration of heparin. OC administration decreases both the level and the activity of this enzyme, and thus could cause elevated triglyceride levels.

The cause of hypertriglyceridemia is unclear at this stage. The increase in triglyceride levels may relate to increased plasma triglyceride input from dietary or hepatic (endogenous) sources, reduced triglyceride removal mediated primarily by lipoprotein lipase, or a combination of both. However, combining the two most likely causes of hypertriglyceridemia caused by OCs, namely the decline in heparinlipolytic activity (decreased lipoprotein lipase) and increase in insulin levels due to OCs, one could postulate a possible mechanism for the hypertriglyceridemia induced by OCs.



SCHEME 4. Possible Scheme for Mechanism of Hypertriglyceridemia Induced by Oral Contraceptives.

The hypertriglyceridemia induced by OCs appears to be reversible as indicated by Wynn and Doar (1969), Hazzard (1969) and Briggs (1970) Triglyceride levels apparently return to normal on discontinuation of the drug. Briggs (1976) indicates that the increase in triglyceride levels is a permanent biochemical change during long term use and does not return to normal on discontinuation of use.

1.10.2 Cholesterol

Earlier studies on the effect of OCs on serum cholesterol seem controversial. Many workers then seemed to indicate a small depression or no change in cholesterol levels (Wynn and Doar 1969; Hazzard et al. 1969). On the other hand, recent studies seem to indicate a definite increase in cholesterol serum levels. Mason et al. (1973), Ygge et al. (1969), Larson-Cohn (1971), Briggs (1976) show that the balance of evidence seems to favour an increase in cholesterol levels in OC users.

It is uncertain at this stage whether this increased serum cholesterol level is permanent or reversible. Aftergood and Alfin-Slater (1969) report that, at least in rats, the return to normal cholesterol level is reversible; however, they found reduced cholesterol levels in Enovid treated rats.

Stokes and Wynn (1971) report that an OC, containing 50 μg of mestranol, when administered daily, had only a minor effect on serum cholesterol as compared to the effect brought about by others containing equivalent doses of ethinylestradiol. This observation seems to correlate with the results reported by Wynn and Doar (1969), who investigated the effects of 0,1 μg mestranol administered daily to six subjects and found a small increase in serum cholesterol levels after treatment. This change was not significant however.

Aftergood and Aflin-Slater (1969) report a significant lowering of plasma and adrenal cholesterol levels and an increase in liver cholesterol content in rats treated with Enovid E. Cholesterol levels in the ovary were found to be reduced. These effects are linked with a reduction in mass of the ovary during OC treatment. This same group also studied cholesterol biosynthesis in liver, adrenal, and ovaries of treated and control female rats for four days. Their investigations indicate that there is a significant increase in cholesterol synthesis in the ovary and adrenals and a depressed hepatic cholesterol biosynthesis. They also noted a decreased excretion of cholesterol in faeces. The increase in the synthesis of cholesterol in ovary and adrenal (organs from which cholesterol are removed) could indicate an increased requirement for cholesterol in these organs during OC therapy since it appears that several aspects of cholesterol metabolism are affected, or there may be a hormone redistribution, as well as anabolic and catabolic changes. Weinstein et al. (1975) found no effect of Enovid on hepatic cholesterol levels on short- or long-term treatment. They also reported no change in adrenal cholesterol for the short-term study, but a significant decrease in adrenal cholesterol in the long-term studies, observations consistent with those of Aftergood and Aflin-Slater (1969).

Weinstein et al. (1975) observed a reduced rate of release of cholesterol from the liver. This could be due perhaps to a reduced synthesis, but they did not observe a reduction of serum cholesterol in long-term studies. These results could only be explained by the assumption that OCs reduces peripheral metabolism of cholesterol.

However, in short-term studies, this group found a significant decrease in serum cholesterol levels without a concomitant reduction in release of hepatic cholesterol. The reason for the conflicting observations of long- and short-term effects of Enovid is unclear.

Tabacchi et al. (1973) also found an increase in liver lipids due to OC treatment for 28 days and a decrease in serum cholesterol. They also studied the effect of dietary lipids on the action of OCs on lipids and concluded that dietary fat significantly influenced the action of OCs on plasma and hepatic lipids. They found that serum cholesterol was decreased by OCs in dietary induced hypercholesterolemic rats and increased by dietary cholesterol. Singh and Swartwout (1972) investigated lipid changes in cervical mucus in conjunction with OC therapy and found that cholesterol levels were raised, while sterol ester levels were lowered.

1.10.3 Phospholipids

Most workers (Bierman 1969; Alvarez 1973; Larson-Cohn et al. 1970) report an increase in phospholipid levels during OC ingestion. Here again, it seems that it is the estrogen component that is responsible. It appears that some phospholipid fractions may be changed to a larger extent than others.

It is known that estrogens affect lipoprotein levels such that high density lipoprotein levels appear to be increased (Bierman 1969; Furman 1969). High density lipoproteins (HDL) contain cholesterol plus phospholipid (Bierman et al. 1969). The studies of Furman (1969) suggested that estrogen administration reduced the increased cholesterol content of the HDL relative to the phospholipid and protein content and the HDL concentration in serum. This conclusion is based on the fact that, during estrogen treatment, relatively more of the cholesterol can be accounted for in the HDL fraction.

Svonborg (1969) found an increase in lecithin, in accordance with the results obtained by Larson-Cohn et al. (1970), who report an increase in lecithin and sphingomyelin fractions.

Singh and Swartwout (1972) indicate that total phospholipids also increase in cervical mucus of treated patients compared to controls.

The alteration of phospholipid concentration is an important factor in the metabolism of drugs since phospholipid levels in hepatic microsomes is an important parameter in drug metabolism (see Section 3.5).

1.10.4 Fatty Acids

Larson-Cohn et al.(1970) measured serum non-esterified fatty acids (NEFA) and found a profound reduction in fasting NEFA in OC users. It appears that the effects of OCs is to reduce the plasma pool size, an effect which seems to be mediated by the progesterone component of the pill. On the other hand, Singh and Swartwout (1972) report an increase in NEFA of the cervical mucus.

The higher lipid levels during OC therapy could indicate an increased lipid transport to depot adipose tissue.

Significance

The clinical importance of the alteration in lipid metabolism produced by OCs is difficult to evaluate especially in view of the conflicting evidence. Initially, the raised lipid levels in OC users were thought to influence the progression of atherosclerosis. However, there does not seem to be any clinical evidence available with regard to OCs on the incidence of this condition.

Presently, no evidence is available to support the concept that lipid changes associated with OC therapy contribute to an increased tendency to develop atherosclerosis. Moreover, Furman (1969) reported that the increase in mortality due to ischaemic heart disease in women after the age of 50

in the U.S.A. is a function of age rather than being due to the menopause (after which serum cholesterol levels increase in women because of a decrease in ovarian estrogen production). This appears to be a logical conclusion since the effect of estrogen treatment on serum cholesterol concentration is far too variable to support the assumption that the increase in heart disease in women beyond the menopause is due to estrogen deficiency.

The increased lipid levels may be implicated in weight gain observed in some women initially starting OC use, but Pincus (1965) suggested that the weight gain might be due to the loss of anxiety about accidental pregnancy. The effectiveness of the pill is thought to improve the psychological state and thus improve appetite. On the other hand, it is unknown whether this increased weight is due to water, fat, muscle or a combination, especially since estrogens are known to cause oedema (Drills 1966) and since studies by Lecocq et al. (1967) indicate that weight gain in OC users may be due to an anabolic effect. Thus, lipid changes may not be important.

SECTION 2

BIOCHEMICAL ACTION OF HORMONES AT MOLECULAR LEVEL

Two general classes of hormones exist, the peptides and steroids; each class seems to function through quite different mechanisms.

To explain the action of hormones, Jensen and Jacobson (1962) initiated the concept of steroid hormone receptors. Physiological amounts of 17β -[^{3}H]estradiol were injected into immature female rats, and it was observed that it was target tissue alone which was capable of maintaining a high dose of radioactive material against a marked concentration gradient in the blood.

This phenomenon will be explained in terms of the activity of progesterone in the chick oviduct and estrogen in the rat uterus since these models have been used to investigate the mechanism of female steroid action and results obtained from these investigations can be used to explain the action of other steroids, even synthetic steroids, since the receptors bind "either naturally occurring or synthetic steroids" (O'Malley et al.1974).

Presented below is a model (Fig. 4) for estrogen action (Shymula et al. 1967; Gorski et al.1968). The model is based on the assumption that:

1) estrogen enters cell
2) target tissues contain receptors
3) the receptors, when estrogen is absent, are found in the cytoplasm
4) estrogen-receptor complex translocates to the nucleus
5) estrogen-receptor complex within nucleus alters gene expression.

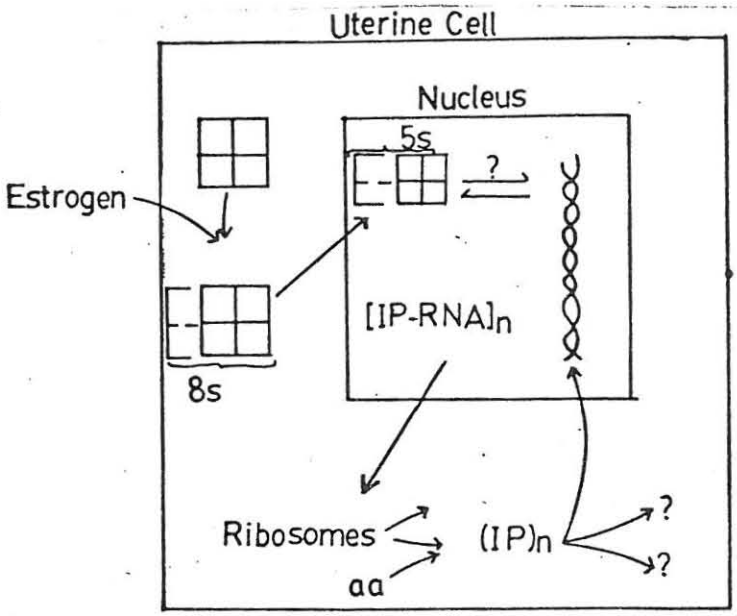


Figure 4. Model for Estrogen Receptor

E-⊞ = estrogen-receptor complex, 8s and 5s refer to sedimentation values for cytoplasmic and nuclear forms. (IP)n refers to various proteins whose synthesis is induced by estrogen. IP-RNA = messenger RNA for IP protein. IP = induced protein.

Since the classical studies of Jensen and Jacobson, receptors for other hormones have been characterized in target tissues. The O'Malley group (1971) and Sherman et al. (1970) have been responsible for characterization of the progesterone receptor in chick oviduct; progesterone binding components have been characterized in the cytosol of guinea pig (Faber et al. 1972a), rabbit (Faber et al. 1972b). Receptors for other hormones have also been confirmed (King and Mainwaring 1974). Fang et al. (1969) investigated androgen receptors, Swaneck et al. (1970) aldosterone, and Wira et al. (1970) glucocorticoid receptors in thymus cells.

2.1 Entry of Steroid into Cell

Steroids are small molecules having a molecular mass of about 300 and it has been assumed that, because of their small size and lipophilic properties, they can easily diffuse through the target cell membrane. The fact that steroids are rapidly taken up by tissues also supports

this theory (O'Malley et al. 1974; Gorski et al. 1976).

Speculation exists as to whether a facilitated transport may be responsible for the uptake of the steroid by the target cell. This question arose since two binding sites for steroids exist, low affinity but high capacity binding sites as well as high affinity, low capacity binding sites being present (King et al. 1974; Peck et al. 1973).

Peck et al. (1973) conclude from results obtained in their investigation that the entry of estrogen into the target cell was a passive process since they could not inhibit the uptake of estrodiol using a -SH blocking agent (N-ethylmaleimide), nor could they saturate the transport mechanism at high concentrations.

Presently, little evidence exists either to support or to refute the concept of a facilitated transport and thus, Peck's work should be considered as being definitive, since there is nothing to prevent a highly lipid soluble molecule such as a steroid from diffusing into the target cell. O'Malley et al. (1976) are also of the opinion that "hormone molecules continue to diffuse into the cells", implying that steroids enter cells by a passive process.

2.2 Intracellular Distribution of Receptors

2.2.1 Nucleus

In majority of estrogen responsive tissues, oestradiol is located mainly in the nucleus after in vivo injection (O'Malley et al. 1976; King et al. 1974).

2.2.2 Liver

King et al. (1974) cite evidence that hepatic microsomes contain sites

which bind [^{3}H] estradiol with Kd 10^{-8}M. These binding sites are thought to exist on the membrane of the endoplasmic reticulum and are more numerous in male than in female rats. This difference is thought to be due to blocking of sites in the female rat by endogenous estrogen, since castration nullified this sex difference.

2.2.3 Cytoplasmic Receptors

Specific attachment of estrogens to cytoplasmic receptors occurs, a specificity which is characterized by a high affinity (Kd $2x10^{-10}$-$1x10^{-9}$M). Kd gives a measure of the concentration of steroid required to half saturate the receptors (Feherty et al. 1970; Giannopoulis et al. 1971; O'Malley et al. 1974). The specificity is for estrogen or estrogen related compounds, such as diethylstilboestrol, a non-steroidal compound which is also capable of binding to the receptor (King et al. 1974). All substances which attach to the receptor mimic the action of estrogen (O'Malley et al. 1974, 1976; Gorski et al. 1968). These properties clearly characterize receptor from non-specific binding.

O'Malley et al. (1974) and King et al. (1974) employed sucrose gradient techniques to separate the estrogen receptor and concluded that the estrogen was bound to a protein molecule.

The receptor was thought to be proteinaceous since the activity of homogenates of uterus incubated with different enzymes was destroyed by proteolytic enzymes but not by deoxyribonuclease or ribonuclease (O'Malley et al. 1974; King et al. 1974).

The receptor is thought to be a globular protein with a molecular mass of 200 000 dalton.

Sucrose density gradients (King et al. 1974) have been employed to characterize the estrogen receptor, which, in low salt concentrations, appears to be an 8S molecule (O'Malley et al. 1974). In high salt concentration 0,4M KCl or urea 3M (Notides and Nielsen 1972), the receptor sediments move slowly on sucrose density gradients and a smaller, 4S (45 000 dalton) estrogen binding unit is obtained (Stancel et al. 1973a,b). Chamness et al. (1972) observed a 6S entity in isotonic medium. The variability of sedimentation values has been observed in many studies (Puca 1971, 1972; Mueller 1972 and Stancel et al. 1973a). The variation in sedimentation rate of the receptor under different conditions is thought to be due to its aggregation with other proteins.

Mueller et al. (1972) have shown that, in a hypotonic saline solution, the receptor sediments at various S values, in agreement with the results of Stancel et al. (1973), who also observed that the S value was dependent on the protein concentration, higher protein concentrations yielding larger sedimentation coefficients.

Mueller et al. (1972) and Stancel et al. (1973) postulate that the 8S receptor is an artifact, consisting of aggregates of several proteins and is formed as a result of homogenization.

Notides and Nielsen (1974) investigated the 4S and 5S cytoplasmic estrogen receptor in the uterine cytoplasm of rats, using sucrose gradient centrifugation and Sephadex G 200 gel chromatography. Using these techniques, they determined molecular mass, sedimentation coefficient and molecular stokes radii of these two receptor species. Some of their results are summarized in Table 5.

TABLE 5. Results Obtained by Notides and Nielsen

	4S Receptor	5S Receptor
Molecular weight	76 200	132 700
Sedimentation coefficient	$4,2 \pm 0,04$	$5,5 \pm 0,02$
Molecular stokes radii	$[44 \pm 0,4] \times 10^{-10}$M	$[58,5 \pm 0,5] \times 10^{-10}$M
Salt concentration: 0,4M KCl pH 7,4		

Using protein standards, Notides and Nielsen (1974) also showed that the 4S receptor could undergo conformational changes.

The investigation of receptors experimentally is beset with problems such as the inability to duplicate the exact conditions within the cell with respect to salt and protein conditions. Studies on steroid receptors have been limited as well by the fact that receptors can only be detected in the presence of bound steroid, meaning that pure receptor can never be isolated. With respect to the estrogen receptor, it appears that, here too, sedimentation rates can vary according to the experimental conditions. Presently, there is no method available to determine the exact size or configuration of the receptor as it exists in the cell.

Estrogen receptors have been shown to exist in a variety of target organs, such as the vagina, mammary gland, pituitary and hypothalamus, such tissues being obtained from rat, mouse and human (King et al. 1974). This demonstrates either the wide distribution or common form of the estrogen receptor.

Progesterone binding globulin have been characterized in cytoplasm of reproductive tracts of guinea pigs (Faber et al. 1972a), rabbit and rat (Faber et al. 1972b), mouse and the human (King et al. 1974).

Experiments with chick oviduct provided evidence for the existence of receptors of progesterone, receptors which seem to involve themselves in similiar interactions as the estrogen receptors. Both 8S and 5S aggregates are present in cytoplasm when sedimented in the absence of KCl (Sherman et al. 1970). The 8S and 5S aggregates are converted to the 3,8S form in the presence of 0,3M KCl.

The oviduct binding component has a high affinity for progesterone as exemplified by a high binding constant Kd = $8x10^{-10}$M at 1 °C (King et al. 1974) and $8x10^{-10}$M at 4 °C (O'Malley et al. 1974). The progesterone receptor can be separated from the binding protein (transcortin) by sedimentation, the binding protein sedimenting as a 4S particle in the presence or absence of KCl. Also, the two types of protein can be separated by agarose gel chromatography and acrylamide gel electrophoresis (King et al. 1974), isoelectric gradient chromatography and protamine sulphate precipitation (O'Malley et al. 1974). This cytoplasmic progesterone receptor, like the estrogen receptor, is destroyed by pronase but not by ribonuclease or deoxyribonuclease. Some of the properties of the receptor are summarised in Table 6 below.

TABLE 6. Properties of Receptors

Parameter	Method sucrose gradient centrifugation	KCl absent	3M KCl
Sedimentation co-efficient		5S, 8S	3,8S
Axial ratio, Prolate ellipsoid	f/fo		14/8
Apparent molecular mass	Agarose filtration		144 000; 190 000
	Octylamide gel	102 000	
	Electrophoresis	357 000	
Apparent molecular mass	20		86 000; 99 000

adapted from Sherman et al. (1970). f/fo = frictional ratio

The receptor seems to exist as an oligomeric acidic protein, asymmetry shaped as a prolate ellipsoid with a molecular weight of 90 000 dalton.

The asymmetry is thought to be part of the reason for the variation in molecular mass obtained under varying experimental conditions. The calculated axial ratio seems to indicate that the receptor has a length 14-18 times greater than its width (Sherman et al. 1970).

O'Malley et al. (1976) indicates that the receptor consists of a dimer, each sub-unit having a different binding site. One sub-unit could bind to DNA while the other could bind to acidic nuclear proteins (O'Malley et al. 1976).

In summary, the binding components in the uterus and the oviduct fitted the criteria for "receptors" since they were only present in the target tissue and each had a high affinity for its particular hormone but a low affinity for other hormones.

2.2.3.1 Changes in Cytoplasmic Receptor Due to Steroid Attachment

Free receptor is predominantly found in the cytoplasm, while steroid receptor complex is found predominantly in the nucleus (O'Malley et al. 1976; King et al. 1974). The studies of Gorski et al. (1968) have led to the idea that an estrogen induced conformational change occurs in the uterine cytoplasmic receptor protein, a change which is followed by the translocation of the steroid-receptor complex to the nucleus, the area regarded as the primary site of hormone action.

Evidence suggests that estrogen-receptor complex differs from free receptor, Peck et al. (1973). Baulieu et al. (1975) and Katzenellenbogen et al. (1973) showed that the estrogen-receptor (e-r) complex was less thermolabile than the free receptor (r). Katzenellenbogen et al. (1975) observed that free

receptor was also more light sensitive than e-r complex. These changes must accompany changes in the physical properties of the receptor and support the theory that a conformational change occurs after the steroid is bound to the receptor (O'Malley et al.1974).

The idea of a conformational change is a theoretical one based partly on lability studies, but such a change has never been proved.

Evidence exists for a change in size of receptor after exposure to estradiol, a change which appears to be temperature dependent (King et al. 1974). The 4S form of the receptor is converted to the 5S form in the nucleus. This change can be observed on sucrose density gradients and in the presence of 0,4M KCl. This 5S nuclear, hormone receptor was undetectable in target tissue not previously exposed to estrogen (O'Malley et al.1974; Giannapoulous and Gorski 1971). Evidence which exists to show that the 5S receptor is simply a changed form of the 4S component are: the cytoplasmic receptor can be transformed (4S) to the 5S form by incubation with estradiol at 25 °C (Katzenellenobogen et al. 1975).

The 5S form accounts for most of the nuclear fraction. The fact that the 4S receptor was converted to the 5S form at 25 °C (Katzenellenbogen et al. 1975) and not at 0 °C (Gschewendt et al.1972) is significant since it indicates that the transformation of e-r from cytoplasm to nucleus is temperature dependent.

Exactly where in the cell this transformation of 4S to 5S occurs remains to be proved. Notides and Nielsen (1974)(see Table 5), on the basis of molecular sieve chromatography and density gradient centrifugation, maintain that the 5S form has a molecular weight of 132 700 dalton.

Notides and Nielsen (1974) and Stancel et al. (1973) showed that the 5S form of the receptor can be reconverted with urea to the 4S form which has a molecular weight of about 76 200. Notides and Nielsen (1974) concluded that the 5S form had a molecular weight of about 132 700 and they postulated that the 5S form is composed of a 4S unit attached to a 56 000 unit of protein.

The majority of conclusions discussed thus have been drawn either from an experiment with uterine tissue in vitro or from results of cell free experiments.

Anderson et al. (1972), using living tissue, tried to correlate the translocation process with the biologic responses of uterine tissue to various estrogenic compounds. This group developed the [^{3}H] estradiol exchange assay to determine the number of binding sites in vivo. This assay relies on the exchange of [^{3}H] estradiol with estradiol bound to nuclear sites and allows an evaluation of the concentration of specific estradiol sites in vivo. This assay is of special significance in view of the demonstrations that the amount of estrogen that is bound in the uterus is that present in the nuclear fraction. This method enabled the number of nuclear sites to be determined as a function of either endogenous estradiol or non-labelled estradiol and a correlation between the number of sites and biological response could be obtained.

To summarise, the e-r complex in cytoplasm is found to exist in a variety of forms (ranging from 4S to 8S), and it is still uncertain which is the form that binds estradiol and where the locus of entry into the nucleus is. Each sedimentation value indicates a change in conformation or composition of the receptor, and it is not known whether these changes are necessary for steroid action to ensue. Most workers however consider

these changes essential for steroid action.

A macro-molecular receptor for progesterone in the nucleus of the target cell was also identified (O'Malley et al, 1971). It was also found that this receptor was almost identical to the receptor found in the cytoplasm of the same cell.

As with estrogen in the rat uterus, it was shown that very little nuclear receptor was present in non-stimulated tissue, but exposure to progesterone greatly increased the extractable nuclear receptor protein. Concomitant with this increase was a decrease in cytoplasmic receptor (O'Malley et al. 1971), and, in the presence of progesterone, this resulted in an accumulation of receptor bound intracellular [^{3}H] progesterone in the target cell nucleus.

2.3 Interaction of Steroid Receptors with Nuclear Components

2.3.1 Estrogens

Most workers indicate that the estradiol is bound to chromatin in the nucleus (King et al. 1974). When the e-r enters the nucleus, it attaches to a nuclear component called the "acceptor" (King et al. 1974). O'Malley et al. (1976) are of the opinion that approximately 5000 such acceptor sites exist per nucleus.

This "acceptor" has some specificity, as target tissue nuclei bind more e-r complex than non-target tissue nuclei (Gschewendt et al, 1972; Musliner et al. 1970). The concept of an acceptor is again a hypothetical one. This concept originated from the binding of repressors with a very high affinity to a limited number of sites on the lac operon in Escherichia coli. The evidence to support such a concept can be summarized as (a) the requirement of high concentrations of salt to release nuclear bound receptors;

(b) the fact that saturable interactions between receptor hormone complexes and nuclei or nuclear components have been observed *in vitro*; and (c) the apparent specificity, both with regard to the source of the nuclei and particular nuclear fraction, to which the hormone-receptor complex binds. The questions to be answered are: what is the acceptor and how is its specificity determined?

DNA has many of the characteristics (see a, b, c) of the nuclear acceptor. However, in contrast to chromatin, DNA alone cannot be considered the physiological acceptor, since hormone-receptor will attach to DNA from several sources (King *et al.* 1974). Thus, with DNA, a lack of specificity is evident, and O'Malley (1974) mentioned that estrogen-receptor from rat uterus can interact with DNA from calf thymus, salmon sperm, *Escherichia coli* and *Bacillus subtilis*. Thus, it seems as though the acceptor site is a complex structure and that other components of chromatin may act to modify receptor binding to DNA.

According to King *et al.* (1974), guanine residues are implicated in the binding of steroid receptor to DNA. The significance of receptor complex attaching to DNA is still obscure since the exact site of attachment of the steroid-receptor complex is still unclear. The possibility should not be ruled out therefore that the DNA-receptor attachment could be the physiologically important one.

A limited number of binding sites for DNA do exist. According to O'Malley et al. (1974), two receptors saturate 10^7 nucleotides, which means that 500 receptor molecules would saturate the quantity of DNA in a single cell nucleus (King *et al.* 1972).

An opposing view is that the histones, highly alkaline proteins, might be

the acceptor, but this suggestion has now been rejected due to the efforts of Spelsberg et al. (1971). This group, using chick oviduct, showed that the two candidates for the role of acceptor are DNA and the non-histone proteins. This problem will be discussed in greater detail in the next section. Other components which the e-r complex have been shown to bind to in target tissue are ribonucleoprotein (Liao et al. 1973) and nuclear membranes (Jackson et al. 1974).

In summary, it seems that some doubt is cast on the simple idea that the binding of estrogen to a few high affinity sites (acceptors) provides an ample answer to both the mechanism of translocation of the steroid-receptor complex and the mechanism of gene activation since it appears that the acceptor site is a complex structure and other components of chromatin may act to modify receptor binding to DNA. This seems to be the most controversial aspect of the model for steroid receptors.

2.3.2 Progesterone

As mentioned earlier, a protein receptor that binds progesterone has been revealed in the reproductive tracts of guinea pig (Faber et al. 1972a); rabbit, rat, (Faber et al. 1972b) and mouse and human (King et al. 1974).

O'Malley et al. (1972) showed that, after binding, the hormone-receptor complex (progesterone-receptor, p-r) translocated to the nucleus. The significance of, or reason for, this translocation is no clearer in the case of this hormone than in the case of estrogen.

Schrader et al. (1972a) showed that [^{3}H]progesterone receptor complex could bind and be retained by purified chick DNA. In a further series of experiments using a diethylaminoethyl cellulose column (DEAE) and eluting the p-r-DNA mixture through this column, they found that the

progesterone receptor was a dimer consisting of an A and B sub-unit (Schrader et al.1972b), each sub-unit being an independent strand of amino acids. Furthermore, both sub-units had a molecular mass greater than 100 000. Each sub-unit was eluted at different positions in DEAE cellulose columns, sub-unit A was eluted at 0,15M KCl and B was eluted at 0,22M KCl.

Both sub-units bound progesterone and biologically active progestins (O'Malley et al.1974). There were only a few (low capacity) sites, but they bound the hormone strongly (Kd = $3x10^{-10}$M, high affinity). Schrader et al.(1972a) observed that only the A sub-unit was bound to naked DNA; the B sub-unit did not. The receptor (A sub-unit) had a high affinity for the binding sites on the DNA and the DNA had only a limited number of sites. It was found that one receptor bound for every 106 nucleotide pairs (O'Malley et al.1974).

Spelsberg et al.(1971) showed that the p-r complex binds to oviduct chromatin to a greater degree than it does non-target chromatin obtained from spleen, heart, lung, liver or erythrocytes.

Spelsberg et al.(1971), in attempting to determine the function of chromatin responsible for the binding of p-r complex to chromosomes, showed that the histones were not responsible for the specificity of receptor binding. This finding is in accordance with the results obtained by King et al.(1972) for estradiol-receptor. They found that removal of histone prior to incubation of e-r complex with chromatin obtained from rat uterus exposed a considerable number of binding sites. Mainwaring et al.(1971) obtained similiar results for androgen-receptor interaction with male target organs (i.e. prostate gland and its binding to [^{3}H]5α-dihydroxytestosterone to chromatin) and non-target tissues

Spelsberg et al. (1971) also found that the native histone which was removed could be replaced by histones of non-target tissues without affecting the binding of the p-r complex.

Furthermore, using chromatin reconstituted histone and non-histone proteins dissociated from chromatin of oviduct and erythrocytes, this group showed that it was the non-histones which showed a selective affinity for the p-r complex, since, during the reconstitution step if the non-histones were omitted, the chromatin lost most of its ability to bind p-r complex.

Spelsberg et al. (1971) also showed that, if oviduct non-histone proteins were replaced by non-histone obtained from erythrocytes (non-target tissue), binding of the p-r complex to reconstituted oviduct hybrid chromatin did not occur. However, hybridisation of oviduct non-histone protein with erythrocyte non-histone protein resulted in a binding of the p-r complex resembling that of native oviduct (O'Malley et al. 1972, Spelsberg et al. 1972).

Spelsberg et al. (1972) localized this binding site (acceptor site?) on a certain subfraction of the non-histone protein of the chromatin. This fraction was designated the Ap_3 fraction. When the Ap_3 fraction was extracted, the residual chromatin lost most of its ability to bind p-r complex, while replacement of Ap_3 fraction restored binding activity. Hybridisation of Ap_3 fraction of non-target tissue with that of target tissue again bestowed binding capacity on the hybrid chromatin resembling native oviduct.

Spelsberg et al. (1972) showed that it was sub-unit B of the receptor which was bound to the Ap_3 fraction.

Since the B sub-unit binds to a specific fraction of chromatin, it may be regarded as a "specifier" and may be the determining factor in determining the exact position of binding to the chromatin, since only an unused number of binding sites exist and the A sub-unit binds to any nearby sequence of DNA.

In summary, it appears that both the DNA and non-histone protein fraction (Ap_3) of chromatin play a role in forming the acceptor sites for uterine chromatin and progesterone receptor in oviduct chromatin. However, it must be borne in mind that, in contrast, the estrogen receptor has not been purified to such an extent that it can be resolved into sub-units as the progesterone receptor complex, nor has the e-r been characterized by specific binding to a single chromatin fraction such as the Ap_3 fraction.

2.4 Steroid Hormones and Gene Regulation

Steroid hormones are found only in eukaryotic cells. In higher vertebrates, the major classes of steroid hormones, estrogens, progesterone, androgens, glucocorticoids and minerolocorticoids, have multiple actions in sensitive tissues, while at the same time a single hormone may have quite different effects on different tissues.

Evidence has accumulated suggesting that hormones regulate the metabolic activities of mammalian cells by controlling RNA and protein synthesis (Katzellenobogen and Gorski 1975; Mueller et al. 1958; Knowles et al. 1971).

2.4.1 Effects of Steroid Hormone on RNA Synthesis

The concept that the effects of estrogen on protein synthesis is mediated

by changes in ribonucleic acid is based on the work of Mueller et al. (1958), who noted changes in RNA build-up prior to alteration in protein synthesis. They also noted that estrogen increased the rate of incorporation of precursors into total, cell RNA.

There seems to be general agreement that one of the first, or at least early, effects of estrogen is the stimulation of synthesis of RNA in its target cells (Baulieu et al. 1971; O'Malley et al. 1974). This increase in RNA synthesis is probably conducive to protein synthesis which follows estrogen administration. The nature of the RNA formed during the first few minutes of estrogen administration is still uncertain; however, evidence suggests that the bulk of it may be ribosomal in nature with some m-RNA and t-RNA formed as well. Hamilton (1968) and Knowles et al. (1971) showed that, a few hours after estrogen administration, both ribosomal precursor RNA (28S, 18S and 5S ribosomal RNA) and 4S t-RNA were stimulated. Jensen and De Sombre (1972), in agreement with Hamilton et al. (1968), cite evidence that this ribosomal type RNA produced, either in vivo or in vitro following estrogen administration, appears to be different from the normal RNA produced in the absence of estrogen. There is also evidence that new species of RNA have been formed in rabbit uterus, hen oviduct and the liver of hen, rabbit and lizard following estrogen stimulation.

Knowles et al. (1971), using gel electrophoresis to fractionate the various types of RNA, found that the synthesis of 28S and 18S RNA of the ribosome was increased one hour after estrogen injection and that the RNA tended to become more AU-rich (adenine and uracil). The mechanism by which the estrogen stimulates RNA synthesis is uncertain. It could arise from an effect of the hormone on chromatin template activity, RNA polymerase

activity, RNA transport from nucleus to nucleolus or a combination of factors (evidence supporting all these have been documented).

Thus, many approaches have been used to study the effect of estrogen on RNA activity. Among these have been DNA-hybridization experiments and chromatin template activity studies.

Church et al. (1970) used DNA-RNA hybridization to investigate the effect of estrogen on rabbit endometrium. Their findings indicate that, one hour after estrogen administration, a new population of RNA appeared in the liver and uterus but not in the lung. They concluded that not more than 25% of the estrogen stimulated RNA was common to the liver and the uterus and suggested that estrogen was qualitatively altering the pattern of transcription in these tissues. This method, employed by Church et al. (1970) seems rather of limited use since only change in repetitive sequences of DNA can be analysed, unique sequences responsible for the synthesis of m-RNA were not determined.

According to O'Malley and Rosen (1976), 70% of the chick genome is composed of unique sequences or single copy sequences; thus, a method of analysis of unique sequences of DNA transcription is essential for the understanding of estrogen action.

O'Malley (1968 and 1974) indicated that estrogen may result in a change in chromatin template, and this change may allow an increased number of binding sites for RNA polymerase.

Chromatin template activity makes possible the estimation of the percentage of total genome available for transcription. Glasser et al. (1972), using rat uterus, noted an increase in chromatin template activity one hour after estrogen administration. This was accompanied by a concomitant stimulation

of RNA polymerase I.

In summary, it is clear that steroid hormone-receptor complex enters the nucleus of the target cell and binds to the chromatin. Thus, it can be assumed that synthesis of RNA plays a major role in the primary mechanisms of action, but it has not been possible to show directly that binding of steroid hormone-receptor to chromatin stimulates the rate of nuclear transcription.

2.4.2 Steroid Hormones and RNA Polymerase Activity

The first step in the sequence of events that leads eventually to protein synthesis is thought to be the binding of the enzyme RNA polymerase to DNA. Very briefly the protein synthesis hypothesis can be summarized as:

DNA ⟶ RNA ⟶ Protein

The sequence of genetic "letters" A (adenine), T (thymine), C (cytosine), and G (guanine) in the DNA is first transcribed into the corresponding sequence of letters A, U (uracil), C, G in the messenger RNA; this occurs through the action of RNA polymerase. Two polymerases are thought to separate in eukaryotic RNA synthesis, a nucleolar polymerase which appears to be responsible for synthesis of r-RNA (polymerase I) and polymerase II, a nucleoplasmic polymerase, that synthesizes DNA-like RNA.

Gorski (1964), using uteri from immature rats, noted that, one hour after estrogen administration, magnesium dependent, RNA polymerase activity was increased, an increase in activity which was blocked by prior administration of puromycin or cycloheximide and which indicated that the estrogen response occurred through the continued synthesis of protein. Subsequently it was shown that the Mg^{2+} dependent enzyme was polymerase I. Hamilton et al. (1968) showed that a second polymerase activity was also

stimulated by estrogen, but this activation required twelve hours to take effect. This enzyme was polymerase II and it was Mn^{2+} dependent. Barry and Gorski (1971) investigated the effects of estrogen on uterine polymerase I, activity. They compared nucleotide incorporation into 3'-chain termini and into internucleotide positions of RNA chains. The RNA chains were obtained by synthesis of DNA-dependent RNA polymerase in isolated uterine nuclei. They concluded that estrogen causes an increase in the rate of chain elongation within one hour but does not alter the number of growing chains. Thus, it appears from these results that estrogen stimulates the activity of RNA polymerase rather than the absolute amount of enzyme. This end could be achieved by increasing the rate of read-off of the enzyme.

Evidence at variance with that of Barry and Gorski (1971) has been cited by Scharwz et al.(1975) and Tsai et al.(1975), who showed that estrogen serves to increase the number of initiation sites per cell. They observed a seven-fold increase in the number of chromatin initiation sites after four days of estrogen administration. As the number of chromatin bound polymerase molecules increased, so did the amount of newly synthesized RNA chains. Scharwz et al.(1975) found no increase in RNA chain elongation since they observed that chromatin prepared from estrogen-treated or control oviducts both supported a chain elongation rate of six nucleotides per second and a chain size of about 700 nucleotides.

The hypothesis of Scharwz et al. (1975) and Tsai et al.(1975) is an attractive one since it supports the view that steroid hormone does directly alter chromatin template activity, further substantiating the claim that it is the chromatin that is the "acceptor site". The direct action by steroids on chromatin template could be the mechanism for induced

transcription at new gene sequences and for the appearance of new m-RNA molecules in target cell. This hypothesis also supports transcriptional control as the primary mechanism of steroid action. (Perhaps the weakness of this theory is that initiation sites have not been shown to exist in eukaryotic organisms.)

Glasser et al. (1972) noted that estrogen had a biphasic effect on polymerase II activity. A rapid but transient rise in polymerase II activity occurred fifteen minutes after estrogen injection and reached a peak at 30 minutes, RNA polymerase II levels returning to normal at 1-2 hours. A peak was again noted at 6 hours. The cause of this biphasic effect is unclear, but the first peak of RNA polymerase II at 30 minutes could trigger off synthesis of RNA, which in turn may induce the later response of the cell to the hormone, i.e. the second increase of polymerase II activity, the rise in polymerase I activity initially, and the increase in protein synthesis. This initial increase of polymerase activity (at 15 minutes) occurred in conjunction with the stimulation of labelled RNA synthesis and could be blocked by α-chromatin, an inhibitor of polymerase II activity. Moreover, the increase in RNA polymerase levels occurred prior to any increase in template or polymerase activity.

Glasser et al. (1972) found that the stimulation of polymerase I activity occurred after one hour and reached its peak at four hours. These results are in agreement with those of other groups (Barry and Gorski 1971; and Gorski 1964), who also found that RNA polymerase I activity increased after one hour and peaked at 4 hours. The reason for the biphasic response of polymerase II activity to estrogen treatment is obscure (see above), but from the results of actinomycin D and protein synthesis blocking experiments it seems possible that this early increase

in RNA polymerase II activity could be necessary for the subsequent biochemical events in the action of estrogen on its target cells.

It has been demonstrated in vivo (Katzenellenbogen and Gorski 1972) and in vitro (Mayol and Thayer 1970) that an early and specific event in the induction of a specific protein (induced protein, abbreviated IP) by estrogen is blocked by actinomycin D. Moreover, this actinomycin D sensitivity manifests itself only during the first thirty minutes, a fact suggesting that RNA synthesis occurs very early after estrogen reaches the target cell.

The actinomycin D sensitive step is unaffected by protein synthesis inhibitors (puromycin and cycloheximide), meaning that the response to the hormone is not dependent on protein synthesis.

In summary, several lines of evidence have been presented to show that estrogen increases the RNA synthesis, which seems to be tied up with the increased RNA polymerase activity, most workers having observed that estrogens increase the activity of polymerase I activity. Also the results of studies in which Escherichia coli polymerase is used should be viewed with caution since the exogenous polymerase may not transcribe in the same positions as endogenous polymerases. However, these experiments do allow one to see that there is a qualitative difference in the chromatin from estrogen treated rats.

2.4.3 Effects of Steroids on Production and Transcription of Messenger RNA

Available data indicate that estrogen acts in the nucleus to stimulate the synthesis of m-RNA, which presumably codes for specific proteins. In the case of the uterus, these proteins are required for its growth

and differentiation. O'Malley et al. (1976) demonstrated that the particular genes transcribed were those coding for proteins normally associated with the response to the hormone. To demonstrate this, they made use of RNA-DNA hybridization techniques. Pure samples of the m-RNA to be detected were prepared, in this case, m-RNA that coded for ovalbumin. Complementary labelled strands of DNA were prepared from the m-RNA by use of the enzyme, reverse transcriptase obtained from a virus.

Using the labelled DNA strands as a probe to determine how much m-RNA was synthesized in chick oviduct after hormone injection, they found that the cellular contents of m-RNA rise from zero to 10 000 molecules per cell in a few hours.

O'Malley et al. (1976) also found during experiments in cell free systems that only RNA synthesized from chromatin exposed to purified hormone-receptor complexes contained ovalbumin messenger RNA. From these experiments it can be concluded that steroid hormone receptors have the ability to bind to chromatin and stimulate total gene transcription and express DNA sequences coding for specific messenger RNA molecules.

O'Malley et al. (1972) measured avidin m-RNA activity in oviduct in response to progesterone and found a good correlation between avidin m-RNA activity and avidin synthesis following administration of a single injection of 1 mg progesterone to chicks which had received estrogen for 12 days. After injection of progesterone, avidin m-RNA activity was evident after six hours and continued to increase until 24 hours after progesterone administration.

Avidin m-RNA levels increased considerably, before the maximum amount of

avidin appeared in the tissues. These results indicate that progesterone and estrogen act in the nucleus of target tissues to stimulate synthesis of m-RNA.

2.6 Dissociation of Receptor from Steroid

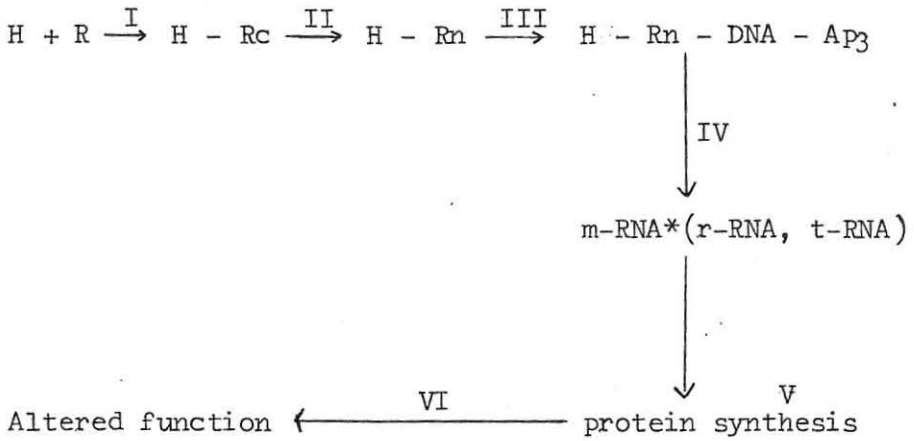
After the hormone has elicited its response, the hormone-receptor components must dissociate from one another and from the chromatin. This process seems to be the least understood aspect of steroid hormone action. It is still uncertain what happens to the steroid once it has dissociated from its receptor, the mechanism by which it is lost from the cell, and what happens to the receptor. O'Malley et al.(1976) suggest that the receptor molecule may not be destroyed. Instead, it may be recycled for use and return to the cytoplasm to form complexes with other hormone molecules and return to the nucleus to continue its action. The hormone is thought to be inactivated and somehow diffuses out of the cell. King et al.(1974) proposed that the released steroid leaves the cell by a non-cytoplasmic route, possibly making its exit through channels of the endoplasmic reticulum; or they proposed that if this does not occur, the steroid must still be attached to some reminant of the receptor thus preventing it from being bound anew to other macromolecules.

Sarff and Gorski (1971) suggest that receptors which have released steroid are not available for binding immediately.

Summary

In the preceding sections, the paths of two steroid hormones, estrogen and progesterone, have been traced from the entry into target cells to the time of release from receptor.

The events occurring can be summarized in Scheme 5.



SCHEME 5. Sequential Steps in Steroid Hormone Action

I) Entrance of hormone (H) into target cell and binding to specific cytoplasmic receptor (R).

II) Formation of hormone-receptor-complex (H-Rc) which translocated to nucleus (H-Rn).

III) Binding of H-Rn to specific acceptor sites (chromatin DNA and non-histones (or acidic proteins Ap_3)).

IV) Activation of transcriptional apparatus resulting in appearance of new RNA species.

V) Transport of hormone-induced RNA to cytoplasm.

VI) Functional response mediated by steroid and characteristic of target tissue.

It has been shown that, between these two events, steroid hormones give rise to a large array of responses in their target cells, the female sex steroids seemingly exerting regulatory effects on synthesis and activity of tissues enzymes and other proteins.

Some problems encountered in the elucidation of the mechanism of steroid hormone action have been mentioned. It seems that the problems associated

with an understanding of steroid hormone action at the molecular level are technological rather than conceptual. The areas of investigation of protein synthesis at the molecular level will include the dismantling and reassembly of biochemical components at the transcriptional level to make possible the study of direct effects of hormones in cell free systems.

Questions which come to mind when considering the expression of a predetermined set of genes are:

(a) will the switch of this mechanism be found at the level of gene transcription or at any of the post-transcriptional steps?

(b) how is the regulated gene turned off when sufficient RNA has been synthesized?

The material presented have been extracted mainly from estrogen and progesterone receptor studies. Most reviews of steroid hormones recognize the universal applicability of these mechanisms to the action of other hormones. Thus, it may be assumed that synthetic steroids, because of their structural similiarities to estrogen and progesterone and because of the similiarity of their effects as compared with natural steroids, bind to these same receptors to exert their hormonal action. It could also be that they block the receptor sites and, thus, suppress response to endogenous hormone and preclude an effect on ovulation.

SECTION 3

DRUG METABOLIZING SYSTEM

The hepatic, drug metabolizing enzymes are membrane bound and are located on the microsomal membranes of mammalian liver cells. These membranes are derived from the rough and smooth endoplasmic reticulum (Claude 1969).

The hepatic drug metabolizing enzymes function as a multicomponent electron transport system responsible for the metabolism of various endogenous substrates (such as steroids, fatty acids and bile acids) and exogenous substrates (such as many drugs, carcinogens and other foreign compounds) (Conney et al.1967; Gillette et al.1972). The drug metabolizing enzyme has an absolute dependence on NADPH and molecular oxygen for its reactions and is known as microsomal hydroxylase, monooxygenase or mixed function oxidase.

3.1 Functional Components , see Scheme 6.

Enzymes involved in the system include:

(a) NADPH-dependent cytochrome P-450 reductase or NADPH-dependent cytochrome c reductase (a flavoprotein)

(b) NADH-dependent cytochrome c reductase

(c) NADH-dependent cytochrome b_5 reductase

(d) Cytochrome b_5 (a haemoprotein)

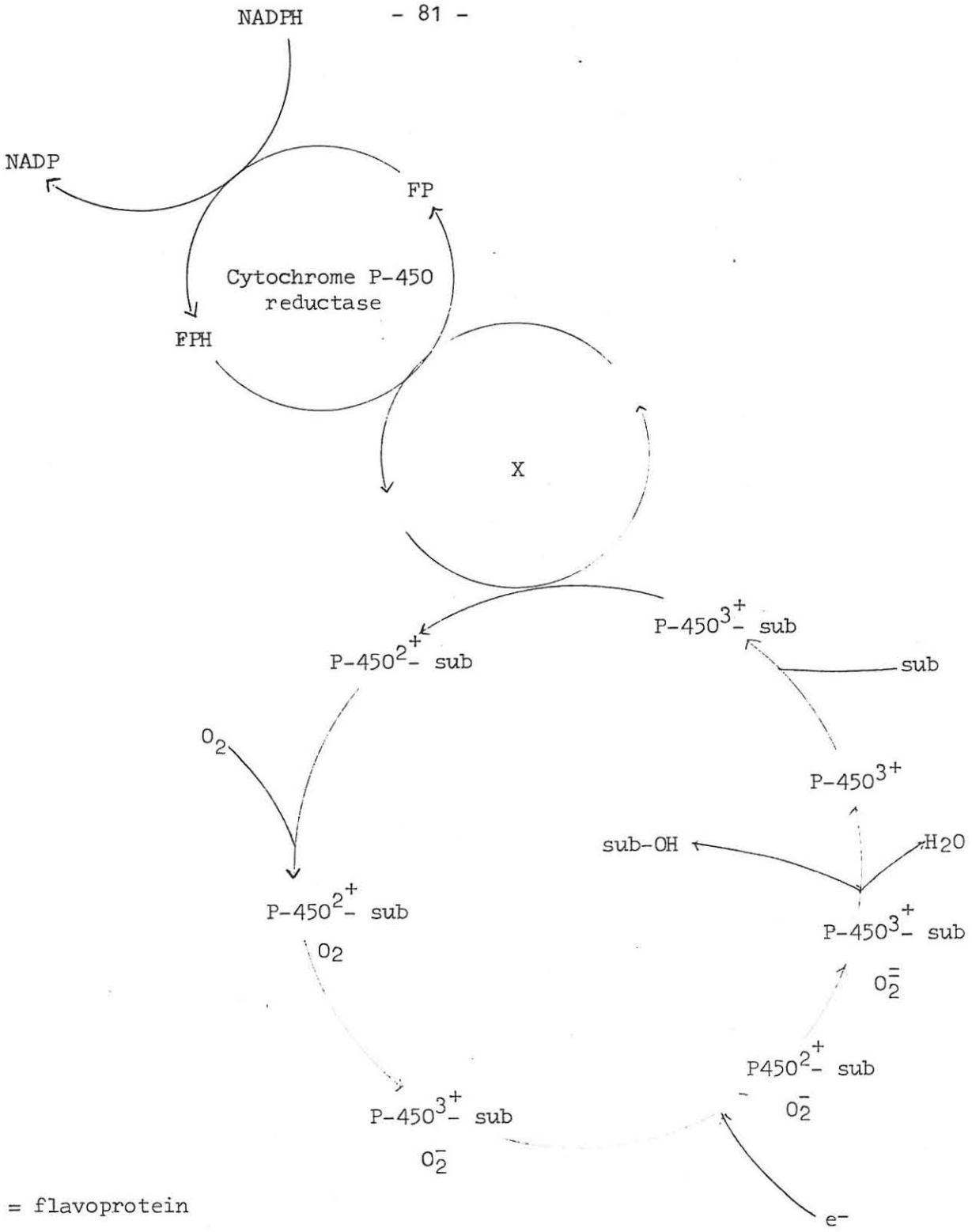
(e) Cytochrome P-450 (a haemoprotein).

Other enzymes, such as phosphatases (mainly ATP-ases) and UDP-glucuronyl transferases, are also associated with the endoplasmic reticulum. Certain phospholipids must also be present, possibly also membrane bound for the system to function (Gillette et al.1972; Eling et al.1971).

Cytochrome P-450 has been isolated from a variety of cellular sources (other than hepatic), for example, the mitochondria and microsomes of adrenal cortex, where it functions in the hydroxylation of steroids (Omura et al.1965), insects, yeasts, plants (Mannering 1972), in bacteria, mainly Pseudomonas putida (Gillette et al. 1972).

Cytochrome P-450 is thought to be the terminal oxidase, which binds both the substrate and molecular oxygen and catalyses the reaction between them, while the reductase serves as an electron carrier, shuttling electrons from NADPH to cytochrome P-450.

In the majority of oxidative reactions catalysed by hepatic microsomes, the substrate first combines with the oxidized (Fe^{3+}) form of cytochrome P-450. The complex is reduced by an electron from the flavoprotein, NADPH cytochrome P-450 reductase. This step is facilitated by the binding of the substrate to P-450 and is thought to be the rate limiting step in the oxidation of the compound (Ishimiae et al. 1971). The reduced complex reacts with molecular oxygen to form a cytochrome P-450-substrate-O_2 complex, which is reduced by a second electron to form a highly active, oxygen intermediate. The exact steps are unknown, but Ishimuae et al. (1971) and Estabrook (1971) give evidence for the existence of this oxygen complex. Decomposition of the complex results in the oxidized substrate and oxidized cytochrome P-450. The substrate can then be conjugated to form the glucuronide or sulphate, which is readily excreted. The scheme of electron transport chain and mechanism of oxidation of cytochrome P-450 is shown in Scheme 6. The source of this second electron is also controversial, and several postulates have been recorded to explain the origin of this second electron. The balance of evidence seems to favour the postulate that the second electron is transferred to oxygenated



Fp = flavoprotein

x = unknown factor

P-450^{2+} = cytochrome P-450 in reduced form

P-450^{3+} = oxidized P-450

P-450-sub = enzyme-substrate complex

SCHEME 6. Functional Components of a Drug Metabolizing System

cytochrome P-450 *via* cytochrome b_5 (Hildebrandt *et al.* 1971; Estabrook 1969; Mannering 1972 and Lu 1976).

If indeed cytochrome b_5 transfers the second electron to oxygenated cytochrome P-450, cytochrome b_5 would be an obligatory component of the mixed function oxidase system.

Levin *et al.* (1974) are at variance with the above postulate on the basis of evidence obtained from their studies. This group investigated the metabolism of benzamphetamine and benzpyrene in the absence or presence of cytochrome b_5. In order to do this, they used a reconstituted, partially purified system of cytochrome P-450 or P-448 obtained from rat liver. Their results are summarized in Table 7.

TABLE 7. Effect of Cytochrome b_5 Concentration on Metabolism of Benzamphetamine and Benzpyrene

Reaction studied	Preparation	Cytochrome P-450 or P-448 n moles	Enzymatic activity m moles/ min	Ratio of cytochrome P-450 to cytochrome b_5
Benzamphetamine	Cytochrome	0,2	9,6	200
Demethylation	P-450	0,2	9,2	25
Benzpyrene	Cytochrome	0,2	0,31	250
Hydroxylation	P-448	0,2	0,32	40

These results show that, when the ratio of cytochrome b_5 is 25 to 1 or greater than 200 to 1, there is hardly any change in the catalytic activity of the reconstituted system.

These results indicate that cytochrome b_5 is not an essential component of the reconstituted system for the metabolism of benzamphetamine or benzpyrene. However, these results do not rule out the possible role of cytochrome b_5 in microsomal oxidation since other factors might be operating in a reconstituted system as opposed to a suspension of microsomes, which was used to

study electron transfer.

Lu et al. (1968) solubilized the hepatic hydroxylating enzyme with detergent and resolved it chromatographically into three components, cytochrome P-450, NADPH cytochrome c reductase, and a lipid fraction called phosphatidylcholine (Lu et al. 1969; Strobel et al. 1970). The lipid fraction could be replaced by other phospholipids, of which phosphatydylcholine was the most effective.

Lu et al. (1972) and Lu et al. (1974) showed that all three components were essential for metabolism of foreign compounds and fatty acids.

Recently, both cytochrome P-450 and NADPH cytochrome c reductase have been purified (Iyanagi et al. 1973; Levin et al. 1974).

These purified preparations have made it possible to show that there exists a complete dependence on cytochrome P-450, NADPH cytochrome c reductase, and lipid for the metabolism of various drugs, steroids, alkanes and polycyclic hydrocarbons (Levin et al. 1974; van der Hoeven et al. 1974).

3.2 Cytochrome b_5 and Drug Metabolism

The involvement of cytochrome b_5 in drug metabolism can be summarized briefly as follows:-

(a) cytochrome b_5 is thought by some to be the source of the second electron in microsomal oxidation

(b) cytochrome b_5 could possibly be involved in some NADPH - dependent microsomal hydroxylations since some antibodies against cytochrome b_5 inhibit some of these reactions (Gillette et al. 1972)

(c) cytochrome b_5 may be involved in some NADPH - dependent reactions but not all (Lu et al. 1974)

(d) cytochrome b_5 is required in the interaction between the NADPH and and NADH mediated electron pathways (Lu et al. 1974 and Gillette et al. 1972).

The purification of any hepatic cytochrome P-450 would make it possible to clarify the extent of involvement of cytochrome b_5 and thus cast more light on the origin of the second electron in microsomal oxidation.

3.3 NADPH Cytochrome c Reductase

Cytochrome c is absent in microsomes so it appears strange that there is a cytochrome c reductase involved in the sequence of reactions. It has been shown however that NADPH cytochrome c reductase plays a role in the transfer of an electron from NADPH to cytochrome P-450 during microsomal hydroxylation. NADPH cytochrome c reductase can be induced by some inducers such as phenobarbital (Mannering 1972).

3.4 Non-haem Iron Protein

Adrenodoxin, a non-haem iron protein, is an essential component of the electron transport chain that functions in the hydroxylation of steroids by adrenal mitochondria (Omura et al. 1966).

In the steroid hydroxylating system of the adrenal gland, adrenodoxin causes the transport of an electron between adrenodoxin reductase, and cytochrome P-450 (Huang et al.;1971; Omura et al., 1966).

Cooper et al. (1965) noted that some similarities between the adrenal hydroxylating system and that of the liver exist, in that the spectral and oxidation-reduction properties of the P-450 complex obtained from beef adrenal microsomes are similar to those of beef liver microsomes. From these similarities between adrenal and hepatic hydroxylating systems, it is assumed that some non-haem iron protein also plays a role in hepatic microsomal hydroxylation reactions.

However, direct evidence has not been presented to allow the conclusion that electron transfer of the adrenal cortex is the same as that found in

the liver.

A similiar non-haem iron protein has been found to play a role in steroid hydroxylation in the mitochondria of testis (Kimura 1968), of ovary, and of placenta (Baron 1973). Kimura et al. (1968) concluded that steroid producing tissues contain adrenodoxin, but liver and other tissues do not.

No non-haem iron protein has been shown to be essential for microsomal steroid hydroxylation; in fact, Sweat et al. (1969) observed that adrenodoxin inhibited bovine adrenocortical microsomal 21-hydroxylase.

Evidence for the essentiality of a non-haem iron protein in microsomal drug metabolizing systems seems inconclusive at this stage, but the balance of evidence obtained from reconstitution studies of partially purified cytochrome P-450 systems seem to favour the concept that the non-haem iron protein is not obligatory for hydroxylations to occur since purified hydroxylating systems seem to metabolize a variety of substances just as well as microsomes do. On the other hand, Mull et al. (1975) indicate that a non-haem iron protein may be obligatory for drug metabolism. Using SDS-polyacrylamide gels for the separation of the components, they assigned one major protein to the same electron transport component from control, phenobarbital and 3-methylcholanthrene treated mice and to the so called "Factor X", which is thought to be the link between the NADPH-dependent flavoprotein coupled with cytochrome P-450 and a NADPH dependent flavoprotein coupled with cytochrome b_5. The "Factor X" protein, according to Mull et al. (1975), has a molecular weight of 48 000 daltons and appears to be a non-haem iron protein, and probably is an electron carrier intermediated between flavo- and haemo-proteins.

3.5 Role of Lipid in Drug Metabolism

Strobel et al. (1970) observed that the lipid fraction of hepatic microsomal

enzymes could be replaced by various phospholipids, of which phosphatidylcholine was the most effective.

The role of the lipid fraction in hepatic microsomal enzyme system is still uncertain, but its presence enhances the reduction of cytochrome P-450 even though the lipid does not contain components that undergo redox reactions (Strobel et al. 1970). Lipid seems to be essential also in induction of cytochrome P-450 in vivo. Marshall et al. (1971) have shown that, in rats, dietary lipids and choline, as well as adequate synthesis of phosphatidylcholine, are all essential for phenobarbital induction of cytochrome P-450.

Norrod and Wade (1972) also noted increases in the amount of cytochrome P-450 and in microsomal drug metabolism in hepatic microsomes from rats maintained on high levels of corn oil (10% in diet).

Norrod and Wade (1972) concluded that a source of polyunsaturated fatty acid is essential for maximal functional activity of the mixed function oxygenase system. The microsomal fatty acid of the phospholipid in rats maintained on a diet lacking in lipid were quantitatively different from those of rats fed on a diet rich in lipid and concomitant with these quantitative changes in phospholipids are a reduced metabolism of hexobarbital, aniline and ethylmorphine, reduced content of P-450, and decreased binding of aniline and hexobarbital to microsomes.

This group also report a qualitative difference in the microsomal preparations obtained from lipid deficient rats as manifested by an alteration in the K_M and K_s for certain substrates and a shift of the ethylisocyanide binding spectrum.

The results of Hopkins and West (1976) concerning the in vivo effects of dietary lipids on the mixed function oxidase system are at variance with

those of Norrod and Wade (1972) and Marshall et al. (1971). Hopkins and West (1976) found an increase in the amount of cytochrome P-450 per kilogram of body mass in rats maintained on a tallow diet.

Some investigations have indicated that the phospholipid may be essential for the binding of type I substrates to cytochrome P-450 (Chaplin and Mannering 1970; Eling et al.1971). Chaplin and Mannering (1970) removed 70% of the total phospholipid from hepatic microsomes of rats, using phospholipase c. Such treatment abolished the ability of the substrates ethylmorphine and hexobarbital (typical type I substrates) to bind to type I sites. However, the microsomes lost only about 40% of their ability to oxidize ethylmorphine and hexobarbital. On the other hand, aniline (typical type II substrate) caused an increase in type II binding and only 15% loss of aniline oxidation. The results obtained by Chaplin and Mannering (1970) are difficult to interpret since addition of phospholipid in their experiments did not restore enzyme activity. The impairment of type I spectral change could have been due to the destruction of binding sites on the removal of phospholipid. These workers explain this result by saying that type I binding may not be obligatory for hydroxylation to occur and postulate that perhaps an alternative route of hydroxylation may exist in the absence of type I binding. Alternatively, two types of cytochrome P-450 exist, both of which could be involved in hydroxylations, but only one of which is associated with type I binding, and it is the more efficient of the two. Thirdly, (and least likely) type I binding has no association with drug metabolism.

The results of more recent studies by Vore et al. (1974), who removed 80-90% of phospholipid by repeated extractions of lyophilized microsomes with 1-butanol and acetone, are at variance with those of Chaplin and Mannering (1970). Vore et al. (1974) observed that removal of 80-90% of phospholipid actually increased the magnitude of hexobarbital and benzamphetamine

induced type I spectra per nanomole of cytochrome P-450.

Vore et al. (1974) concluded that phospholipid may be required for enzymatic activity but was not essential for binding of substrates to P-450, a conclusion with which this author agrees.

In summary, it seems that phospholipid may not be obligatory for substrate binding to cytochrome P-450 but may enhance this binding.

3.6 Cytochrome P-450 and Spectral Changes

To obtain an indication of the type of binding between substrate and cytochrome P-450 during drug metabolism by the mixed function oxidase system, spectroscopic studies can be applied.

3.6.1 Carbon Monoxide Difference Spectrum

A difference spectrum is generated when a microsomal fraction is divided into two cuvettes, the cuvettes placed in a spectrophotometer and a baseline run between 500 nm to 360 nm. A compound (in this case carbon monoxide) is added to the sample cuvette and the resulting spectrum measures the difference between the reference and sample cuvette(see Figure 5).

The ability of cytochrome P-450 to bind to carbon monoxide led to its discovery. The absorption spectrum of the reduced (Fe^{2+}) form, when bound to carbon monoxide, gives a maximum at a wavelength of 450 nm (Klingenberg 1958 and Garfinkel 1958). A smaller peak at 420 nm also exists because some P-450 is converted to a new complex with a maximum absorption at 420 nm (Estabrook 1963).

Sladek and Mannering (1966, 1969) and Shoeman et al. (1969) termed this haemoprotein with altered activity cytochrome P-420.

Carbon monoxide competitively inhibits the enzyme since it competes with oxygen for the binding site on reduced (Fe^{2+}) cytochrome P-450. Moreover, this inhibition is reversible with irradiation with light at wavelength of 450 nm (Cooper et al. (1965).

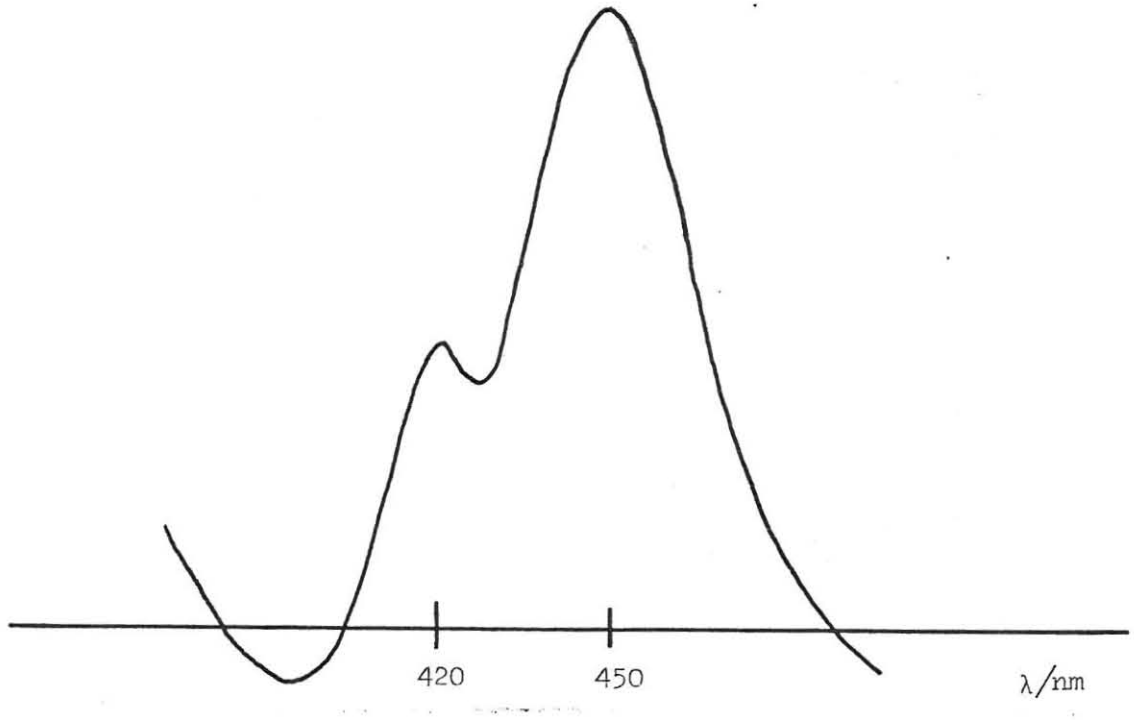


Figure 5. Carbon Monoxide Difference Spectrum

3.6.2 Spectral Changes and Binding of Cytochrome P-450 to Drugs

The binding of many compounds to the microsomal mixed function oxidase system produces two distinct types of difference spectra. These spectra are termed type I and type II spectra. Compounds giving rise to type I and type II difference spectra with hepatic microsomes have come to be known as type I and type II compounds (or drugs). A third, less common spectrum, a reverse type I is also possible. The various types of spectra are depicted in Figure 6.

The binding of substrates to microsomal cytochrome probably precedes enzymatic oxidation, and the manifestation of a spectral change is regarded as evidence that an enzyme-compound complex is formed. The type I spectrum has a maximum at 390 nm and a minimum at 420 nm (Figure 6a). Compounds which give rise to a type I spectrum include barbiturates, aminopyrine, testosterone, β diethylaminoethyl diphenylpropylacetate (SKF 525A) (Schenkman et al. 1967). Type 1 compounds are metabolized by the

enzyme system and hence may be termed substrates. The type II spectrum is characterized by a peak at approximately 425 - 435 nm and trough at 390 nm (Figure 6b).

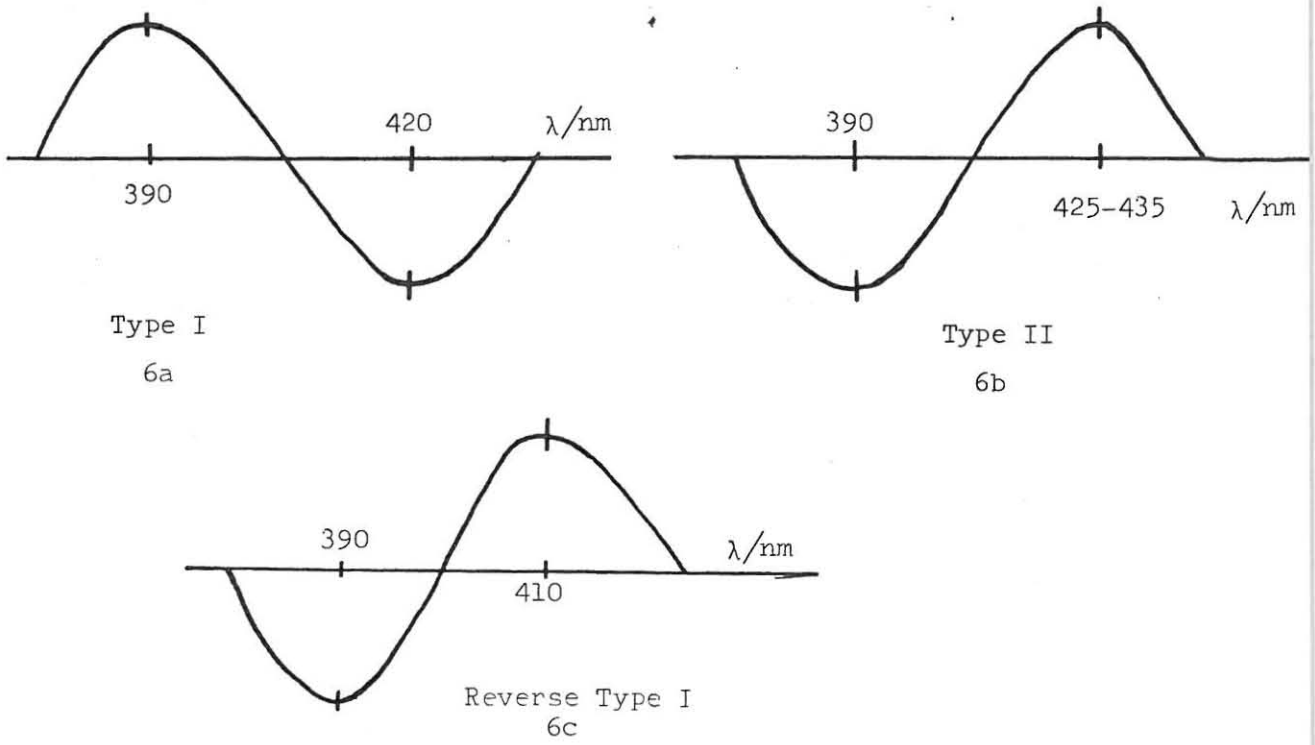


Figure 6 Difference Spectra

Type II substrates normally contain a basic nitrogen atom and include compounds such as aniline, pyridine, and 2,4-dichloro-6-phenylphenoxy-ethylamine hydrochloride (Schenkman et al. 1967; Gillette et al. 1972). These compounds are generally not metabolized by cytochrome P-450.

Shoeman et al. (1969) and Schenkman (1970) presented evidence that many compounds may combine in varying degrees with both binding sites and this may explain the metabolism observed with some type II compounds.

The reverse type I (RI) spectrum (also known as modified type II) has a peak at 420 nm and a trough at 392 nm (Figure 6c). The cause of this spectrum has been explained in several ways but one explanation is that

compounds (methanol, ethanol, 1-butanol, acetone and phenacetin) which elicit such a spectrum do so by displacing endogenous substrates from cytochrome P-450 (Schenkman et al. 1972), i.e., the RI spectral change is caused by reversal of structural state of P-450 caused by prior binding of endogenous substrates to the enzyme in vivo.

Since some reverse type I substrates are metabolized, one explanation is that they are bound first to the type I site, then, with increasing concentration to another site as well, giving rise to a new spectrum which obscures the type I component (Wilson and Orrenius 1972; Schenkman 1970).

The results obtained by Vore et al. (1974) tend to dispute the conclusion of Schenkman et al. (1972), that the RI spectrum is due to displacement of endogenous substrate from cytochrome P-450. The results of Vore et al. (1974) indicate that the phenacetin and ethanol induced RI spectrum might not be due to prior binding of endogenous substrates since they observed that, after one 1-butanol extraction followed by repeated extractions of microsomes with acetone, the phenacetin induced RI spectrum was increased at low phenacetin concentrations but decreased at high concentration in the extracted microsomes as compared with untreated microsomes from 3-methylcholanthrene treated rats. If the ethanol and phenacetin induced RI binding spectra are due to displacement of endogenous substrates, then organic solvent extraction should reduce RI binding, with the result that the phenacetin induced RI spectrum should have decreased at all concentrations.

It must be pointed out that Vore et al. quantified the recovery of cytochrome P-450/mg of protein after extraction and Schenkman et al. (1972) did not correct for the loss of cytochrome due to the extraction procedure, and it could be that this lack of correction could account for decreased RI spectral change due to repeated extractions.

Clearly, the displacement of an endogenous substrate from the cytochrome P-450 to give rise to the reverse type I spectrum has not been demonstrated conclusively.

Since cytochrome P-450 has only been partially purified, little is known about the chemical bonds of the haem moiety or of the co-ordination bonds of the fifth and sixth ligands of the haem iron. (Figure 7).

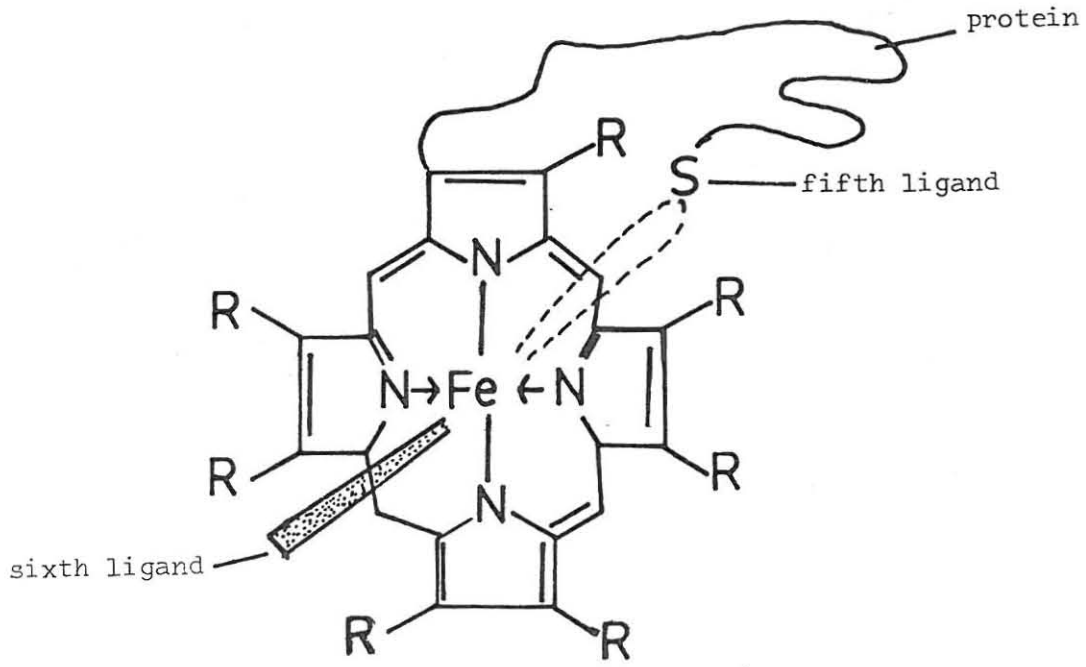


Figure 7. Haem Moiety Showing Bonds of Fifth and Sixth Ligands

For these reasons, the nature of the binding sites at which the type I and type II compounds interact is still largely unresolved and speculative.

Schenkman et al. (1967) favours the hypothesis that type I and type II compounds affect different ligands of the haem of the enzyme, causing a spectral shift.

Imai and Sato (1967) suggest that the substrate combines with a specific site located on the protein moiety of cytochrome P-450, giving rise to a conformational change in the haemoprotein molecule to change the ligand

site. Various types of substrates may or may not combine with different sites on the protein moiety.

The type I spectral change is generally accepted as being indicative of the formation of an enzyme substrate complex (Schenkman et al. 1967 and Schenkman 1972) and has been shown to parallel enzyme activity (Schenkman 1972). The resulting spectral change appears to be characteristic of the protohaem itself and is thought to be caused by an increase in the electronegativity of one ligand of the haem (Schenkman et al. 1968), indicating that, in microsomes, the spectral changes are due to the displacement of the fifth or sixth ligand from a hydrophobic region of the apoenzyme to a more hydrophillic one by the substrate.

Schenkman et al. (1967) attributed the type II spectral change to ferrihaemochrome formation, a conclusion based on the characteristics of the spectrum, the fact that type II compounds were amines and the observation that aniline, a type II compound, displaced carbon monoxide from cytochrome P-450, meaning that type II compounds might be attached to the same site as oxygen (Schenkman et al. 1967; Chaplin and Mannering 1970).

Binding of type I and type II substrates may occur as depicted in Figure 8.

Binding of a type I compound, e.g. aminopyrine, is thought to change the conformation of the protein, so that the Fe-binding shifts from the sulphur of one amino acid of protein to a nitrogen of a nearby amino acid. This shift from sulphur to nitrogen might cause the type I difference spectrum. Type II compounds, e.g. aniline, are thought to bind as a ligand to the 6-position, which is the site for oxygen, i.e., ferrihaemochrome formation.

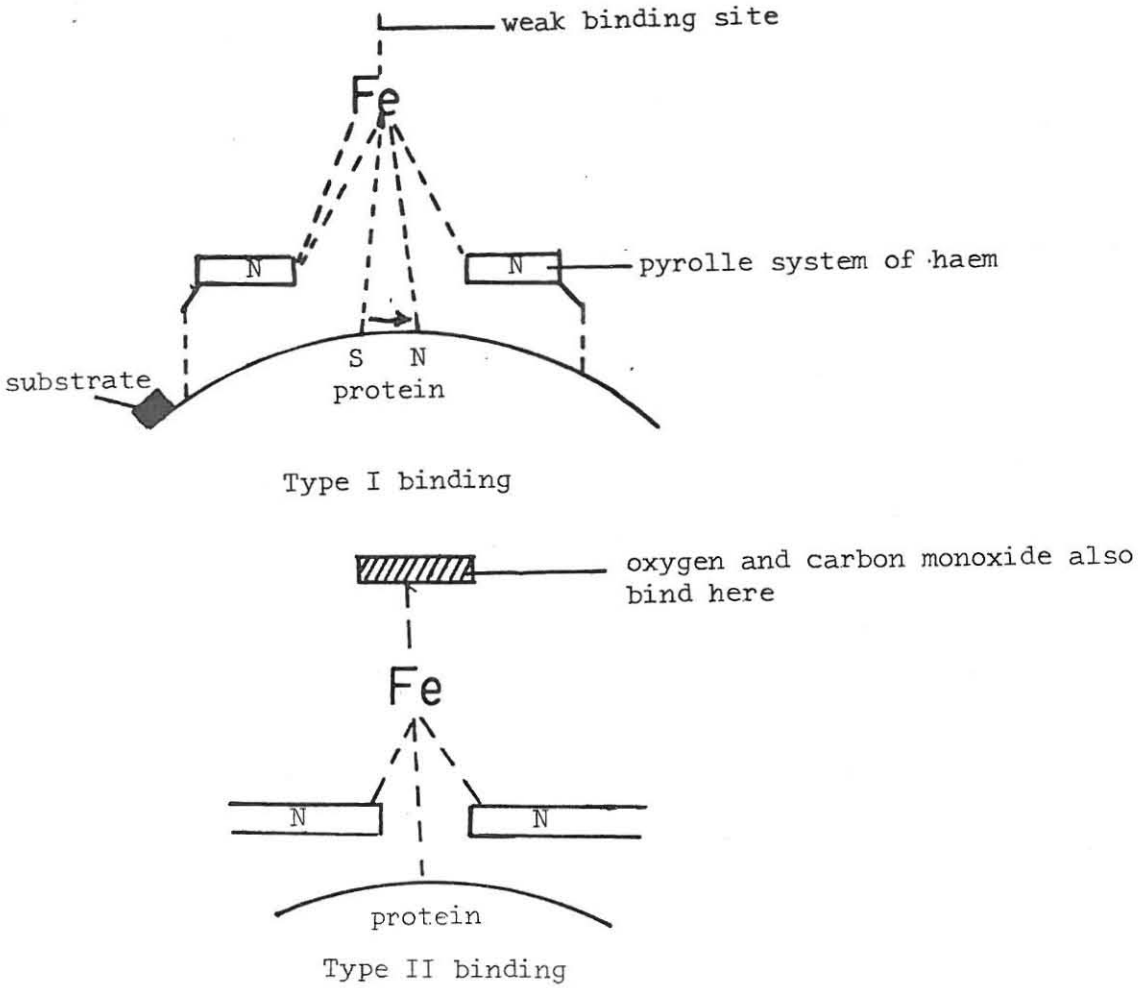


Figure 8. Proposed Binding Sites of Type I and Type II Compounds

3.7 Heterogeneity of Cytochrome P-450

During the last few years, numerous studies have indicated that different forms of cytochrome P-450 exist in hepatic microsomes. Alterations in catalytic activity towards different substrates and in spectral properties of cytochrome P-450 in hepatic microsomes prepared from animals pretreated with various inducers have been observed (Conney 1967; Gillette et al. 1972; Remmer et al. 1966; Schenkman 1970).

Sladek and Mannering (1966, 1969), Kuntzman et al. (1969) observed that marked differences exist in the behaviour of reduced cytochrome P-450 obtained from the livers of rats pretreated with phenobarbital as compared

with that from rats pretreated with 3-methylcholanthrene, with respect to binding to ethylisocyanide, a liganding reagent.

Alvares et al. (1967) and Hildebrandt et al. (1968) observed that the position of the λ max. of a microsomal preparation obtained from livers of rats pretreated with polycyclic hydrocarbons, differed slightly from that from untreated controls when the preparations were reduced and then saturated with carbon monoxide. Alvares et al. (1967) reported a λ max. at 448 for the pretreated preparation. The cytochrome was referred to as cytochrome P_1-450, or cytochrome P-448. Hildebrandt et al. (1968) observed a λ max. at 446 nm, but since microsomes from untreated rats were placed in the reference cuvette of a double beam spectrophotometer, the spectrum obtained by this group is actually a composite spectrum that results when cytochrome P_1-450 (λ max. 448) is superimposed on the inverted spectrum of cytochrome P-450 (λ max. 450).

Some workers (Schenkman et al. 1969; Hildebrandt 1968) have proposed that the changes seen in the haemoprotein after treatment with polycyclic hydrocarbons are due to the binding of inducer, or its metabolites, to cytochrome P-450; these workers thought that the complex of P-450 and inducer (or metabolite) shifted the peak to 448 nm. The basis for their suggestion is that the absolute spectrum (see Figure 9) of liver microsomes from animals pretreated with polycyclic hydrocarbon exhibit a type I-like spectrum with a shoulder at 390 nm. It appears unlikely, however, that the P-448 peak is due to a P-450-inducer (metabolite)-complex because there is a time lag of 24-48 h before the peak at 448 nm can be detected in induced animals. Such a lag is more indicative of de novo haemoprotein synthesis as no such lag would be expected if P-448 were a complex of P-450 with inducer. Moreover, the induction of cytochrome P-448 can be blocked by protein synthesis inhibitors such as actinomycin D and ethionine (Gillette et al. 1972; Lu 1976) and cytochrome P-450 cannot

be converted *in vitro* to cytochrome P-448 by the addition of 3-methylcholanthrene.

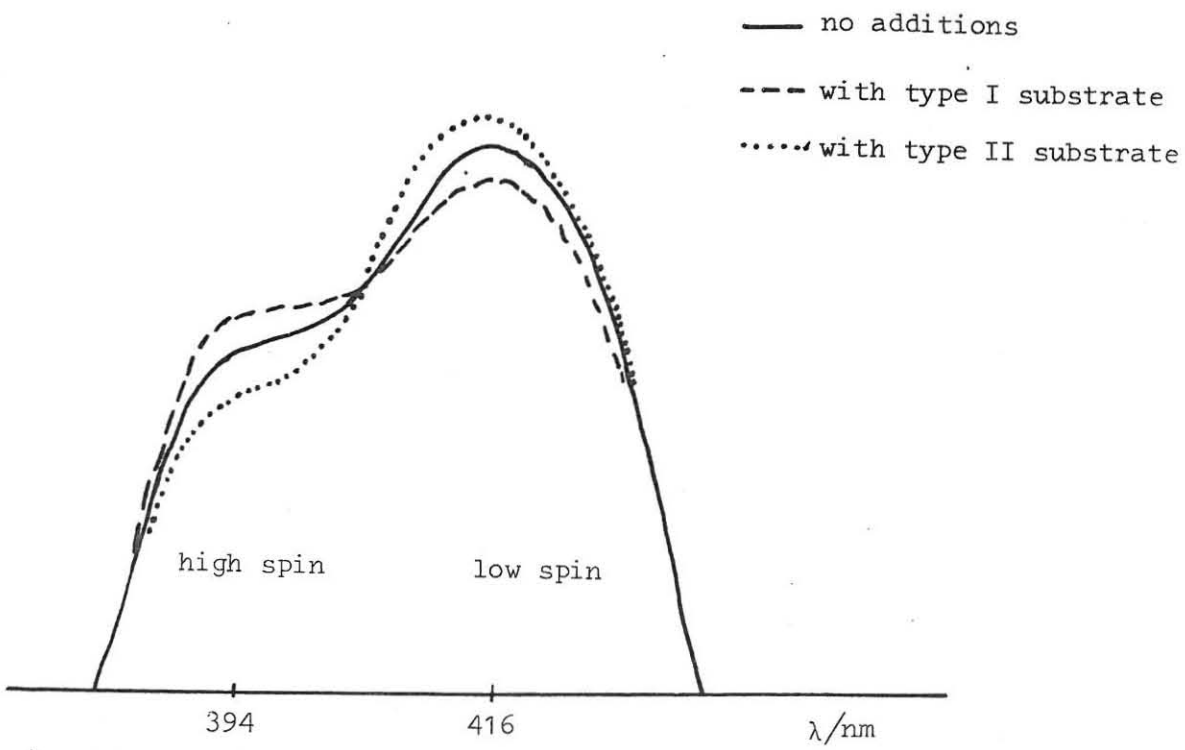


Figure 9. Absolute Spectrum

An absolute spectrum is obtained by first inducing the enzyme system to increase the levels of cytochrome P-450 (cytochrome b_5 levels are not markedly affected by induction). Non-induced microsomes are used as a control to balance the absorption in the spectrophotometer on the basis of protein concentration.

The change in the spectrum on the addition of a Type-I substrate indicates that some of the cytochrome P-450 is being converted from the low spin to the high spin state, absorbing at 410 nm and 394 nm respectively. The opposite occurs with Type-II substrates (Peisach *et al.* 1972).

Mannering (1972) also cited arguments which weaken the hypothesis of Schenkman et al. (1969), the hypothesis that only two forms of P-450 exist, the native enzyme and the enzyme-substrate complex.

There is general agreement that the administration of polycyclic hydrocarbons causes a change in the haemoprotein. However, there is controversy about whether the change occurring is due to a new molecular species being formed, or due to the existence of several interconvertible forms which are selectively induced depending on the inducer.

The concept that cytochrome P-450 and P-448 are different entitites is an attractive one. A review of these differences is beyond the scope of this work, but the differences between these two haemoproteins can be briefly listed.

(1) Electron paramagnetic resonance studies show that polycyclic hydrocarbons selectively induce the high spin form of cytochrome (P448), Jefcoate et al. (1969) assigning a g value of 6,6 to cytochrome P-448.

(2) The Soret peak at 418 nm. observed in the reduced absolute spectrum of cytochrome P-450 is observed at 414 nm. in the spectrum of cytochrome P-448 (Mannering 1972; Fujita et al. 1973).

(3) Both haemoprotein can be converted to the inactive form, P-420, but those from different sources were found to have rather different properties (Mannering 1972).

(4) Molar extinction coefficient of cytochrome P-448 is different from that of P-450 (Fujita et al. 1973).

(5) The reduced P-450-carbon monoxide difference spectrum of the haemoprotein occurs in slightly different positions, cytochrome P-450 occurs at 450 nm ; P-448 at 448 nm.

Thus, the balance of evidence seems to favour the assertion that cytochrome P-450 and P-448 are different entities. This concept seems plausible since the existence of both haemoproteins could be accounted for in the following ways:-

(1) A native cytochrome P-450 exists in several interconvertible forms which are selectively inducible by various inducers.

(2) The inducer could generate a new cytochrome which is different from cytochrome P-450 and is not normally present in abundance.

(3) There might be two cytochrome systems for metabolizing polycyclic hydrocarbons, one using P-450 and the other P-448.

(4) The ratio of the peaks produced by the reduced ethylisocyanide difference spectrum of cytochrome P-448 is altered, as is the pH intercept. The ethylisocyanide difference spectrum is explained below. The ethylisocyanide difference spectrum of reduced cytochrome P-450 produces two peaks, one at approximately 455 nm and the other at 430 nm (Imai et al. 1967) and the relative magnitudes of the peaks are dependent on pH (Figure 10). The two peaks are equal in magnitude from microsomes obtained from female rats at a pH about 7,4 , but the peak at 455 nm is higher than that at 430 nm at pH values above 7,4 and lower at pH values below pH 7,4. With microsomes obtained from rats pretreated with 3-methylcholanthrene, the two peaks are almost equal at pH 6,8 (Sladek and Mannering 1966). The fact that two spectral species can be observed when reduced cytochrome P-450 is reacted with ethylisocyanide (Imai et al. 1967 and Sladek and Mannering 1966) supports the concept that more than one cytochrome P-450 exists. It has been conclusively shown that 3-methylcholanthrene and other polycyclic hydrocarbons induce a new kind of cytochrome P-450, i.e. P-448.

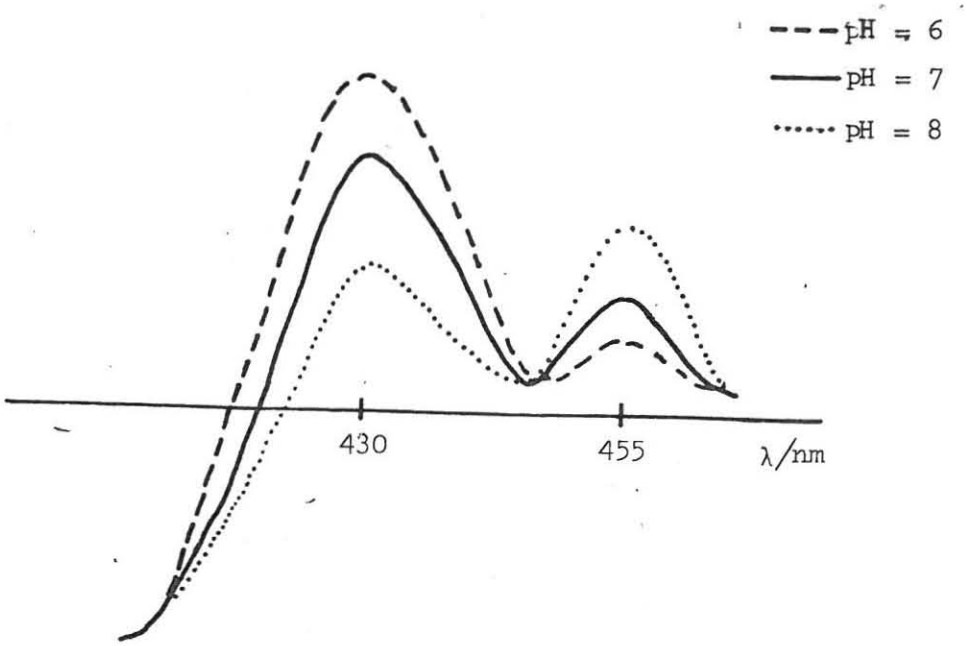


Figure 10. Ethyl Isocyanide Difference Spectrum (Imai and Sato 1967).

The more complicated question is if different species of haemoprotein exist in a single preparation of hepatic microsomes such as microsomes obtained from a 3-methylcholanthrene- or from a phenobarbital-pretreated rat.

Comai et al. (1973), using hepatic microsomes obtained from untreated rats or from rats induced with phenobarbital or 3-methylcholanthrene, have identified and separated three forms of P-450, which have different cyanide binding affinities. The relative amounts of the three forms of P-450 fluctuated on pretreatment with inducers; phenobarbital increasing form II, and 3-methylcholanthrene increasing form III. It is uncertain whether these three forms show structural dissimilarities. More recently, Ryan et al. (1975), using rat liver, and Haugen et al. (1975), using rabbit liver, separated several forms of cytochrome P-450 and P-448 differing in various properties such as molecular weights, spectral characteristics and catalytic properties.

The discovery of different forms of cytochrome P-450 existing in the hepatic microsomes of the same animal is interesting since, in many investigations, it has been assumed that the P-450 of uninduced animals and phenobarbital induced cytochrome P-450 is the same.

The precise number of cytochromes P-450 that occur in hepatic microsomes is unknown presently, but from the more recent work of Ryan et al. (1975), Levin et al. (1974), Haugen et al. (1975), and Kawalek et al. (1975), it appears that perhaps more than six types of cytochrome P-450 may exist. Some of the results obtained by these workers are summarized in Table. 8. The existence of various forms of cytochrome P-450 in untreated and induced animals is an attractive concept, since it could assist in the revision of the interpretaion of results obtained in drug metabolism investigations with hepatic microsomes and provide a means for explaining apparent anomalies.

The multiple forms of cytochrome P-450 or P-448 may have marked differences in substrate specificities, or there may be considerable overlap.

The occurrence of these multiple forms within a single liver in various proportions may help to explain the species, age, sex differences observed in drug metabolism.

Exposure of an animal to various inducing substances may alter the relative proportions of these various forms of cytochrome P-450, and this could change the spectral and catalytic properties of the microsomes of the induced animal as opposed to the uninduced animal.

In summary, the heterogeneity of cytochrome P-450 can be observed in three different instances in vivo:-

(a) Multiple forms of cytochrome P-450 occur in different species

Table 8. Possible Types of Cytochrome P-450

Parameters	Rat (Pb)[a]		Rat (3-MC)[a]		Rabbit (Pb)[b]	Rabbit 3-MC[c]
	Fraction A	Fraction B	Fraction A	Fraction B	LM_2	
Specific content, nmoles/mg	3-4	14-16	2-3	18-22	18	22-24
CO-difference spectrum, absorption maximum	450	450	449	447	451	447
Absolute spectra, maximum at nm Oxidized	418 535 568	418 535 568	390,414 530-564 642	417 535 568	417 534 568	416,533 566
Reduced CO-reduced	414,545 450,550	4. .,545 450,550	411,542 449,550	411,542 447,550	414,542 451,552	410,540 447,552
[d]Ethylisocyanide difference spectrum Absorption maxima, at nm ratio of 455 peak to 430 peak at pH 7,4	 430,455 1,8-2,0	 430,455 0,4-0,5	 430,455 1,2-1,4	 430,455 1,8-2,0	 - -	 428,452 1,4
pH Intercept (when 455 peak = 430)	6,90	7,85	7,10	6,85	-	7,25
Molecular weight determined by sodium dodecyl sulfate gel electrophoresis	50,000	48,000	-	53,000	50,000	51,000
Benzo[a]pyrene hydroxylation, nmoles/minute per nmole cytochrome	0,1	0,2	0,1	2-3	0,04	0,1
Benzamphetamine N-demethylation, nmoles/minute per nmole cytochrome	7	40	-	1-2	66	0,5

Fraction A and B refer to different forms of cytochrome P-450 obtained from the liver of the same animal.

a = Ryan et al. 1975; and
Levin et al. 1974

b = Haugen et al. 1975

c = Kawalek et al. 1975

d = Lu 1976.

LM_2 refers to electrophoretic behaviour in which different forms are distinguished.

3-MC = 3-methylcholanthrene

Pb = Phenobarbital

of animals pretreated with the same inducer, e.g. 3-methylcholanthrene treated rats and rabbits (Kawalek et al. 1975).

(b) Within the same animal, either treated with inducer or untreated, there are different forms of cytochrome P-450 (Werringloer et al. 1975; Ryan et al. 1975; Comai and Gaylor 1973; Haugen et al. 1975).

(c) Multiple forms of cytochrome P-450 are apparent when a particular species of animal is treated with different inducers, i.e., cytochrome P-448 in 3-methylcholanthrene rats versus cytochrome P-450 in phenobarbital treated rats (Ryan et al. 1975).

SECTION 4

INDUCTION OF CYTOCHROME P-450 ENZYME SYSTEMS

The duration and intensity of action of many drugs depend on the rate at which they are metabolized within the body by microsomal enzymes. Dietary and nutritional factors, ingestion of xenobiotics and hormonal changes influence the activity of the enzyme system.

The increase in the activity of the hepatic microsomal system is termed enzyme induction and reflects an increased concentration of enzyme protein. This increased concentration of enzyme could be due to an enhanced rate of synthesis, a reduced rate of degradation, or both.

The amount of enzyme protein formed is thought to be genetically controlled. The "operator model", a hypothesis which has general applicability, can be used to explain induction and repression of enzymes (Wold 1971; Davson 1970). The structure of the enzyme protein is determined by the structural gene. Information from regulator genes is transferred via the operator to the structural genes through the repressor-inducer system (Davson 1970). The overall effects of induction on the mixed function oxidase system are increased activity of drug metabolizing enzymes, an increase in protein synthesis in the endoplasmic reticulum, proliferation of the smooth endoplasmic reticulum and, eventually, hypertrophy of the liver (Mannering 1968; Conney 1967; Remmer 1970).

There is apparently no relationship between either the pharmacological action or structure and the ability of a compound to induce enzymes, since the compounds which are known to cause induction are structurally unrelated and these compounds have a diverse pharmacological action. Conney (1967) and Remmer (1969) recognize that most inducers have a high

lipid solubility, concentrate in the liver and have a long duration of action.

4.1 Classification of Enzyme Inducers

Inducing agents can be divided into two groups, exemplified by phenobarbital and polycyclic hydrocarbons, of which 3-methylcholanthrene is an example (Gillette et al. 1972; Sladek and Mannering 1968). Among the inducers in the phenobarbital group are insecticides and some drugs. The phenobarbital group of inducers activates many different pathways of metabolism, while the polycyclic hydrocarbon group appears more specific and stimulates the metabolism of certain substrates only.

Phenobarbital has a delayed and prolonged action as an inducing agent and causes an increase in the concentration of cytochrome P-450, NADPH- cytochrome P-450 reductase and no cytochrome b_5 (Greim et al. 1970; Conney, 1972). There is a general increase in protein synthesis, and in haem synthesis. This is indicative of the inducing agent acting on an operator gene controlling the synthesis of several proteins a suggestion supported by the fact that the inducing effect is blocked by puromycin and actinomycin D (Conney and Gilman 1963; Dehlinger and Schimke 1972). Phenobarbital also increases the magnitude of type I and type II spectral changes as well as the rate of cytochrome P-450 reduction (Schenkman et al. 1967). Phenobarbital also causes a marked proliferation of smooth endoplasmic reticulum and increase in the liver mass.

The polycyclic hydrocarbons have a more specific inductive action. They activate the metabolism of a limited number of compounds such as the N-demethylation of azo-dyes, the hydroxylation of aniline and of 3,4-benzpyrene (Conney 1967). The polycyclic hydrocarbons have a much

more rapid onset of induction and shorter duration of action than the phenobarbital group. Proliferation of smooth endoplasmic reticulum is not observed, nor is there any increase in NADPH-cytochrome P-450 reductase (Alvares et al. 1973). The polycyclic hydrocarbons increase the activity of aryl hydrocarbon hydroxylase in many tissues besides the liver, while phenobarbital acts mainly on the hepatic enzyme system (Gelboin 1972). Aryl hydrocarbon hydroxylase is one of the few activities stimulated by both the phenobarbital group and polycyclic hydrocarbons, however, phenobarbital seems to stimulate general proliferation and increase in enzyme activity, while polycyclic hydrocarbons appear to have a more selective and specific action. These differences indicate that there are two enzyme systems under separate control and that the inducers have different mechanisms, or loci, of action (Mannering 1968).

It appears that steroids can be classified in the phenobarbital group of inducers since they seem to give rise to the phenobarbital type of induction, and for this reason in this review phenobarbital will be used as a prototype to try to explain the mechanism of induction.

4.2 Mechanism of Enzyme Induction

Enzyme induction was defined as an adaptive increase in the number of molecules of a specific enzyme, secondary to either an increase in the rate of synthesis of the enzyme, or a decrease in the rate of enzyme degradation. It should be borne in mind that enzyme activity may also be changed without an alteration in the amount of enzyme, and that hormones and other ligands may bring about such changes. For the purposes of this discussion, enzyme induction will be considered to mean primarily a selective increase in the rate of synthesis of a specific enzyme.

4.2.1 Enzyme Induction in Micro-organisms.

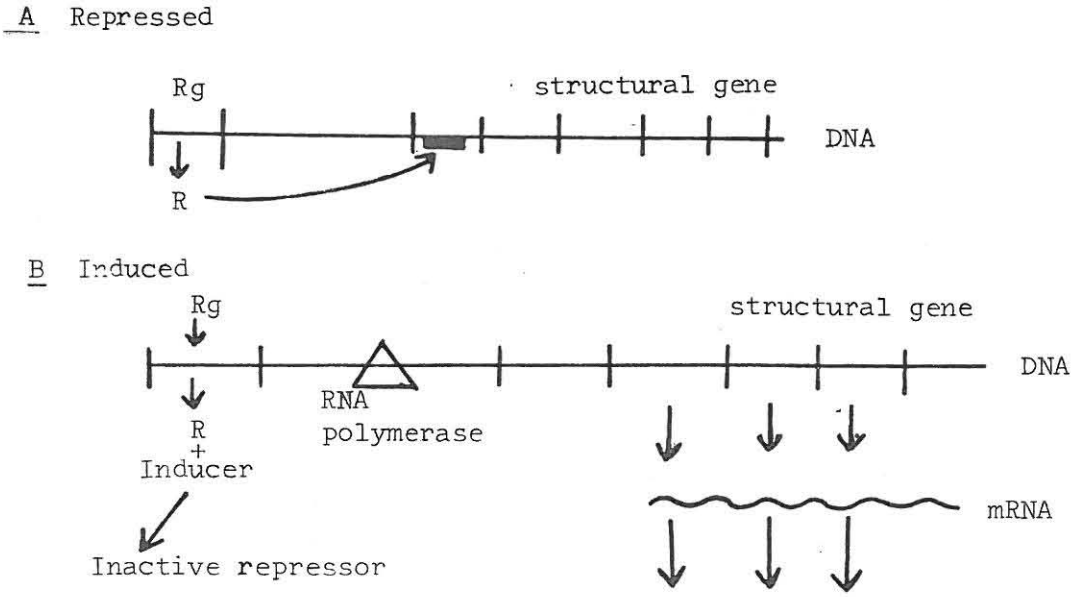
A brief discussion of enzyme induction in micro-organisms will be considered since most models of the mechanism of enzyme induction in mammalian systems have been based on models of enzyme induction from prokaryotic organisms (Conney 1972). The model discussed here is based on the studies by Jacob and Monod on the lac operon of Escherichia coli.

According to the hypothesis put forward by Jacob and Monod (1961) to account for the genetic aspects of bacterial synthesis, the control operates from a definite part of the cell's genome, the regulator gene. Genetic analysis led to the concept of an operon, which can be defined as a group of adjacent structural genes and their closely associated controlling sites, the regulatory gene, Rg, the promotor, P and the operator, O. (see Scheme 7). When bacterial genes are being actively expressed, their corresponding messenger ribonucleic acids (mRNA) are being synthesized. The rate of synthesis of the proteins encoded by these genes is dependent on the concentration of mRNA s. The transcription of adjacent structural genes into a single mRNA molecule and its sequential translation account for the co-ordinate expression of the operon.

The material produced by the regulator gene, the repressor, acts on a strip of DNA adjacent to that portion concerned with the synthesis of a given group of proteins and when it is attached to this region of DNA, it inhibits the transcription of the whole operon, i.e., the production of messengers for this particular group of proteins (Scheme 6A).

Inducers are postulated to be small molecules that can interact with the repressor to alter its configuration and thus prevent it from binding to the operator, thereby allowing transcription of the structural genes to occur (Scheme 7B).

The important features of this model are that regulation of specific protein synthesis is modulated by distinct regulatory genes, that regulation operates at the level of gene transcription, and that it operates by controlling the rate of synthesis of a labile mRNA.



Scheme 7. Schematic Representation for Enzyme Induction in Bacteria Modified from Davson (1970).

Rg = regulatory gene

R = repressor

P = promoter

O = operator

4.2.2 Induction in Higher Organisms

Increased levels of hepatic microsomal drug metabolizing enzymes could arise in several ways.

The inducing agent could activate the synthesis of more enzyme by directly interacting with DNA (to stimulate the synthesis of induction - specific RNA), or with repressor synthesized by a regulator gene (Jacob

and Monod hypothesis), or with other regulators of gene function such as histones or non-histone proteins (O'Malley et al. 1976). Alternatively, the translation of the induction specific RNA on the ribosomes could be stimulated by activation. The inducer could also interact with the end product of gene activity, the hepatic microsomal drug metabolizing enzyme itself, inhibiting degradation of the enzyme in situ or preventing a feedback inhibition of synthesis, both of which could lead to an increased enzyme level.

Studies with inhibitors of protein synthesis indicate that induction involves both protein and RNA synthesis and, possibly, activation of specific genes (O'Malley et al. 1976; O'Malley 1974).

Induction of induced enzymes is completely prevented if either actinomycin D, which acts at the level of transcription, or cycloheximide or puromycin, which act at the translational level, are concomitantly administered with the inducing agent.

Gerlehrter (1973) mentions that stimulation of enzyme activity (in this case that of tyrosine aminotransferase) becomes insensitive to actinomycin D during treatment with inducer, and Nebert and Gelboin (1970) found that the inducing system became insensitive to actinomycin D but continued to be cycloheximide sensitive, i.e., protein synthesis is required continuously. Thus, two processes for induction appear necessary:

(a) synthesis of induction-specific RNA which is cycloheximide insensitive and, thus, translation independent.

(b) translation related to the induction-specific RNA which is insensitive to inhibition by actinomycin D and, thus, transcription independent.

The induction-specific RNA may accumulate at the nuclear site bound to DNA. This accumulation of RNA causes the induction process to pass

from the transcription-dependent to the transcription independent stage. The second, the translational step, involves the synthesis of either enzyme protein or a rapidly synthesized and degraded protein activator of the enzyme complex.

4.3 Mechanism of Enzyme Induction by Steroids

The molecular basis of hepatic microsomal enzyme induction by steroids is unknown, but it is possible that the mechanism of enzyme induction by steroids in the liver is a similar mechanism to the mechanism of induction of enzymes elicited by steroid hormones in the target tissue.

Steroid hormones enter target cells and bind to receptor proteins with high affinity and stereospecifity. Part, if not all, of these complexes undergo an obscure "activation" process wherein they become capable of migrating and binding to the nuclei (King and Mainwaring 1974; O'Malley 1976; Gorski et al. 1976). The steroid hormone receptor complex (sh-rc) binds to specific acceptor sites in the nuclei of the target cells, namely chromatin DNA, and to the Ap_3 fraction of non-histone protein (O'Malley et al. 1976).

Recently, estrogen receptors (probably steroid binding proteins) have been isolated from chicken liver nuclei (Joss et al. 1976) and from adult female rats (Eisenfeld et al. 1976). In fact, Eisenfeld et al. (1976) postulate that "the estrogen-binding macromolecule in the liver supernatant is the estrogen receptor and that it modulates hepatic synthesis of plasma protein that may be involved in mediating side effects of estrogen-containing contraceptives". Thus, it is possible that the protein in the liver of adult female mammals which bind estrogen may be estrogen receptors involved in modulating hepatic synthesis of selective proteins, in this case cytochrome P-450.

The effect of steroids on gene regulation has been reviewed in detail elsewhere (section 2.3) and will only be considered briefly here in an attempt to explain the increase in protein synthesis (induction) during steroid administration. Katznellenbogen and Gorski (1975) and Knowles et al. (1971) have shown that steroid hormones regulate the metabolic activities of mammalian cells by controlling RNA and protein synthesis. Thus, it is possible that the inducing steroid may operate at the level of gene-regulation affecting RNA synthesis. If the mechanism of hepatic microsomal enzyme induction by steroids is similar to that of enzyme induction in target tissues, then the inducing steroid will mediate its effect by directly acting with DNA. The direct binding of steroid hormone complex to the chromatin DNA and Ap_3 fraction of non-histone protein in hepatocytes could activate a selected segment of DNA and thus stimulate the gene to synthesize RNA for the synthesis of specific protein. The binding of steroid hormone receptor to DNA would stimulate RNA polymerase activity, activate the rate of nuclear transcription, and stimulate the synthesis of mRNAs which presumably code for specific proteins, which, in the case of the liver, are those enzymes required to detoxify drugs (O'Malley 1974; King and Mainwaring 1974; O'Malley et al. 1976).

4.4 Steroid Induction of Protein Synthesis

Steroid induction of protein synthesis has been discussed in detail in a previous section (Section 2) and will only be briefly considered here.

Steroid induced protein synthesis has been detected in several systems, such as estrogen-mediated synthesis of ovalbumin in the chick oviduct (O'Malley et al. 1976).

Two responses to the synthetic estrogen diethystilboestrol (DES) have been studied. The primary response occurs when DES is administered to newly hatched chicks. Administration results in oviduct growth and differentiation towards cell types which produce ovalbumin.

A secondary response occurs when DES treatment is withdrawn for a short time and the tissue shrinks in size and ovalbumin synthesis stops. Re-stimulation with DES (secondary response) gives rise to a more rapid sequence of estrogenic responses inducing ovalbumin synthesis.

4.5 Primary Site of Steroid Action during Induction

Gerlehrter (1973) and Tomkins et al. (1969) proposed a post-transcriptional control of steroid-induced protein synthesis. However, it appears likely that steroids exert their primary effects at the level of gene transcription rather than at a post-transcriptional level. Although knowledge of steroid receptors and gene action is still fragmentary, one may speculate on models for steroid hormone-receptor regulation of gene transcription. Post-transcriptional control at a primary regulatory site for steroid hormone action cannot be completely ruled out, but the bulk of evidence presented in this section and in Section 2 points to an enhancement in gene transcription as the mechanism for steroid hormone action in target cells.

In addition to that mechanism mentioned, others which could be postulated to explain steroid induced increase in RNA synthesis are:

(1) The activity of RNA polymerases may be altered by direct interaction of steroid hormone complexes - steroid hormone complexes have been identified in the liver (Joss et al. 1976; Eisenfeld et al. 1976).

(2) New initiation sites may be created on the genome, or the steroid-complex may enhance the rate of RNA chain elongation.

The above are but tentative postulates, since ultimate proof of the mechanism by which steroids modify RNA synthesis must await the absolute purification of steroid receptors.

The studies of Scharwz et al. (1975) and Tsai et al. (1975) are more consistent with transcriptional stimulation rather than post-transcriptional stimulation. These workers established a temporal relationship between levels of nuclearly bound, estrogen receptor, polymerase binding to RNA chain initiation sites on chromatin and ovalbumin message production in the stimulated withdrawn chick given a secondary estrogen challenge (section 2.5.2).

From the work of O'Malley (1974), and O'Malley et al. (1976), it appears then that the earliest response to receptor-hormone binding to chromatin is initiation of mRNA synthesis. This could be due to an increased availibility of RNA polymerase-binding and initiation sites (O'Malley et al.1976).

4.6 Models for Enzyme Induction by Steroids and Gene Action

Processes controlling the transcription of DNA into messenger RNA and those regulating the translation of the messenger RNA to protein are obscure.

However, it is possible that certain genes may influence the activity of other genes and that the control of one gene by another is influenced by various molecules in the environment of the cell. These molecules may be of endogenous origin, such as hormones, or of exogenous origin, such as drugs. In this way, environmental factors influence the genetic activity of the cell.

Knowledge obtained from the study of prokaryotic systems can be combined with facts about steroid hormone receptor interaction with the eukaryotic genome in target cells in order to formulate a hypothesis for steroid mediated, transcriptional control during hepatic microsomal enzyme induction.

The model shown (Figure 11) involves steroid(s) combining with cytoplasmic receptor (Rc) to form steroid-cytoplasmic receptor (S-Rc). S-Rc undergoes a process of "activation" (O'Malley 1976), which somehow alters the complex and facilitates translocation into the nucleus. Here, receptor-complex (S-Rn) accumulates. The chromatin, consisting of DNA, histone and non-histone proteins [(Ap_3 fraction) NHP], contain specific acceptor sites for steroid nuclear receptor (S-Rn) - the mechanism of location of S-Rn is discussed earlier. Perhaps acidic proteins (a) serve to direct S-Rn complexes to acceptor sites (O'Malley 1976).

Binding of the S-Rn complex to the chromatin induces a facilitation of RNA polymerase action (B) in the promoter region (P). This causes transcription, through the structural gene (SG_1) of specific mRNA - in this case those which code for cytochrome P-450. Inducing response would be terminated by dissociation of the steroid from the receptor made in the nucleus of the hepatocyte.

After the mRNA is made in the nucleus of the hepatocyte, it is transferred to the cytoplasm and functions on the ribosome-membrane complex of the endoplasmic reticulum (Dallner et al., 1976). In this ribosome-membrane complex, the mRNA directs the assembly of amino acids into specific proteins (cytochrome P-450). The process would require a variety of factors such as guanosine triphosphate and transferases and initiation factors.

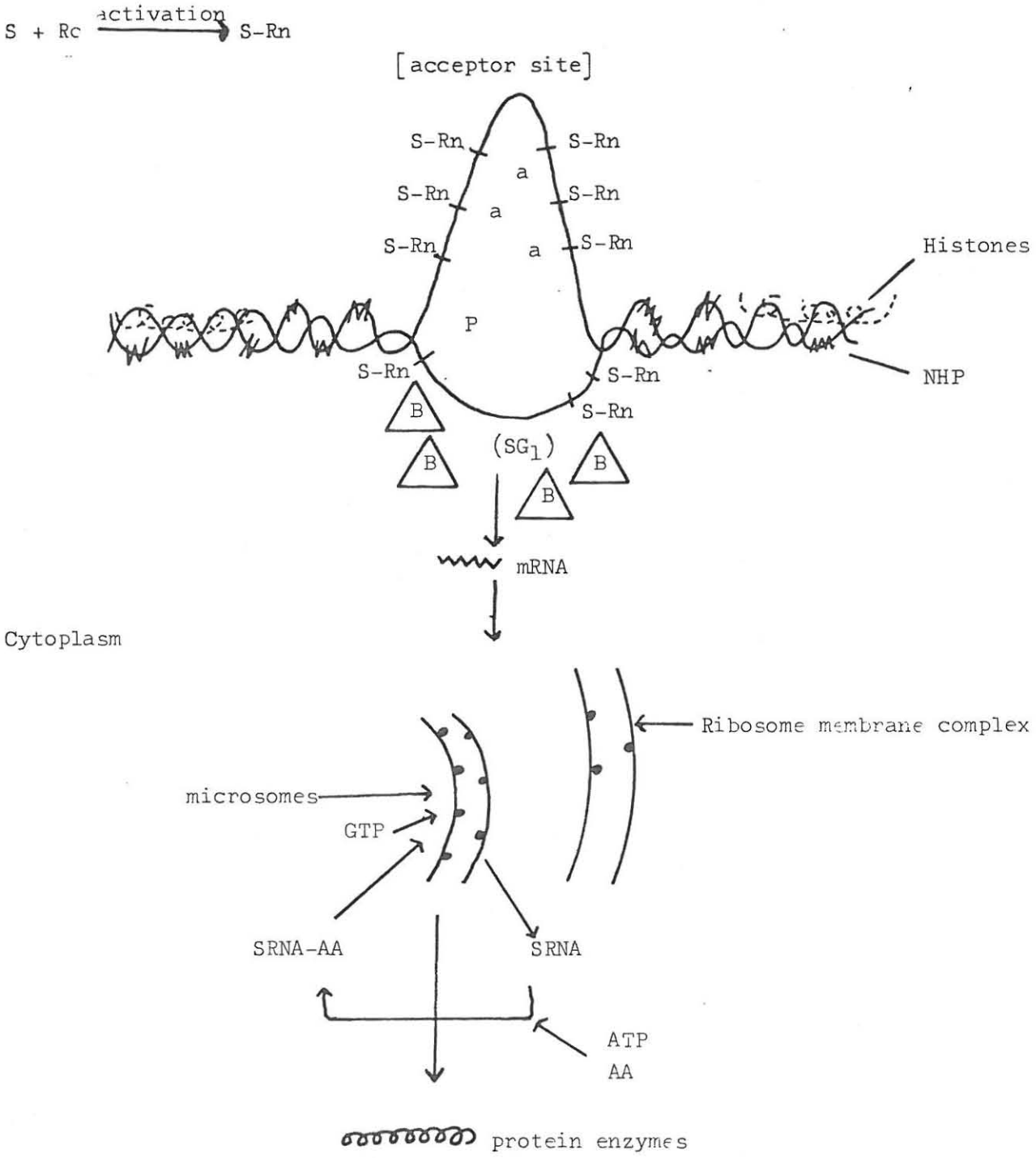


Figure 11. Model for Enzyme Induction by Steroids

S-Rn = receptor complex in nucleus

a = acidic protein

B = RNA polymerase

P = promoter region

SG_1 = structural gene

NHP = non-histone proteins

GTP = guanosine triphosphate

4.7 Effect of Inducers on the Turnover and Activity of Microsomal Protein

Jick and Shuster (1966) investigated the turnover of microsomal, reduced NADPH cytochrome c reductase and other microsomal protein in liver of mice treated with phenobarbital and concluded from these studies that phenobarbital causes both an increased rate of synthesis and a decreased rate of decomposition of microsomal NADPH cytochrome c reductase. Kuriyama et al. (1969) studied the effects of phenobarbital on the degradation and synthesis of total microsomal protein, and specifically NADPH cytochrome c reductase and cytochrome b_5. The effect of phenobarbital on the synthesis and degradation of the proteins was studied with labelled leucine C^{14} and guadino labelled arginine C^{14}. These workers observed that a single administration of phenobarbital increased the rate of total microsomal protein and NADPH cytochrome c reductase synthesis but had no effect on the rate of cytochrome b_5 synthesis. Repeated doses of phenobarbital caused a further increase in NADPH cytochrome c reductase and a slight increase of cytochrome b_5 - these studies indicate that an increased synthesis and decreased degradation of these two proteins are occurring. During the induction of the mixed function oxidase system, an increase of amino laevulinic acid synthetase (ALAS) activity occurs (Granick 1966; Granick and Kappus 1967). The ALAS is a mitochondrial enzyme and is considered to be the rate limiting enzyme of haem biosynthesis (Granick 1966). The hepatic synthesis of haem is restricted since only small amounts are required for haem turnover in cytochromes, catalase and other haemoproteins (Granick 1966; De Matteis 1967; Granick and Kappus 1967). During induction, an increase of cytochrome P450 follows an increase of ALA. Thus, it is suggested that haem biosynthesis plays an

important role in the induction of microsomal enzymes (Marver 1969; Remmer 1970) (see Figure 12).

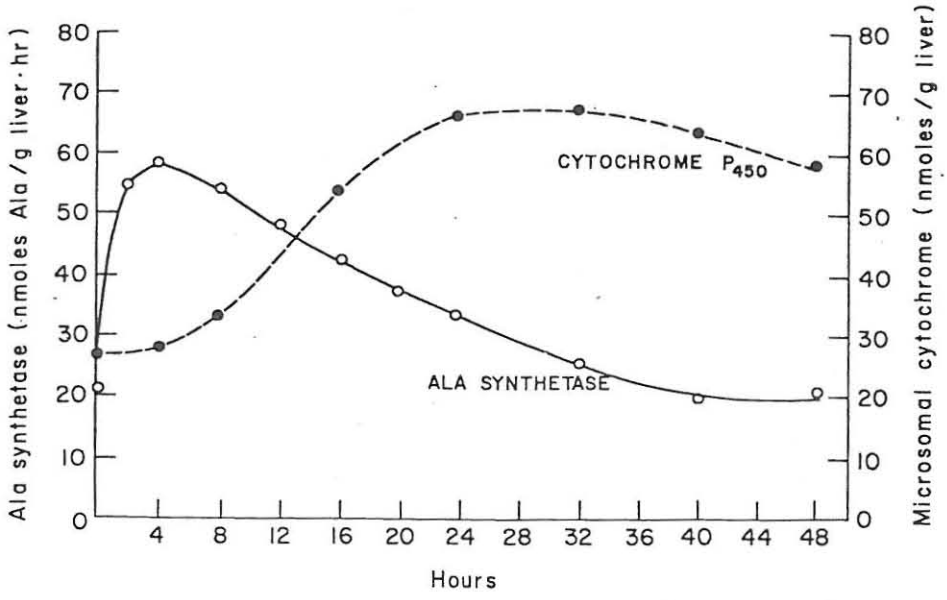


Figure 12. Hepatic δ - Amino laevulinic Acid Synthetase (ALA synthetase) and Cytochrome P-450 after a Single Injection of Phenobarbital (Marver 1969).

Granick (1966) used the concept of feedback repression to explain the control of enzyme formation. This model is based on that of Jacob and Monod (1961) in that a specific repressor molecule is considered to inhibit a specific DNA region (operon) from being decoded into mRNA, thereby preventing the synthesis of an enzyme during the uninduced phase.

The repressor is considered to be a protein, the aporepressor, to which is bound the co-repressor, haem. The aporepressor, which has two binding sites, is synthesized by the regulator gene, becomes activated, and engages itself with the corresponding operator following its combination with the specific co-repressor. The operator closes whenever it is engaged by the repressor (Davson 1970; Gerlehrter 1973). Repressors have the ability to bind the specific co-repressor and to bind to a specific site on the DNA molecule.

Haem acts as the specific co-repressor in the regulation of the synthesis of ALAS. According to De Matteis (1967) and Marver (1969), it is the "intracellular" concentration of free haem that is the factor which determines whether the operator site is open or closed or whether the transcription process (thus the synthesis of the enzyme) is switched on or off.

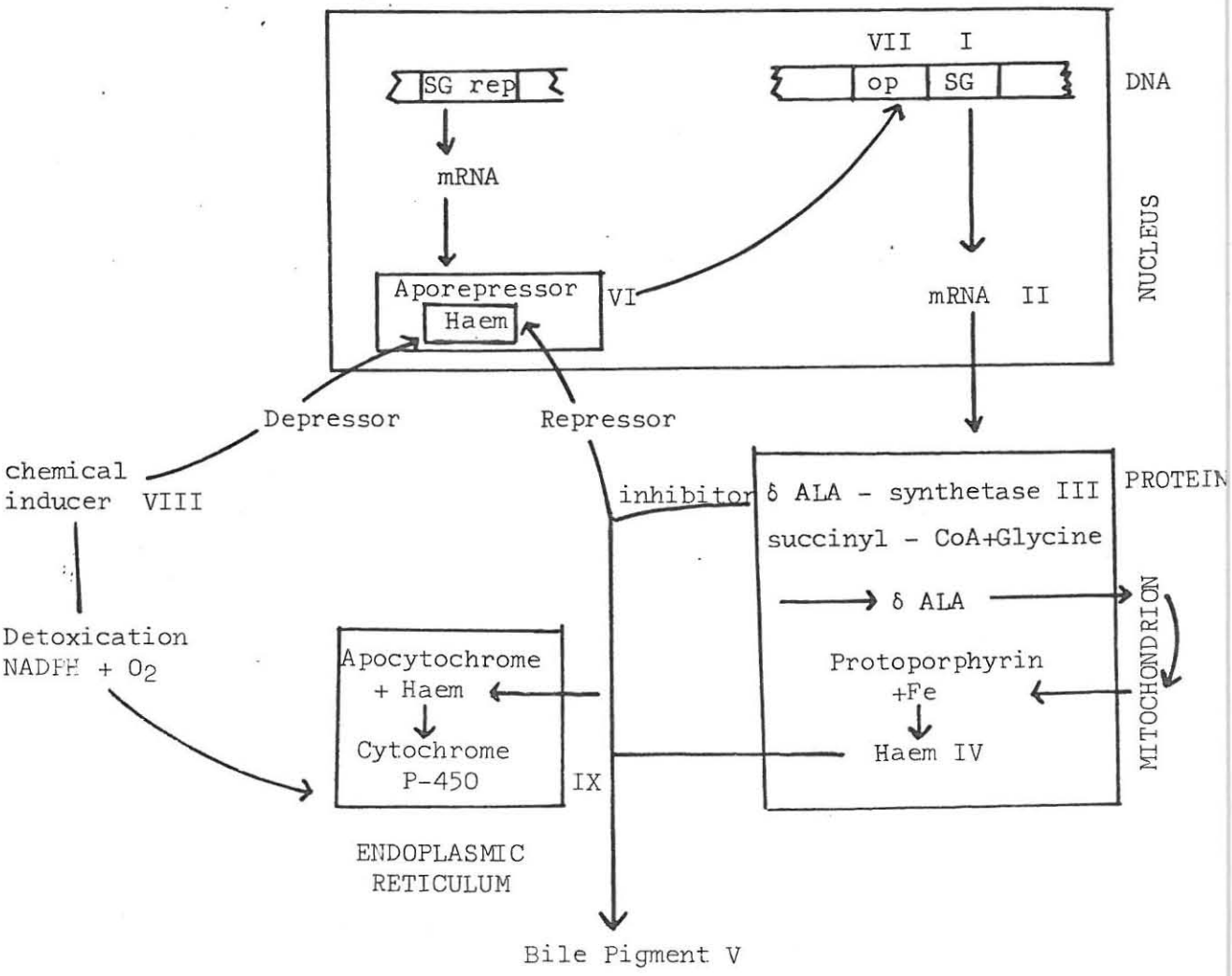
The repressor will be activated and engage the operator site, thus preventing the synthesis of specific mRNA, when the concentration of haem increases above a threshold level. The excess haem will be used in the synthesis of various liver haemoproteins or broken down to bile pigments and, when the concentration of haem falls to a significantly low level, the repressor will be inactivated and thus, disengage the operator site, leaving it open for the synthesis of new mRNA, specific for ALAS synthesis.

Another possibility is that inducing agents interfere with the mechanism of repression that normally controls the synthesis of ALA synthetase, thus causing larger amounts of ALA synthetase to be synthesized (Granick 1966).

Granick (1966) and De Matteis (1967) point out that possibly only one site is involved. It is suggested that inducing agents interact with the binding site of the aporepressor molecule that normally binds haem and that they are in competition with haem for this site. This would prevent haem from engaging the aporepressor and the operator would be kept open for continuous synthesis of specific mRNA and of enzyme protein.

4.7.1 Summary of Hypothesis on Induction of Haem in Hepatocytes

Scheme 9, shown on the following page, summarizes the various hypotheses on induction of synthesis of haem, a precursor of cytochrome P-450, by inducing agents.



Scheme 9. Scheme for the Detoxication of Chemical Inducers in Liver Derepression of the Repressor Control on the Synthesis of ALAS

The control of haem biosynthesis in the liver involves a competitive process between haem and a chemical inducer, e.g.,a steroid, for a binding site on the aporepressor that controls the synthesis of ALA synthetase. The chemical inducer causes more haem to be synthesized, the extra haem being utilized for oxygenase reactions to "detoxify" the inducer. This Scheme incorporates the interaction of the nucleus, mitochondria and the endoplasmic reticulum. Within the nucleus,at I there is a structural gene (S.G.) that codes for ALAS. This code

is transcribed into a mRNA at II. The mRNA is translated into the polypeptide chain of ALS synthetase which enters the mitochondrion at III.

The synthesis of ALA synthetase is the rate limiting step in the pathway for haem biosynthesis. Enzymes in the biosynthetic chain convert the ALA in the mitochondrion to haem. Some of the haem is transferred outside the mitochondrion.

The newly synthesized haem enters the endoplasmic reticulum and becomes part of the oxygenase enzymes (IX) that detoxifies inducing agents with the aid of NADPH and oxygen, or haem may be converted to bile pigment (V), or the haem may enter the nucleus to form part of the repressor (VI) that controls the synthesis of ALA synthetase.

When haem engages the aporepressor, then the operator gene (Op) is inactive and no mRNA can be formed. When the chemical inducer (VIII) displaces the haem from the aporepressor, the operator becomes active, ALA synthetase can now be synthesized.

4.8 Sex Differences in Drug Metabolism by Rat Liver Microsomes

Liver microsomes from male rats metabolize various drugs more rapidly than those from female rats, thus, the duration of activity of certain drugs persists longer in females than males (Booth and Gillette 1962; Conney 1967; Gillette *et al*. 1972).

The production of androgens appears to be the important factor in determining the sex variation. Up to the age of puberty, rats exhibit no sex difference in response to hexobarbital, but during puberty the sleeping times of male rats decrease while those of females remain constant (Quinn *et al*. 1958).

Jellinick et al. (1965) report an increased metabolism of estrogens by male rat liver microsomes compared to female rat liver microsomes.

In all studies on the effect of sex differences on drug metabolism, castration of rats, or administration of testosterone to female rats, influence the ability of liver microsomes to metabolize drugs (Quinn et al. 1958; Jellinick et al. 1965; Conney et al. 1967; Gillette et al. 1972). In the female rat, Booth and Gillette (1962) found that stimulation of drug metabolizing enzymes by testosterone derivatives paralleled their anabolic effect more closely than their androgenic activity.

4.9 The Clinical Significance of Enzyme Induction

Enzyme induction in man:

The stimulatory effects of drugs and other xenobiotics on drug and steroid metabolism in animals have been reproduced in a few cases in man (Beckenbridge et al. 1970). It is common practice to extrapolate inductive effects of drugs and xenobiotics from animal studies to man. This may be an unreliable procedure since species and strain differences are factors which affect drug metabolism. Nevertheless, it is important to recognize that, whenever hepatic microsomal enzymes are induced in man, one can more or less expect similar effects in the duration and intensity of drug effects to occur, as observed in animals.

Since in many cases, drugs are administered concomitantly, and in many cases without proper consideration of the possibility that one drug may interact with another at the metabolic site, adding or subtracting a drug from a regimen can have serious consequences for the metabolism and action of other co-administered drugs.

Many of the hypnotics and sedatives in clinical use are known to give rise to induction of microsomal enzymes in animals (Conney 1967), e.g.,

phenobarbital and glutethimide. Stimulation of metabolism of the barbiturates resulting in some degree of tolerance in man, is a frequent observation (Conney 1967; Mannering 1969).

4.9.1 Barbiturates and Anti-coagulants

Co-administration of hypnotics and oral anti-coagulants probably represent the most serious interaction, with the danger of excessive bleeding occurring if the administration of hypnotic is stopped. Conney (1967) cites evidence of death of a patient from haemorrhage after having received chloral hydrate and bishydroxycoumarin in combination. On withdrawal of chloral hydrate therapy, the prothrombin time of the patient increased and haemorrhage occurred.

Chronic administration of inducing agents such as steroids or barbiturates could give rise to a state of chronically high hepatic enzyme levels. The epileptic patient may ingest large doses of the inducer; or patients on OCs therapy may accumulate drugs within the body as a result of chronic ingestion, and, as a result, the activity of widely differing substances, both endogenous and exogenous may be altered.

Conney (1967) and Beckenridge et al. (1976) mention that phenobarbital and diphenylhydantoin cause induction of the hepatic microsomal enzyme system. Laengner et al. (1974) cite evidence that these two drugs may accelerate the metabolism of estrogens and failure of oral contraceptives in epileptics may be the result.

Another consequence of enzyme induction is that chronic administration of a drug may enhance its own metabolism and this effect may explain some instances of drug tolerance. Protracted glutethimide administration stimulates its own metabolism (Conney, 1967). This effect is important when it causes drugs to become less toxic and less effective

during prolonged administration. On the other hand, if a metabolite is more active than the parent drug, then enzyme induction will enhance the drug's action, or its toxicity if the metabolite is more toxic.

An important consequence of enzyme induction is the effect of accelerated metabolism of normal body substrates such as physiological sex hormones, which are metabolized by the same drug metabolizing system. These effects have yet to be worked out, but it is possible that changes in pattern of metabolites might arise due to induction. Many more interactions between inducing agents and other drugs exist, and this subject has been extensively reviewed by Conney (1967) and Beckenridge et al. (1976).

In summary, it is vital that these interactions occurring at the metabolic site be identified and their ability to increase the rate of metabolism in man be investigated, because inducing agents are often administered concurrently to patients who are given potent, specific drug therapy.

4.10 Specific Effects of Inducing Agents on Hepatocytes and their Relation to Drug Metabolism

The overall effects of induction on the mixed function oxidase system are an increase in the activity of drug metabolizing enzymes, increase in protein synthesis, proliferation of smooth endoplasmic reticulum and, eventually, hypertrophy of the liver (Remmer and Merker 1965; Mannering 1968; Conney 1967).

4.10.1 Stimulatory Effect of Inducers on Protein Synthesis and Liver Growth in Liver Microsomes

Evidence for increased synthesis of microsomal protein after administration of inducer is provided by the fact that an increased rate of incorporation of amino acids into microsomal proteins occurs in studies

carried out *in vivo* and *in vitro* (Gelboin and Sokoloff 1961; Dehlinger *et al.* 1972). Dehlinger *et al.* (1972) showed that the incorporation of labelled leucine into cytochrome P-450 was increased in animals induced with phenobarbital or 3-methylcholanthrene as compared to uninduced controls. Conney (1967) cites evidence that the incorporation of arginine, leucine, lysine, phenylalanine and valine was increased *in vitro* by livers pretreated with phenobarbital.

More recent evidence of increased incorporation of amino acid into microsomal protein following induction is the work of Cappon *et al.* (1975), who reported an increased rate of incorporation of [^{14}C] phenylalanine into protein in microsomes obtained from livers induced with diphenyldichlorotoluene (DDT).

Administration of phenobarbital or other inducing agents results in a marked enlargement of the liver (Conney 1967; Mannering 1968; Remmer 1970). The hypertrophy of the liver is preceded by a gross proliferation of the smooth endoplasmic reticulum and is accompanied by protein synthesis.

Prolonged phenobarbital administration to rats leads to an increase of both the liver mass and microsomal protein per gram of liver, but 3-methylcholanthrene, which stimulates induction of fewer enzymes, does not (Conney 1967). Accompanying this increase of protein is an overall increase in the hydroxylating capacity of the liver as well as in the amount of drug-metabolizing enzymes (Remmer 1970).

The physiological importance of enhanced liver growth and function in animals treated with various inducers has not been determined. However Gilbert and Goldberg (1965) studied the effect of butylated hydroxytoluene and found a close correlation between increased liver mass and enhanced

drug metabolism. The ability of inducing agents to enhance liver growth and liver regeneration without signs of toxicity may contribute to the enhancement of liver function in patients with impaired liver function.

4.10.2 Proliferation of Smooth Endoplasmic Reticulum in Animals Treated with Drugs

The endoplasmic reticulum of the hepatocyte represents a highly specialized system. It is present in almost all regions of the cytoplasm. Two types of endoplasmic reticulum are found, rough endoplasmic reticulum consisting of long parallel rows with many adherent ribosomes, and smooth endoplasmic reticulum which differs morphologically from rough endoplasmic reticulum mainly by the lack of associated ribosomes (Fawcett 1967). The main feature of endoplasmic reticulum is its adaptive ability (Dallner et al. 1976; Remmer and Merker 1965).

Remmer and Merker (1963, 1965) and Fouts and Rogers (1965) reported that treatment of animals with phenobarbital, tolbutamide or chlordane gave rise to a marked proliferation of smooth endoplasmic reticulum of the hepatocyte. More recently, Garg et al. (1972) demonstrated a similar increase in smooth endoplasmic reticulum in rat liver cells following treatment with pregnenolone-16α-carbonitrile. Administration of inducing agents almost always causes a proliferation of smooth endoplasmic reticulum, and Remmer (1968) has described this as a process of adaptation by the body to rid itself of a foreign compound. Recent studies demonstrate an increase in phospholipid during the inductive process (Lu 1976; Gillette et al. 1972). It is possible that the increased phospholipid synthesis is an early step in the proliferation of smooth endoplasmic reticulum.

Accompanying the increase in smooth endoplasmic reticulum is a parallel increase in drug metabolizing enzymes, protein and lipid in smooth

endoplasmic reticulum (Remmer and Merker 1963, 1965). These workers also showed that drug metabolizing enzymes occur in higher concentrations in smooth endoplasmic reticulum than in rough endoplasmic reticulum (Remmer 1969). Recent sub-fractionation studies by Nillson and Dallner (1977) show that cytochrome P-450 is the enzyme present in greatest abundance in the smooth endoplasmic reticulum and is homogenously distributed on the inner and outer membrane. These workers estimate that approximately 5 - 15% of membrane is composed of cytochrome P-450, which, as was mentioned earlier, probably occurs in different forms.

Depending on the inducing agents used and conditions of the experiments, stimulation of drug metabolizing enzymes can occur at varying rates and to different extents. The inductive effect is reversible (Conney 1967; Depierre 1976). Usually complete reversal of the inductive effect occurs in a matter of days. Conney (1967) indicates that fifteen days is normally sufficient for rats to recover from the manifestations of phenobarbital induction. Depierre (1976) demonstrated that NADPH cytochrome c reductase, aminopyrine demethylation and microsomal phospholipid returned almost to normal values six days after the cessation of phenobarbital treatment.

Correlating the biochemical data and taking morphological data into account, Remmer (1970) proposed a hypothesis for detoxification of inducing agents.

The drug metabolizing enzymes are thought of as being enclosed in a sheath of lipid and are situated in the smooth endoplasmic reticulum. Xenobiotics with a high lipid solubility enter easily and accumulate in the cell and come into contact with the enzymes which decompose them into more polar water soluble metabolites.

A high concentration of inducing agent at the site of drug metabolizing enzymes could enhance enzyme synthesis on the ribosome with an enhanced lipid formation, thus new smooth membrane structures are formed.

In summary, a chain of events led to the discovery of a previously unrecognized effect of many drugs, namely induction. Work leading to this discovery had its origins in attempts to explain the concept of drug tolerance of barbiturates (Mannering 1968). The liver can adapt itself to many drugs, and the individual hepatocyte becomes capable of decomposing a drug more rapidly after the drug has induced the synthesis of drug metabolizing enzymes. The inductive process is not limited to this effect but extends to the synthesis of new membranes in the endoplasmic reticulum, membranes which are extremely rich in enzymes which decompose foreign substances. The proliferation of the smooth endoplasmic reticulum is associated with increased protein synthesis, with the result that hypetrophy of the liver ensues.

SECTION 5

EFFECT OF ORAL CONTRACEPTIVES ON DRUG METABOLISM

Progestational synthetic steroids combined with estrogen derivatives are widely used as oral contraceptives and, in many cases, are co-administered with other drugs.

Similarities between drug and steroid hydroxylases in liver microsomes (Table 9) suggested that drugs and steroids are substrates for the same hydroxylating enzymes (Kuntzman et al. 1964). Thus, it is possible that the metabolism of co-administered drugs and OCs may be in competition with each other.

Table 9. Similarities between Hepatic Hydroxylases that Metabolizes Semi-synthetic Steroids and Drugs

(1)	Localized in liver microsomes; require NADPH and oxygen for activity.
(2)	Higher activity in adult male rats than in adult female rats.
(3)	Little or no sex difference in mice.
(4)	Higher activity in male rats than in female mice.
(5)	Higher activity in adult male rats than in immature female rats.
(6)	Inhibition in vitro by addition of SKF 525A.
(7)	Activity inhibited by carbon monoxide.
(8)	Activity increased after treatment of rats with drugs or halogenated insecticide.
(9)	Steroid hormones are competitive inhibitors of drug metabolizing enzyme.

Many steroid hormones, including synthetic progestins and estrogens, have been shown to inhibit in vitro metabolism of several drug substrates.

Tephyl and Mannering (1968) observed that estradiol-17β, testosterone and progesterone competitively inhibited oxidation of ethylmorphine and hexobarbital in male rats. Juchau and Fouts (1966) found that norethynodrel and progesterone added to incubations *in vitro* caused a significant inhibition of side chain oxidation of hexobarbital, ring hydroxylation of zoxazolamine and hydroxylation of aniline.

Carter *et al.* (1974) reported that norethynodrel or mestranol competively inhibited *in vitro* demethylation of ethylmorphine.

Reports on the regulation of drug metabolism by various OCs in rats have been conflicting. Juchau and Fouts (1966) studied the effects of norethynodrel and progesterone on the hepatic drug metabolizing enzyme system. Short term, somewhat longer, and prolonged studies were undertaken.

In the short term studies, a single administration of 50 mg/kg of steroid was administered at time intervals ranging from 1 h to 48 h before sacrifice of the experimental animals. The longer studies were terminated after 3 days, and animals were treated once daily with 50 mg/kg of steroid. In the case of prolonged studies, the animals were treated with 50 mg/kg of steroid once daily for twenty-one days.

These workers found that, when the steroid was administered 1 or 2 h before the sacrifice of the animals, the steroids inhibited the metabolism of hexobarbital and zoxazolamine *in vitro.* If norethynodrel was administered 18 to 48 h prior to sacrifice, hexobarbital metabolism was stimulated. Moderate and prolonged treatment with norethynodrel also stimulated hexobarbital metabolism. Stimulation of zoxazolamine metabolism occurred when norethynodrel was administered between 18 to 36 h prior to sacrificing of the animals and on prolonged treatment of the animals.

These workers also observed that, when norethynodrel and progesterone, at concentrations of both 10^{-4} M and 10^{-5} M, were added directly to incubation mixtures containing the 9 000 g supernatant fraction of hepatic homogenates, co-factors and drug substrates, the side chain oxidation of hexobarbital, ring hydroxylation of zoxazolamine and hydroxylation of aniline were significantly inhibited. This finding correlates well with results obtained in the pretreatment studies, in which it was shown that only hexobarbital and zoxazolamine metabolism could be markedly altered by pretreatment of intact animals with norethynodrel or progesterone.

The results of Juchau and Fouts (1966) indicate that the nature of inhibition of drug metabolizing enzymes by norethynodrel and progesterone were competitive.

Jori et al. (1969) investigated both short term and long term treatment with synthetic steroids (norethynodrel and medroxyprogesterone acetate) or with progesterone, each administered separately or in combination with estrogens (ethinyl estradiol and mestranol), on the metabolism of foreign compounds in vivo and in vitro. In short term studies the steroid was administered 2 or 24 h before pentobarbital and sleeping times were evaluated. The experiment was terminated to measure brain pentobarbital levels 90 min after pentobarbital administration. Long term studies lasted for 30 days, and sleeping time and brain pentobarbital levels were also determined. The results of Jori et al. (1969) indicate that pretreatment of rats with medroxyprogesterone acetate, administered separately or in combination with ethinyl estradiol, increased the metabolism of p-nitroanisole, aminopyrine and aniline. The sleeping time of the rats treated with pentobarbital did not differ significantly from those of

controls however, but analysis of brain pentobarbital levels showed that the steroid pretreated rats had a lower brain concentration of pentobarbital than the controls. Norethynodrel (10 mg/kg) administered in combination with mestranol (18 mg/kg) for 30 days neither altered pentobarbital narcosis, nor significantly altered the hydroxylation rate of aniline, the N-demethylation of aminopyrine, nor the O-demethylation of p-nitroanisole. These steroid pretreated rats showed a significantly lower brain pentobarbital concentration as compared to controls, yet the sleeping time was not altered. Rats treated with medroxyprogesterone acetate or medroxyprogesterone acetate and ethinyl estradiol showed a decreased brain pentobarbital concentration. Only those rats treated with medroxyprogesterone acetate showed a significantly reduced sleeping time. These workers found no statistical correlation between brain pentobarbital concentration and sleeping times. The results of Jori et al. (1969) are in accord with those of Juchau and Fouts (1966). Both groups found that norethynodrel in high doses 50 mg/kg competitively inhibited the metabolism of pentobarbital. Jori et al. (1969) observed increased levels of pentobarbital in the brain after its co-administration with 50 mg/kg norethynodrel.

Carter et al. (1974) reported a significant (30% to 70%) increase in the activity of ethylmorphine demethylase after prolonged administration of a norethynodrel-mestranol combination for ten days or six weeks to female rats. Doses of 1,08 mg/kg norethynodrel plus 21,6µg/kg mestranol were administered. This group also showed that cytochrome P-450 concentrations increased after six weeks, liver mass also increased, indicating an inductive effect. However, microsomal protein failed to increase.

Carter et al. (1974) also observed that high concentrations of norethynodrel and mestranol (10^{-4} M and 10^{-5} M) completely inhibited

ethyl morphine metabolism directly to incubation flasks containing appropriate co-factors.

More recently Briatico et al. (1976) investigated the effects of OCs on drug metabolism in rats, mice and guinea pigs. They compared the effects of three steroid contraceptive drug (SCD) combinations, lynestrol plus mestranol, norethisterone plus mestranol and norethynodrel plus mestranol, at various dose levels. Their studies were conducted for 4 days (short) or 30 days (prolonged treatment). The results of these workers indicate that, in rats and mice, all the combined treatments (except norethynodrel plus mestranol in mice) induce liver microsomal enzymes, as reflected by an increase in the metabolism of aminopyrine, aniline and p-nitroanisole in microsomes prepared from rats administered these SCD combination.

The increased enzyme activity in these studies was not accompanied by increases in microsomal protein or cytochrome P-450 concentrations, parameters which are used as indices of enzyme induction. These results are difficult to explain, since most workers who have demonstrated an activating effect by a drug have observed either changes in cytochrome P-450 or protein levels, or both, depending on the type of inducer tested. Moreover, it has been shown more or less conclusively (Table 9) that drugs and steroids share a common metabolizing enzyme system. Thus, one would expect that any stimulation of this system by inducers would be reflected in either an increase in cytochrome P-450 or protein concentration, depending on the inducer studied. A rather unlikely explanation for the constant levels of cytochrome P-450 would be that these steroids are stimulating one of the multiple forms of cytochrome P-450 discussed earlier.

Hamrick et al. (1973), who studied the effects of methyltestosterone, cortisone and spironolactone on drug metabolism, observed that these steroids stimulated the hepatic microsomal drug metabolizing system without causing an increase in cytochrome P-450 levels, and their explanation of their results was that, perhaps, these steroids regulate the rate of drug metabolism by factors which are not related to cytochrome P-450 and cytochrome c reductase.

Sachan (1976) investigated the effects of a norethynodrel-mestranol combination, dose 10 mg /kg of norethynodrel plus 18 µg /kg of mestranol, on the induction of drug metabolizing enzymes in rats of different nutritional status. Rats were maintained on a low protein, on normal protein and, on high protein diet. Sachan (1976) observed the greatest stimulation of microsomal drug metabolizing enzymes by OCs in rats fed on a normal protein diet.

The investigations of the above workers, of Juchau and Fouts (1966), Jori et al. (1969), Carter et al. (1974), Briatico et al. (1976), Hamrick et al. (1973) and Sachan (1976) indicate that OCs, at least the progestational components thereof, are capable of causing a stimulation of hepatic microsomal drug metabolizing enzymes.

Studies on the effects of estrogens on drug metabolism in vivo and in vitro (Jori et al. 1969, Blackam and Spencer 1969, Briatico et al. 1976, and Freudenthal et al. 1974 b) indicate that estrogens have no effect on the hepatic microsomal enzyme system. Brooks (1976) is of a similar opinion since, in his review on metabolism of steroid hormones in breast cancer, he maintains that "estrogens have demonstrated little or no effect on this microsomal enzyme system".

Several OCs were also found to reduce drug metabolism. Juchau and Fouts (1966), Jori et al. (1969) observed that high doses of norethy-

nodrel inhibited drug metabolizing enzymes.

Freudenthal et al. (1974b) found that norethynodrel and norethindrone increased pentobarbital sleeping time and decreased the metabolism of several drugs in vivo. Tüttenberg et al. (1974) observed that four progestins, norethisterone acetate, d-norgestrel, lynestrol and allylestrol inhibited the demethylation of p-nitroanisole and hydroxylation of aniline in phenobarbital stimulated rat liver microsomes. Feuer et al. (1977) observed a differential action of progesterones on hepatic microsomal activity in rat liver. This group found a significant inhibition of drug metabolizing enzymes (aminopyrine demethylase and coumarin hydroxylase) in hepatic microsomes after the administration of reduced derivatives of progesterone to rats. On the other hand, 16α-hydroxyprogesterone and pregnenolene-16α-carbonitrile significantly increased drug metabolism and enhanced phosphatidylcholine synthesis.

Earlier it was discussed that phospholipid played an important role in the formation of endoplasmic reticulum membranes and in the induction or inhibition brought about by xenobiotics (Marshall et al. 1971). Pregnenolone and pregnanediol reduced these processes. These results imply that steroids may affect drug metabolism by altering the synthesis of membrane-bound phospholipid. The reduced drug metabolizing activity of the female rat associated with a reduced microsomal phospholipid synthesis (Belina et al. 1975) may be associated with the presence of pregnenolone and pregnanediol.

The above studies appear contradictory. These conflicting results arise partly from different conditions employed in different laboratories, conditions such as different dose levels applied, and different species or strains of animals used.

Relatively little additional information on the effects of OCs on drug metabolism is available.

Crawford and Rudofsky (1966) determined the urinary excretion of meperidine in 5 women on OCs and in 4 controls. They observed that, in 4 out of 5 women on OC therapy, there was a significant increase in unchanged meperidine in the urine. These observations led them to conclude that OCs inhibited drug metabolism.

O'Malley et al. (1972) determined the plasma half-life of antipyrine and phenylbutazone in a group of women on various OC therapy and a control group. They observed that the mean plasma half-life of antipyrine was 81% longer in women on OC therapy than in controls. Although phenylbutazone half-lives were longer (14%) in the OC group, this difference was not statistically significant. In addition, three women had antipyrine or phenylbutazone determinations made while on OC therapy and 2 months after cessation of therapy. In all three, the half-lives were shorter after cessation of OC therapy - these results indicate that OCs impair drug metabolism in the human.

More recently, Carter et al. (1974) investigated the effect of OC therapy on drug metabolizing activity by monitoring antipyrine and phenylbutazone half-lives in women. These workers found that, after 3 months of oral steroid administration (norethynodrel plus mestranol), the antipyrine half-life was increased in 5 out of 8 subjects (as compared to their previous half-lives); phenylbutazone half-lives were not significantly altered. These results also indicate that OCs cause inhibition of drug metabolism in humans.

In summary, it appears that the investigations of the effect of OCs on drug metabolism has produced conflicting results. However, in work on animals

the balance of evidence seems to favour the concept that OCs, especially the progestins, may cause a stimulation of hepatic microsomal enzymes, while estrogens seem to be ineffective.

In humans, the available limited data gives some indication that OCs inhibit drug metabolism showing that findings in studies on animal and human do not correlate well. The use of the rat as a model for the study of the effects of OCs on drug metabolism in women will be discussed later (see discussion).

SECTION 6

MATERIALS AND METHODS

6.1 The Test Animals

Albino female rats acquired from South African Institute for Medical Research (SAIMR) were used as the test animals. The animals were between 4 and 5 months old and weighed 180 $\pm$ 20 g. Animals from a common source and stock were used since strain differences have been reported to be related to differences in drug metabolizing enzymes (Conney 1967).

Housing of the Animals

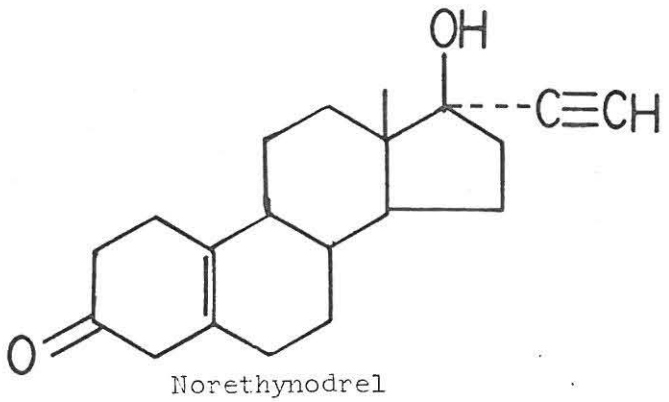
Each group (see methods) of four animals were housed in single, stainless steel, rod type cages, located in a room with temperature and humidity control. The temperature and humidity could be controlled by an air conditioning system and the temperature throughout the period of experimentation was kept constant at 20^{o}C.

Animal bedding for the cages was not used since Mazel (1972) reported that various types of bedding (red cedar or white pine) were found to increase the metabolism of certain drugs. Insecticides were never used for the control of pests as it is known that a number of commonly used insecticides induce the synthesis of microsomal enzymes (Fouts and Rogers 1965). Animals were allowed to settle-in for a period of three weeks before being used for any assays and close attention was paid to the health of these rats and any rats that showed signs of ill health were removed from the experiment.

Rats were allowed food and water *ad libidum* since Kato *et al.* (1967) observed that starvation increased the metabolism of drugs.

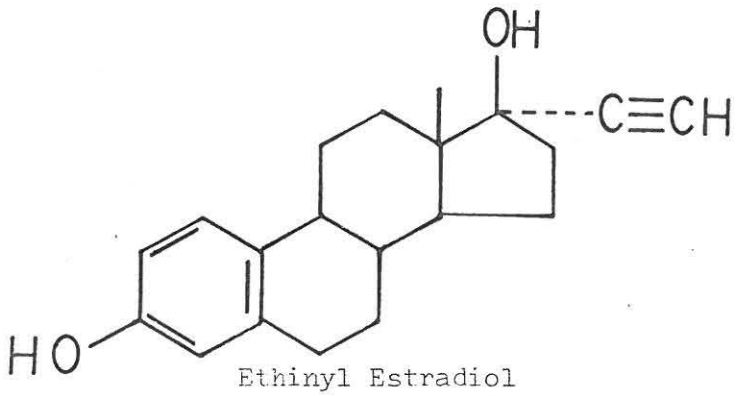
6.2 The Steroids Used

The following steroids, norethynodrel (a progestin) and ethinyl estradiol (an estrogen), were used in the experiments, their structural formulae are indicated below.



Norethynodrel

17α-Ethinyl-17-hydroxy-estra 5(10)-en-3-one



Ethinyl Estradiol

17α-Ethinyl estra 1,3,5(10) triene-3,17β-diol

6.3 Dosing The Animals

Two dosage levels, 1 mg and 20 mg/kg, of each steroid were administered to the rats for various lengths of time, intra-peritoneally.

The steroids were dissolved in an appropriate volume of ethyl oleate and convenient volumes administered to animals intra-peritoneally between 08h30 to 09h00 daily for various lengths of time. Control animals received equivalent volumes of ethyl oleate.

The animals were weighed at various time intervals, and the volume of fluid injected was adjusted on a weight basis.

6.4 Preliminary Experiments

The aim of these experiments was to determine optimal conditions to be applied to the main study. These are briefly set out below.

Experiment 1

Aim: to determine the optimal dose of pentobarbital sodium ("Sagatal"®) for the sleeping time studies.

Method:

Ten animals were used for this study; these were divided into five equal groups and the following dosages of Sagatal® administered to each group, 15 mg/kg, 30 mg/kg, 45 mg/kg, 60 mg/kg and 75 mg/kg.

The group of animals receiving 15 mg/kg of Sagatal were sedated but did not sleep, the animals receiving 30 mg/kg slept for various lengths of time, the animals receiving 45 mg/kg slept, some of the animals receiving 60 mg/kg and 75 mg/kg died.

A dose of 25 mg/kg was later tried, but this only served to heavily sedate the animals.

A dose of 30 mg/kg was thus chosen for all the experiments.

Experiment 2

Aim: to determine if ethyl oleate had any effect on the hepatic drug metabolizing system.

Method:

Eight animals were used for this study; these were divided into two equal groups, animals in one group receiving 0,5 ml of ethyl oleate daily for one month, while the other group remained untreated.

At weekly intervals, sleeping times were performed and the mass of each animal was recorded. At the end of a period of one month, the animals of both groups were sacrificed and protein and cytochrome P-450 concentrations of the hepatic microsomal enzyme system of both groups of animals were determined. No change was found to occur.

Electron microscopy of tissues from these animals was not undertaken, but electron micrographs of livers, adrenals and ovaries of animals receiving ethyl oleate (control groups in main study) were compared to a large number of electron micrographs obtained from the electron microscope unit and to references in standard texts (Fawcett 1967) and it was concluded that ethyl oleate had very little, or no, effect at all on the ultrastructure of these organs.

6.5 Definitive Experiments

6.5.1 Sampling of Animals

The design of each experiment was the same and, since two dosage levels of each steroid were administered, four experiments were performed.

In each experiment, thirty-six animals were used and these were divided into nine equal groups and sampled as is indicated in Table 10.

Animals were sorted into their respective groups on the basis of their oestrus cycles, which were monitored by vaginal smears. During the settling-in period of 3 weeks, the oestrus cycles of the animals were recorded (for method see 6.5.2) for 3 consecutive cycles to determine its regularity. The average cycle length of most of the animals was

4 days. Only animals with a regular 4 day cycle were selected. Animals with late oestrus occurring on the same day were placed into one group.

Table 10. Sampling Procedure for Sacrifice of Animals

GROUP	EXPERIMENTAL							CONTROL	
	A	B	C	D	E	F	G	H*	I*
Days of steroid treatment before sacrifice	2	4	6	8	12	16	30	-	-
Number of animals sacrificed 2 h after the last steroid treatment	2	2	2	2	2	2	2	2	2
Number of animals sacrificed 24 h after the last steroid treatment	2	2	2	2	2	2	2	2	2
Total number of animals per group	4	4	4	4	4	4	4	4	4

* H and I = control animals received only ethyl oleate daily until the termination of the experiment at 30 days.

Administration of steroid to the various groups of animals was commenced when all the animals of a particular group were at late oestrus. This procedure was followed for the sake of uniformity since differences in metabolic activity may be observed if steroid treatment were intiated in a group of rats at various phases of the oestrus cycles.

The effect of treatment with OCs was compared in subsequent experiments with the activity of the control groups, the oestrus state of the animals being taken into account. Animals in groups A to G were treated with steroid, and these animals were sacrificed by a blow on the head after various time intervals. Group A was sacrificed 2 days, group B 4 days, group C 6 days, group D 8 days, group E 12 days, group F

16 days and group G 30 days after the intiation of steroid treatment. Two animals in each group were sacrificed (on the days indicated) 2 h after the last steroid administration; one of these animals was used for electron microscopy and the other to determine the effect of steroid on hepatic microsomal enzyme activity. The remaining two animals in each group mentioned were sacrificed 24 h after the last steroid injection; one was used for electron microscopy and the other for determining hepatic microsomal activity. Thus, for the experimental animals at each sampling, two animals (one 2 h after the last OCs administration and the other 24 h) were used to determine alterations in hepatic microsomal enzyme activity, and, similarly, two animals were used for electron microscopy.

The animals in the control groups H and I were sampled as in experimental groups, except that, after samples were taken from the liver for electron microscopy, the livers in the same treatment group (2 h or 24 h after ethyl oleate) were pooled and protein and cytochrome P-450 concentrations determined.

The effect of short-term and long-term administration of OCs on the hepatic microsomal enzyme system in experimental animals was compared to that of controls by comparing the changes in sleeping time, in protein levels and cytochrome P-450 concentrations - these are all tests for enzyme induction.

The effects of OCs on the changes in mass of the animals, form of cells of the vagina and of organelles of the hepatic, adrenal and ovarian cells as evidenced by electron microscopy, were also compared. The methods used for the determination of the above mentioned parameters are discussed below.

6.5.2 Vaginal Smears

The oestrus cycle of rats was monitored by observing the changes in cell types in the vagina, the so-called "vaginal smear". A "Johnsons swab" was used to obtain the smear. The swab was moistened with water and gently inserted and slightly rotated within the vagina. The swab was then pressed into a drop of water on a microscope slide and the slide examined under the low power of a microscope. Selected slides were stained in Mayers Haemotoxylin and Eosin and photographed (see Results).

6.5.3 Sleeping Times

Sleeping times of the steroid treated rats and control rats were determined for groups E, F and G. Sleeping times were determined once a week up to the time of sacrifice, thus one sleeping time determination for groups (E and F) and three for group G were obtained. Thirty mg/kg of pentobarbital sodium ("Sagatal"®) was administered intra-peritoneally. The "sleeping time" is defined as the time lapse between the disappearance and reappearance of the righting reflex. As a precaution to prevent interference with later determinations, an interval of one week was allowed between determinations in order to allow the rats to clear their system of the pentobarbital. The sleeping time was used as an index of in vivo drug metabolism since the duration of action of pentobarbital is directly related to its biotransformation. These results are indicated in Table 20.

6.6 Preparation of the Rat Liver Microsomal Fraction

The method used for the preparation of the microsomes was that of Cinti et al. (1972).

Chemicals :

Phosphate buffer made up of KH_2PO_4 and $K_2HPO_4.3H_2O$ at pH 7,4. After the buffer was prepared, the pH was checked with a pH meter.

0,25M Sucrose

1,15% KCl

0,9% NaCl

$CaCl_2.2H_2O$

All the reagents used were analytical reagent grade. These were accurately weighed out on a analytical balance and accurately made up to volume in a volumetric flask with distilled water. All solutions were stored in a refrigerator.

The distilled water used for preparation and analyses of rat liver microsomal fraction was glass distilled and stored in a 5 litre glass container. Water used to make up solutions was always freshly distilled to minimize the risk of contamination.

All glassware used was thoroughly washed and rinsed in hot water, then soaked in chromic acid for 24 h. After this, the glassware was rinsed three times in distilled water and dried in an oven at 75 ^{o}C.

This cleaning procedure was necessary to ensure that the glassware was scrupulously clean to prevent contamination of the hepatic microsomal preparation.

Sacrificing The Animals:

Animals were sacrificed by a blow on the head with a metal bar. To prevent circadian variation in hepatic microsomal activity, rats were sacrificed at the same time every morning, between 09h00 to 09h15 Once the animal had been sacrificed, the liver was quickly excised and weighed. Once the liver had been removed, all operations were

performed between 0 °C - 4 °C, to prevent degradation of the enzymes.

When the liver was removed, care was taken not to cut any of the lobes, since any damage would interfere with perfusion of the liver.

Since haemoglobin would interfere with spectral examinations of cytochrome P-450, it was removed from the liver by perfusion with 0,9% NaCl until the liver became light tan in colour. A 25% w/v liver homogenate was always prepared (1 g liver + 3 volumes sucrose); thus, after perfusion the liver was placed into 3 volumes of cold 0,25 M sucrose solution and minced into small pieces with a clean pair of scissors.

6.6.1 Tissue Homogenization

After the liver had been minced, it was homogenized in a Thomas tissue homogenizer which is illustrated in Figure 13.

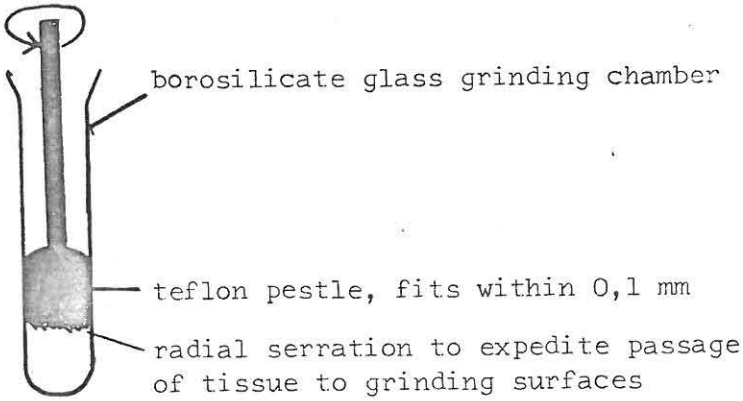


Figure 13. Thomas Tissue Homogenizer

The steel rod of the teflon pestle was attached to an electric drill which allowed the teflon pestle to be rapidly rotated while in the glass homogenizer. The electric drill was connected to a rheostat; thus, the speed of rotation of the electric drill could be controlled.

To homogenize the tissue, the glass homogenizer tube was moved up and down relative to the pestle through 6 - 8 excursions. Rapid rotation

of the pestle and the vertical movement of the glass tube cause disintegration of the tissue and disruption of the cells, yielding a homogenate consisting of diluted cell sap, intracellular particles (mitochondria, nuclei and microsomes) and some unbroken cells. These components may be separated by fractional centrifugation.

The following precautions were observed during tissue homogenization.

The liver was minced as finely as possible. Since small pieces of tissues homogenize easier than large pieces, and since less time is required when small amounts of tissue are homogenized, small quantities of finely chopped tissue were homogenized to prevent overheating of the tube and subsequent enzyme destruction.

The homogenizer tube was kept cold throughout the homogenization process by means of ice wrapped in paper.

6.6.2 Centrifugation

The homogenate was transferred to two plastic centrifuge tubes and placed in a MSE 18 High Speed Centrifuge (maximum speed 18 000 revolutions per min) at 4 °C. The required radial centrifugal force (RCF) had to be converted into revolutions per min, taking into account the head type. The following formula was used.

$$RCF = 0{,}0000284 \times R \times N^2$$

where R = radius (inches) from the centre of the centrifugal shaft to the tip of the centrifuge tube (R in this case = 4,2)

N = revolutions per minute

The differential centrifugation was started at 4 000 g ($5{,}8 \times 10^3$ revolutions per minute) for ten minutes to allow cell debris and nuclei to sediment; this treatment was followed by 20 min at 10 000g ($9{,}2 \times 10^3$ revolutions per min), to allow the mitochondria

to sediment. These two speeds were employed since it was found that during this gradual sedimentation fewer microsomes were trapped by the sedimenting particles. The supernatant liquid was poured into a clean measuring cylinder and made up to 50 ml with cold 0,25 M sucrose, this liquid was then adjusted to a final concentration of 8 mM in $CaCl_2$ by addition of 58,8 mg of $CaCl_2.2H_2O$ in it.

The Ca^{2+} makes aggregates of the endoplasmic reticulum membranes and, thus, causes the microsomes to sediment in 15 - 20 min at a force of 27 000 g (15 500 revolutions per min) [without the Ca^{2+}, one would have to apply a force of 105 000 g for one hour (Cinti et al. 1972)].

The resulting microsomal pellet was washed by resuspending it, in an equal volume of 1,15% KCl and resedimented at 27 000 g. The new pellet was washed to remove excess Ca^{2+}, which may interfere with subsequent metabolic tests.

The final microsomal pellet was re-homogenized in phosphate buffer of pH 7,4 and the preparation appropriately diluted for studies. For spectral studies and protein determinations, the microsomal pellet was diluted to 100 ml with buffer, and this procedure usually resulted in a suspension with a protein concentration of approximately 2,0 $\pm$ 0,5 mg/ml.

6.7 Determination of Microsomal Protein

The colorimetric determination of Lowry et al. (1951) as modified by Miller (1959) was used for the determination of microsomal protein with the modification that microsomes were prepared by the method of Cinti et al. (1972) using an MSE 18 High Speed Centrifuge.

The colour formed is thought to be due to a complex between the alkaline copper-phenol reagent and the tyrosine and tryptophan residues of the protein. This complex formation is directly proportional to the protein concentration only in the range 50 - 200μg/ml. Bovine serum albumin was used as the standard.

6.8 Determination of Cytochrome P-450

The method used for this determination was that of Omura and Sato (1964).

Reagents:

Prepared hepatic microsomal suspension

carbon monoxide

$Na_2S_2O_4$

Instrument: Beckman Acta M VI Spectrophotometer

Method

Carbon monoxide difference spectra of cytochrome P-450 were run on a Beckman Acta M VI Spectrophotometer in the range from 500 nm to 360 nm. The spectrophotometer was equipped with a scatter transmission accessory (see Figure 14).

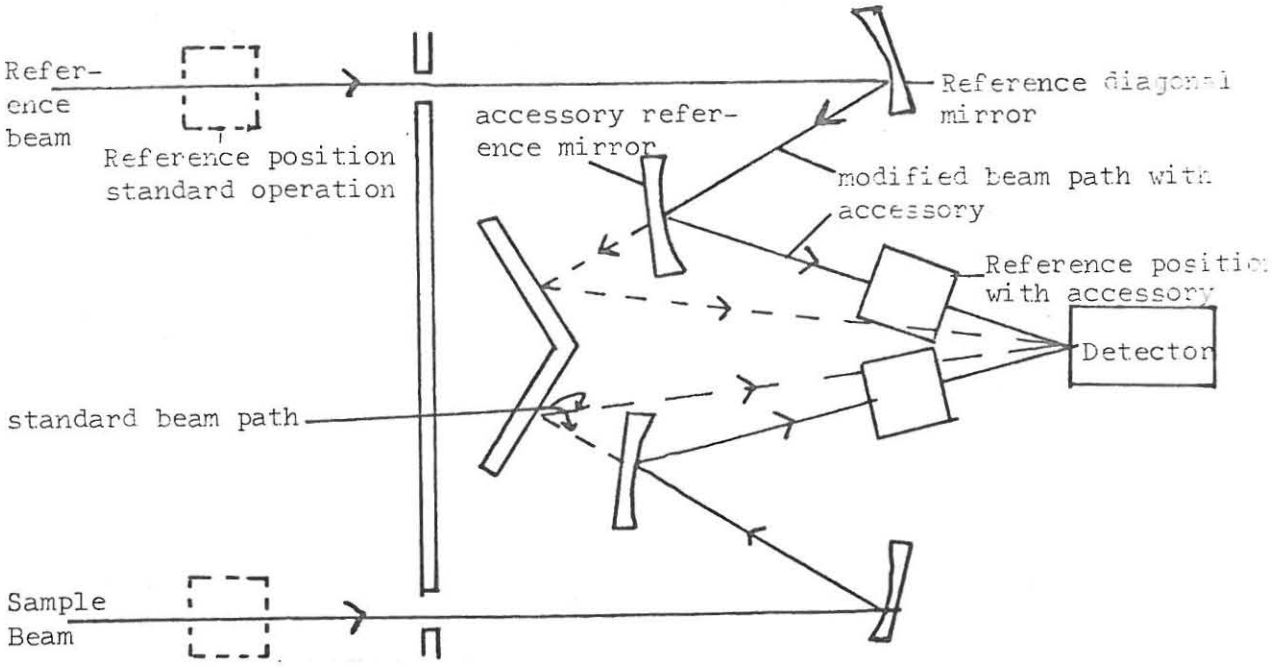


Figure 14. Scatter Transmission Accessory

In the normal instrument's sampling position, very turbid samples scatter the incident radiation to such an extent that very little transmitted radiation falls on the detector. The result is high background absorption and loss of detail in the spectrum. The scatter transmission accessory eliminates these difficulties by placing the sample close to the end-on photomultiplier detector so that nearly all of the scattered radiation falls upon the detector (the accessory mirrors give a bigger angle between the two incident beams, so that the cuvettes can be placed next to each other, close to the detector.

All subsequent procedures were carried out at room temperature.

(1) Three ml of microsomal suspension was placed into each of two matched cuvettes of 1 cm optical path length.

(2) A baseline was prepared with the aid of a set of potential adjustments every 20 nm from 500 nm to 360 nm.

(3) The microsomal suspension from each cuvette was pooled and the solution reduced with a few mg of $Na_2S_2O_4$.

(4) Three ml was pipetted out into the sample cell and the remaining 3 ml returned to the reference cuvette.

(5) The sample cuvette was then treated with carbon monoxide by bubbling the gas through for one minute.

(6) The spectrum was then recorded from 500 nm to 360 nm.

(7) Each spectrum was then traced out and suitable adjustments for irregularities in the baseline effected.

(8) The amount of cytochrome P-450 can be calculated from the optical density difference (450 nm - 480 nm) and the molar extinction coefficient of 91 $mM^{-1}cm^{-1}$.

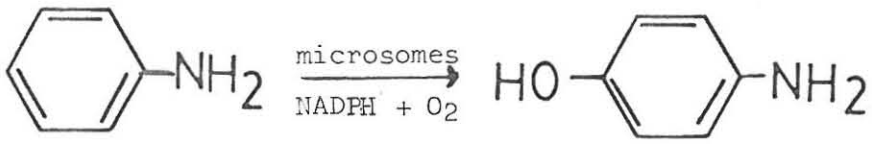
For each cytochrome P-450 determination the values represent the average of three peaks.

6.9 Enzymatic Assays

The microsomes were resuspended in 0,2 M Tris-HCl buffer (pH 7,8) and used the enzymatic assays (the animals used are indicated under the respective studies undertaken). In this study, cytochrome P-450 and protein concentrations were also determined. The purpose of determining enzymatic activity was to compare the rate of metabolism of aniline and aminopyrine by the microsomal enzymes of rats treated with steroid and of control rats. The rate at which the metabolites, p-aminophenol and formaldehyde, are formed is an index of drug metabolism.

6.9.1 The Determination of Microsomal Aniline Hydroxylase

The rate of aniline metabolism in vitro may be determined by measuring the quantity of p-aminophenol formed according to the following reaction.



Reagents:

Tris buffer pH 7,8

$MgCl_2.6H_2O$

$MnCl_2.4H_2O$

NADP

Isocitric Acid

Isocitrate Dehydrogenase

The following NADPH generating system, which was found to be optimal for this laboratory, was used.

NADP	2 µmoles
DL-Isocitric Acid	10 µmoles
$MgCl_2$	25 µmoles
$MnCl_2$	0,010 µmoles
Isocitric Acid Dehydrogenase (ICDH)	0,4 IU

The NADP and DL-isocitrate were made up together in distilled H_2O immediately before use. The $MgCl_2$ and $MnCl_2$ were combined in a stock solution and stored in a refrigerator. Aniline was redistilled freshly before use; 5 μM(0,47 μl) was used.

Details of the composition of the assay mixtures employed in the assay of aniline is shown in Table 11.

Table 11. Composition of Assay Mixture for Aniline

	Flasks	Drug	Mg^{++} Mn^{++}	NADP Isocitric Acid	ICDH	Microsomes	
						Cont.	Exp.
Tissue blank, control animals	1 - 3	-	0,4 ml	0,4 ml	20 μl	1	
Substrate, control animals	4 - 6	0,47 μl	0,4 ml	0,4 ml	20 μl	1	
Tissue blank, steroid	7 - 9	-	0,4 ml	0,4 ml	20 μl		1
Substrate, steroid	10 -12	0,47 μl	0,4 ml	0,4 ml	20 μl		1

Buffer 0,2M Tris-HCl pH 7,8 was used to bring the final, total mixture volume to 4 ml, microsomes equivalent to 250 mg of wet liver was added last with 20 μl of ICDH to start the reaction.

The flasks were incubated with rapid shaking for 20 min at 37 ^{o}C in a metabolic shaker. Aromatic hydroxylation of aniline was determined by measuring p-aminophenol formation according to the method of Mazel (1972) with the above modifications, colourimetric determinations were performed using a Beckman DB Spectrophotometer.

6.9.2 Kinetics of Aminopyrine Demethylase

Aminopyrine is N-demethylated giving formaldehyde as one of its metabolites. The Nash reaction has been extensively used to determine

formaldehyde concentrations. The formaldehyde formed during the incubation is trapped as the semicarbazone (by semicarbazide in the incubation mixture) and determined by the colourimetric procedure of Nash, based on the Hantzsch reaction (Nash 1953).

Formaldehyde reacts quantitatively with acetyl acetone and ammonium salt to form a yellow complex, diacetyl dihydrolutidine at an acid pH (5.5 to 6,5). The reaction product is relatively stable, but is affected by prolonged exposure to light and oxidizing agents.

Demethylation of aminopyrine was determined by measuring formaldehyde formation according to the method of Mazel (1972) using the same generating system in the metabolism as for the determination of aniline hydroxylase activity. The only procedural modification was that the final volume of the incubate was adjusted to 6 ml. The colourimetric determination was performed with a Beckman DB Spectrophotometer.

6.10 Electron Microscopy

Following sacrificing, a portion of the right lateral lobe of the liver, left adrenal and left ovary were removed as soon as possible and placed in cold 5% phosphate buffered, glutaraldehyde fixative and cut into small pieces 1 - 4 mm^3. The finely cut tissues were placed in specimen tubes and left to fix for 24 h at 4 °C. A temperature of 4 °C was maintained until the tissues were placed into 80% acetone.

Following glutaraldehyde fixation, the tissues were washed twice for 10 min each in 0, 1 M phosphate buffer. Secondary fixation was carried out for 90 min in 1% osmium tetroxide (Millionig 1961, 1962 from Hayat 1970). This process was followed by two further 10 min washes in phosphate buffer. Dehydration was achieved by passing the tissues through a series of acetone concentrations (10 min in each); 30%, 50%,

70%, 80%, 90%, the rest of the solution being made up of distilled water. The tissues were finally dehydrated by immersing them in 100% acetone twice for 10 min each time.

Penetration of Epon into the tissue was achieved by transferring the tissue from 100% acetone to mixtures; 75 : 25 Acetone : Epon, 50 : 50 Acetone : Epon, 25 : 75 Acetone : Epon. The issue remained for 20 min in each of the mixtures and was transferred with pure Epon. The Epon was allowed to polymerise in an oven at 450 °C for 12 h and then at 60 °C for 24 h.

Sections of approximately 50 nm were cut on an LKB UM3 ultra-microtome, using glass knives. Sections were collected on 400 mesh uncoated copper grids.

Staining was carried out in 5% aqueous uranyl acetate followed by lead citrate (Reynolds 1963 from Hayat 1970). Standard precautions were taken to avoid contamination of grids by lead carbonate precipitation during staining (Hayat 1970).

Viewing :

The sections were viewed in a Hitachi HU II B Electron Microscope.

Three grids were observed for each tissue category. General changes were noted and recorded on Kodak Electron Image Plates (Kodak Limited, Rochester, New York).

SECTION 7

RESULTS

For the sake of convenience, the results have been divided into the effect of oral contraceptive steroids on:-

1 Change in mass of animals

2 Change in cell types of the vagina

3 Change in sleeping times of animals

4 Protein concentrations

5 Cytochrome P-450 concentration

6 Metabolic studies

7 Cytological features

(a) Liver

(b) Adrenal

(c) Ovary

7.1 Gross Effects of Norethynodrel

Both dose levels of norethynodrel caused the animals to lose mass markedly; at 20 mg/kg the coat colour of the animals changed from white to a creamy colour, the animals also seemed "greasy in appearance" (Figures 15 and 16). The change in coat colour at 20 mg/kg appeared after about two days of steroid treatment and, even in the 30 day treatment group, progressed until they were sacrificed.

At 1 mg/kg similar changes were noted, however the change in coat colour occurred after about 6 days of steroid treatment. Hair loss, which was not severe, occurred in some of the animals treated with 20 mg/kg norethynodrel.

Except for these above changes the animals appeared normal.

Figure 15. A photograph showing the marked mass loss of a rat receiving 20 mg/kg of norethynodrel, compared to a control animal after 3 weeks of treatment. C is the control animal and E is the experimental animal.

Figure 16. A photograph showing the marked mass loss of three animals receiving 20 mg/kg norethynodrel. Both photographs were taken while a sleeping time was being performed, and this was after 3 weeks of treatment.

7.2 The Effect of Oral Contraceptives on Change in Mass of Animals

Although the original body masses of the treated groups were within 10% of the masses of the controls, the final body masses of the rats receiving norethynodrel and ethinyl estradiol at both dose levels were markedly decreased in comparison with control.

7.2.1 Effect of 20 mg/kg Norethynodrel

Norethynodrel at this dose level caused the largest reduction in mass of the animals.

The results for the change in mass of individual groups of animals sacrificed at various time intervals are shown in Table 12.

Animals treated with norethynodrel lost mass within the first two days and a loss in mass continued throughout the experiment. At the termination of the experiment at 30 days, masses of the animals were 82% of original (Figure 17).

Table 13 shows the mean loss in mass of the 30 day, 16 day and 12 day groups of animals at weekly intervals up to the time of sacrifice.

The change in mass at weekly intervals of the 30 day group of animals is represented graphically (Figure 18). Experimental animals lost mass markedly, while control animals increased in mass. At the termination of the experiment, there was a significant difference between the final body masses of the treated animals and control animals.

Photographs giving a good indication of the marked mass loss of the experimental animals compared to a control animal is shown (Figure 15). Figure 16 shows three experimental animals which have lost mass markedly.

Table 12. Effect of 20 mg/kg Norethynodrel on Rat Body Mass.

Days of Treatment	Original Body Mass g	Final Body Mass g	Per Cent of Original Mass g/g x 100
2	178,75 ± 4,64	177 ± 8,36	97,02
4	171,75 ± 7,27	166,50 ± 7,50	96,90
8	179,75 ± 10,68	166,00 ± 12,35	92,0
12	188,50 ± 7,4	171,00 ± 12,80	90,72
16	182,15 ± 6,18	168,80 ± 8,04	88,42
30	186,00 ± 8,28	154,75 ± 9,28	81,86

Each figure represents the mean value and range of variation of four animals. See FIGURE 17.

Table 13. Effect of 20 mg/kg Norethynodrel on Weekly Change in Rat Body Mass

Experiment	Per Cent of Original Mass			
	1 Week	2 Weeks	3 Weeks	4 Weeks
Controls	105,35	108,12	112,80	113,82
30 Day	93,95	87,03	84,20	81,86
16 Day	92,34	88,42*		
12 Day	92,77	90,72*		

Each figure represents the mean value and range of variation of four animals. See FIGURE 18.

*Animals were weighed on the day of sacrifice.

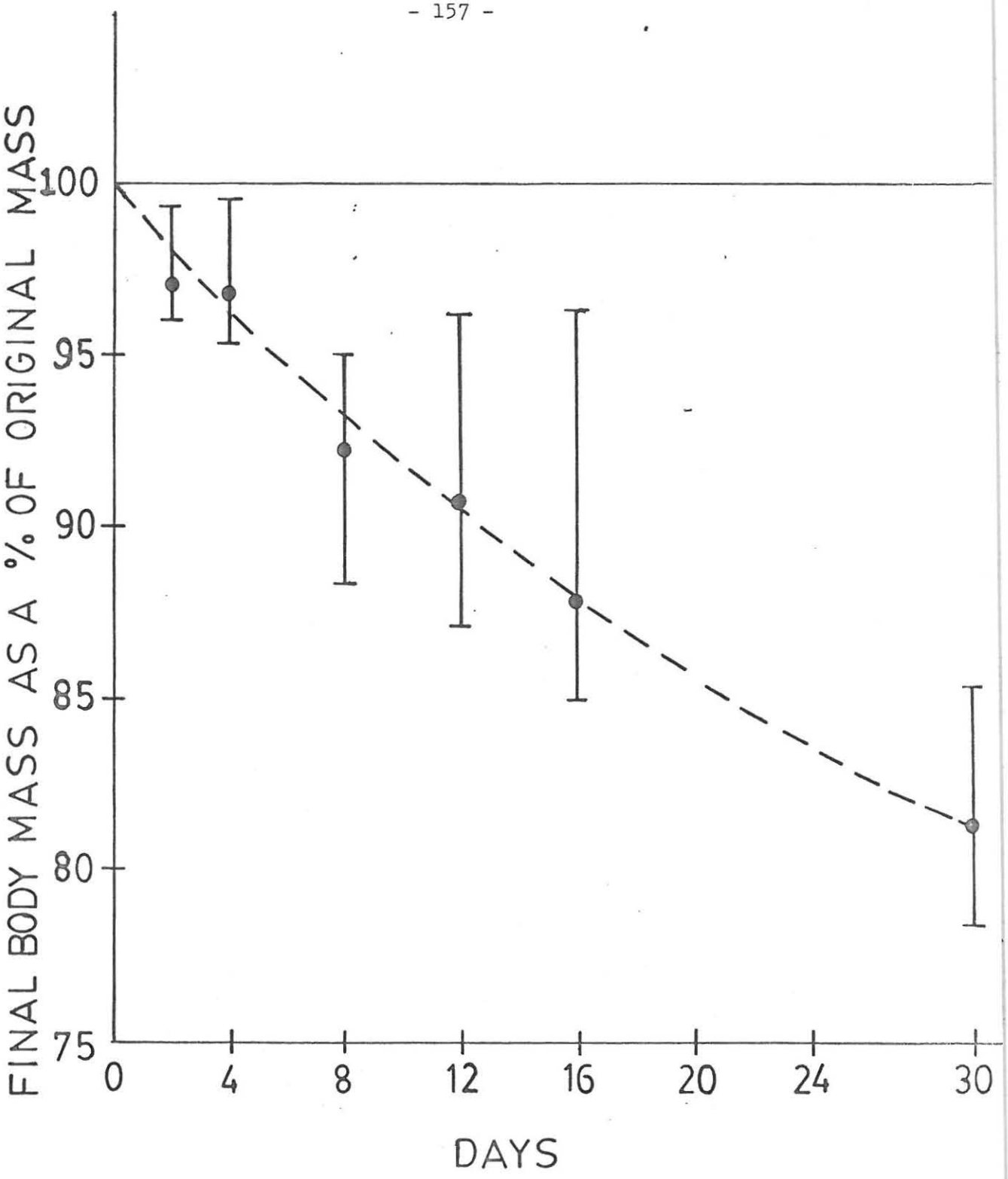


Figure 17. Graph showing the change in body mass of each group of animals on the day of sacrifice. Each point represents the mean value, and range of variation, of four animals expressed as a percentage of original mass.

Dosage: 20 mg/kg norethynodrel.

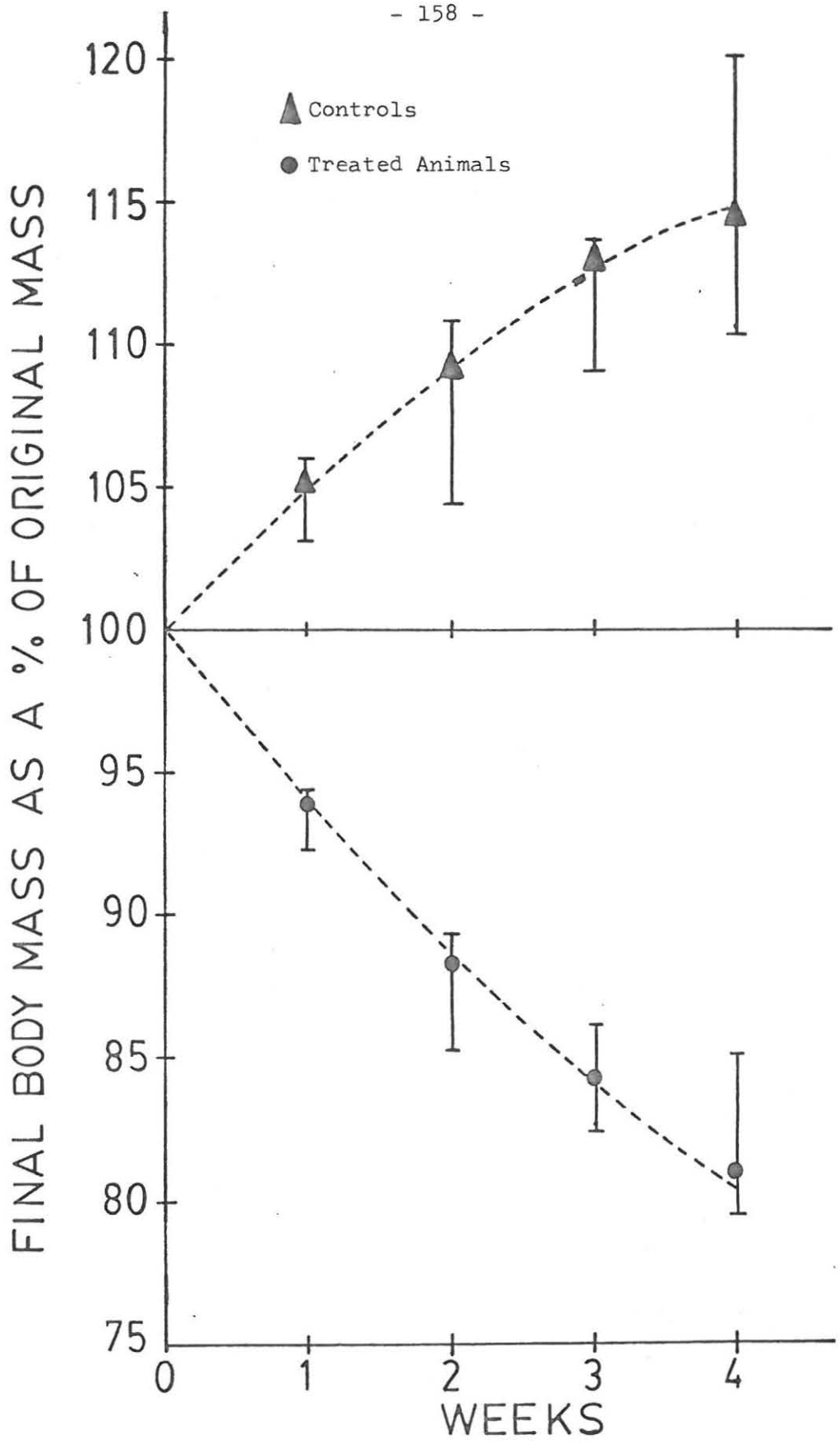


Figure 18. Graph showing the change in body mass, over a period of four weeks, of one group of experimental animals and of the control group. Each point represents the mean value, and range of variation, of four animals expressed as a percentage of original mass. Animals were weighed at weekly intervals.
Dosage: 20 mg/kg norethynodrel. Controls received ethyl [illegible]

7.2.2 Effect of 1 mg/kg Norethynodrel

The effect of the lower dose of norethynodrel on change in the mass of animals show similar trends to those of the high dose, except that the effect of the steroid is less marked. At the end of the experiment (30 days), the mass of the animals was 95% of their original. This mass loss was not significant. It seems, however, that at the lower dose the steroid inhibits the growth of the animals, since there was a significant difference between the final masses of the control animals and those of the experimental animals since the control animals increased in mass. The change in mass of animals on 1 mg/kg norethynodrel is illustrated by means of Table 14 and 15 and Figures 19 and 20.

7.3 Gross Effects of Ethinyl Estradiol

Treatment of animals with both dose levels of ethinyl estradiol caused animals to lose mass.

Coat colour of animals in the 20 mg/kg treatment group changed to an off-white colour while those of the 1 mg/kg groups remained normal. The coats of animals did not at any stage appear greasy; however, 20 mg/kg of ethinyl estradiol caused a more severe hair loss than was the case with norethynodrel.

7.3.1 Effect of Ethinyl Estradiol on Rat Body Mass

At both dose levels of ethinyl estradiol there was a resulting decrease in the mass of experimental animals; however, the effect was less marked than when norethynodrel was administered at a corresponding dose.

Tables 16 and 17 and Figures 21 and 22 indicate the change in mass of animals receiving 20 mg/kg of ethinyl estradiol.

Tables 18 and 19 and Figures 23 and 24 indicate the change in mass of animals receiving 1 mg/kg of ethinyl estradiol.

Table 14. The Effect of 1 mg/kg Norethynodrel on Rat Body Mass

Days of Treatment	Original Body Mass g	Final Body Mass g	Per Cent of Original Mass g/g x 100
2	181,25 ± 6,94	182,50 ± 8,06	100,60
4	172,50 ± 7,32	173,50 ± 10,47	100,25
6	178,50 ± 7,85	175,75 ± 8,53	98,41
8	180,25 ± 11,90	176,75 ± 10,90	98,06
12	178,25 ± 8,95	175,75 ± 9,91	97,56
16	183,25 ± 8,50	179,25 ± 7,27	96,97
30	179,75 ± 7,63	170,25 ± 6,84	94,70
Controls	175,50 ± 7,93	202,75 ± 10,04	113,82

Each figure represents the mean value and range of variation of four animals. See FIGURE 19.

Table 15. Effect of 1 mg/kg Norethynodrel on Weekly Change in Rat Body Mass

Experiment	Per Cent of Original Mass			
	1 Week	2 Weeks	3 Weeks	4 Weeks
Controls	102,60	105,92	111,74	115,11
30 Day	98,55	97,35	96,11	94,70
16 Day	97,75	96,97 *		
12 Day	98,55	97,56 *		

Each figure represents the mean value and range of variation of four animals. See FIGURE 20.

* Animals were weighed on the day of sacrifice.

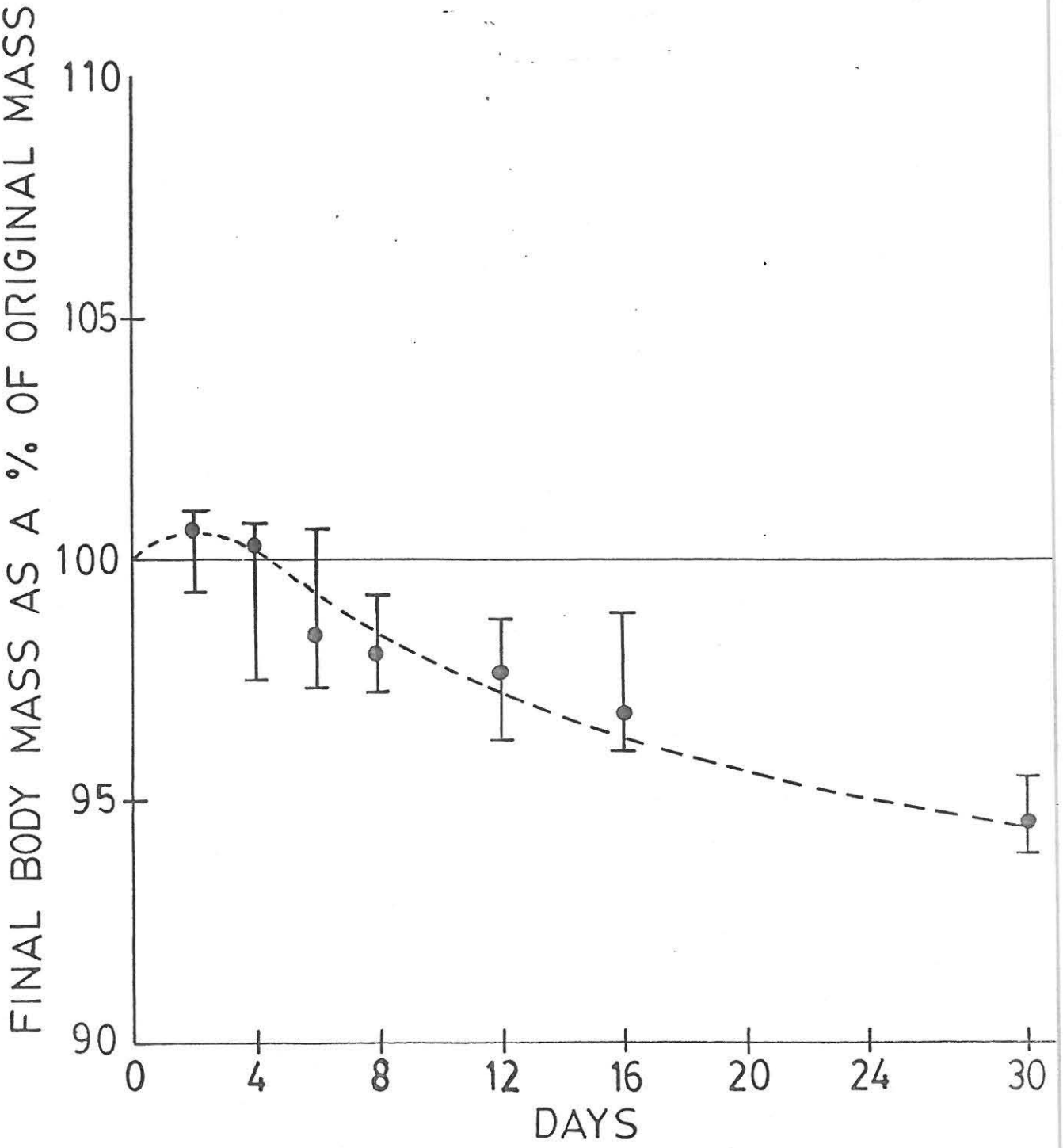


Figure 19. Graph showing the change in body mass of each group of animals on the day of sacrifice. Each point represents the mean value, and the range of variation, of four animals expressed as a percentage of original mass.

Dosage. 1 mg/kg norethynodrel.

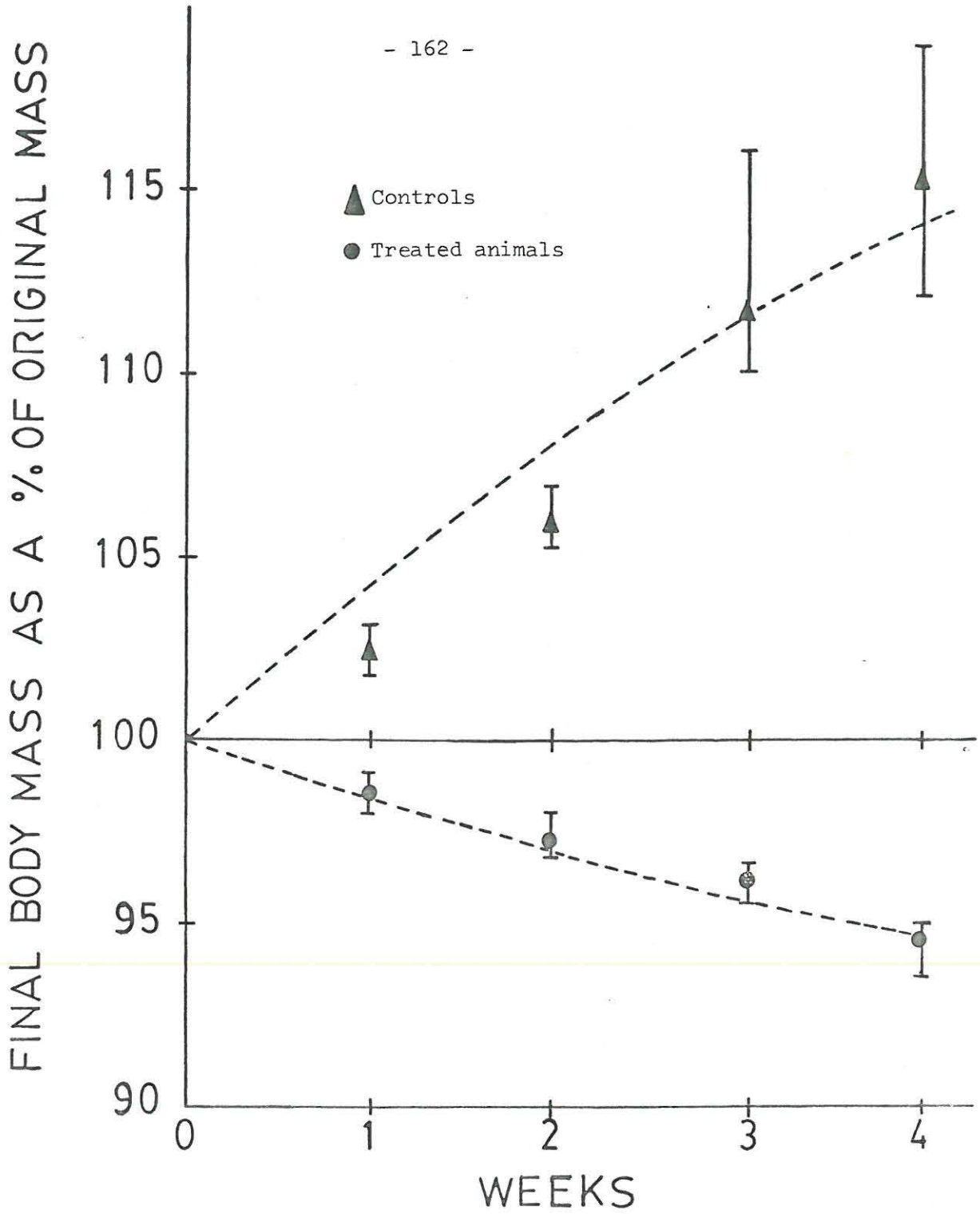


Figure 20. Graph showing the change in body mass over a period of four weeks of one group of experimental animals and of the control group. Each point represents the mean value, and range of variation, of four animals expressed as a percentage of original mass.
Animals were weighed at weekly intervals.
Dosage: 1 mg/kg norethynodrel.
Controls received ethyl oleate.

Table 16. Effect of 20 mg/kg Ethinyl Estradiol on Rat Body Mass

Days of Treatment	Original Body Mass g	Final Body Mass g	Per Cent of Original Mass g/g x 100
2	189,00 ± 4,96	190,00 ± 3,74	100,50
4	176,00 ± 5,94	171,60 ± 7,60	98,20
6	175,50 ± 7,32	171,75 ± 6,89	97,37
8	174,00 ± 5,71	170,75 ± 6,18	97,00
12	182,25 ± 5,90	177,50 ± 3,87	97,40
16	176,25 ± 6,13	167,50 ± 5,50	95,03
30	173,00 ± 6,78	162,25 ± 9,0	93,65
Controls	179,50 ± 6,55	209,00 ± 3,55	116,37

Each figure represents the mean value and range of variation of four animals. See FIGURE 21.

Table 17. Effect of 20 mg/kg Ethinyl Estradiol on Change of Rat Body Mass at Weekly Intervals.

Experiment	Per Cent of Original Mass			
	1 Week	2 Weeks	3 Weeks	4 Weeks
Controls	103,82	108,37	111,95	116,37
30 Day	97,04	95,17	94,80	93,65
12 Day	97,77	95,55*		
16 Day	96,72	95,03*		

Each figure represents the mean value and range of variation of four animals. See FIGURE 22.

* Animals were weighed on the day of sacrifice.

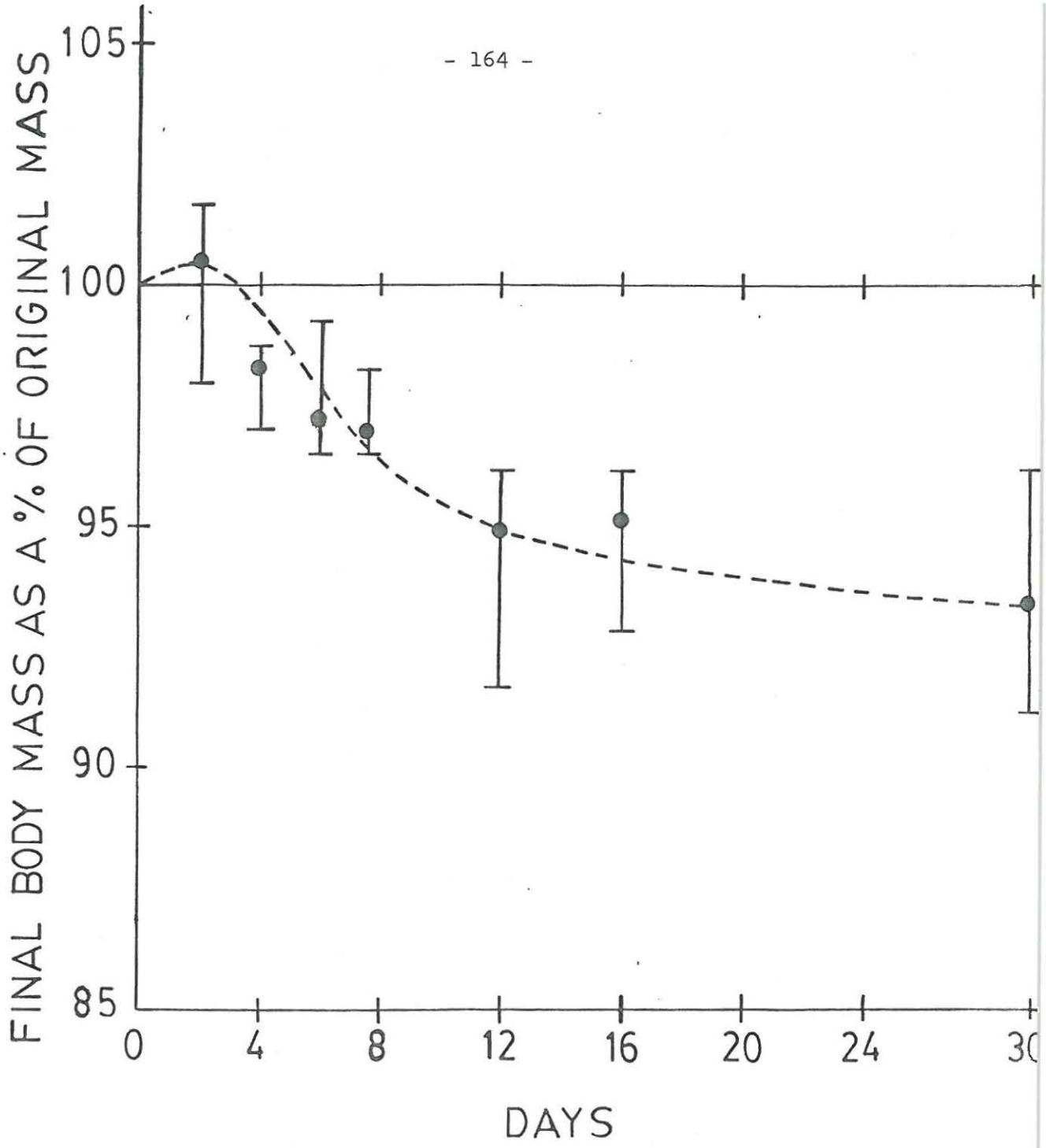


Figure 21. Graph showing the change in body mass of each group of animals on the day of sacrifice. Each point represents the mean value, and range of variation, of four animals expressed as a percentage of original mass.

Dosage: 20 mg/kg ethinyl estradiol.

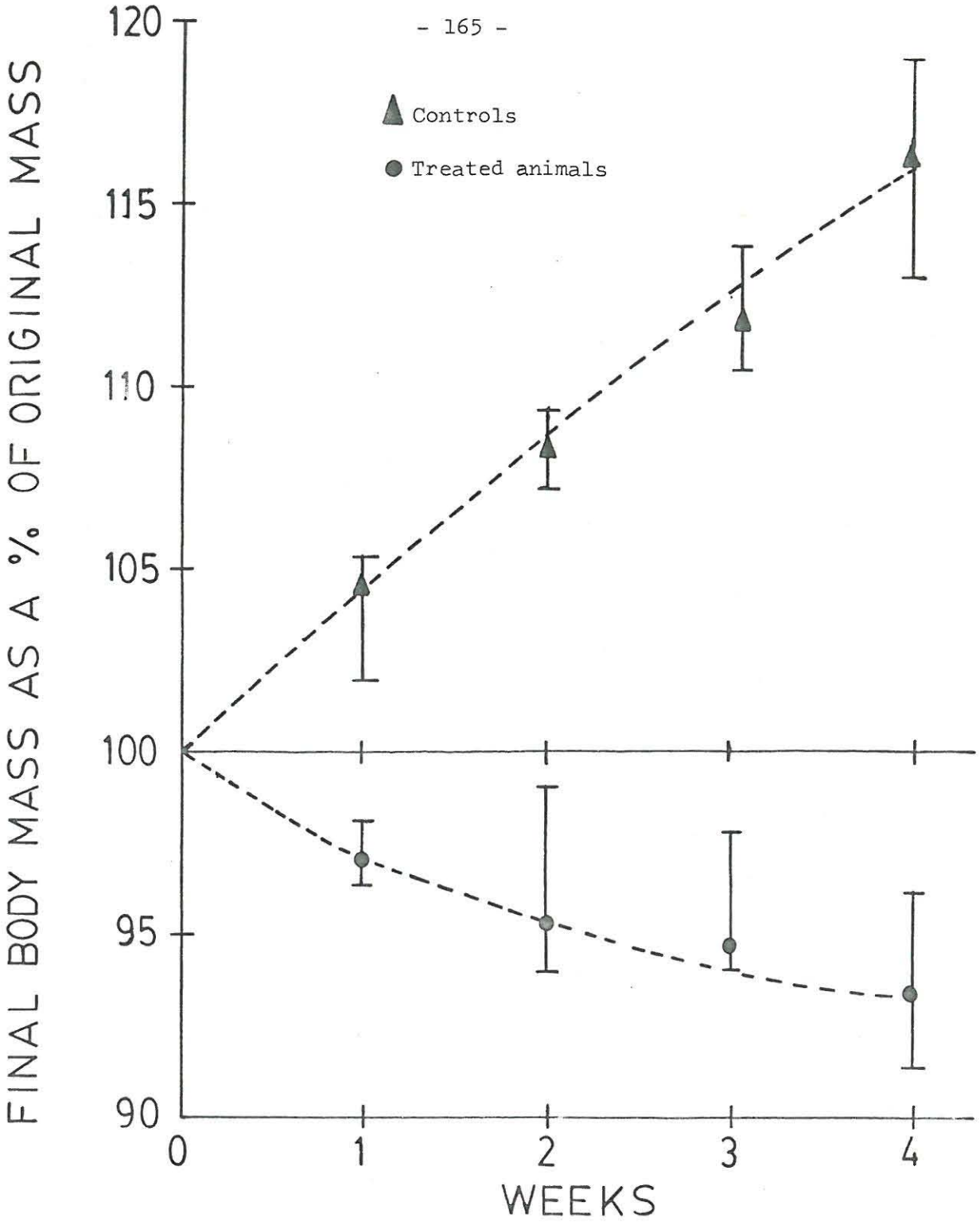


Figure 22. Graph showing the change in body mass over a period of four weeks of one group of experimental animals and of the control group. Each point represents the mean value, and range of variation, of four animals expressed as a percentage of original mass.
Animals were weighed at weekly intervals.
Dosage: 20 mg/kg ethinyl estradiol.
Controls received ethyl oleate.

Table 18. Effect of 1 mg/kg Ethinyl Estradiol on Rat Body Mass

Day of Treatment	Original Body Mass g	Final Body Mass g	Per Cent of Original Mass g/g x 100
2	179,75 ± 8,18	181,5 ± 8,96	100,93
4	174,50 ± 8,06	178,25 ± 7,71	102,17
6	178,0 ± 9,38	178,50 ± 7,50	100,20
8	170,25 ± 8,77	171,25 ± 7,84	100,60
12	182,25 ± 8,18	180,75 ± 6,94	99,00
16	177,75 ± 9,28	175,00 ± 9,66	98,40
30	179,00 ± 12,40	175,25 ± 12,73	97,89
Controls	173,00 ± 4,96	199,50 ± 2,87	115,00

Each figure represents the mean value and range of variation of four animals. See FIGURE 23.

Table 19. Effect of 1 mg/kg Ethinyl Estradiol on Change of Weekly Rat Body Mass

Experiment	Per Cent of Original Mass			
	1 Week	2 Weeks	3 Weeks	4 Weeks
Controls	103,30	105,66	112,00	115,00
30 Day	98,97	98,48	97,87	97,89
16 Day	99,20	98,40*		
12 Day	99,48	99,00*		

Each figure represents the mean value and range of variation of four animals. See FIGURE 24.

* Animals were weighed on the day of sacrifice.

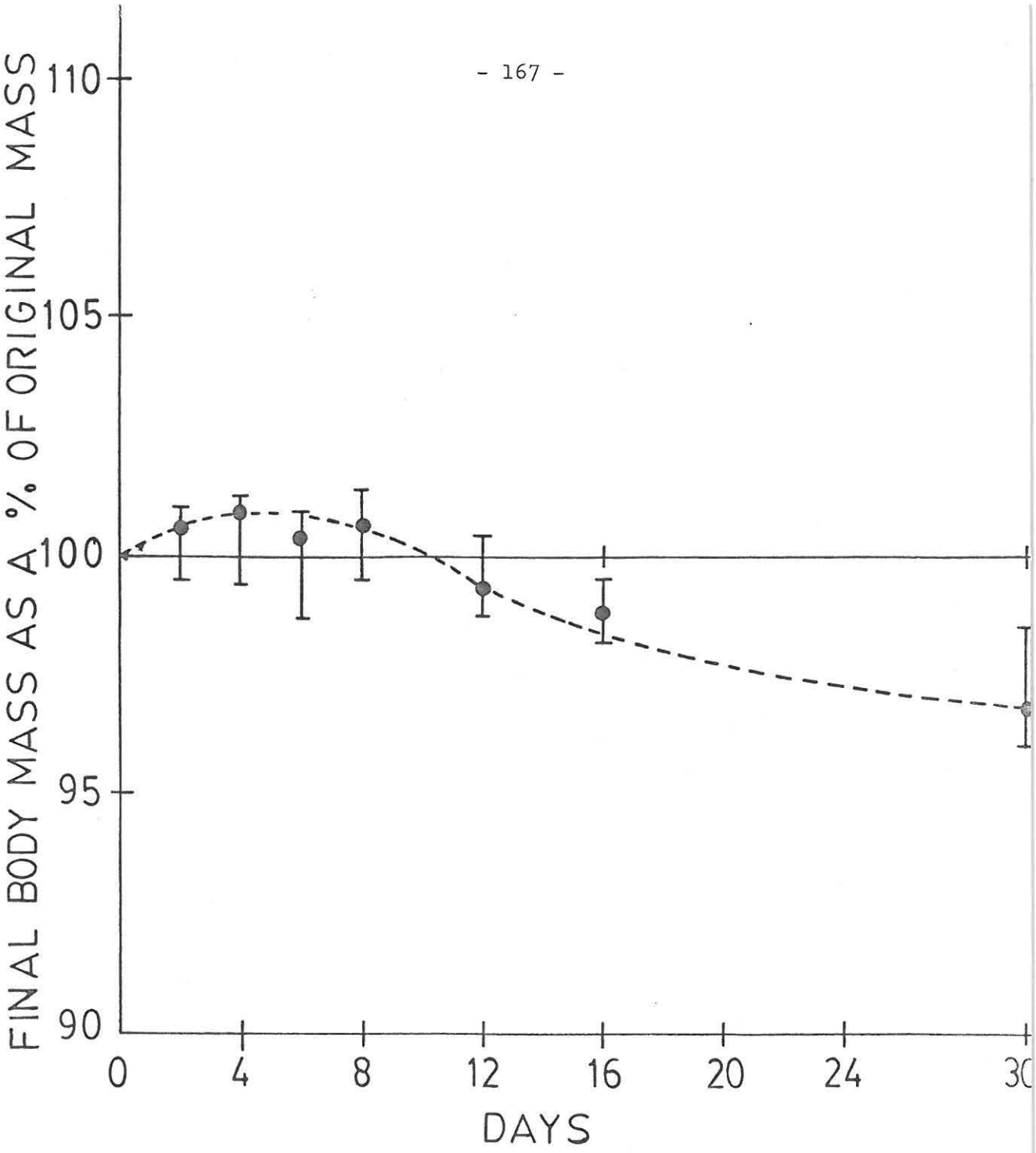


Figure 23. Graph showing the change in body mass of each group of animals on the day of sacrifice. Each point represents the mean value, and range of variation, of four animals expressed as a percentage of original mass.

Dosage: 1 mg/kg ethinyl estradiol.

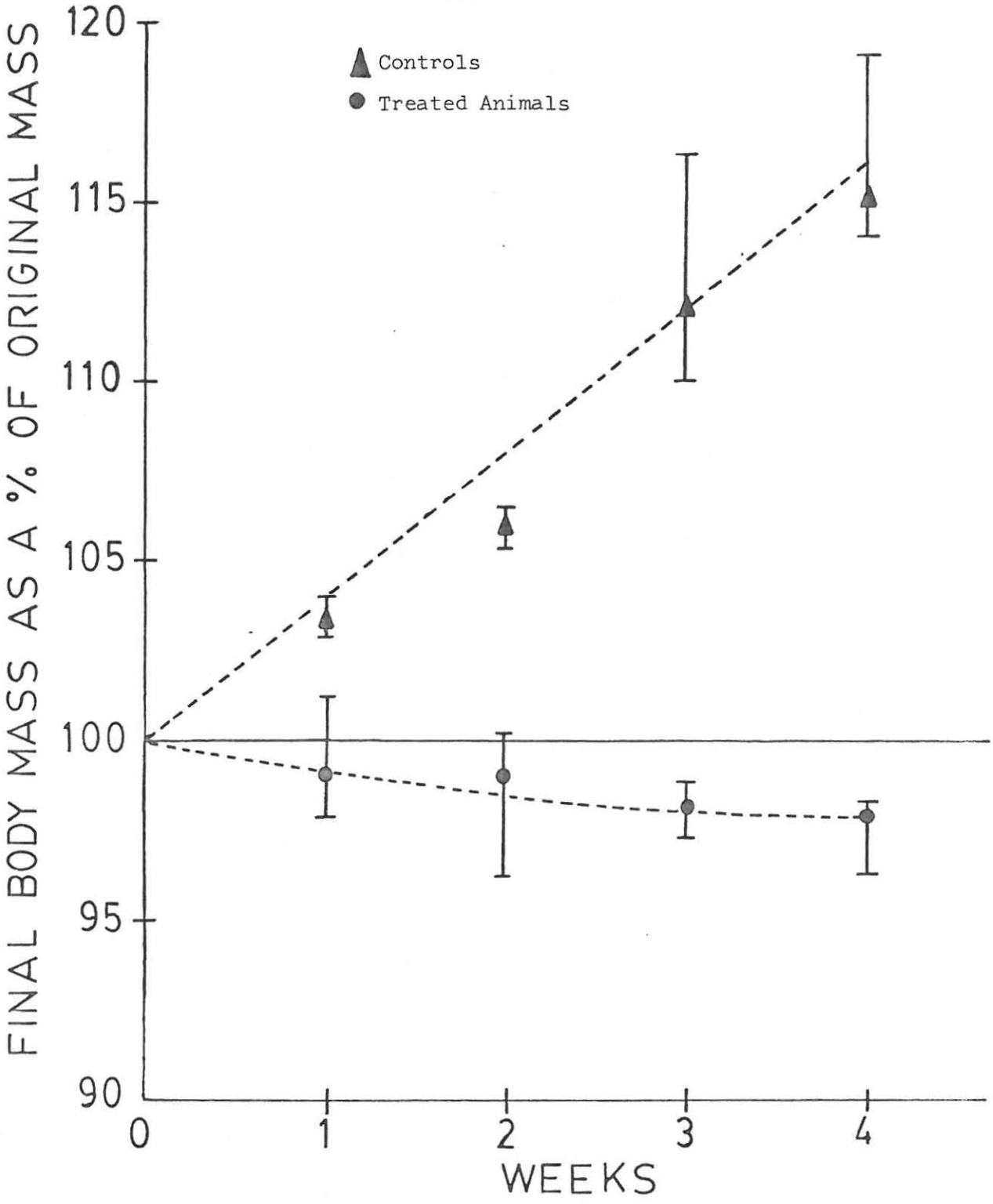


Figure 24. Graph showing the change in body mass over a period of four weeks of one group of experimental animals and of the control group. Each point represents the mean value, and range of variation, of four animals expressed as a percentage of original mass. Animals were weighed at weekly intervals.
Dosage: 1 mg/kg ethinyl estradiol.
Controls received ethyl oleate.

7.4 Effect of Oral Contraceptive Steroids on Sleeping Times

To determine the effect of oral contraceptive steroids on pentobarbital metabolism *in vivo*, sleeping times were measured every week for the 30-day, 16-day and 12-day experiments as described in Methods (6.5.3.).

The results in Table 20 show that treatment with both doses of norethynodrel caused marked shortening of the sleeping time, the 20 mg/kg dosage level being more effective. Although treatment with ethinyl estradiol at both dosage levels shortened sleeping times the difference between controls and treated animals was not significant according to the students "t" test, $p < 0,01$. In the case of the animals treated for 16 days, at the end of one week, these rats slept longer than the controls, but this difference (6 min) was not significant.

The effect of the steroid on the duration of action of pentobarbital is a reflection of its effect on the pentobarbital oxidizing system in hepatic microsomes.

7.5 Effect of Oral Contraceptives on Cells of the Vagina

7.5.1 Control Groups

From day to day the appearance of the cells in the smear was noted and the following observations recorded:

During the anoestrus, the smear consists predominantly of leucocytes, with an occasional cornified epithelial cell. The first occurrence of oestrus was marked by mucification of the vagina, followed quickly by the complete, or nearly complete disappearance of leucocytes and their replacement by large numbers of round, nucleated epithelial cells. These cells are about three times as large as the leucocytes. Once this change has taken place and for about 12 h thereafter, the animals are fertile.

Table 20. Effect of Oral Contraceptives on Sleeping Time

Experiment	Steroid	Sleeping Time (Minutes) Duration of Treatment (Weeks)		
		1	2	3
30 Day*	20 mg/kg norethynodrel	101,50 ± 37,90	104,50 ± 17,80	110,75 ± 27,40
	Controls	190,75 ± 20,08	172,75 ± 22,20	170,75 ± 30,30
16 Day*	20 mg/kg norethynodrel	105,50 ± 10,24		
	Controls	163,50 ± 23,64		
12 Day*	20 mg/kg norethynodrel	120,25 ± 30,29		
	Controls	164,00 ± 23,98		
30 Day*	1 mg/kg norethynodrel	127,50 ± 9,70	112,50 ± 11,32	128,50 ± 9,94
	Controls	171,50 ± 7,50	163,75 ± 10,50	168,75 ± 7,50
16 Day*	1 mg/kg norethynodrel	124,00 ± 20,20		
	Controls	152,50 ± 8,70		
12 Day*	1 mg/kg norethynodrel	137,00 ± 11,90		
	Controls	152,50 ± 15,80		
30 Day	20 mg/kg ethinyl estradiol	109,25 ± 14,11	123,75 ± 11,81	118,75 ± 10,81
	Controls	125,25 ± 6,50	139,50 ± 12,10	132,25 ± 5,73
16 Day	20 mg/kg ethinyl estradiol	125,50 ± 7,50		
	Controls	120,00 ± 9,30		
12 Day	20 mg/kg ethinyl estradiol	113,00 ± 6,27		
	Controls	122,00 ± 8,79		
30 Day	1 mg/kg ethinyl estradiol	135,00 ± 14,10	156,00 ± 18,70	142,00 ± 11,20
	Controls	148,00 ± 15,84	165,00 ± 13,60	159,00 ± 16,20
16 Day	1 mg/kg ethinyl estradiol	140,50 ± 11,60		
	Controls	151,50 ± 7,60		
12 Day	1 mg/kg ethinyl estradiol	148,75 ± 16,13		
	Controls	165,00 ± 8,32		

All rats received pentobarbital (30 mg/kg i.p.).

The figures represent the average obtained from four animals.

* $P < 0,01$ with respect to the controls according to students "t" test.

Desquamation of the epithelial cells occurs next and white cheesy masses of disintegrating squamous cells are found in the smear. Leucocytes now make their re-appearance and the cycle is repeated. These observations are depicted in Figure 25.

The whole cycle in the rat last about 4 days, being divided roughly into $2\frac{1}{2}$ days of anoestrus and the rest in oestrus (Perry 1971). The cycle makes its appearance at puberty, which is reduced in the rat at an age of 60 - 90 days.

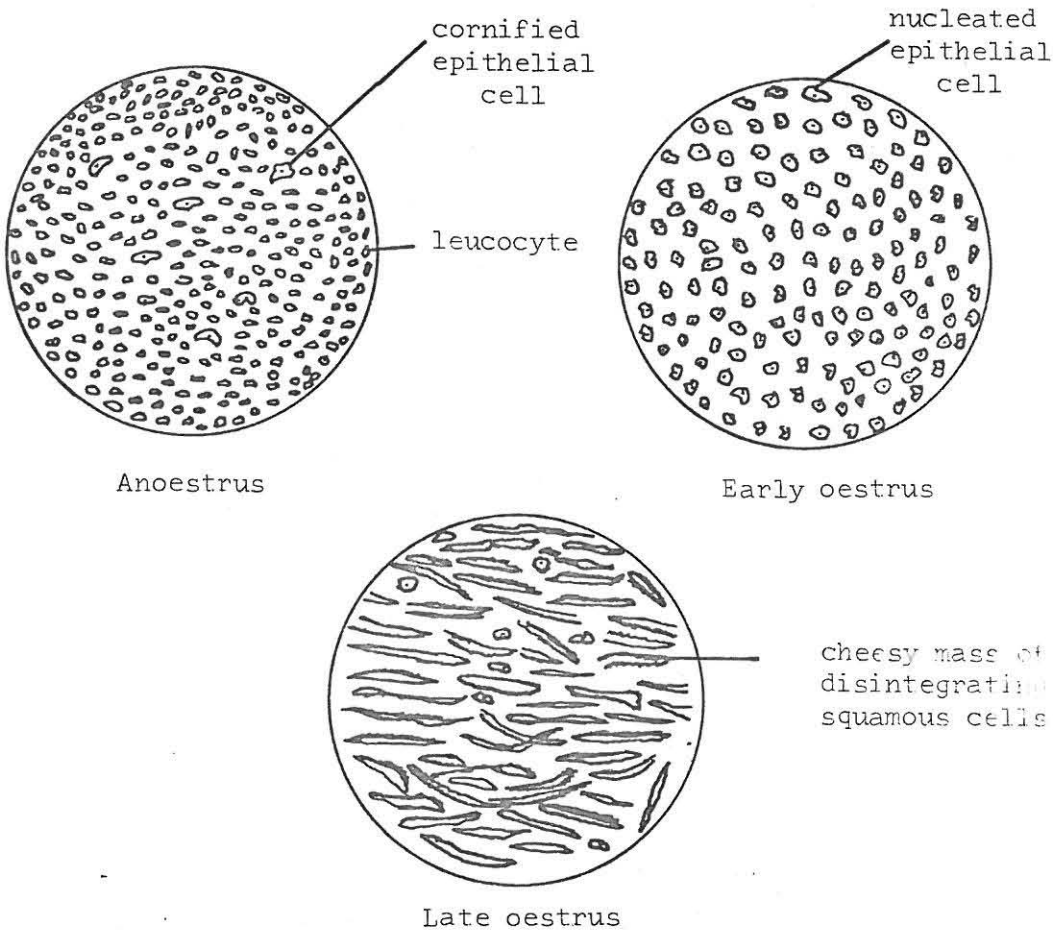


Figure 25. Cytological Changes during Normal Oestrus

7.5.2 The Effect of Norethynodrel on Vaginal Cytology

None of the animals receiving a daily dose of 20 mg/kg of norethynodrel exhibited a typical and regular oestrus cycle, as described above. Most of the rats went through an initial period during which the smears consisted of a mixture of uncornified and cornified epithelial cells with the occasional leucocyte (Figure 26). Following the initial period of "mixed smears", the animals entered a phase of prolonged vaginal cornification (Figure 27). In some animals, this persisted until they were sacrificed. In others, it was followed either by a re-appearance of the mixed smears for an irregular period, or by an atypical dioestrus phase.

The animals injected with 1 mg/kg daily for 30 days showed similar changes to those at the 20 mg/kg dosage level.

All the control animals of both treatment groups experienced regular oestrus cycles of 4 days.

7.5.3 Effect of Ethinyl Estradiol

Animals treated with 20 mg/kg or 1 mg/kg of ethinyl estradiol did not show typical oestrus cycles. For the first few days following initiation of administration of estrogen, the smear appeared to be of the "mixed type" (Figure 28). This was followed by a period of prolonged cornification (Figure 29), which usually persisted in all animals up to the time of sacrifice. The cells during the period of prolonged cornification were much larger than those produced during norethynodrel administration. In these animals, the mixed smear did not recur at any stage.

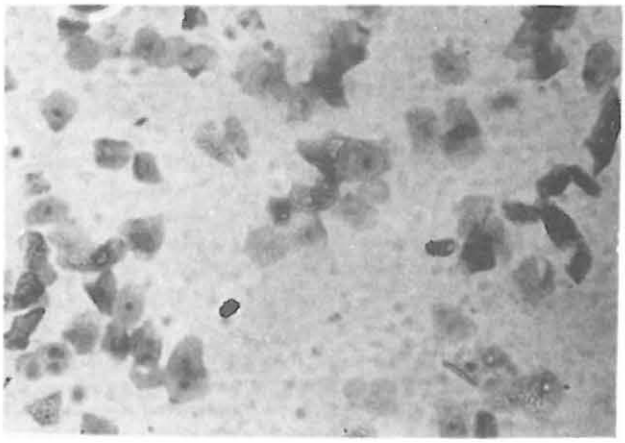


Figure 26. Vaginal smear of rat treated for 4 days with 20 mg /kg norethynodrel. The smear is of a mixed type.

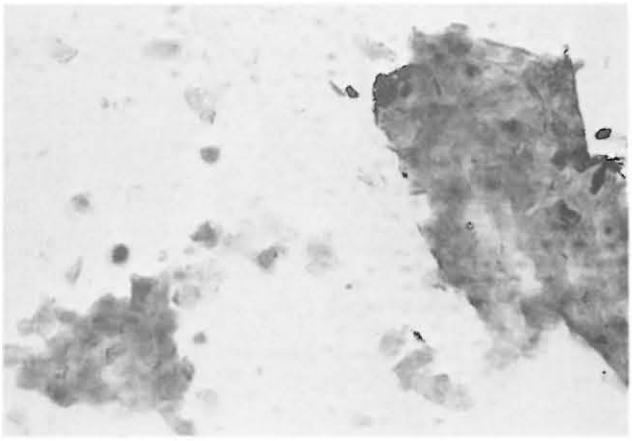


Figure 27. Vaginal smear of rat treated with 20mg/kg norethynodrel for 30 days Prolonged cornification of cells.

Figure 28. Vaginal smear of rat treated with ethinyl estradiol for 6 days. Smear is of a mixed type.

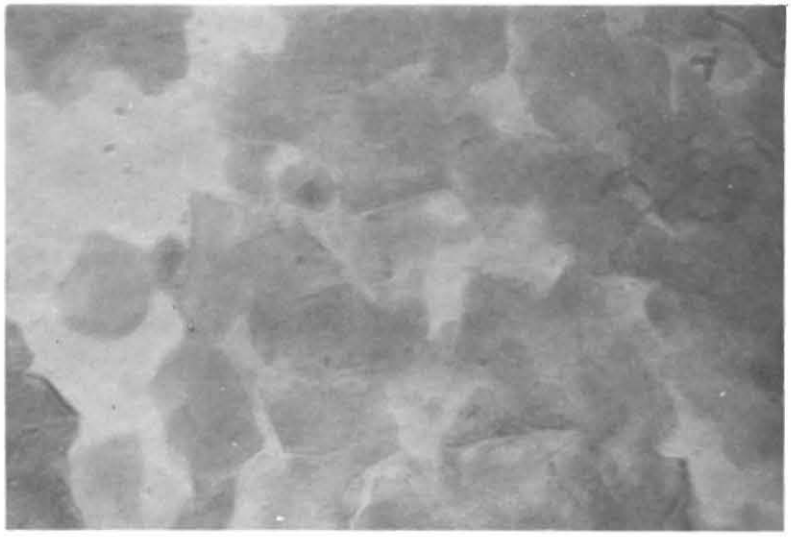


Figure 29. Vaginal smear of rat treated with ethinyl estradiol for 30 days. Prolonged cornification of cells.

7.6 The Effect of Administration of Contraceptives on Rat Liver Mass, Microsomal Protein and Cytochrome P-450 Concentration

7.6.1 Effect of Treatment 2 h before Animals Were Sacrificed

Results obtained from rats sacrificed 2 h after the last administration of steroid in all the experiments showed that such treatment did not modify the activity of these enzymes as compared with the rats sacrificed 24 h after the last steroid treatment.

7.6.2 Effect of Administration of Contraceptives on Rat Liver Mass

The mean relative liver mass (absolute liver mass in g per 100 g body mass) in groups receiving norethynodrel (both dosages, Tables 21 and 22) were markedly increased from the second day onwards, and this increase continued until the thirtieth day. Liver mass in the 20 mg/kg group of rats receiving norethynodrel was higher than that in controls. The liver mass in the group of rats receiving 1 mg/kg norethynodrel also increased, but this increase was not as large as in the 20 mg/kg group.

The animals receiving ethinyl estradiol (Tables 23 and 24) also showed an increase in liver weight relative to body mass, but the actual liver mass in each group of animals receiving a different dosage level did not appear to increase. In the case of ethinyl estradiol, the change in relative liver mass, therefore, was due entirely to decrease in body mass, while in the norethynodrel group of animals, the changes in relative body mass was due to an increase in the size of the liver, as well as to a marked decrease in body mass.

The control group of animals all showed an increase in body mass, as was expected, so that their relative liver masses were lower than in the experimental rats.

Table 21. Effect of 20 mg/kg Norethynodrel on Rat Liver Mass, Microsomal Protein and Cytochrome P-450 Concentration

Experimental Day of Sacrifice	Liver Mass, g	Body Mass, g	$\frac{\text{Liver Mass}}{\text{Body Mass}} \times 100$	Mg of Protein per g of Liver	* Cytochrome P-450 nmoles/mg Protein	+ Cytochrome P-450 nmoles/g of Liver
C2	7,80	179	4,41	23,42	0,566	0,072
D2	6,49	171	3,70	24,02	0,494	0,076
C4	8,33	170	4,90	26,27	0,606	0,072
D4	8,72	175	4,98	27,04	0,660	0,078
C6	8,92	171	5,20	28,78	0,740	0,081
D6	8,21	154	5,33	29,47	0,770	0,093
C8	8,80	178	4,94	27,64	0,726	0,084
D8	8,19	161	5,08	30,24	0,740	0,090
C12	10,90	191	5,70	31,91	0,894	0,082
D12	8,89	160	5,55	27,03	0,802	0,090
C16	8,35	161	5,18	30,03	0,956	0,110
D16	7,35	178	4,12	29,02	0,932	0,126
C30	8,43	150	5,62	31,03	1,486	0,176
D30	10,10	163	6,10	32,47	1,63	0,161
Controls						
C30	7,57	202,75	3,72	21,61	0,530	0,069
D30	8,06	207,50	3,88	21,80	0,587	0,072

C refers to a rat sacrificed 2 hours after the last steroid administration.

D refers to a rat sacrificed 24 hours after the last steroid administration.

Numbers after C or D refer to the days after which the rats were sacrificed.

Figures for experimental animals represent the values from one animal.

Figures for control animals represent the mean values of four animals, the livers of which were pooled.

*See Figure 30.

+See Figure 31.

Table 22. Effect of 1 mg/kg Norethynodrel on Rat Liver Mass, Liver Microsomal Protein and Cytochrome P-450 Concentration

Experimental Day of Sacrifice	Liver Mass, g	Body Mass, g	$\frac{\text{Liver Mass}}{\text{Body Mass}} \times 100$	Mg of Protein per g of Liver	* Cytochrome P-450 nmoles/mg Protein	+ Cytochrome P-450 nmoles/g of Liver
C2	7,22	187	3,80	21,62	0,496	0,068
D2	7,86	173	4,50	24,02	0,560	0,071
C4	7,60	184	4,13	23,72	0,541	0,071
D4	8,20	180	4,55	25,03	0,591	0,069
C6	8,65	169	5,18	25,57	0,703	0,081
D6	8,08	181	4,46	24,50	0,620	0,076
C8	8,09	170	4,75	24,77	0,770	0,095
D8	8,17	185	4,41	17,40	0,689	0,084
C12	8,14	182	4,47	26,27	0,786	0,096
D12	8,76	173	5,06	22,20	0,701	0,080
C16	8,57	172	4,98	24,52	0,753	0,087
D16	7,46	185	4,03	26,41	0,727	0,097
C30	8,60	166	5,18	31,47	0,890	0,103
D30	8,18	165	4,95	28,82	0,880	0,107
Controls						
C30	7,97	199,20	4,0	23,30	0,50	0,062
D30	7,60	200,20	3,79	22,76	0,53	0,068

C refers to a rat sacrificed 2 hours after the last steroid administration.

D refers to a rat sacrificed 24 hours after the last steroid administration.

Numbers after C or D refer to the days after which the rats were sacrificed.

Figures for experimental animals represent the values from one animal.

Figures for control animals represent the mean values of four animals, the livers of which were pooled.

*See Figure 32.

+See Figure 33.

Table 23. Effect of 20 mg/kg Ethinyl Estradiol on Rat Liver Mass, Microsomal Protein and Cytochrome P-450 Concentration.

Experimental Day of Sacrifice	Liver Mass, g	Body Mass, g	$\frac{\text{Liver Mass}}{\text{Body Mass}} \times 100$	Mg of Protein per g of Liver	Cytochrome P-450 nmoles/mg Protein	Cytochrome P-450 nmoles/g of Liver
C2	8,35	185	4,51	23,67	1,92	0,22
D2	8,01	191	4,19	22,69	1,92	0,21
C4	7,67	176	4,35	25,41	1,46	0,19
D4	7,09	180	3,93	21,44	1,29	0,18
C6	9,06	166	5,45	24,70	1,89	0,20
D6	7,72	181	4,26	22,75	1,51	0,195
C8	7,02	163	4,30	21,89	1,45	0,20
D8	7,55	172	4,38	22,59	1,30	0,17
C12	6,93	177	3,91	23,21	1,50	0,21
D12	8,01	183	4,37	23,72	1,86	0,23
C16	6,02	161	3,73	21,17	1,30	0,21
D16	7,28	165	4,41	21,96	1,49	0,20
C30	7,20	170	4,23	23,42	1,83	0,25
D30	7,46	156	4,78	23,67	1,70	0,22
Controls						
C30	7,44	209	3,54	21,70	1,79	0,24
D30	7,50	206,25	3,63	22,29	1,64	0,22

C refers to a rat sacrificed 2 hours after the last steroid administration.

D refers to a rat sacrificed 24 hours after the last steroid administration.

Numbers after C or D refer to the days after which the rats were sacrificed.

Figures for experimental animals represent the values from one animal.

Figures for control animals represent the mean values of four animals, the livers of which were pooled.

Table 24. Effect of 1 mg/kg Ethinyl Estradiol on Rat Liver Mass, Microsomal Protein and Cytochrome P-450 Concentration

Experimental Day of Sacrifice	Liver Mass, g	Body Mass, g	$\frac{\text{Liver Mass}}{\text{Body Mass}} \times 100$	Mg of Protein per g of Liver	Cytochrome P-450 nmoles/mg Protein	Cytochrome P-450 nmoles/g of Liver
C2	6,82	182	3,74	20,96	0,514	0,075
D2	7,59	194	3,91	21,74	0,486	0,064
C4	7,30	175	4,17	21,03	0,560	0,076
D4	8,02	169	4,74	22,76	0,540	0,067
C6	8,42	182	4,62	23,12	0,592	0,070
D6	7,56	170	4,47	20,84	0,492	0,065
C8	7,30	165	4,42	20,23	0,553	0,075
D8	7,57	177	4,27	21,43	0,479	0,063
C12	7,83	173	4,52	21,65	0,526	0,067
D12	8,46	185	4,57	24,21	0,560	0,066
C16	7,93	177	4,48	22,82	0,532	0,067
D16	8,60	161	5,34	23,30	0,570	0,066
C30	7,78	178	4,37	22,60	0,548	0,070
D30	8,75	190	4,60	22,80	0,593	0,068
Controls						
C30	7,62	199,25	3,83	20,06	0,506	0,074
D30	7,71	202	3,81	21,47	0,520	0,067

C refers to a rat sacrificed 2 hours after the last steroid administration.

D refers to a rat sacrificed 24 hours after the last steroid administration.

Numbers after C or D refer to the days after which the rats were sacrificed.

Figures for experimental animals represent the values from one animal.

Figures for control animals represent the mean values of four animals, the livers of which were pooled.

7.6.3 Effect of Contraceptive Treatment on Microsomal Protein and Cytochrome P-450 Concentration

Administration of norethynodrel to rats increases hepatic microsomal protein as well as cytochrome P-450 concentration, 20 mg/kg of the agent being more effective than 1 mg/kg.

From Tables 21 and 22, it appears that there is a concomitant increase in liver mass, protein concentration and cytochrome P-450 concentration. The first notable increases of these parameters when 20 mg/kg of norethynodrel is administered occurs at the fourth day and the values steadily increased up to the thirtieth day. The values from Table 21 are illustrated graphically in Figures 30 and 31 to show the increase in cytochrome P-450 and protein concentration following 20 mg/kg of norethynodrel.

At a dosage of 1 mg/kg norethynodrel per day, the effects appear less marked at first and only become marked after the twelfth day (Table 22 and Figures 32 and 33). Table 23 and Table 24 show the effects of ethinyl estradiol at both dose levels.

Both dose levels increased the relative liver mass in the treated animals. Both groups lost mass, with those receiving 20 mg/kg ethinyl estradiol losing more. Neither of the dose levels of ethinyl estradiol, however, appeared to increase liver mass, microsomal protein or cytochrome P-450 concentrations, and it appears that estrogens have a negligible effect on the hepatic microsomal enzyme system.

Examination of the results of Table 23 shows that the cytochrome P-450 levels of animals dosed with 20 mg/kg of ethinyl estradiol and that of controls appear high throughout the experiment.

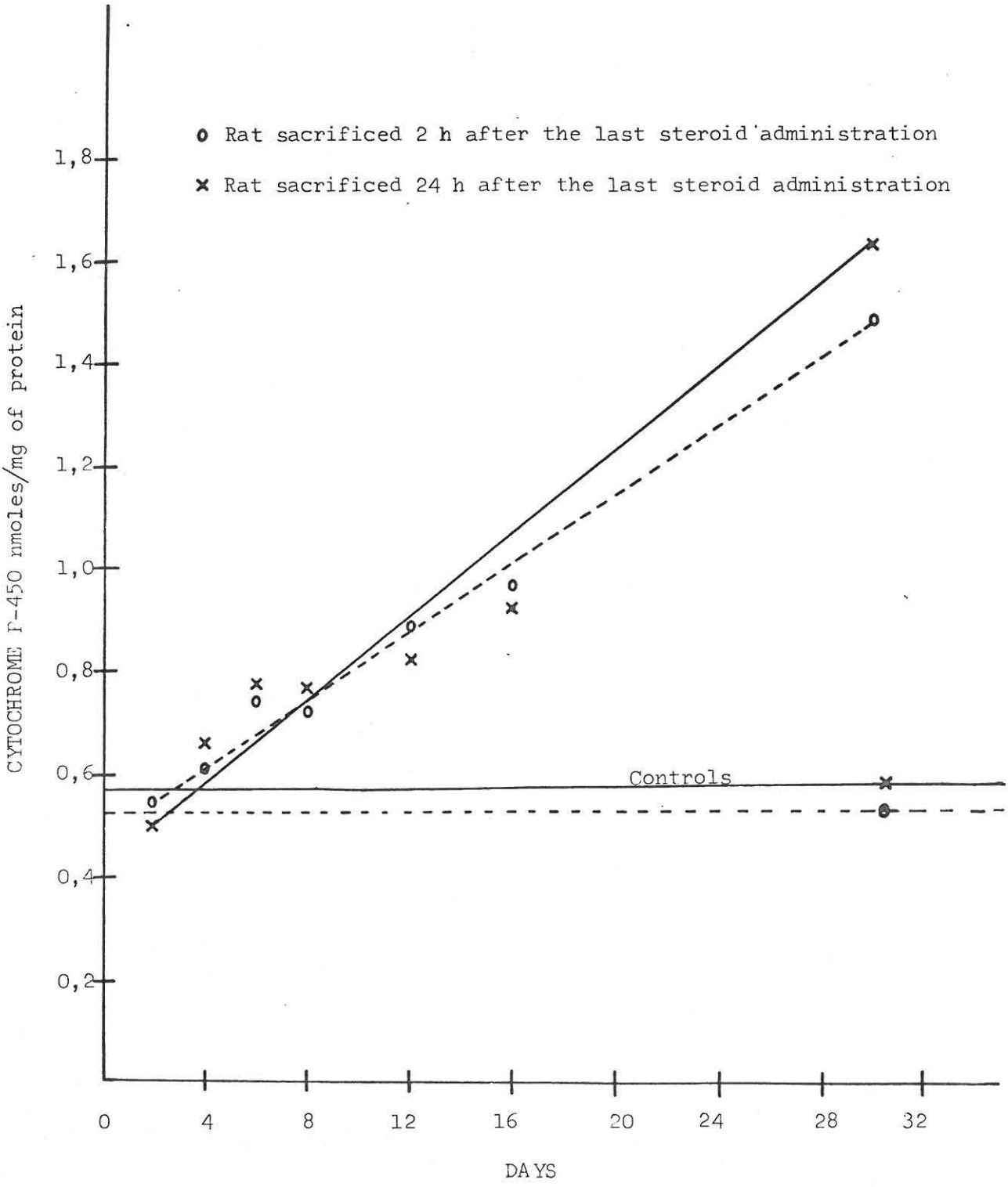


Figure 30. Graph showing increase in cytochrome P-450 per mg of protein after administration of 20 mg/kg norethynodrel. Controls received ethyl oleate.

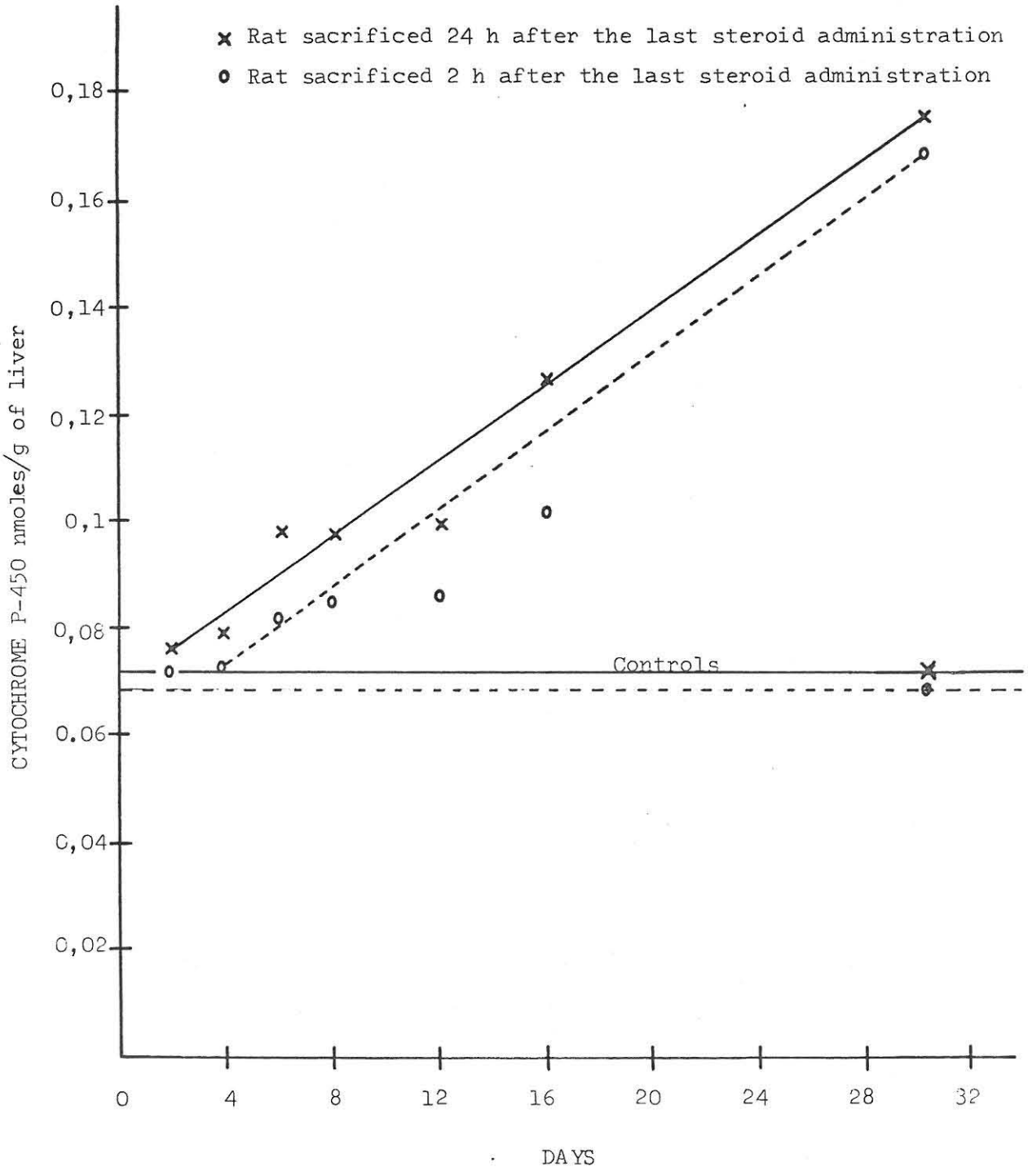


Figure 31. Graph showing increase in cytochrome P-450 per g of liver after administration of 20 mg/kg norethynodrel. Controls received ethyl oleate.

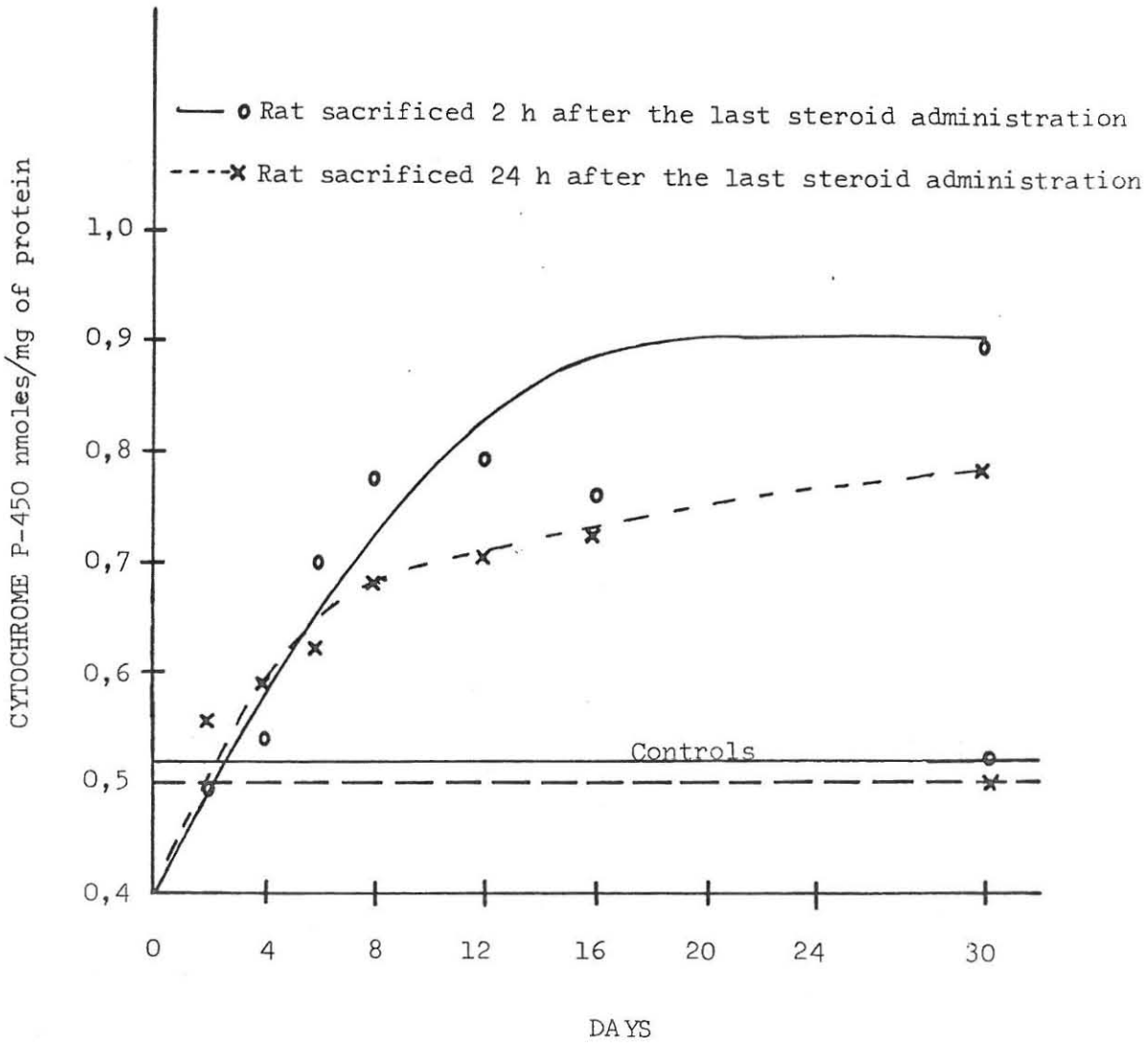


Figure 32. Graph showing increase in cytochrome P-450 per mg of protein after administration of 1 mg/kg norethynodrel. Controls received ethyl oleate.

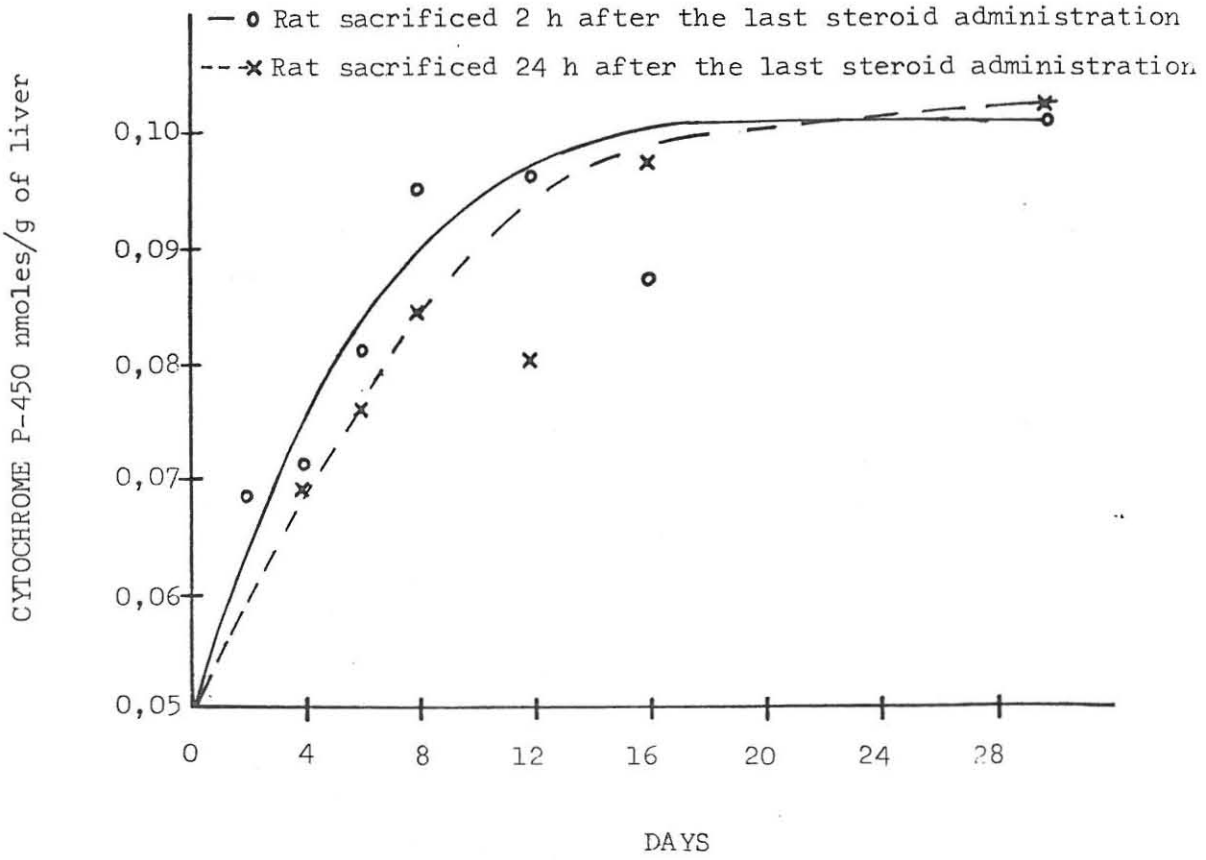


Figure 33. Graph showing the increase in cytochrome P-450 per gram of liver after administration of 1 mg/kg norethynodrel.

The high cytochrome P-450 concentration in the control animals (which were not significantly different from the experimental animals) suggest that these animals were induced prior to the intiation of ethinyl estradiol pretreatment. Maximal induction of the hepatic microsomal enzyme system of these rats by an unknown inducer could have obscured any inductive effect on the part of ethinyl estradiol. For this reason, an experiment was conducted in order to assess whether or not the remaining members of this group of rats had been maximally induced prior to treatment with the estrogen.

A group of eight rats were divided into two equal groups, one group serving as control, the other group receiving a daily dose of 80 mg/kg phenobarbital (a known potent inducer) intra-peritoneally for four days. Control animals received the same amount of distilled water as was used for administering the drug. Twenty-four h after the last phenobarbital injection, the rats in both groups were sacrificed by a blow on the head. Livers from animals in the same treatment group were pooled and microsomes were prepared from the equivalent of 2,5 g of wet mass of liver from each group. Cytochrome P-450 and microsomal protein determinations were carried out and the results are presented in Table 25.

Table 25. Increased Liver Protein and Cytochrome P-450 in Phenobarbital Treated Rats

	Mean Wet Mass of Liver, g	Microsomal Protein mg/Liver	Cytochrome P-450 nmoles/mg Protein	Cytochrome P-450 nmoles/g Liver
Control	7,67	22,10	1,74	0,223
Phenobarbital	9,31	28,87	3,00	0,322

The results from this experiment indicate that the hepatic microsomal enzyme system of these rats was not maximally induced since treatment with phenobarbital elicited a large response. Thus, it was concluded that if ethinyl estradiol could exert any inductive effect, a response would have been noted, provided that the mechanism of induction of ethinyl estradiol is the same as for phenobarbital.

7.6.4 Enzymatic Assays

Table 26 summarizes the effect of OCs on the hepatic microsomal enzyme activity with respect to aniline hydroxylase.

Norethynodrel at both dose levels was effective in increasing aniline hydroxylase activity significantly. Ethinyl estradiol also increased aniline hydroxylase activity but not significantly.

Table 27 shows the effect of 20 mg/kg of norethynodrel and ethinyl estradiol. Enzyme activity for the low dose (1 mg/kg) of these steroids was not determined.

Norethynodrel significantly increased aminopyrine demethylation, while ethinyl estradiol had only a negligible effect.

7.7 Electron Microscopy

Samples of liver, adrenal and ovary were subjected to electron microscopic investigation.

Animals 2 or 24 h after the last steroid administration were sacrificed for electron microscopy as described under Methods (6.13). No difference in the morphology was observed between the 2 or 24 h samples; thus, in this report, the effect of the administration of oral contraceptive steroids on the various organs is assessed by comparison with the cytological appearance of the control groups. No allowance is given to the time of sacrifice of the animals (i.e., 2 or 24 h after the last injection).

Table 26. Effect of Dosing with Contraceptive Agents on Aniline Hydroxylase Activity

Treatment	mg/kg for 12 Days	p-aminophenol Formed per mg Microsomal Protein (nmoles/mg/20 min.)	Microsomal Protein mg/g Liver	Cytochrome P-450 nmoles/mg Protein	Cytochrome P-450 nmoles/g Liver
Norethynodrel	1	9,28	23,59	1,92	0,24
Controls		7,39	21,70	1,78	0,22
Norethynodrel	20	10,60	26,40	2,18	0,27
Controls		6,20	22,70	1,66	0,20
Ethinyl Estradiol	1	7,42	22,81	1,71	0,21
Controls		7,09	22,45	1,64	0,20
Ethinyl Estradiol	20	7,14	22,58	1,81	0,22
Controls		6,80	21,30	1,74	0,21

Each figure represents the mean value for four animals.

Rats were sacrificed 24 hours after the 12th day of dosing.

Enzymatic activity is represented by the amount of metabolite formed from microsomes from 250 mg of fresh liver.

Table 27. Effect of Dosing with Contraceptive Agents on Aminopyrine N-Demethylase Activity

Treatment	mg/kg for 12 Days	Formaldehyde Formed per mg Microsomal Protein nmoles/mg/20 min.)	Microsomal Protein mg/g Liver	Cytochrome P-450 nmoles/mg Protein	Cytochrome P-450 nmoles/g Liver
Controls	-	2,67	20,96	1,56	0,19
Norethynodrel	20	3,44	23,21	1,73	0,21
Ethinyl Estradiol	20	2,85	21,40	1,51	0,188

Each figure represents the mean value for four animals.

Rats were sacrificed 24 hours after the 12th day of dosing.

Enzymatic activity is represented by the amount of metabolite formed from microsomes from 250 mg of fresh liver.

7.7.1 The Effect of Administration of Oral Contraceptives on the Liver

Control Groups:

The fine hepatic structure in the control groups receiving ethyl oleate conformed to published descriptions of normal rat liver (Fawcett 1967; Sandborn 1970). Figures 34 and 35 illustrate electron micrographs of hepatocytes from control animals.

7.7.2. Effect of Steroids on the Ultrastructure of the Liver

The gross structure of the livers in all the experimental rats were preserved throughout the experiments; there was no evidence of necrosis, inflammation or fibrosis. However, cytologic changes had occurred in all the norethynodrel treated rats. On the other hand, ethinyl estradiol did not appear to alter the ultrastructure of the liver markedly.

7.7.2.1 Norethynodrel

Treatment with 20 mg/kg of norethynodrel:

An electron micrograph of a section through a hepatic parenchymal cell from control animals sacrificed after 30 days of ethyl oleate treatment is shown in Figures 34 and 35.

Following administration for two days, there was an apparent increase in smooth endoplasmic reticulum (SER).

After four days of treatment, large areas of the cytoplasm were filled with closely packed vesicles of SER. The cisternae of the rough endoplasmic reticulum (RER) were shortened and, in some instances, vesiculated, and sites of continuity between rough and smooth endoplasmic reticulum were noted frequently (Figure 36).

In addition, some of the mitochondria in these cells appeared rounder and swollen after the fourth day of steroid treatment.

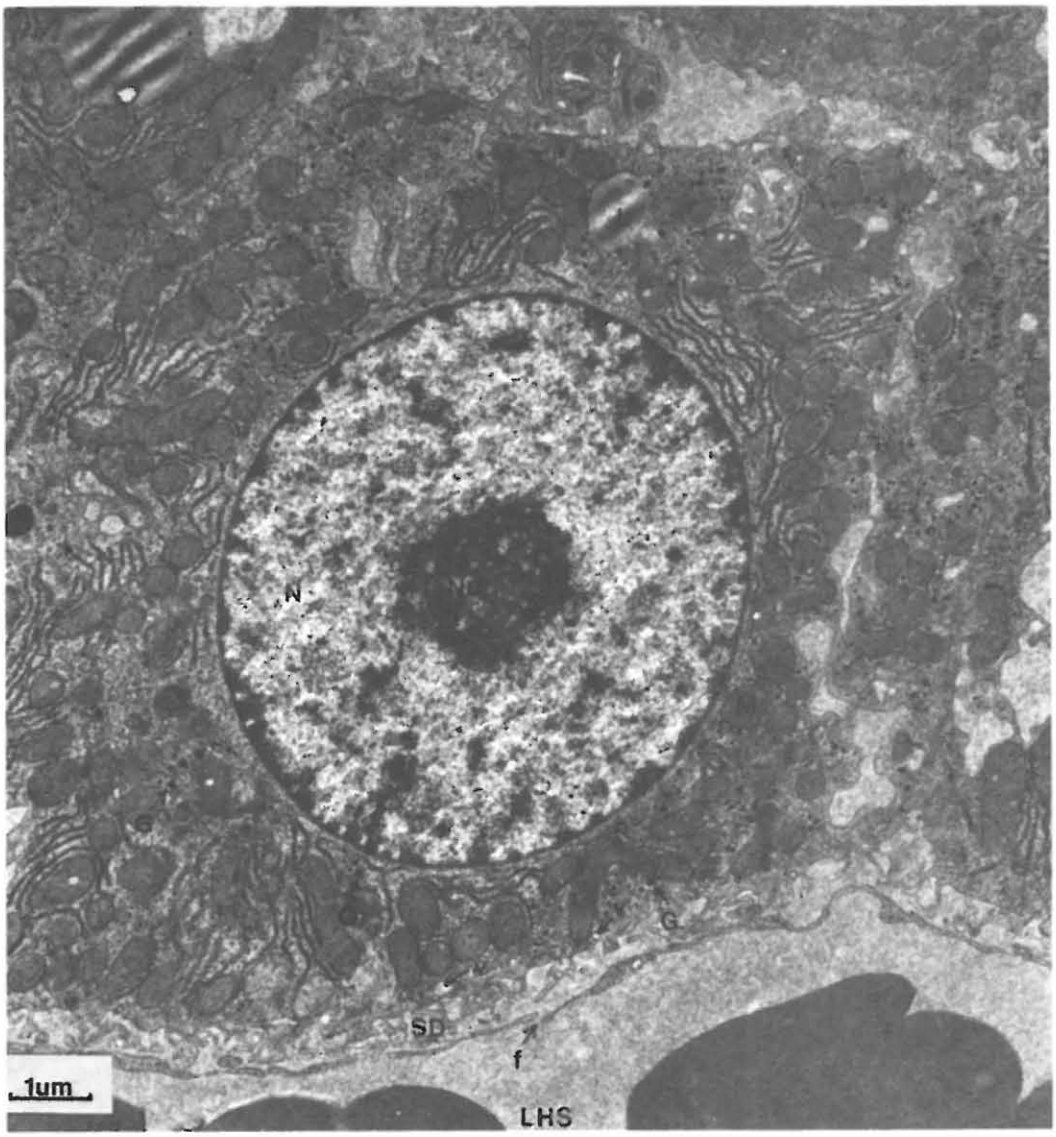


Figure 34. Liver from control rat treated with ethyl oleate for 30 days. Normal histologic portion of a hepatocyte showing characteristic features. x 11 000

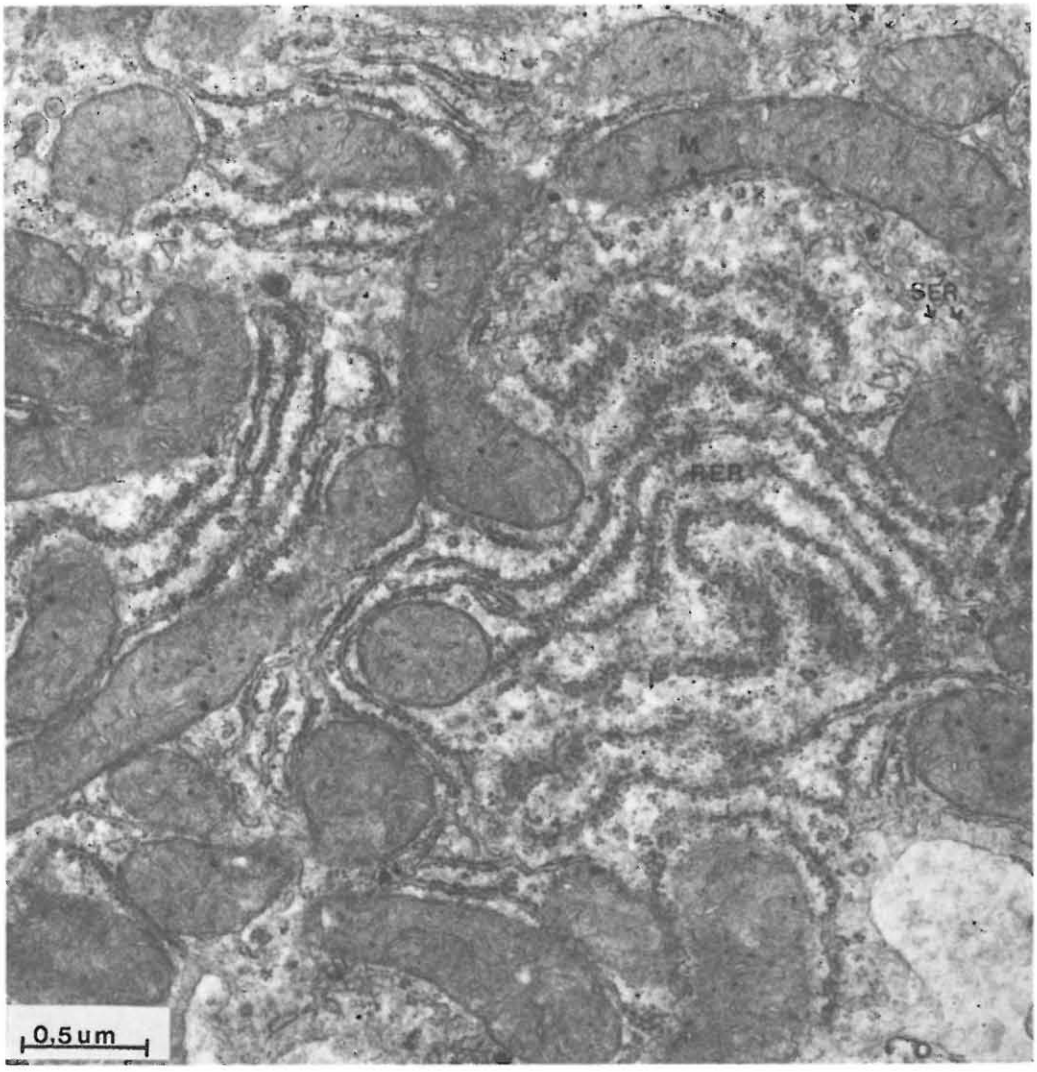


Figure 35. Hepatocyte from a control rat treated with ethyl oleate for 30 days. Note the prominent rough endoplasmic reticulum, the cisternae of which are arranged in more or less parallel arrays. Mitochondria are closely associated with the rough endoplasmic reticulum. x 37 400

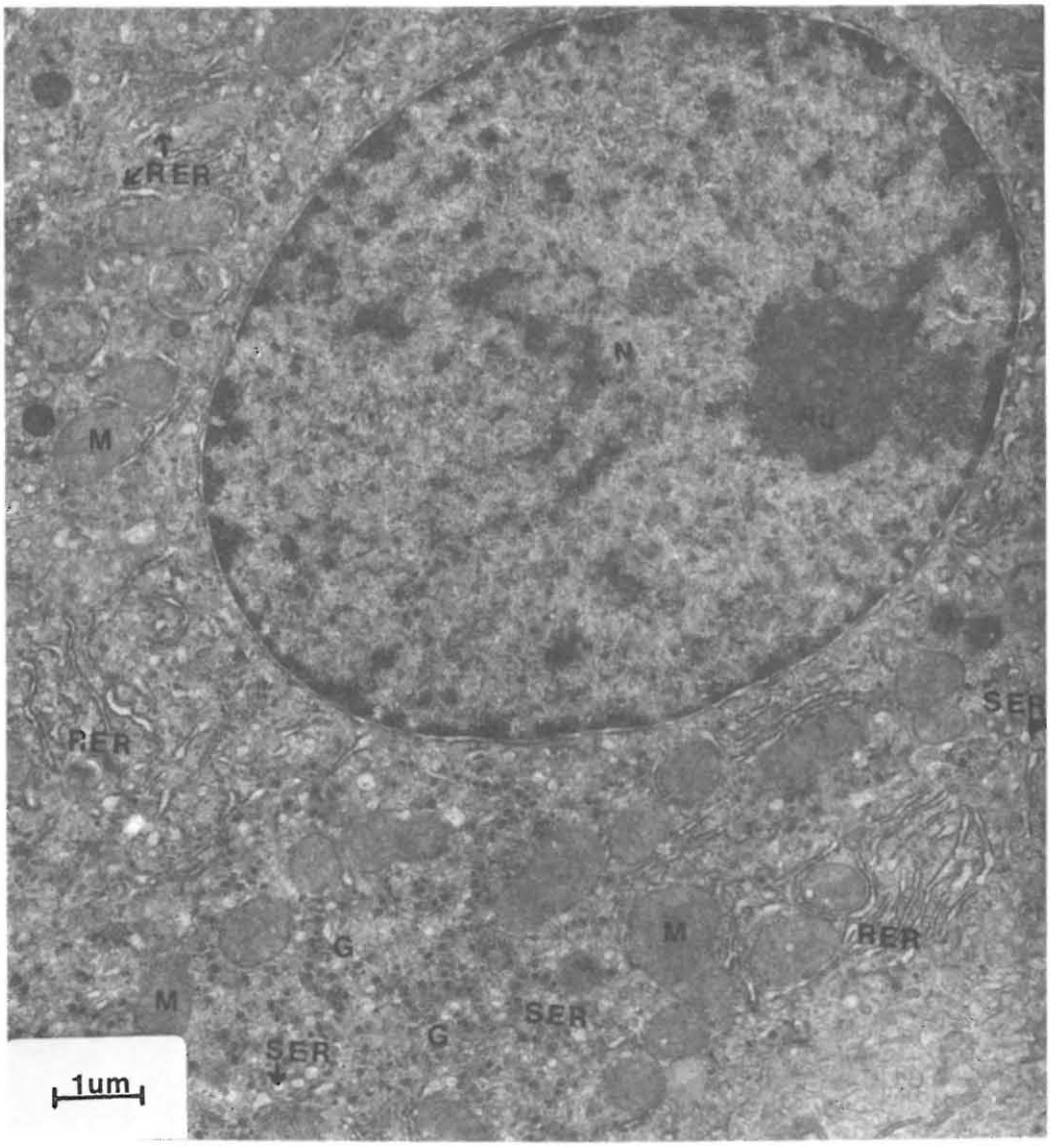


Figure 36. Hepatocyte from a rat treated for 4 days with 20 mg/kg norethynodrel showing the slight increase in smooth endoplasmic reticulum and shortened cisternae of rough endoplasmic reticulum (arrowed). x 10 800

By the sixth day, all these abnormalities present after four days of treatment were seen in cells with the swollen mitochondria now prominent (Figures 37 and 38). Glycogen lakes were no longer present in the altered hepatocytes, although in most of them glycogen particles were scattered between vesicles of the SER (Figure 37). In some cells, however, the regions of vesicles were completely devoid of glycogen. Following treatment for 8, 12, 16 and 30 days, the ultrastructural changes observed were similar to those described for the sixth day. Figure 39 shows a hepatocyte of a rat following 16 days of administration of norethynodrel. The abnormal cells invariably showed a marked proliferation of SER and a decrease in the number and length of cisternae of RER. After 30 days of treatment, the RER was considerably decreased in quantity as well as in length (Figures 40, 41 and 42).

7.7.2.2 Treatment with 1 mg/kg of Norethynodrel

An electron micrograph of a hepatocyte from a control rat sacrificed after 30 days is shown in Figure 43; the cell organelles conformed to those of a normal liver cell.

The effect of 1 mg/kg of norethynodrel on liver ultrastructure shows similar trends as those caused by the 20 mg/kg dose level, except that the effect is less marked and the onset of changes occurs only after the eighth day of steroid regimen and does not become marked until the twelfth day of steroid administration. Figure 44 shows the liver structure after four days of dosing. No apparent change has occurred; glycogen lakes are abundant, mitochondria appear normal and RER is prominent and SER sparse. As steroid treament was prolonged, the increase in SER became more apparent as evidenced on the 6th and 8th day. Following twelve days of treatment, the proliferation of SER became marked (Figure 45). Figure 46 shows the change occurring after 16 days.

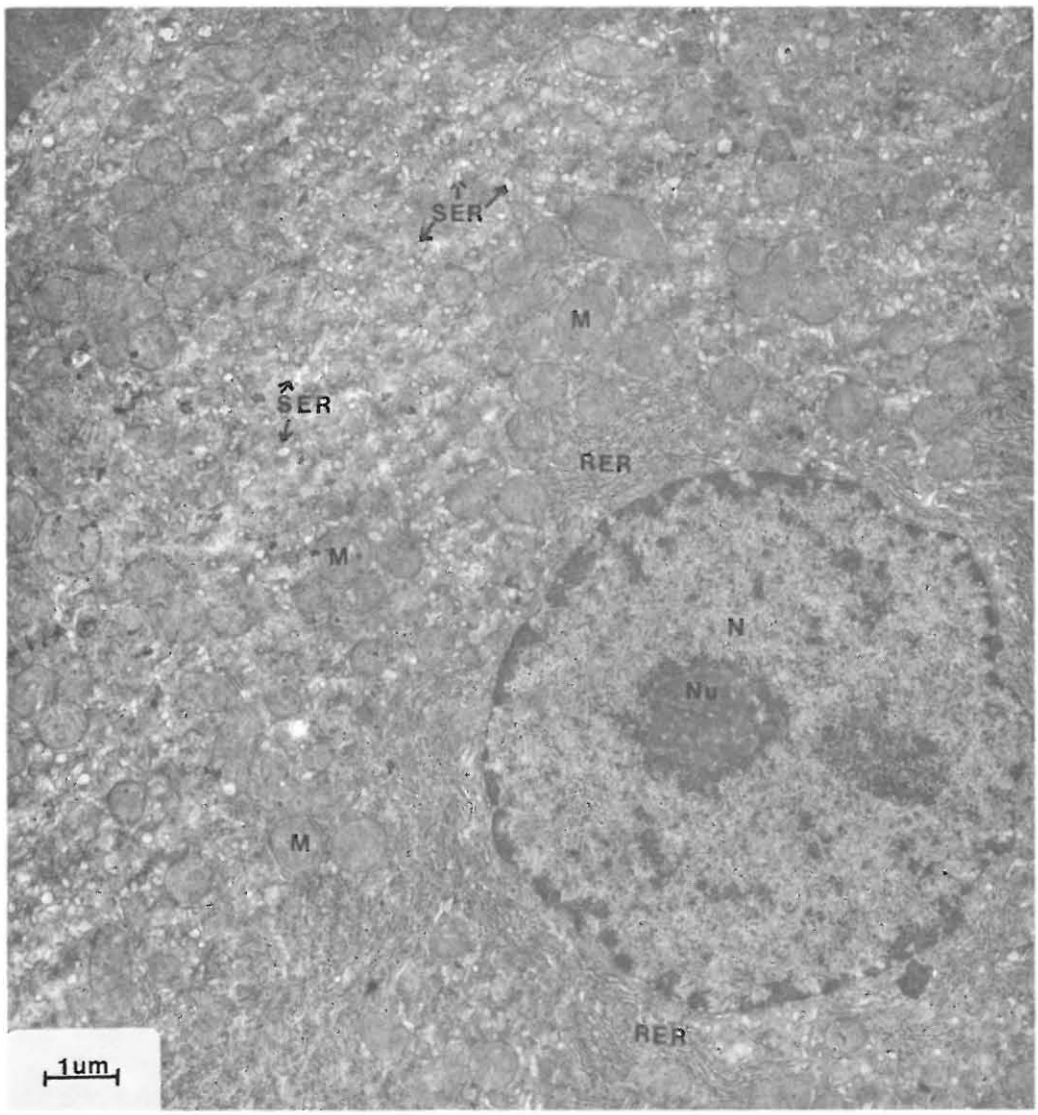


Figure 37. Hepatocyte from rat treated for 6 days with 20 mg/kg norethynodrel. Smooth endoplasmic reticulum has increased markedly, and mitochondria appear more swollen and rounder than controls, glycogen lakes have disappeared. x 10 800

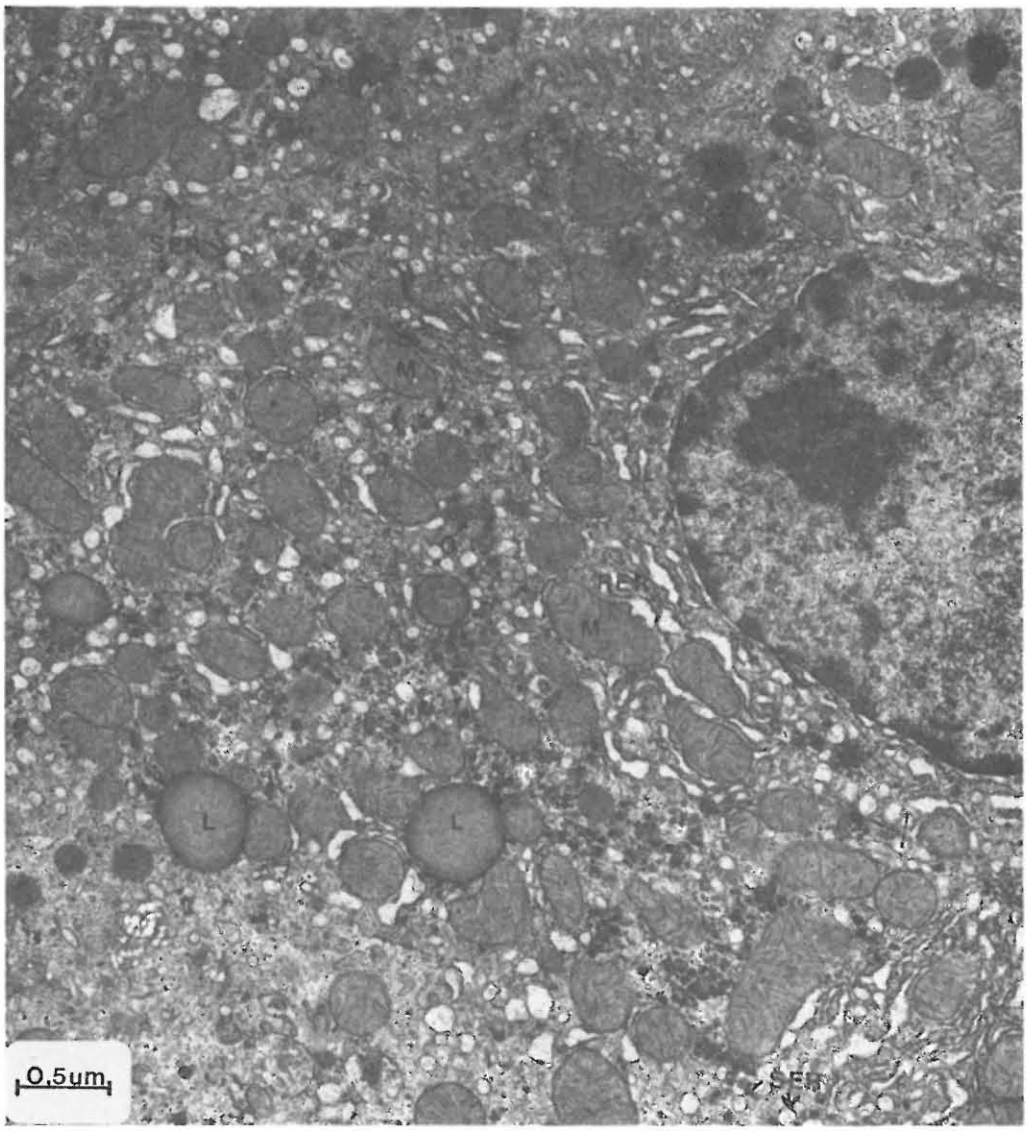


Figure 38. Hepatocyte from a rat treated for 8 days with 20 mg/kg norethynodrel. In this hepatocyte glycogen still appears prominent. Rough endoplasmic reticulum is considerably shortened and smooth endoplasmic reticulum has increased markedly. Note sites of continuity between rough and smooth endoplasmic reticulum (marked c). x 13 000

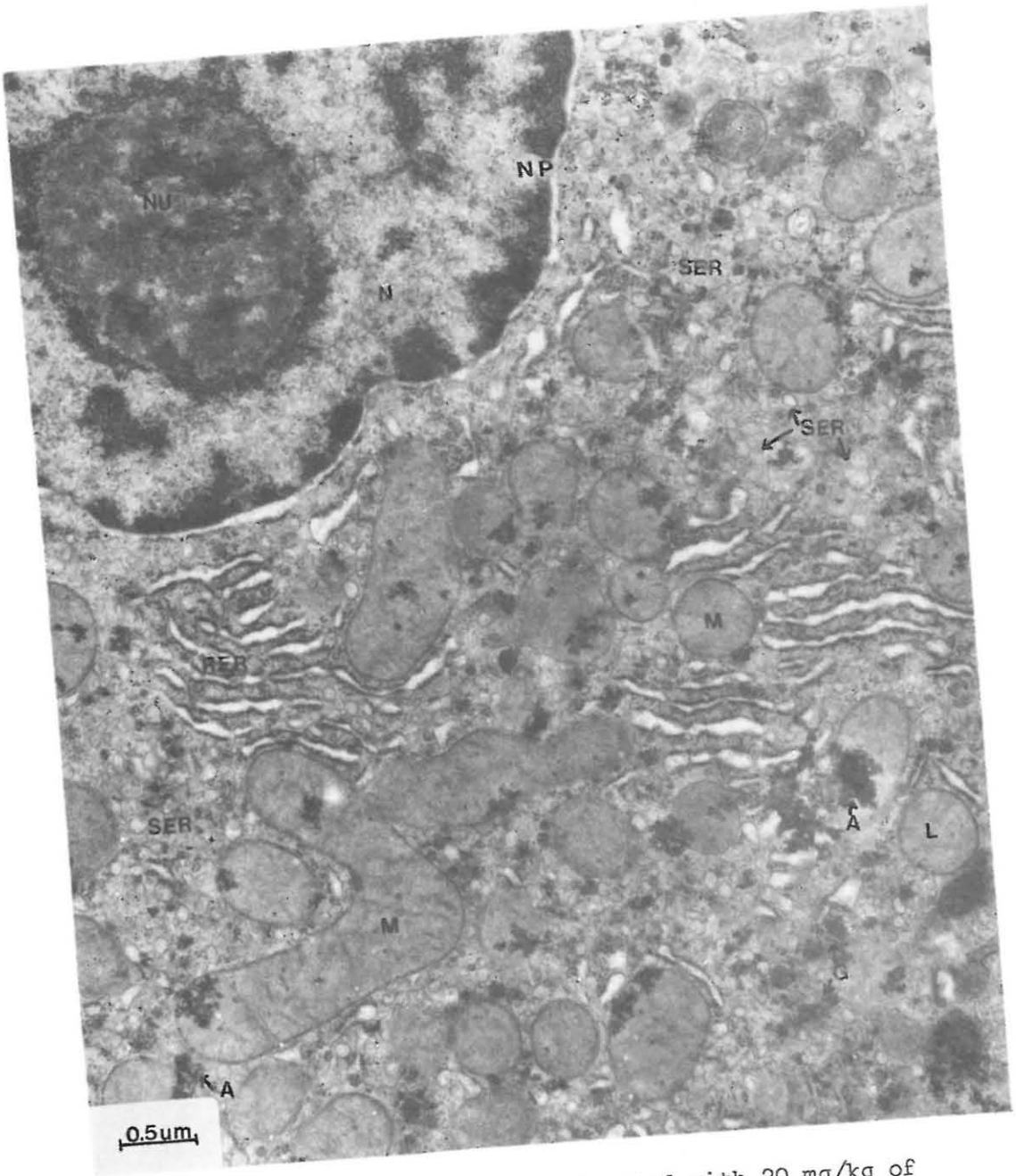


Figure 39. Hepatocyte from a rat treated with 20 mg/kg of norethynodrel for 16 days. Proliferation of smooth endoplasmic reticulum, depleted glycogen stores, much shortened rough endoplasmic reticulum and swollen mitochondria. x 26 400

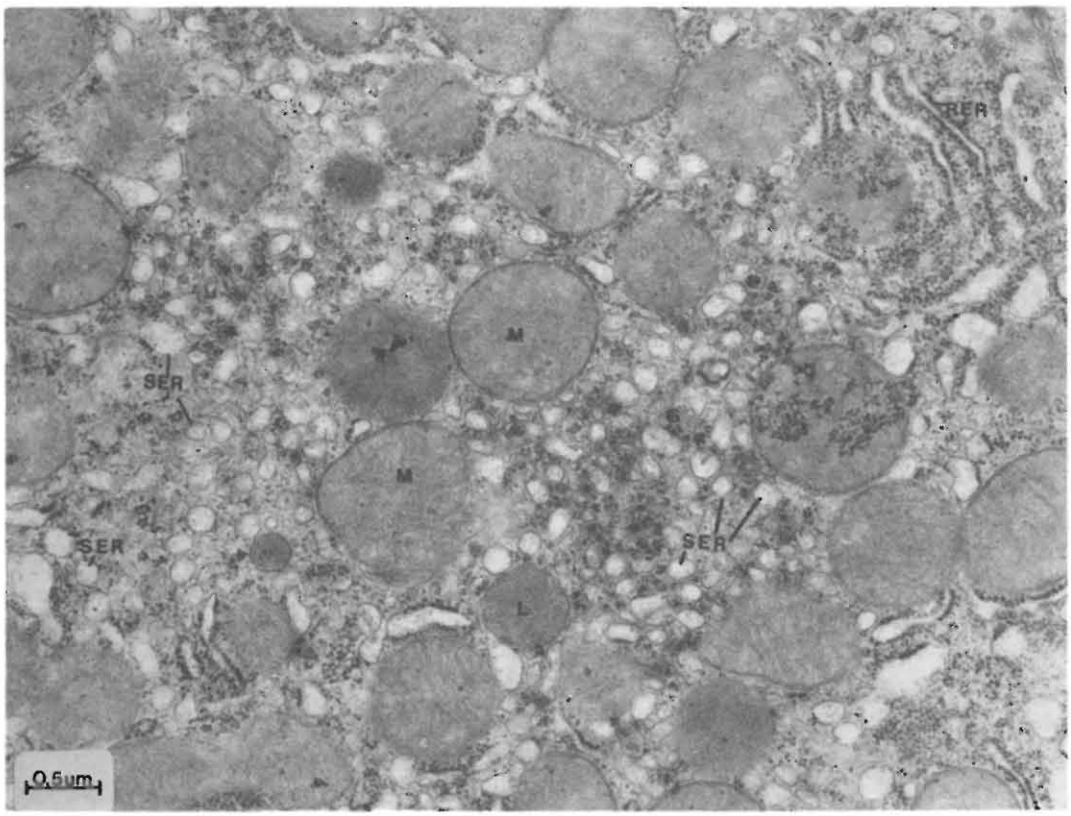


Figure 40. Hepatocyte from a rat treated with 20 mg/kg of norethynodrel for 30 days. Massive proliferation of smooth endoplasmic reticulum, depleted glycogen stores and swollen and rounded mitochondria. x 34 500

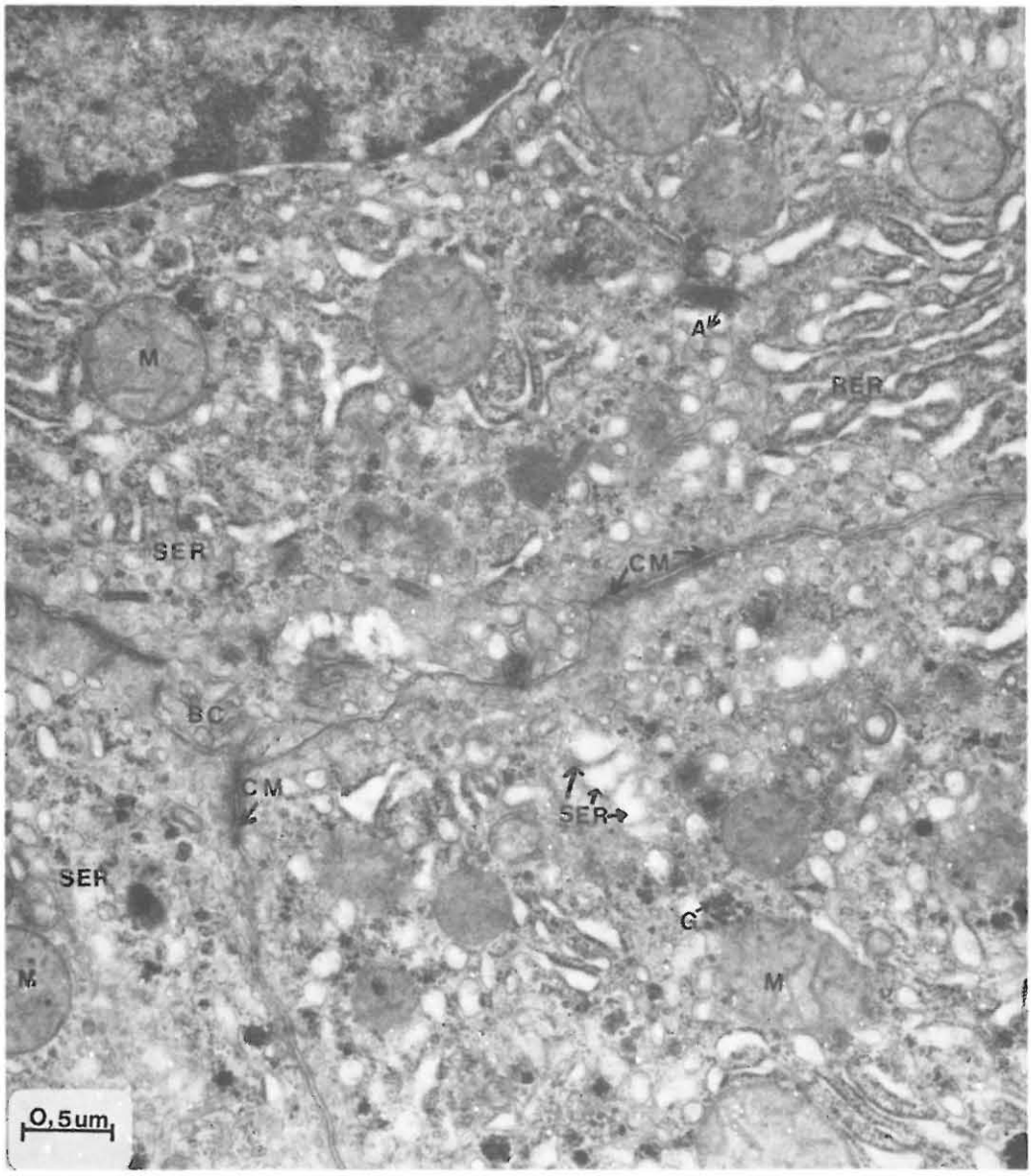


Figure 41. Portion of three hepatocytes from a rat treated with 20 mg/kg of norethynodrel for 30 days. Shortened rough endoplasmic reticulum also markedly decreased in number. x 26 400

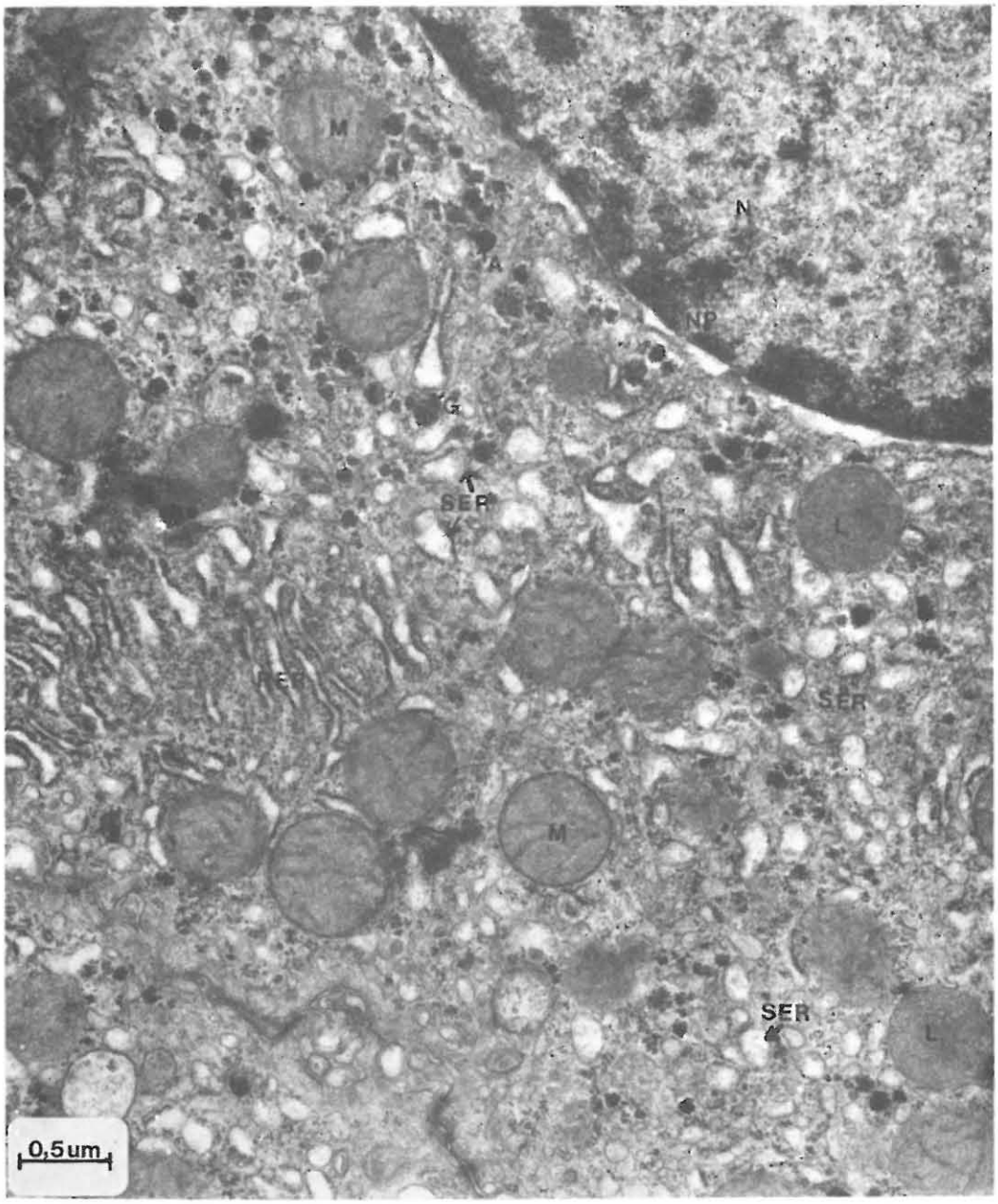


Figure 42. Hepatocyte from a rat treated with 20 mg/kg of norethynodrel for 30 days showing similar changes in ultrastructure to a rat treated for 16 days. x 26 400

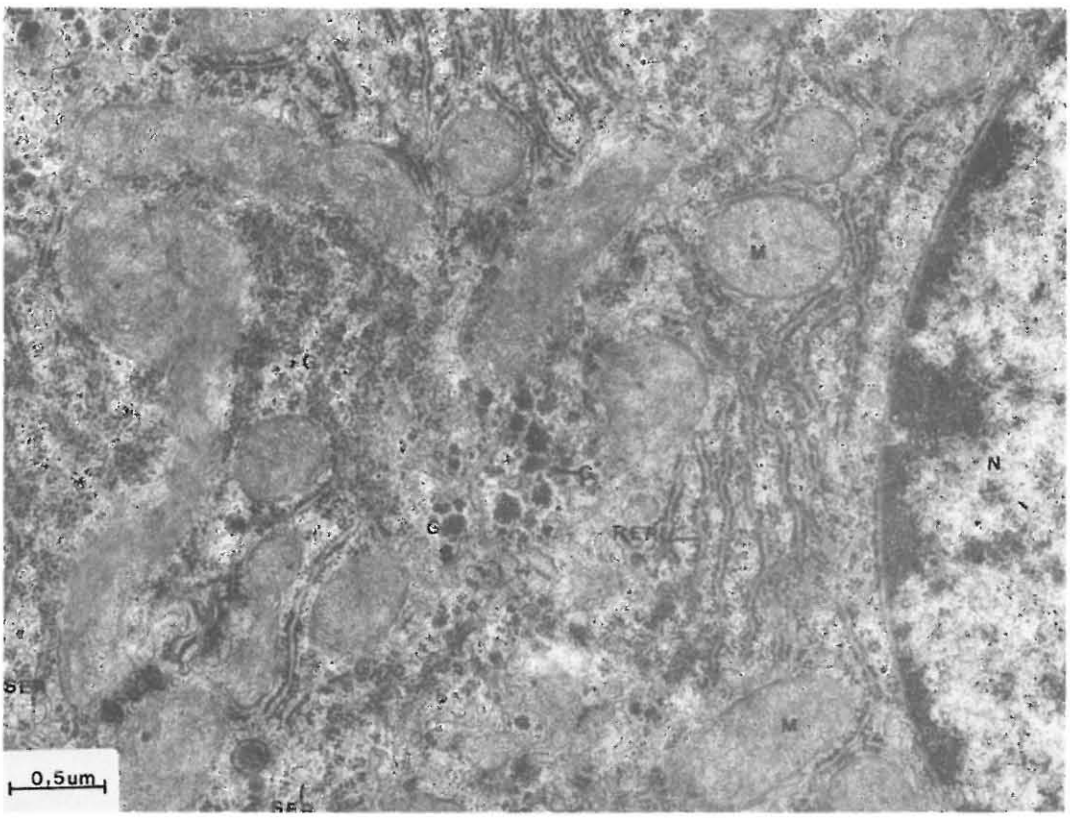


Figure 43. Hepatocyte from a control rat belonging to the group of rats treated with 1 mg /kg of norethynodrel. x 39 000

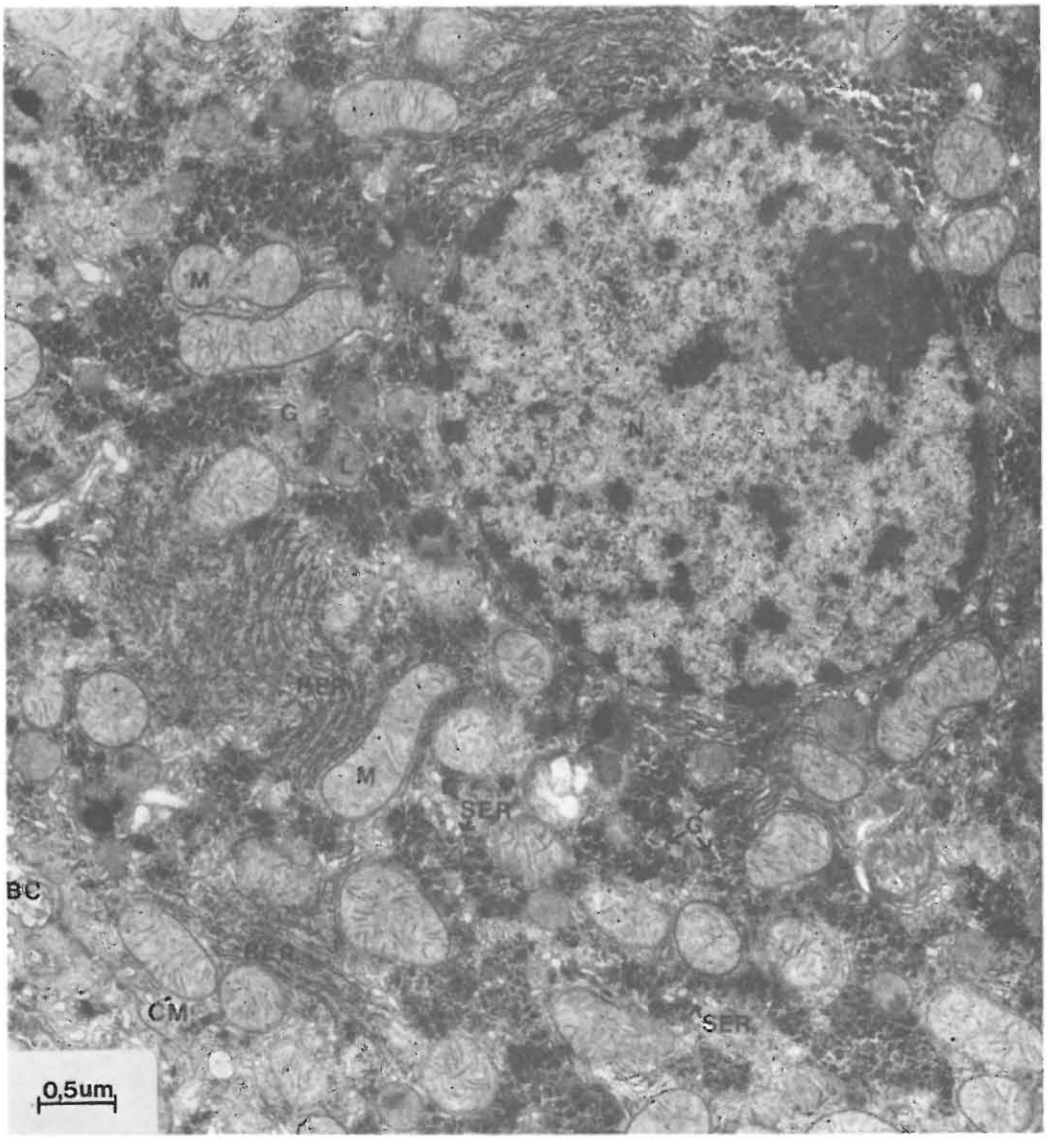


Figure 44. Hepatocyte from a rat treated for 4 days with 1 mg /kg norethynodrel. Glycogen lakes are abundant, mitochondria normal, rough endoplasmic reticulum prominent and smooth endoplasmic reticulum sparse. x 19 000

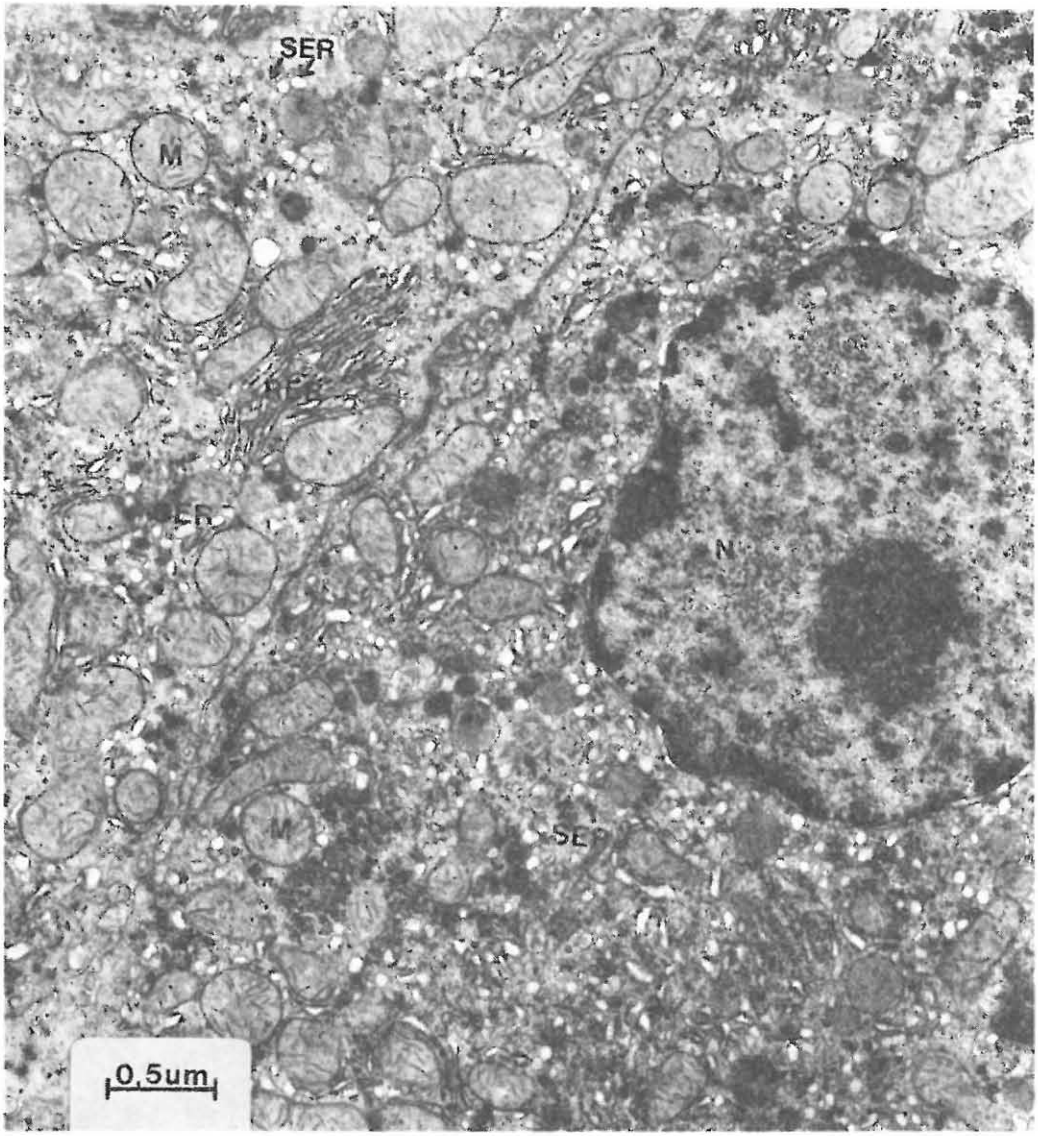


Figure 45. Hepatocyte from a rat treated for 12 days with 1 mg /kg of norethynodrel. Marked proliferation of smooth endoplasmic reticulum, glycogen decreasing and rough endoplasmic reticulum decreasing in extent as well as length. x 13 000

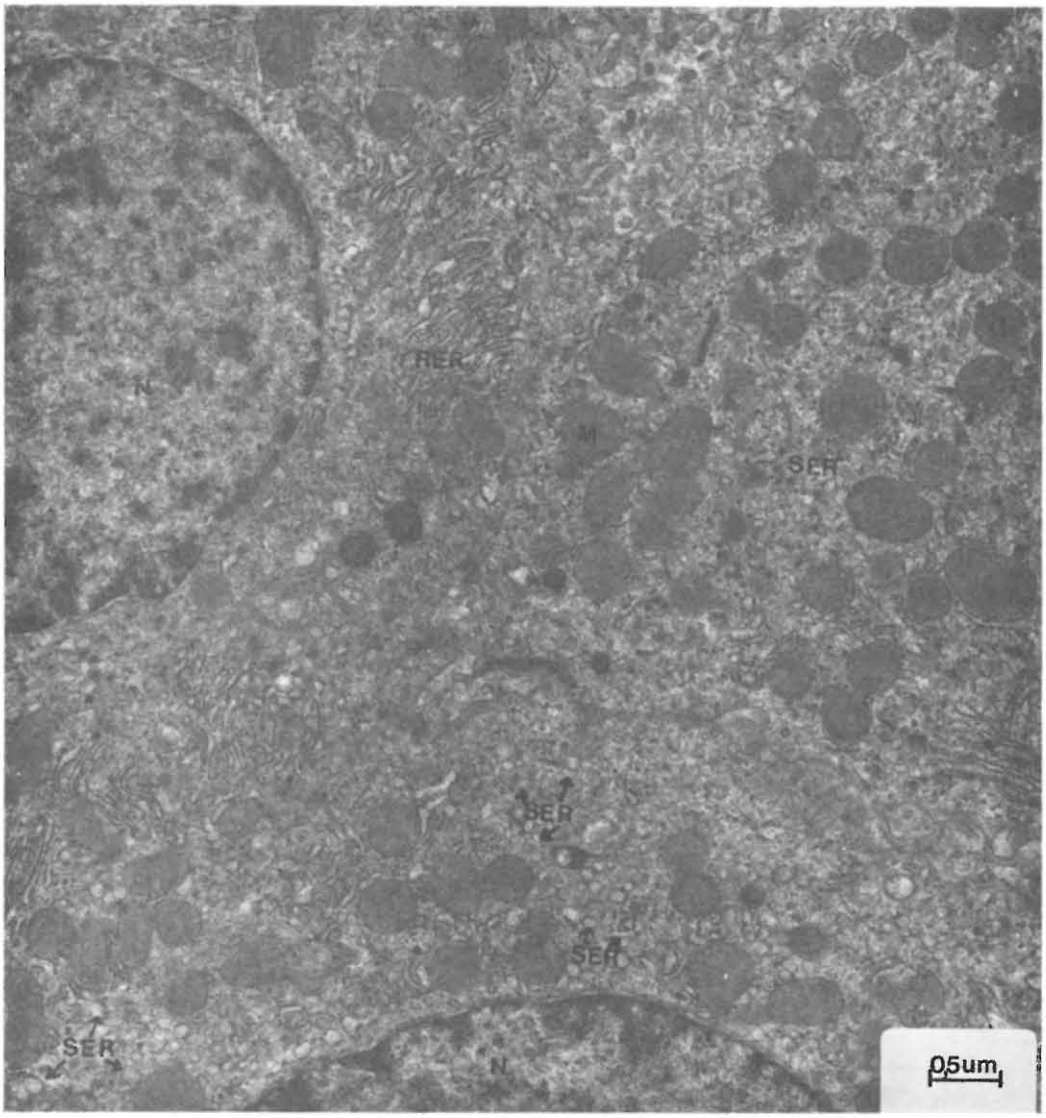


Figure 46. Hepatocyte from a rat treated with 1 mg/kg of norethynodrel for 16 days. Massive proliferation of smooth endoplasmic reticulum, glycogen absent and cisternae of rough endoplasmic reticulum shorter than after 12 days treatment. x 10 800

Figures 47 and 48 show the changes occurring after 30 days of treatment; the SER proliferation appears more abundant than on any of the other days of treatment.

One mg/kg of norethynodrel increased SER markedly, did not seem to have an apparent effect on the mitochondria, generally depleted glycogen lakes, and caused a shortening of RER. However, these alterations of the hepatocytes were less prominent than at the high dosage level.

7.7.3 Effect of Ethinyl Estradiol

7.7.3.1 Treatment with 20 mg/kg of Ethinyl Estradiol

Following treatment with 20 mg/kg of ethinyl estradiol for 2, 4, 6 and 8 days, liver cells from the rats showed characteristic features of control animals. Figure 49 shows liver cell from a control animal. Figure 50 shows a liver cell from an animal after 8 days of treatment. After 12, 16 or 30 days of treatment, increases in SER were noted with the largest increase occurring after 30 days (Figure 51); however, this increase was not marked. The increase in SER occurring after days 12 and 16 were only slight (Figure 52).

Throughout the 30 days of treatment with 20 mg/kg ethinyl estradiol, the RER of the hepatocytes appeared normal (Figure 53). The other cell organelles from treated animals also appeared normal.

7.7.3.2 Treatment with 1 mg/kg of Ethinyl Estradiol

Liver cells from animals following 2, 4, 6, 8 or 12 days of administration of ethinyl estradiol exhibited features virtually characteristic of control animals. Figure 54 shows a liver cell from a control animal and Figure 55 shows the liver of a rat after eight days of treatment.

After 16 or 30 days of ethinyl estradiol regimen, a slight increase in SER occurred (Figures 56 and 57), but this increase in SER was negligible

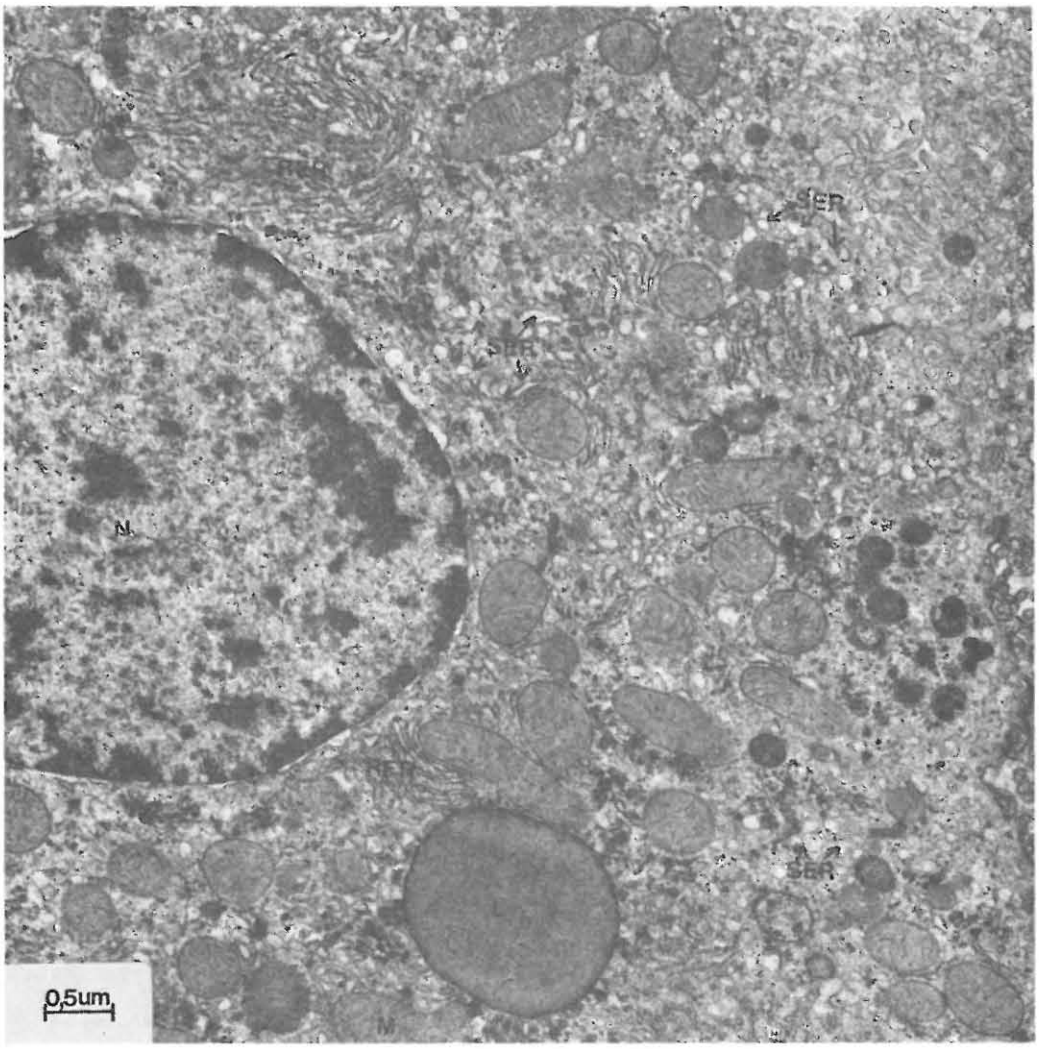


Figure 47. Hepatocyte from a rat treated with 1 mg /kg of norethynodrel for 30 days. Massive proliferation of smooth endoplasmic reticulum (appears more abundant than for any of the other days of treatment). Glycogen stores also depleted, rough endoplasmic reticulum decreased in number and extent. x 13 000

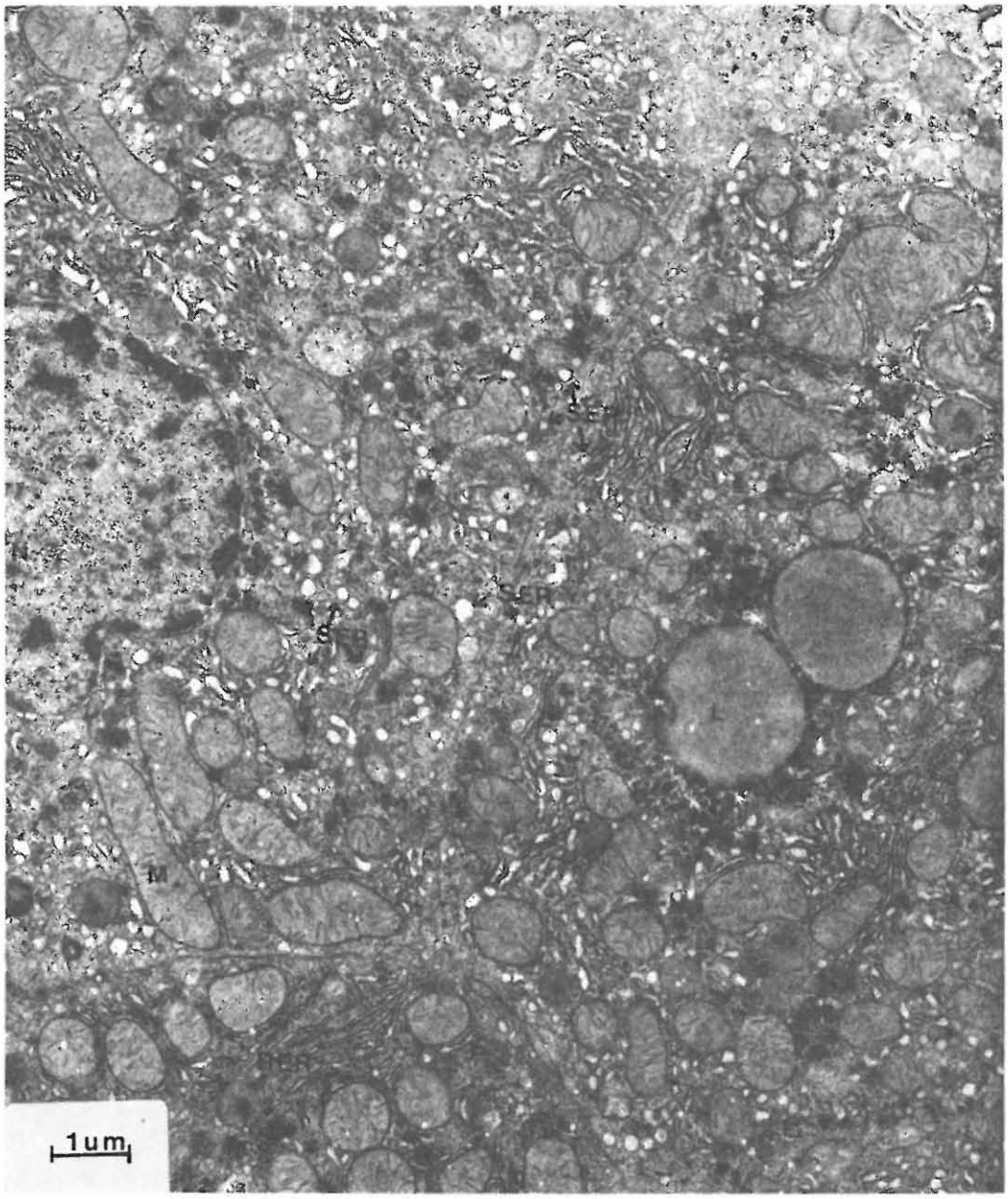


Figure 48. Portion of three hepatocytes from a rat treated with 1 mg /kg of norethynodrel for 30 days. Shows similar changes to Figure 47. x 13 000

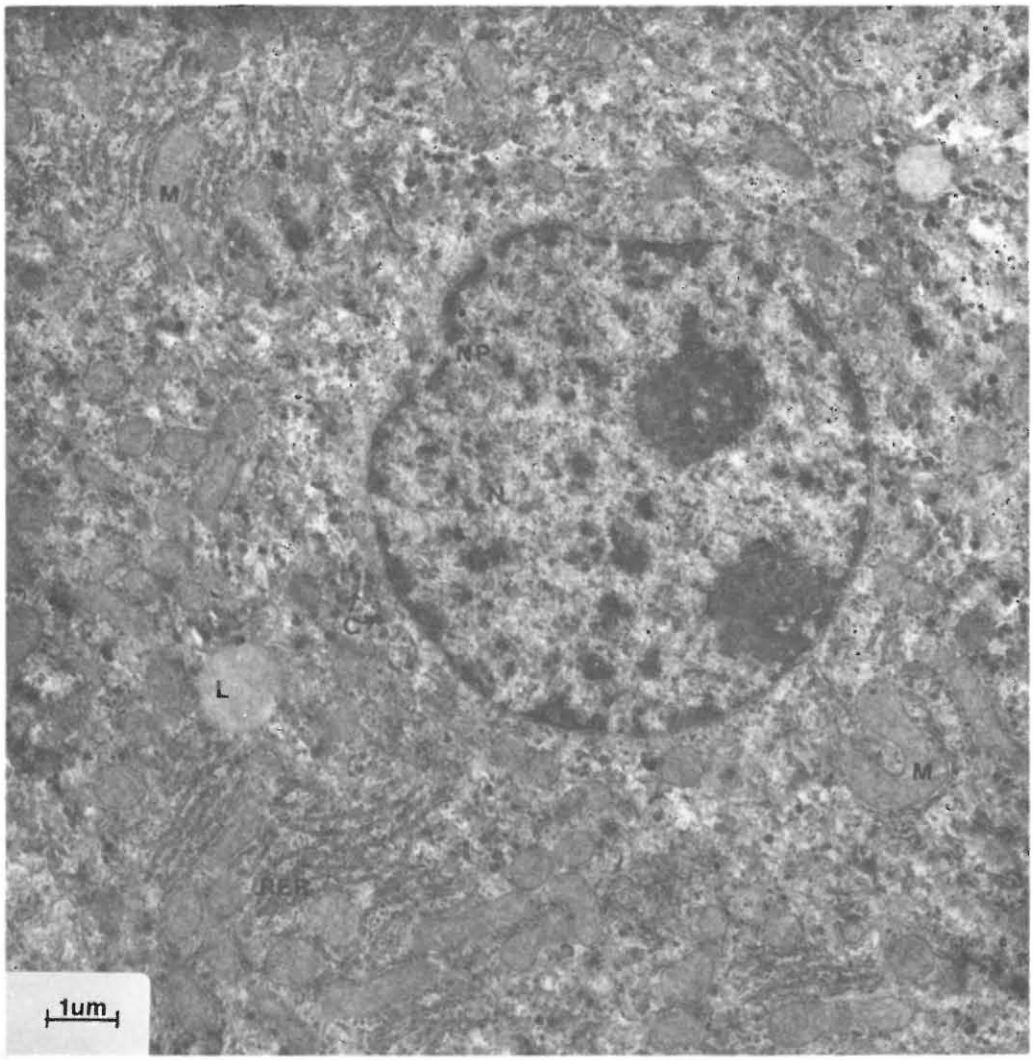


Figure 49. Hepatocyte from a control rat belonging to the group that received 20 mg /kg of ethinylestradiol. Liver cell shows characteristic features of a normal cell. x 10 800

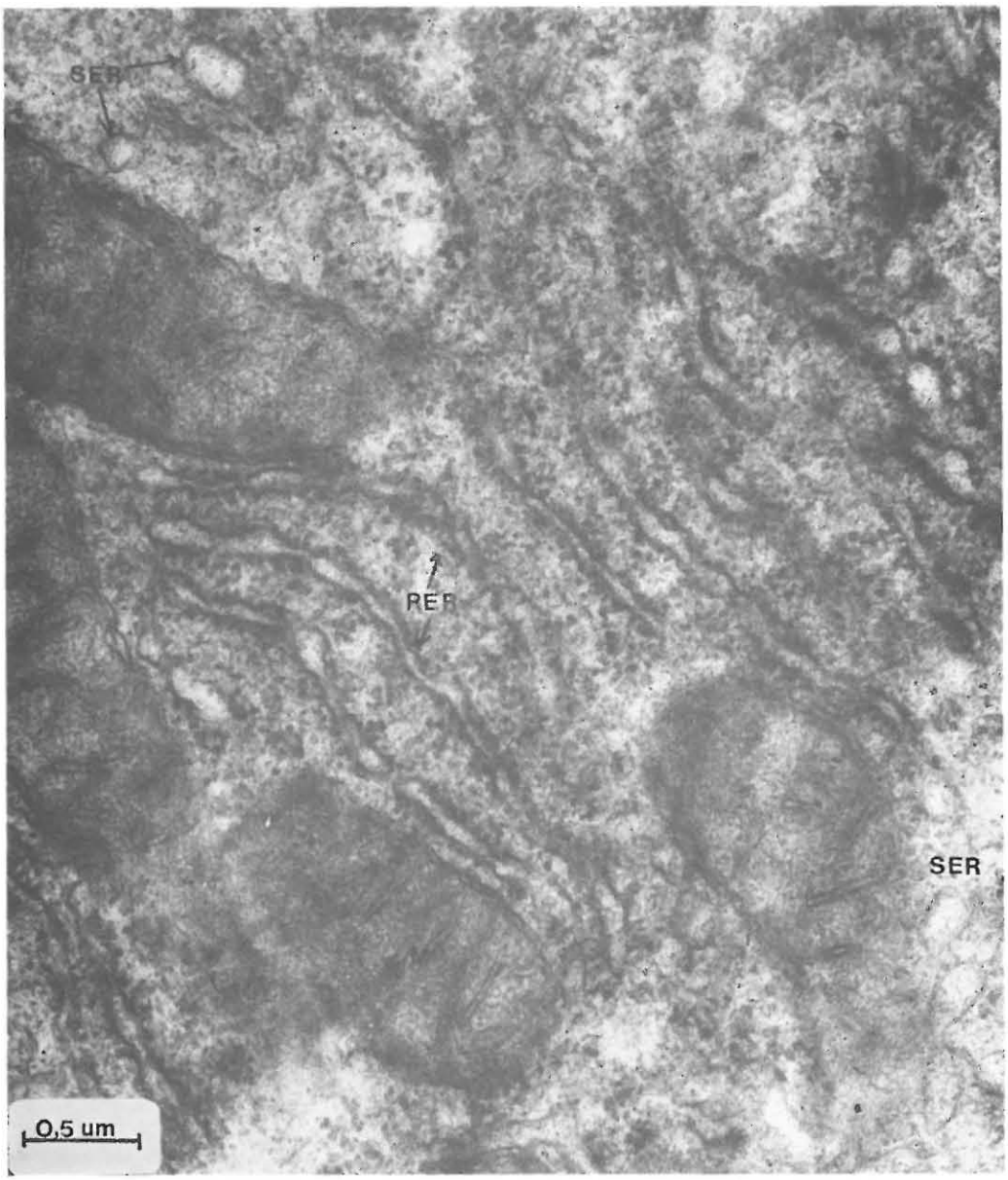


Figure 50. Hepatocyte from a rat treated with 20 mg /kg of ethinyl estradiol for 8 days. Cells normal and rough endoplasmic reticulum characteristically in parallel rows. x 75 700

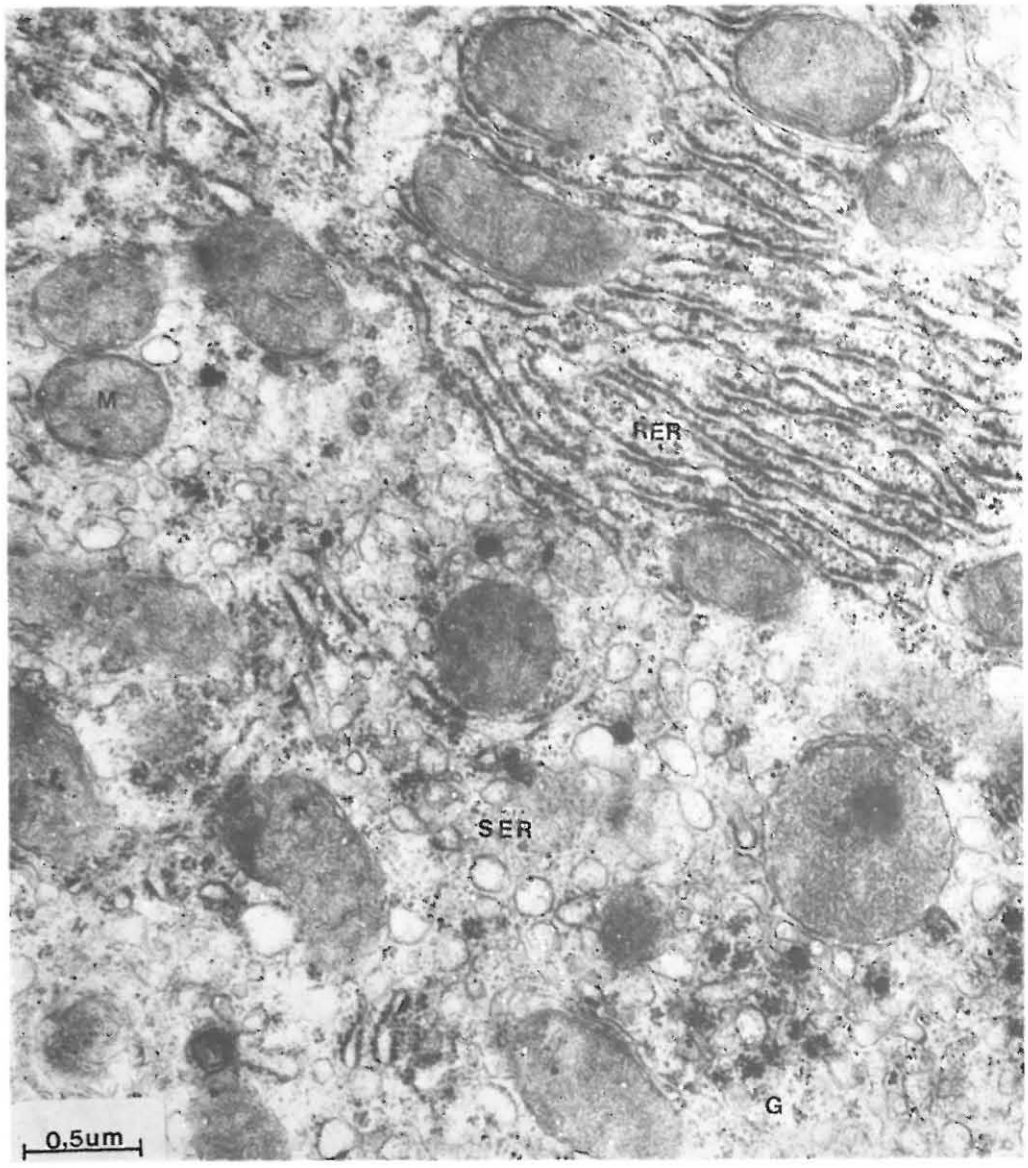


Figure 51. Hepatocyte from a rat treated with 20 mg /kg of ethinyl estradiol. The smooth endoplasmic reticulum increased slightly, otherwise shows characteristic features. Rough endoplasmic reticulum still normal. x 37 600

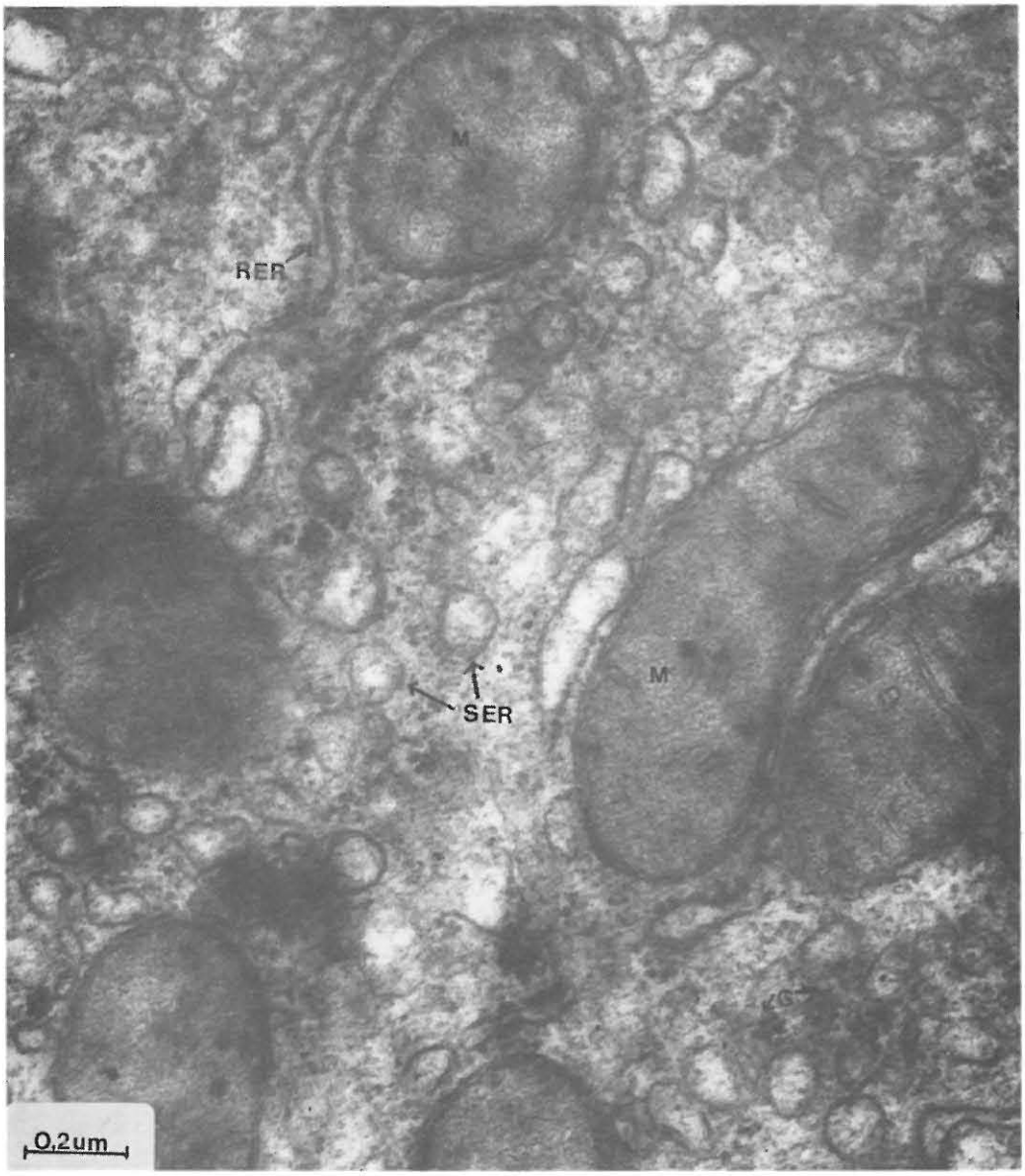


Figure 52. Hepatocyte from a rat treated with 20 mg /kg ethinyl estradiol for 16 days, smooth endoplasmic reticulum has only increased slightly. x 75 700

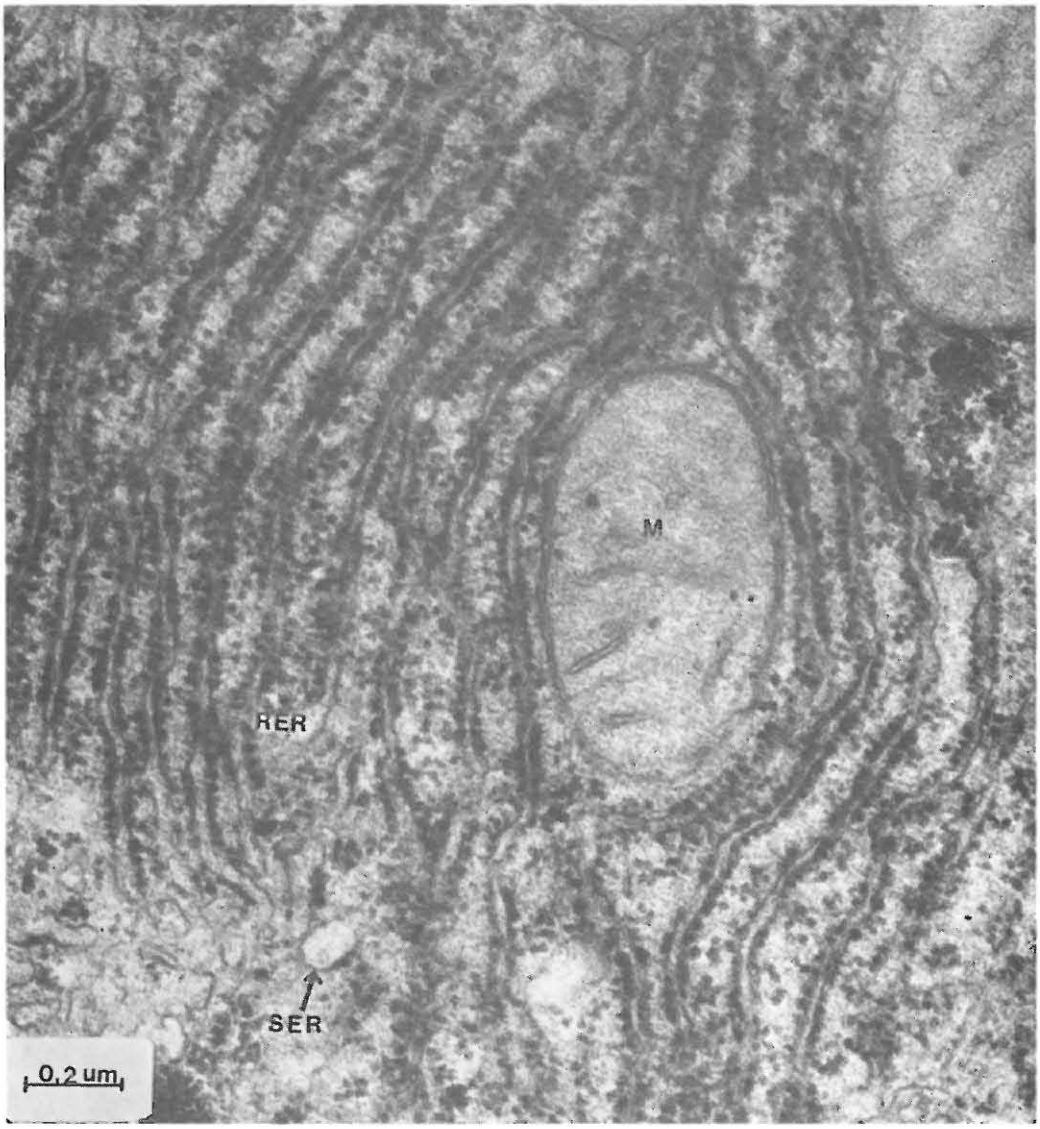


Figure 53. Hepatocyte from a rat treated with 20 mg /kg of ethinyl estradiol for 30 days. Rough endoplasmic reticulum still normal. x 71 600

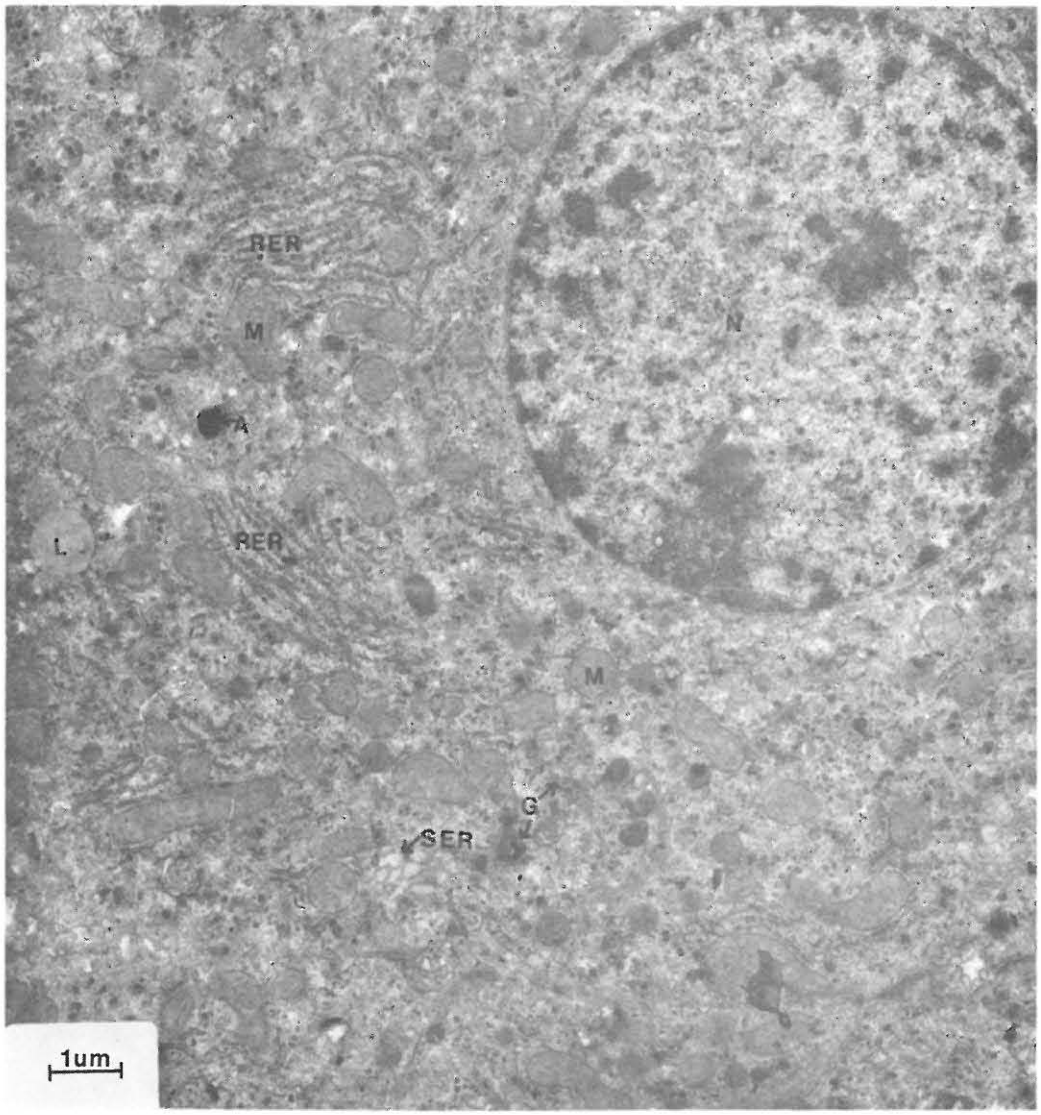


Figure 54. Control hepatocyte from a rat treated with ethyl oleate for 30 days, belonging to the group of rats treated with 1 mg./kg of ethinyl estradiol. The cell exhibits a characteristic appearance. x 10 800

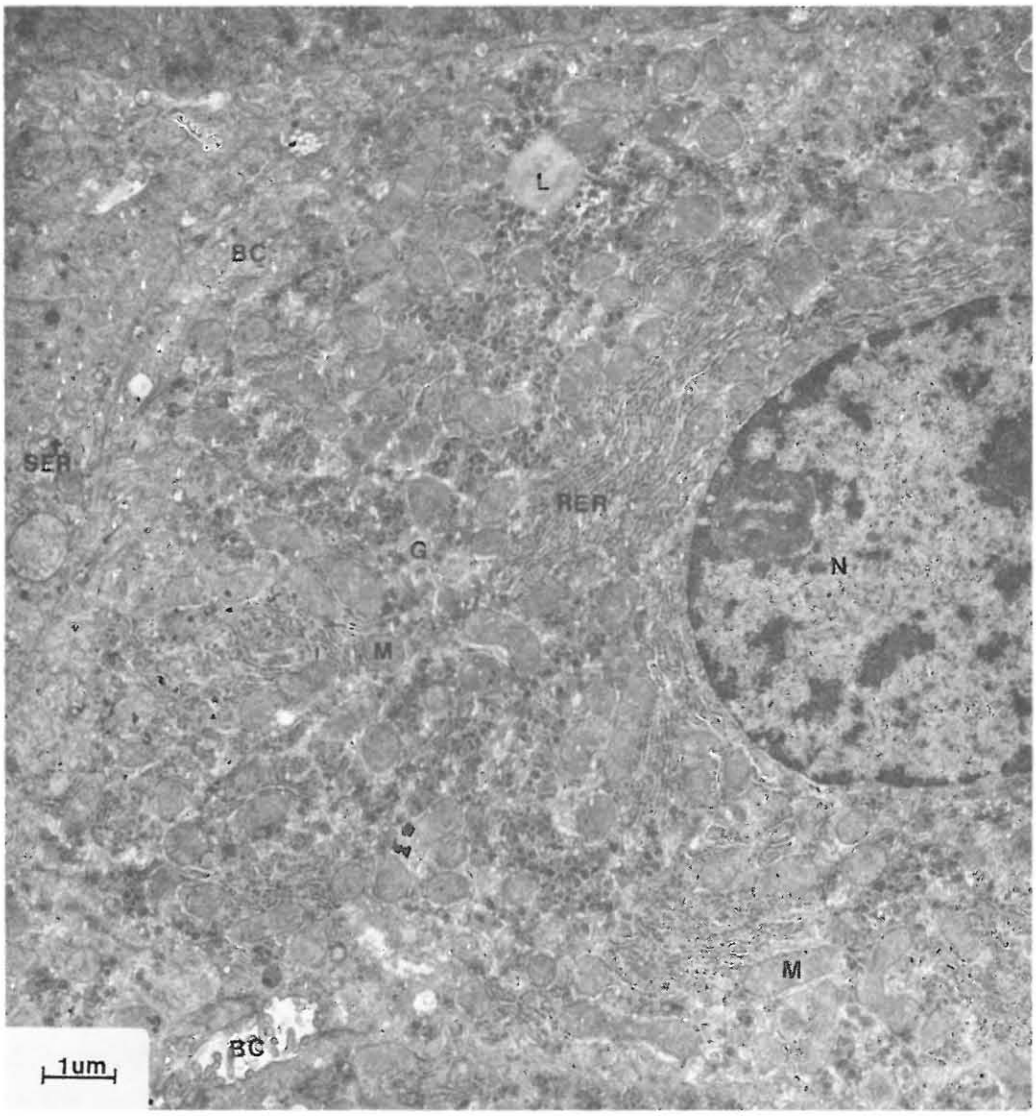


Figure 55. Hepatocyte from rat treated with 1 mg./kg. of ethinyl estradiol for 8 days. The liver cell shows virtually no change. x 10 800

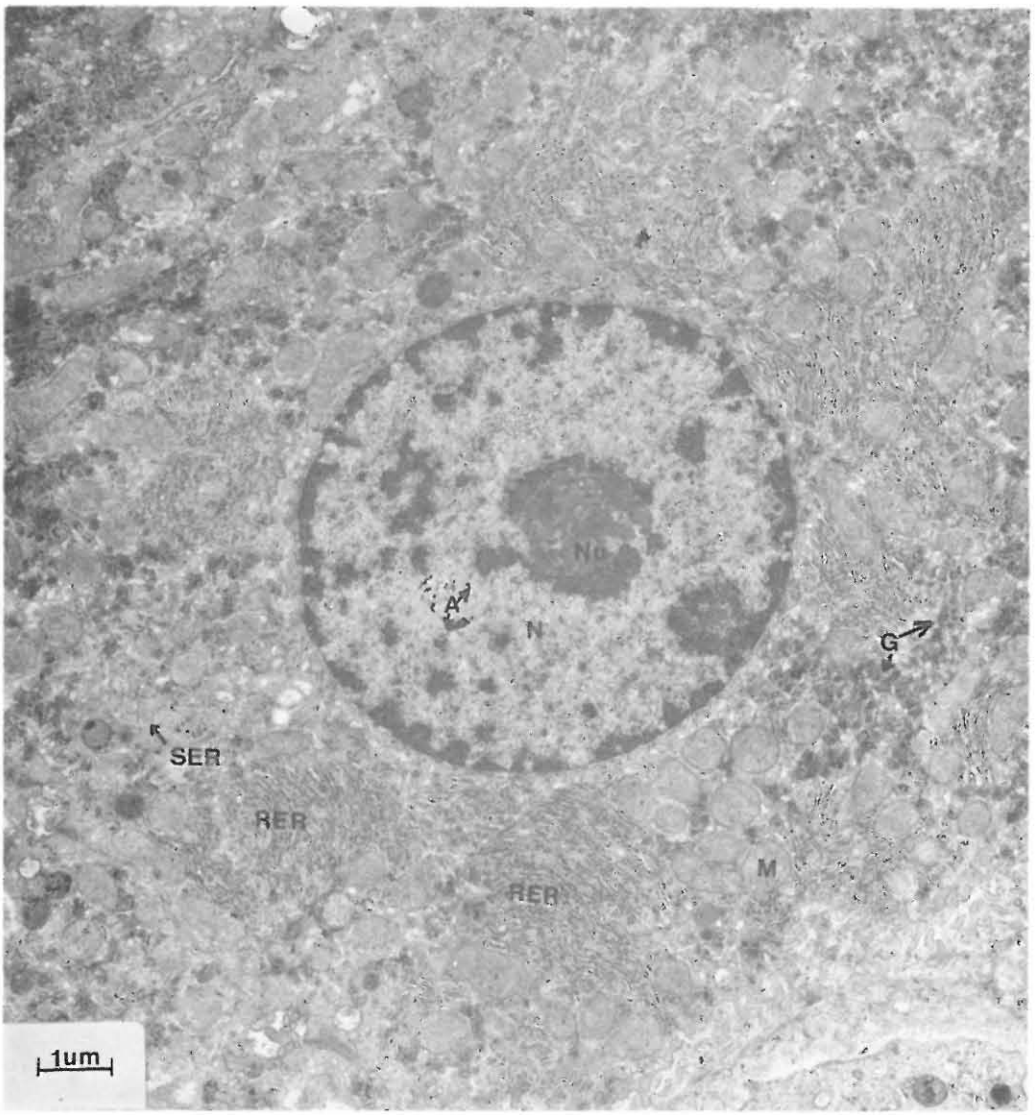


Figure 56. Hepatocyte from rat treated for 16 days with 1 mg /kg ethinyl estradiol, only a very slight increase in smooth endoplasmic reticulum is apparent. x 10 800

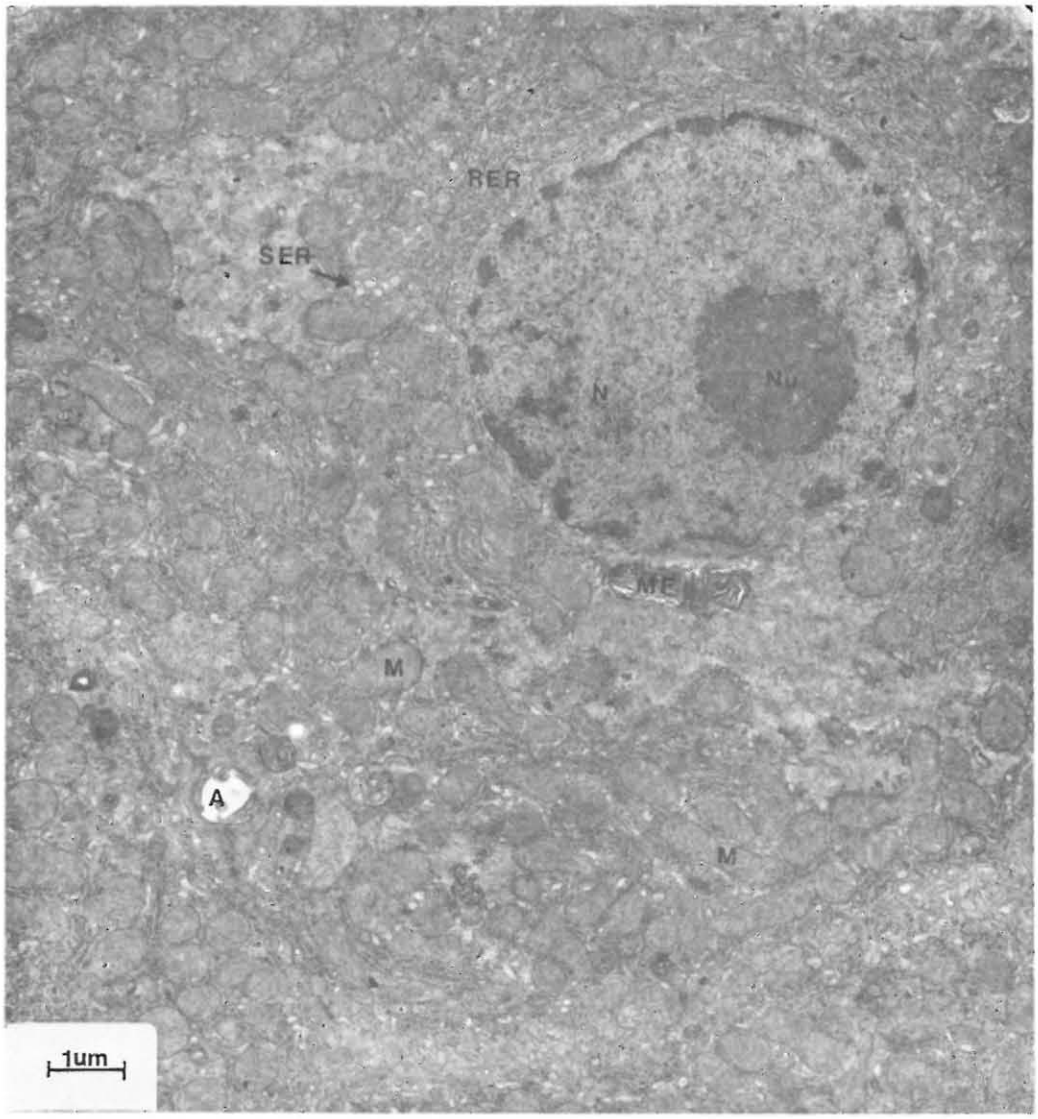


Figure 57. Hepatocyte from rat treated for 30 days with 1 mg./kg ethinyl estradiol showing the slight increase in smooth endoplasmic reticulum. x 10 800

The other cell organelles from treated animals appeared normal, in that the mitochondria appeared normal, and there was no marked decrease in glycogen granules (although individual sections did show slight variation Figure 58).

The ultrastructural changes in hepatocytes after exposure to norethynodrel and ethinyl estradiol are summarized in Table 28.

7.7.4 Effect of Oral Contraceptive Administration on the Adrenal Cortex

7 7.4.1 Control Groups

The appearance of the cellular ultrastructure in the control groups receiving ethyl oleate conformed to previous descriptions of normal adrenal cortex (Idleman 1970 and Malamed 1975) (Figure 59).

Malamed (1975) indicates that electron microscopy has revealed three outstanding ultrastructural features of the mammalian adrenal cortex.

(a) Mitochondria which differ in structure to those of most cells.

(b) The abundance of large fat droplets or "liposomes".

(c) An abundance of SER.

7.7.4.2 Effects of Norethynodrel on the Adrenal Cortex

Gross Effects:

The adrenal glands of rats dosed with 20 mg/kg of norethynodrel daily for four or more days were much darker in colour and more brittle than those of control rats. At 1 mg/kg this change was also apparent following eight days of administration.

Cytological Changes:

Treatment with 20 mg/kg of norethynodrel caused a marked depletion of lipid from the zonae fasciculata and zonae reticularis in every animal.

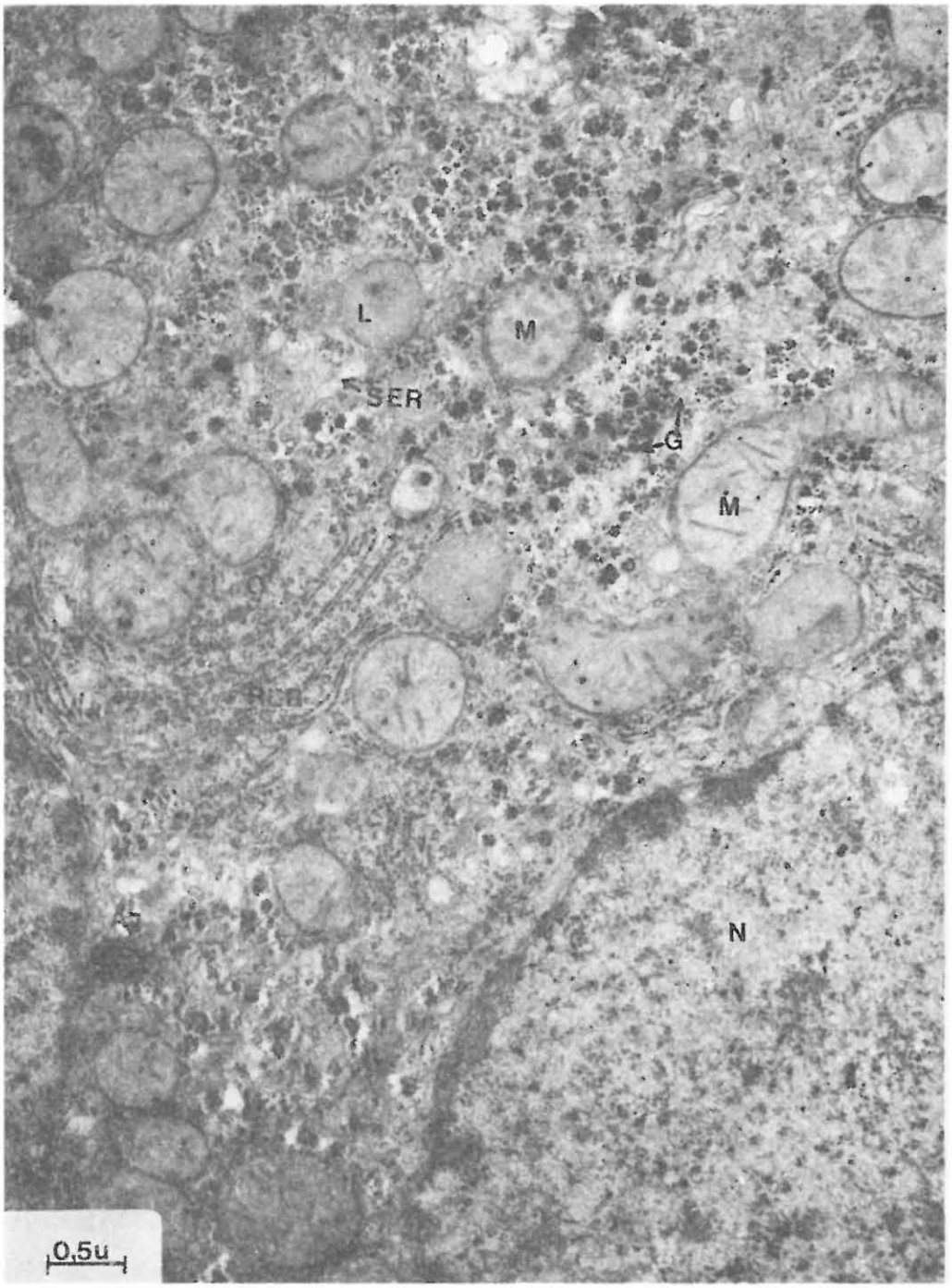


Figure 58. Hepatocyte from rat treated for 30 days with 1 mg /kg ethinyl estradiol showing normal glycogen, normal rough endoplasmic reticulum and sparse smooth endoplasmic reticulum. x 19 400

Table 28. Summary of the Ultra-structural Changes Caused by Steroids Used in Experiments

	RER	SER	Ribosomes+	Mitochondria	Glycogen	Lipids	Golgi Apparatus	Nucleus	Bile Caniculus
Controls	Prominent lamellae, not dilated. Occurs in parallel rows .	Scattered throughout the cell	Normally attached to RER but free rosette polysomes seen	Round and oval with cristae.	Usually present in fairly large amounts, occurs as "lakes"	Erratic sometimes 4 or 5 droplets per cell. Sometimes none	Lamellar Golgi	No chromatic margination, usually round.	Not dilated, micro-villi fairly abundant
Norethynodrel 20 mg/kg and 1 mg/kg *	Less than in controls, shortening and dilatation of lamellae visible in all stages	Extensive, especially at 20 mg/kg, became extensive following 6 days of treatment	More free ribosomes than in controls	Somewhat swollen	Somewhat depleted	As in controls perhaps less	Moderately dilated	Somewhat crenulated with a margination	Enlarged with micro-villi
Ethinyl Estradiol 20 mg/kg and 1 mg/kg	At 1 mg/kg as in controls, at 20 mg/kg slightly less and shortened	Slight increase at both dosages	As in controls	As in controls	Slightly less than controls	As in controls	As in controls	As in controls	As in controls

*Effects of higher dose was more marked.

+ Not sure whether they are the α-glycogen cells or rosettes of free polysomes, since they are difficult to distinguish.

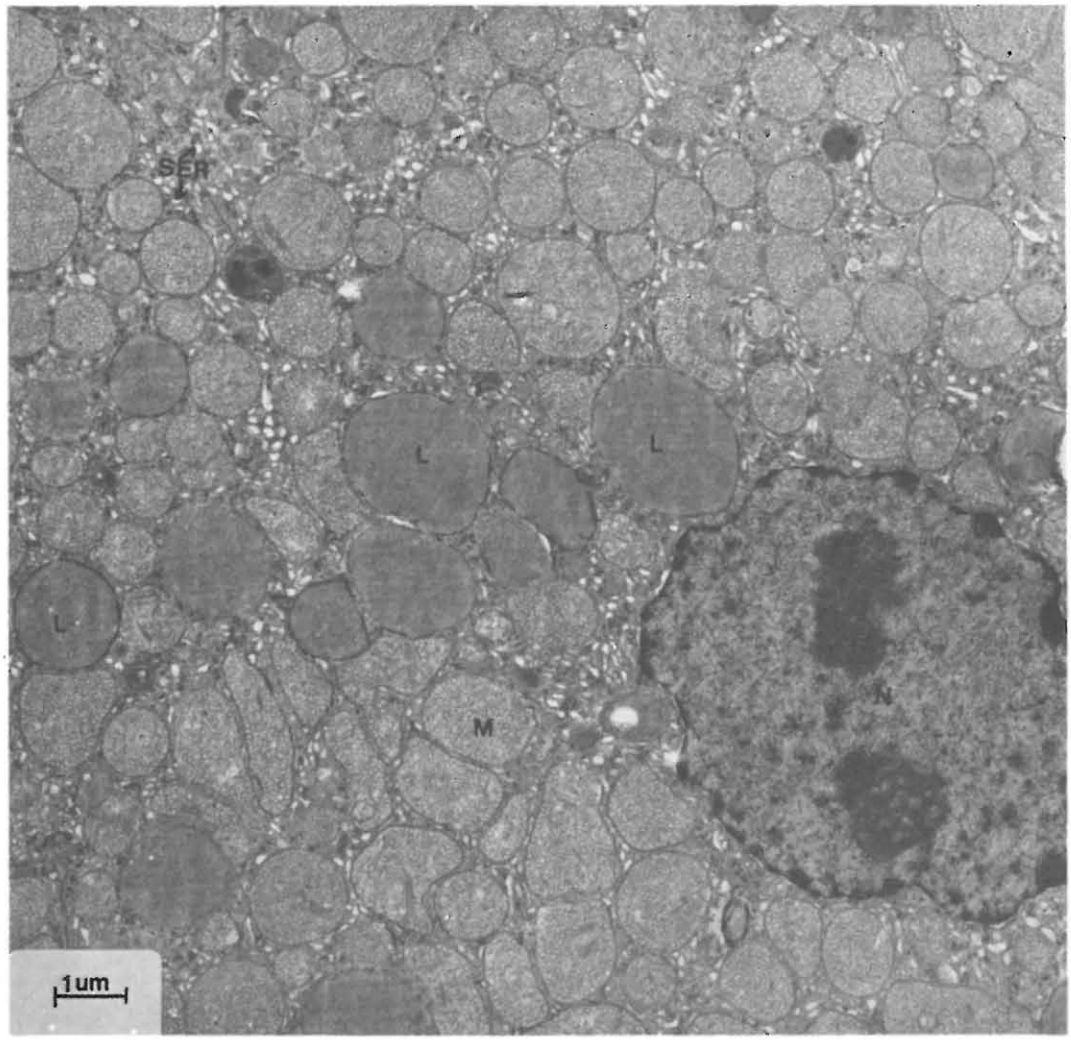


Figure 59. Adrenocortical cell obtained from a control animal showing characteristic features. Lipid droplets, mitochondria and smooth endoplasmic reticulum are present. x 10 800

Lipid depletion became apparent after four days of administration of 20 mg/kg norethynodrel and depletion became significant after six days of treatment, persisting to sixteen days. In these cases, only fine, scattered, sparse droplets of lipid remained (Figures 60 and 61).

In the tissue from animals sacrificed after thirty days of treatment, electron dense spherical "organelles" of variable size were visible (Figures 62 and 63). These "organelles" could be lipid droplets, which would then indicate that, between the sixteenth day and the thirtieth day, lipid again increases. However, no investigation of these droplets has been carried out to determine their true nature. These "organelles" were absent in control adrenals which were fixed and stained simultaneously.

With a daily dose of 1 mg/kg norethynodrel, lipid depletion occurred after six days of administration and this effect became marked after eight days (Figure 64). Unlike the case with the higher dosage, no electron-dense organelles appeared in any of the animals after 30 days of treatment. Generally, the change at 1 mg/kg resembled those at 20 mg/kg but was less prominent, and these changes occurred later.

7.7.4.3 Effect of Ethinyl Estradiol

Treatment with 20 mg/kg ethinyl estradiol caused a marked depletion of lipid in the zonae fasciculata and zonae reticularis in every animal. This lipid depletion became marked after two days of treatment (Figure 65). In the tissues of animals sacrificed after 4, 6, 8, 12, 16 and 30 days of treatment with 20 mg/kg of ethinyl estradiol lipid depletion resembled that of day two.

At 1 mg/kg lipid depletion was marked after only four days of treatment and the tissues of animals sacrificed after 6, 8, 12, 16 and 30 days of treatment resembled that of day four. Figure 66 illustrates an electron micrograph of an adrenocortical cell from an animal treated with 1 mg/kg ethinyl estradiol for 30 days.

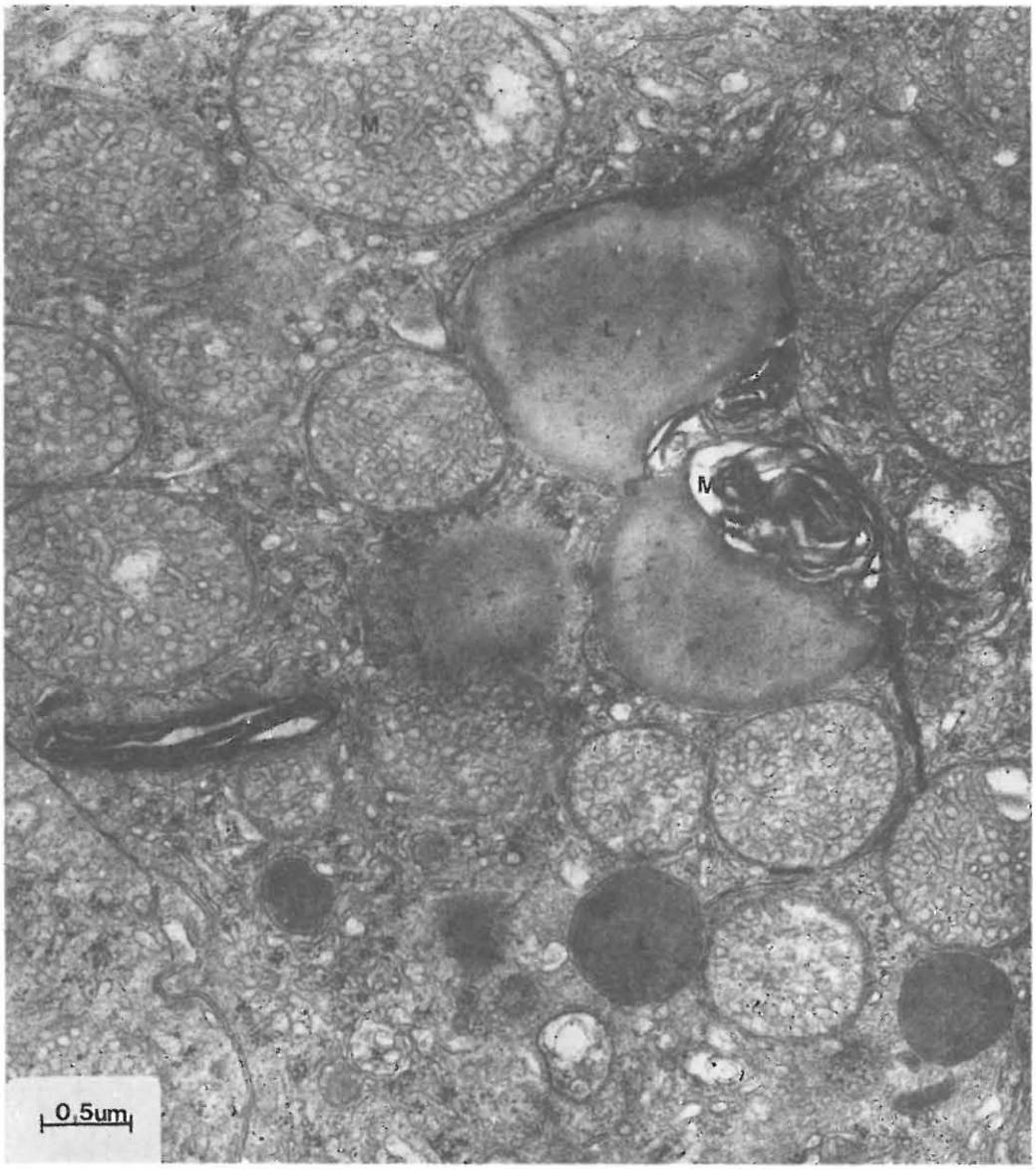


Figure 60. Adrenocortical cell from rat treated with 20 mg /kg of norethynodrel for 6 days. Few lipid droplets remain. x 26 000

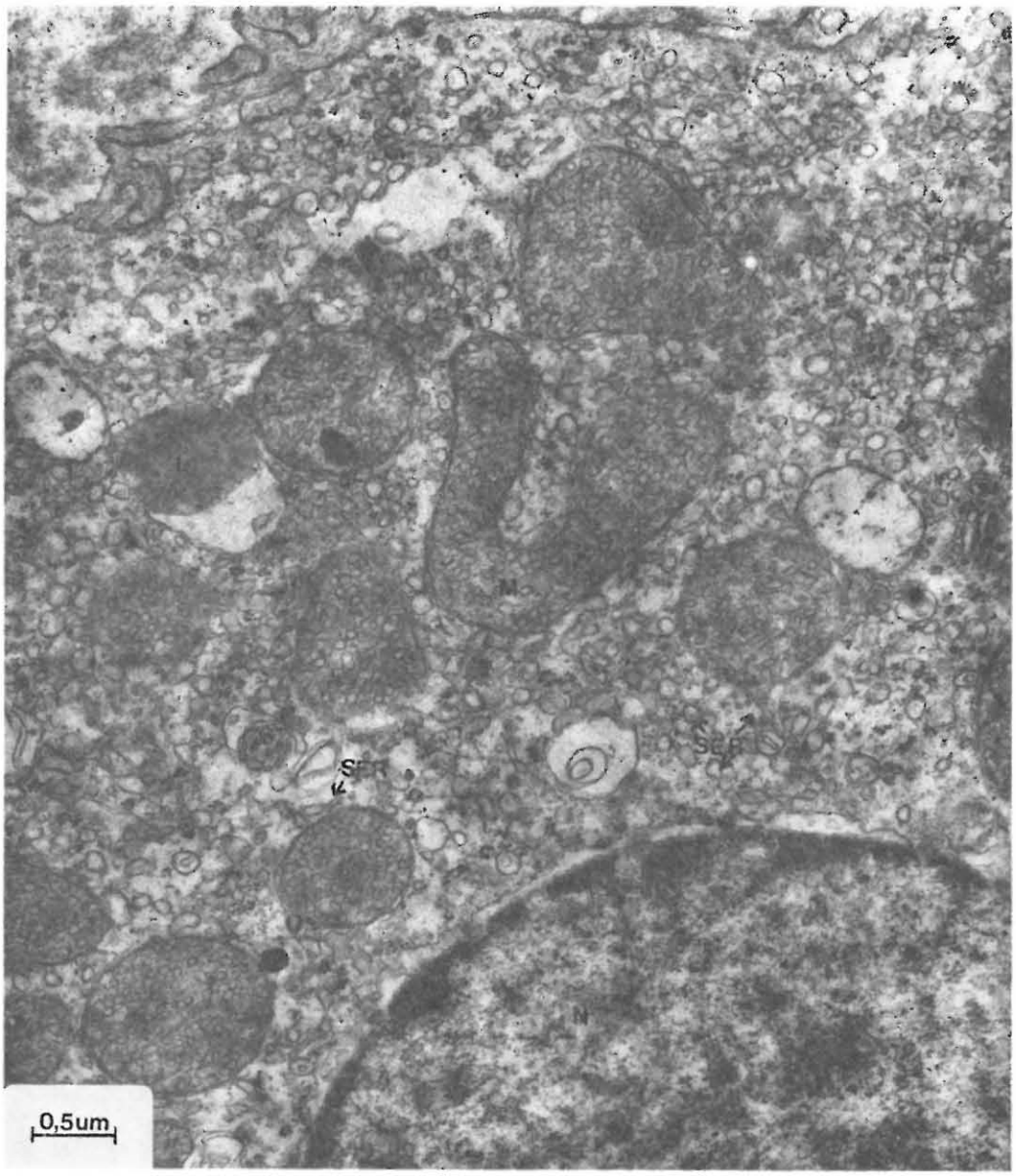


Figure 61. Adrenocortical cell from rat treated with 20 mg /kg of norethynodrel for 16 days. Note virtual absence of lipid droplets. x 24 300

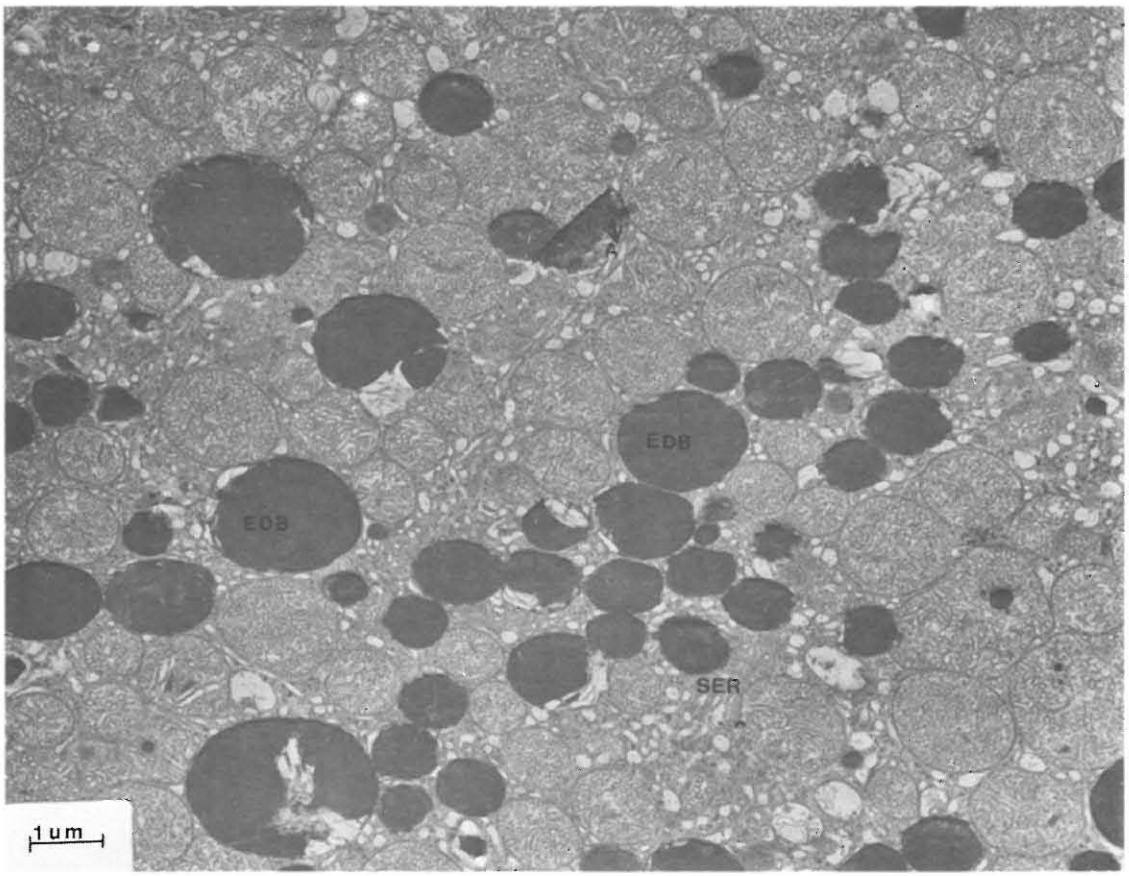


Figure 62. Adrenocortical cell from a rat treated with 20 mg/kg of norethynodrel for 30 days. Electron dense spherical bodies appear and may be lipid. x 11 400

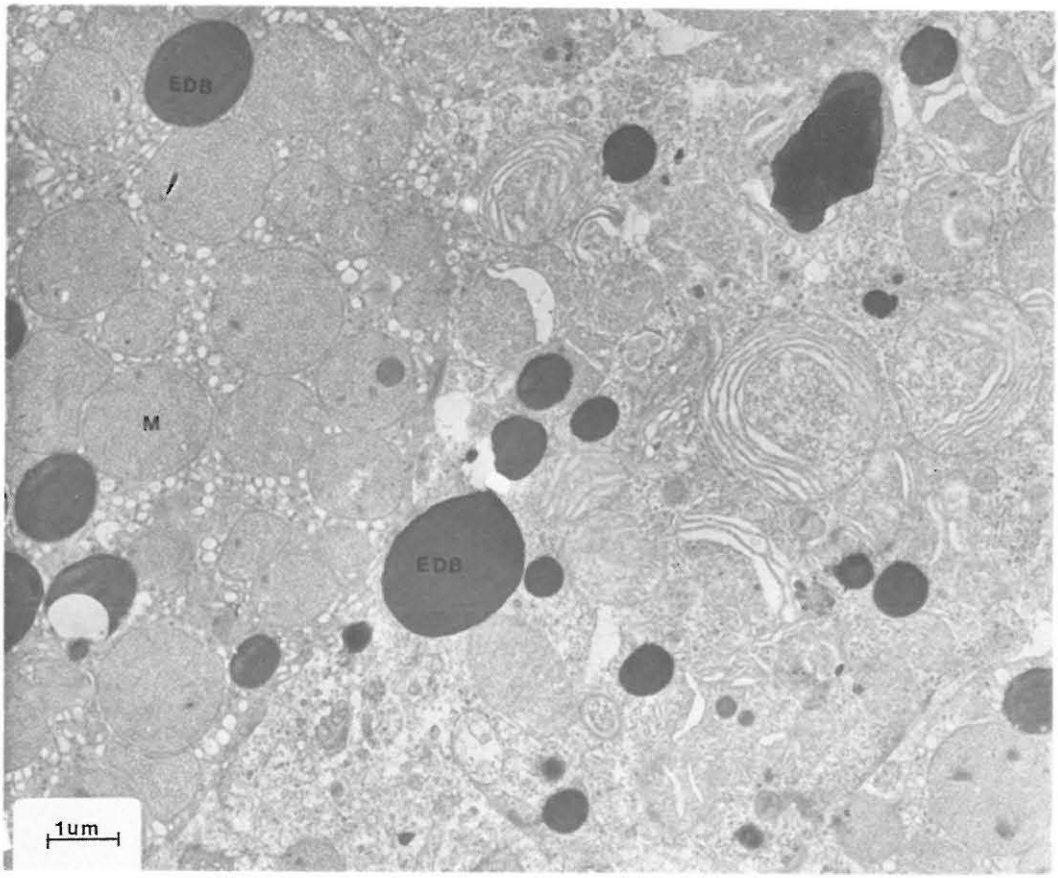


Figure 63. Adrenocortical cell from rat treated with 20mg/kg norethynodrel showing variable size of electron dense bodies. Electronmicrograph from Figures 62 and 63 are from different rats. x 11 400

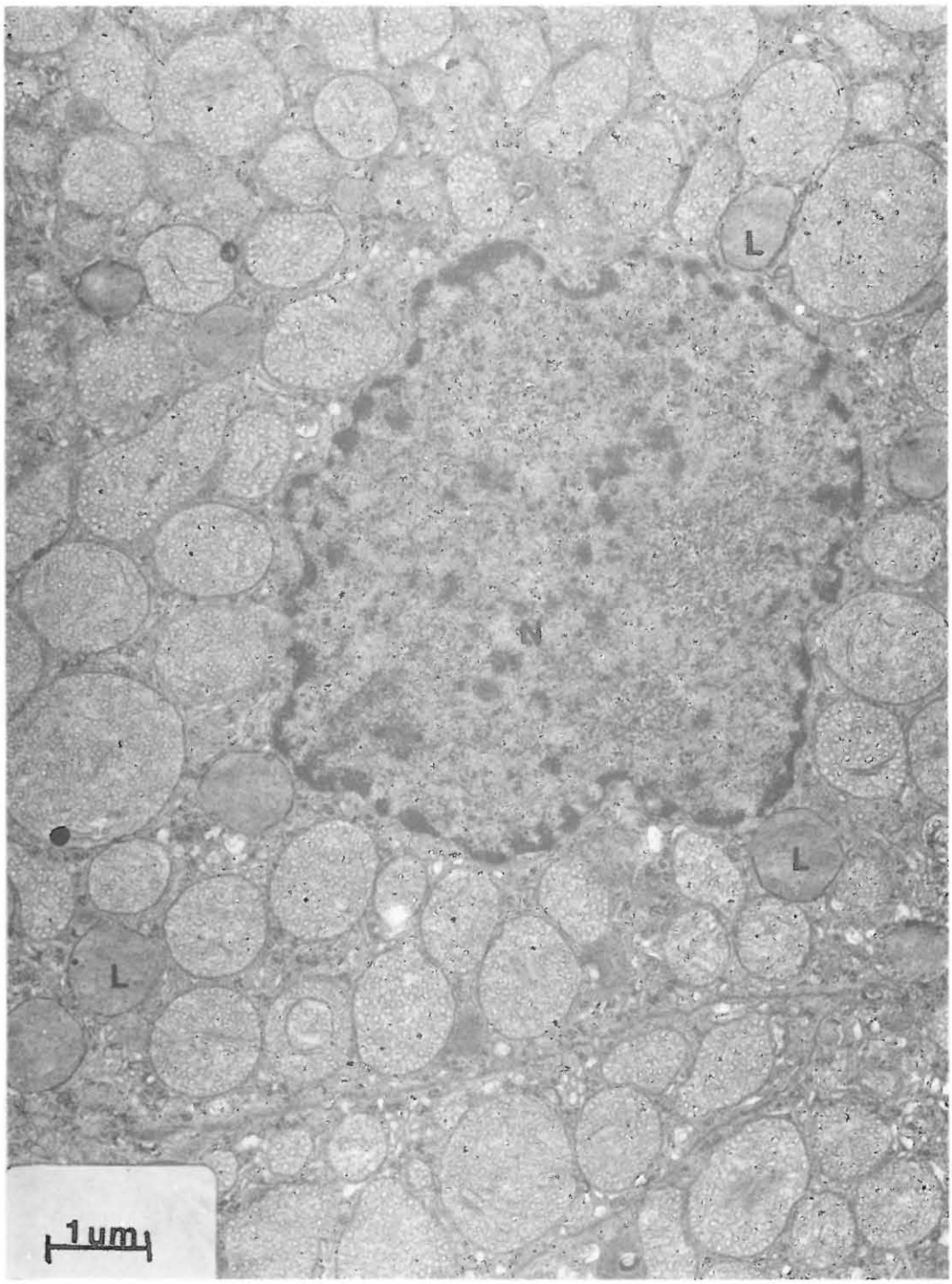


Figure 64. Adrenocortical cell from rat treated for 8 days with 1 mg./kg of norethynodrel. x 10 800

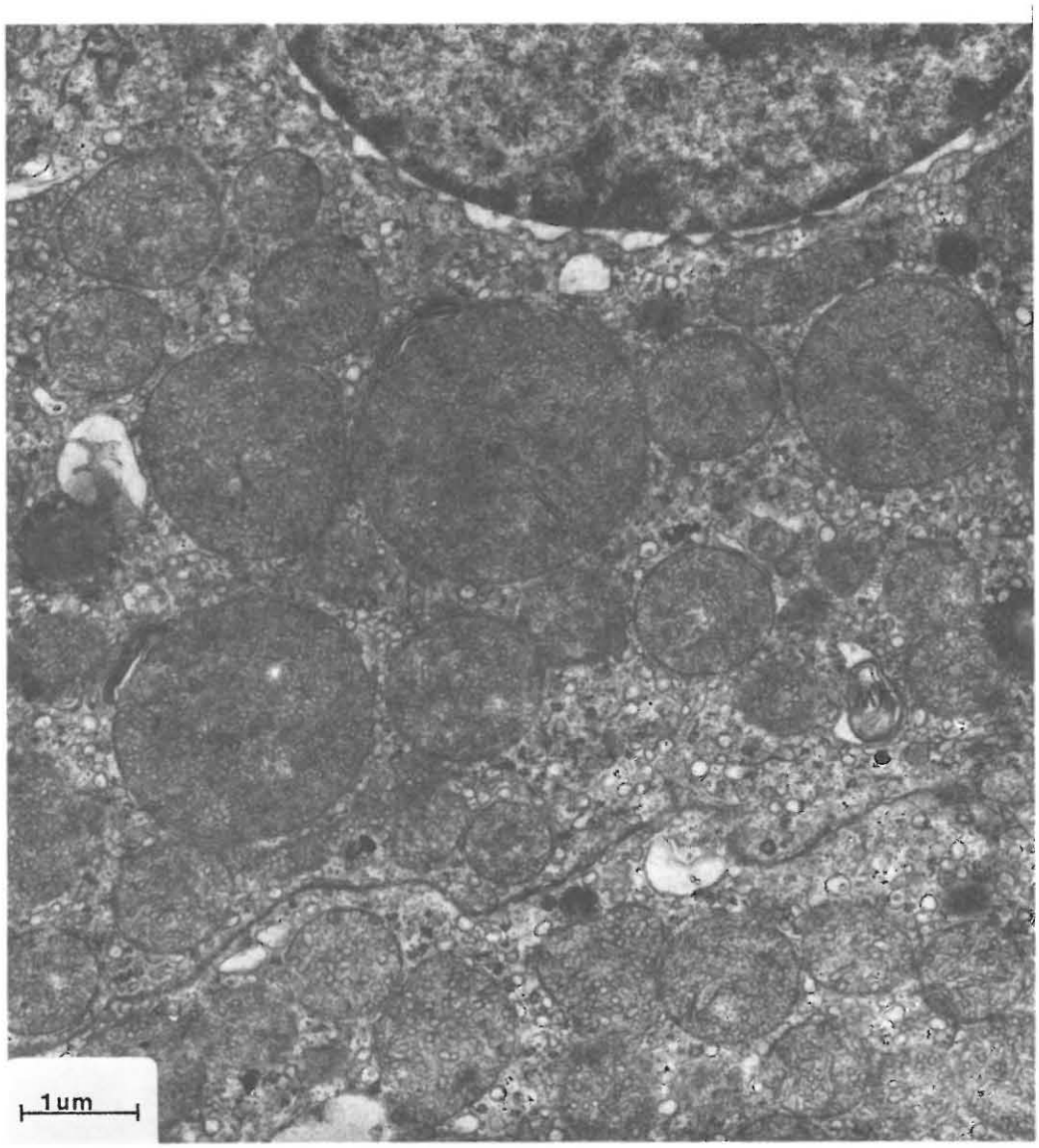


Figure 65. Adrenocortical cell from rat treated with 20 mg /kg. of ethinyl estradiol for 30 days. Lipid depletion became marked after 2 days of administration. x 17 800

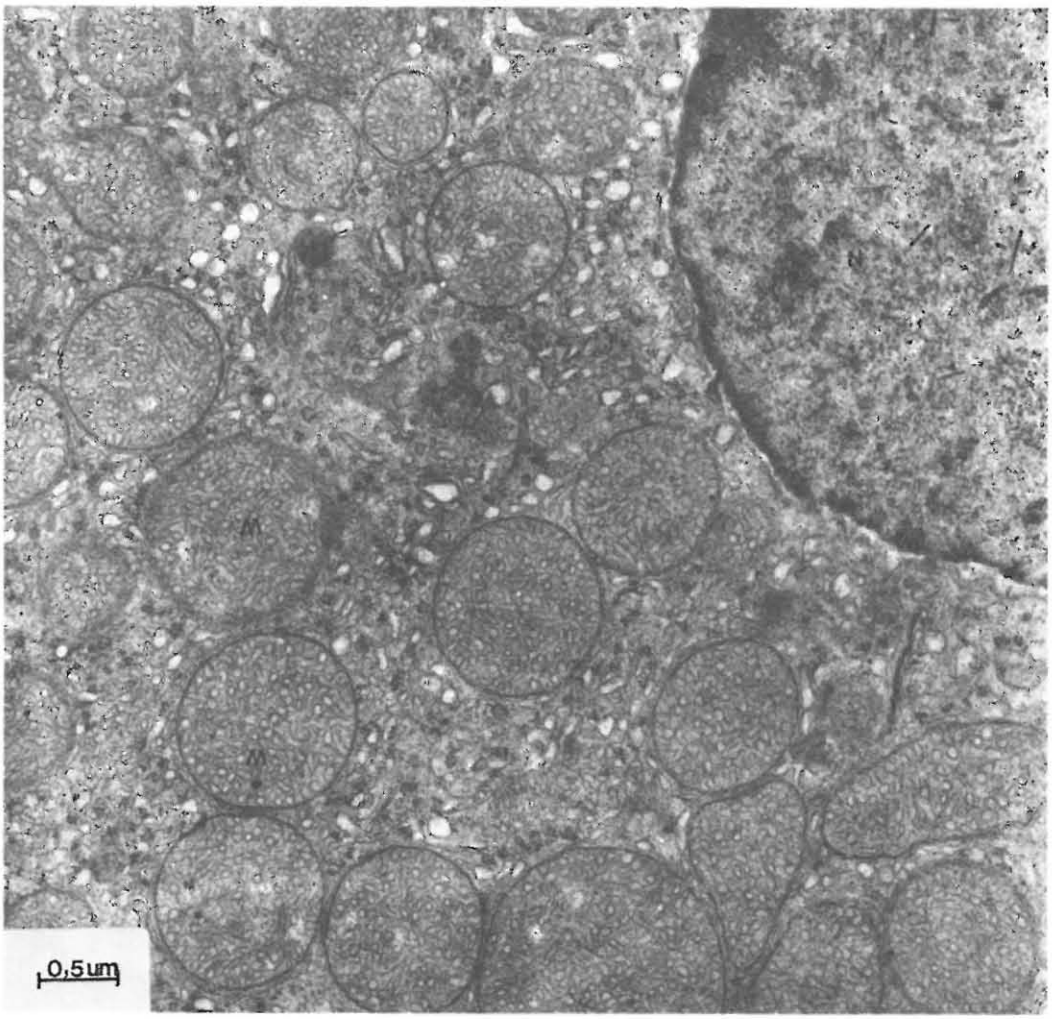


Figure 66. Adrenocortical cell of rat treated with 1 mg /kg ethinyl estradiol for 30 days, lipid is absent. x 20 900

The effect of oral contraceptive administration on the adrenal cortex is summarized in Table 29.

Table 29. Summary of Ultrastructural Changes of Adrenal Cortex following OC Administration

Steroid	Number of Lipid Droplets		Number of Mitochondria		Smooth Endoplasmic Reticulum (Extent)	
	Zona fasciculata	Zona reticularis	Zona fasciculata	Zona reticularis	Zona fasciculata	Zona reticularis
Norethynodrel	-	-	-	-	$\frac{+}{-}$	$\frac{+}{-}$
Ethinyl estradiol	-	-	-	-	$\frac{+}{-}$	$\frac{+}{-}$

- = Decrease

$\frac{+}{-}$ = No change

7.8 Effects of Oral Contraceptive Treatment on the Ovary

7.8.1 Effects of Norethynodrel

The appearance of the cellular structure in the control groups receiving ethyl oleate for 30 days conformed to previous descriptions of normal ovary (Figure 67), Sandborn (1970).

Treatment of rats with both dosage levels of norethynodrel caused the appearance of very light staining (non-electron dense)"organelles"which occurred in clusters (Figure 68). Control ovaries fixed and stained simultaneously were without these light staining organelles. If these organelles are lipid droplets, then it indicates that norethynodrel causes a change in the chemical composition of the lipid droplets.

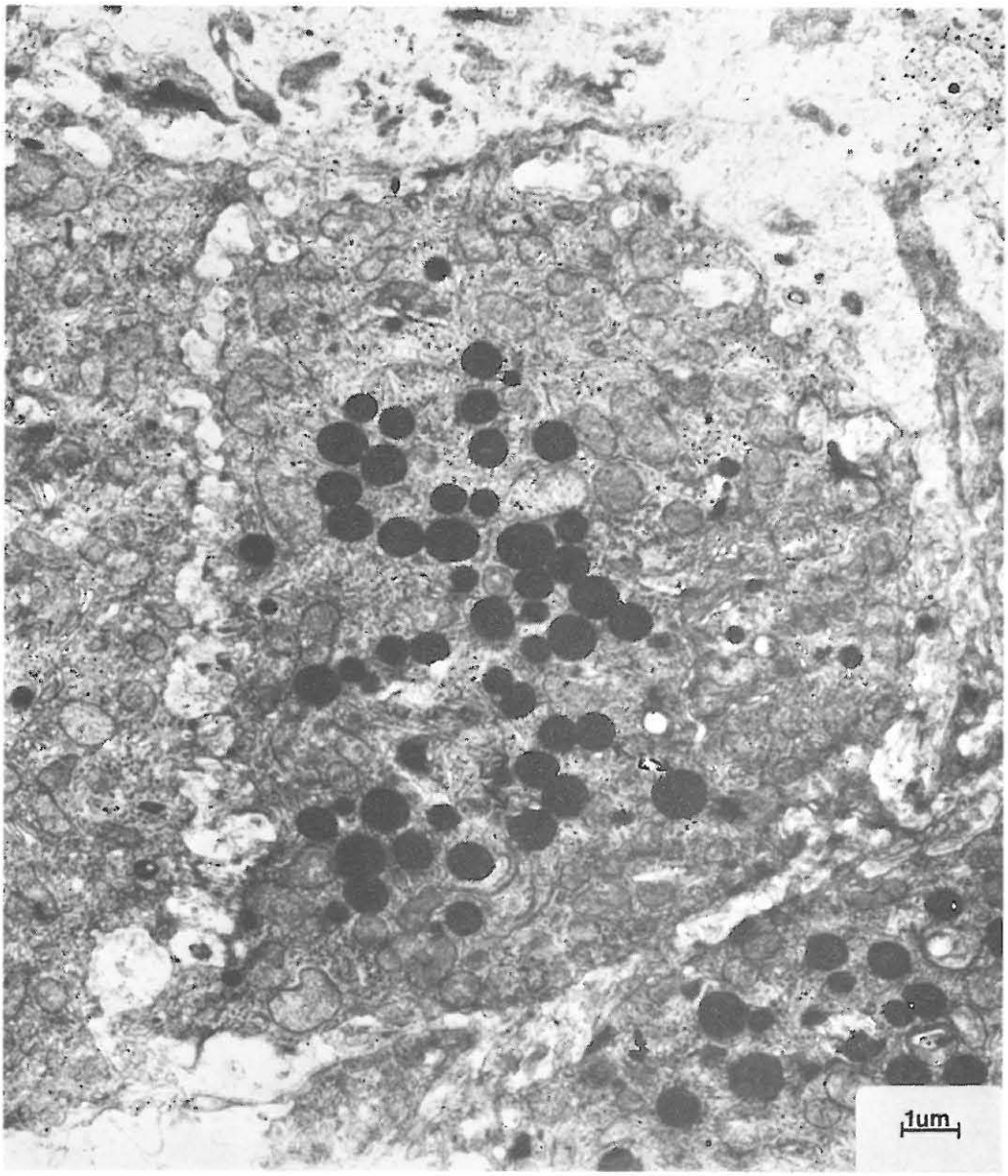


Figure 67. Control ovary showing characteristic features of corpus luteum. The dark staining bodies (arrowed) are lipid droplets. x 8 840

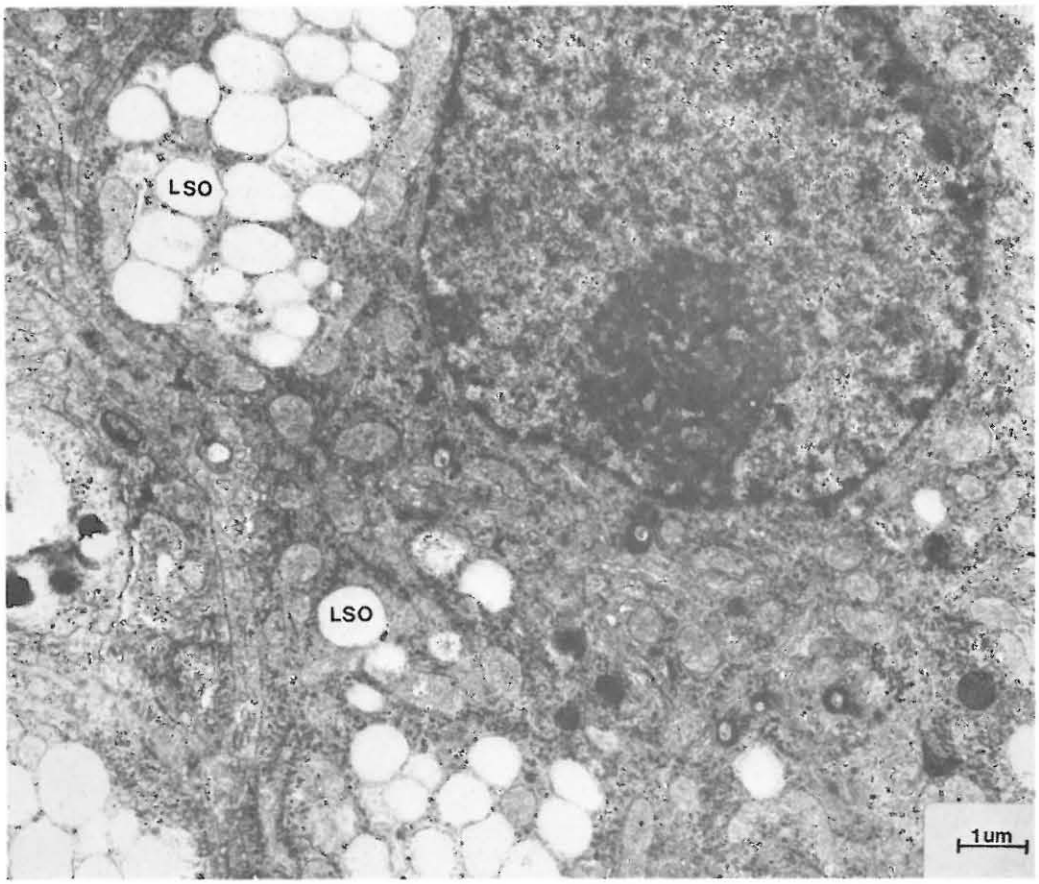


Figure 68. Portion of a corpus luteum from rat treated with 20 mg /kg norethynodrel for 30 days, light staining organelles (probably lipid droplets) are present in clusters. x 11 300. Dosing with 1 mg /kg norethynodrel caused a similar appearance of light staining organelles.

Corpora lutea from animals dosed for shorter periods of time with 20 mg/kg showed that this change occurs by the sixth day.

7.8.2 The Effects of Ethinyl Estradiol

Only the group to which the steroid had been administered for thirty days was examined and, in this case as well, the only definitive change was the appearance of these light staining organelles discussed above (Figure 69).

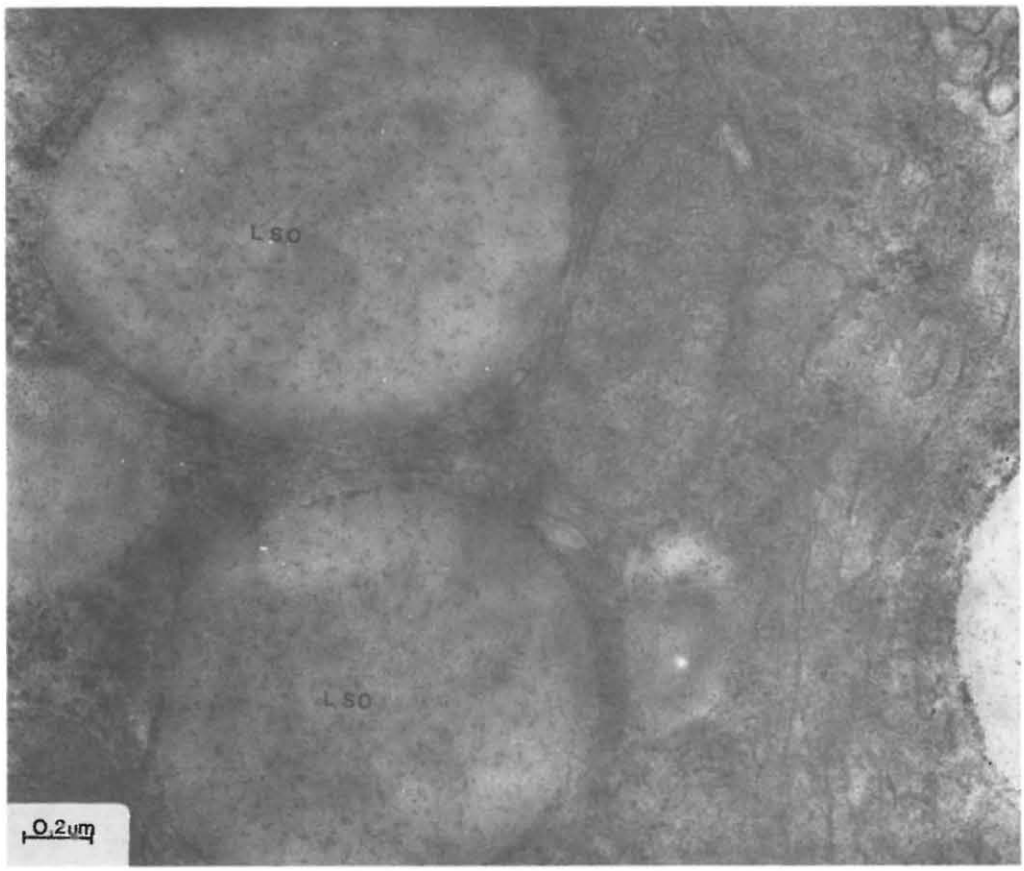


Figure 69. Portion of a corpus luteum from a rat treated with 20 mg /kg of ethinyl estradiol for 30 days, light staining organelles are present. x 54 500

SECTION 8

DISCUSSION

The results described here indicate that norethynodrel (a progestin) enhances the activity of the hepatic microsomal enzyme system in female rats at both levels (1 mg/kg and 20 mg/kg) tested. Ethinyl estradiol (an estrogen) appears devoid of activity or, if it has any effects, they are negligible.

Effect of Treatment with Norethynodrel

Associated with the induction of the hepatic microsomal enzyme system as a result of treatment with norethynodrel was a significant decrease in sleeping time of rats $p < 0,01$ according to the students "t" test (Table 20), an enlargement of the liver, which, especially when coupled with a concomitant decrease in total body mass, caused the relative mass of liver to increase (Tables 21 and 22), an increase in hepatic protein and in cytochrome P-450 (Tables 21 and 22), an increase in aniline hydroxylase and aminopyrine demethylase activity (Tables 26 and 27) and a marked increase in SER.

The decrease in sleeping time caused by norethynodrel is at variance with the results of Jori et al. (1969), who found that 30 days of treatment with norethynodrel (10 mg/kg daily) had no effect on pentobarbital narcosis. The decrease in sleeping time caused by norethynodrel is, however, in agreement with the results of Juchau and Fouts (1966), who found an increase in in vitro hexobarbital metabolism in male rats sacrificed 24 h after dosing

with 50 mg/kg of norethynodrel for 3 days or 3 weeks. However, it may not be valid to compare the results of the present study with those of Juchau and Fouts (1966) since they used male rats, and sex differences, especially in rats, have been recognized in the metabolism of drugs (Conney 1967).

Several explanations may exist for steroid-induced reduction in sensitivity of the rat to pentobarbital:

(1) there may be changes in tissue distribution or plasma binding of pentobarbital

(2) renal handling of pentobarbital may be altered

(3) there may be changes in the inherent sensitivity of the cerebro-spinal axis to pentobarbital

(4) finally, there may be changes in the ability of the rats to metabolize pentobarbital.

The role of the first three factors is undecided since no experiments were performed to determine these effects. However, the tests for enzyme induction (increase in liver size, in total protein, in cytochrome P-450, increased enzymatic activity and proliferation of SER) indicate that increased biotransformation of pentobarbital seems at least an important cause related to norethynodrel administration.

One further possible explanation for the altered rates of metabolism of pentobarbital observed in steroid pretreated rats is that individual steroids may alter the animal's basal temperature and this can markedly alter the rate of metabolism.

Tables 20 and 21 support the view that increases in microsomal protein and cytochrome P-450 occur at an early stage. On the fourth day of treatment with 20 mg/kg norethynodrel, protein and microsomal levels begin to increase and continue to do so at least throughout 30 days of

treatment and probably thereafter. At this dose level, steady increases were noted from the beginning of steroid treatment to the sixteenth day. At day 30, the cytochrome P-450 and protein levels are significantly higher than at any other time, indicating that, between the sixteenth and thirtieth day, a marked increase in these levels occurred.

One explanation for the high level of protein and cytochrome P-450 at the thirtieth day of treatment with 20 mg/kg of norethynodrel is that the system is attempting, through the hepatic metabolizing enzyme system, to rid itself of the high dose of steroid present.

Even at a dosage level of 1 mg/kg, norethynodrel induces increases in protein and cytochrome P-450 concentrations after a short period of administration, but the response appears to level off by the twelfth day of treatment (Figure 33). It may be that the hepatic metabolizing system of the rat is able to handle the lower dosage of (1 mg/kg) norethynodrel by the inductive adaptation so that a levelling off of enzyme concentration after the twelfth day occurs (Figure 33). The increase in liver mass, in protein and in cytochrome P-450 concentration in the rats dosed with norethynodrel (1 mg and 20 mg) was accompanied by a significant increase in aniline hydroxylase and aminopyrine demethylase activity (Tables 26 and 27), parameters generally indicative of enzyme induction. From these results, it is conceivable then that this increased protein concentration may be accounted for, at least in part, by an increased enzyme synthesis and, if this concept is correct, one could expect that those (microsomal) enzymes which are increased are largely those which are specific for the metabolism of foreign compounds. This, in turn, probably accounts for the reduced sleeping time, increased enzymatic activity and marked proliferation of the SER seen in norethynodrel treated rats.

The most striking changes in fine structure in the hepatocytes following dosing with norethynodrel include proliferation of SER with associated shortening and dispersions of rough endoplasmic cisternae, swelling of the mitochondria, and a depletion of glycogen stores.

Increases in SER and enhancement of the hepatic drug metabolizing enzyme system closely paralleled each other, since in both groups treated with norethynodrel increases in protein and cytochrome P-450 concentrations and increases in SER occurred more or less simultaneously.

Mannering (1968) and Conney (1967) indicate that an increase in SER is indicative of an increase in drug metabolizing enzymes. For a review of the induction of the cytochrome P-450 enzyme system see Section 4.

The significance of the swelling of the mitochondria is unclear. Similar morphological changes in mitochondria have been reported by Cardell et al. (1974).

The significance of the reduction in glycogen stores is also unclear, but this phenomenon could be due to a reduced ability of the liver to retain glycogen after norethynodrel administration, or it could be that norethynodrel treated rats consume less food and this would have led to the depleted glycogen stores.

Classification of Norethynodrel as an Inducer

It is now recognized that there are two principal classes of microsomal enzyme inducers (Conney 1967). This topic has been discussed at length in Section 4.1. Briefly, one class is represented by compounds such as phenobarbital and many insecticides, and the other by some carcinogenic compounds such as 3-methylcholanthrene and benzopyrene.

Inducers of the phenobarbital group promote a hypertrophy of the liver SER as well as, a generalized increase in microsomal activity of many

microsomal enzymes. Inducers of the 3-methylcholanthrene group promote a more moderate proliferation of the SER and induce only a few, rather specific enzymes.

The development of hypertrophy of the smooth membrane, an elevation of cytochrome P-450 and the elevation of protein concentration do not allow a rigid assignment of norethynodrel to either of these categories of inducers; however, norethynodrel's biochemical and morphological profiles are more in keeping with those of the phenobarbital group, and its effect on drug metabolizing capacity would add support to such a view.

The Effect of Ethinyl Estradiol

The experiment using phenobarbital to assess the inducibility of the hepatic mixed function oxidase system of the rats used for the 20 mg/kg ethinyl estradiol tests showed that the rats were not maximally induced.

Therefore, the results concerning the effect of estrogen on the drug metabolizing enzyme system indicate that ethinyl estradiol may be devoid of or have only a negligible inductive capacity.

Small, insignificant fluctuations (increases or decreases) in drug metabolizing parameters occurred after administration of both dose levels of ethinyl estradiol; for instance, sleeping times were shortened in most cases (Table 20). This difference, however, was small (5 min). Ethinyl estradiol also slightly increased aniline hydroxylase activity and 20 mg/kg ethinyl estradiol increased aminopyrine demethylase activity. Again however, these increases were small and insignificant.

The changes caused by ethinyl estradiol on protein and cytochrome P-450 concentrations were small and too irregular to be of any significance. No general trend depicting an increase or decrease in any of these parameters could be detected since the changes were too small.

The only significant change caused by ethinyl estradiol was an increase in relative liver mass and this increase was due largely, if not entirely, to the fact that treated animals lost mass. Liver mass of treated animals did not increase compared to the control animals. Ethinyl estradiol apparently has no effect on the proliferation of SER or the shortening of the lamellae of RER, and the finding that SER is not markedly increased correlates well with the biochemical findings.

The finding that ethinyl estradiol has no effect on the hepatic drug metabolizing system is in agreement with the findings of Blackham and Spencer (1969), Freudenthal and Amerson (1974) and Briatico et al. (1976).

Since small increases in some parameters, e.g., enzymatic activity caused by ethinyl estradiol, were evident, a synergistic effect of the estrogens on the activity of the progestins cannot be ruled out in the case of combined OCs.

The decrease in body mass in animals dosed with either norethynodrel or ethinyl estradiol appeared to be dose related, since the higher dose of both steroids resulted in a more marked decrease in body mass. It is of interest that norethynodrel significantly decreased body mass as well as increased liver mass, and it is probable that this increased liver mass accounted in part for the increased drug metabolism observed in these rats. Gilbert and Goldberg (1965) found a correlation between increase in liver mass and increased drug metabolism.

The estrogen on the other hand had no effect on liver mass and no appreciable effect on the rate of drug metabolism. The greasy appearance of the norethynodrel treated rats may be due to an increase in the activity of the sebaceous glands due to an increased glandular activity, which is known to exist in prepubertal males and females.

An objection might be raised that the alterations in microsomal enzyme activity caused by the administration of oral contraceptive steroids are produced only at very high doses as compared with those used in human contraceptive regimens, but it must be noted that large unphysiological doses were employed in order to accelerate untoward changes or side effects which may occur over a long period of time at lower, more physiological doses. Moreover, the doses employed in these experiments were those recommended by Günzel (1972).

Carter et al. (1974) mention that, since rat liver mass - body mass ratios of rats are greater than those of humans, the metabolizing capacity of the rat liver is approximately 10 times that of humans; on this basis, the low dose of 1 mg/kg does not appear particularly high.

Animal studies indicate that OCs, especially the progestins, may cause stimulation of drug metabolism. Available data on the effects of OCs on the hepatic microsomal enzyme system in humans indicate that OCs inhibit drug metabolism (Crawford and Rudofsky 1966; O'Malley et al. 1972). Thus, studies on animal and on humans do not correlate well. The effect of OCs on human drug metabolizing enzymes, however, should be interpreted with some caution since only isolated studies have been carried out and these have involved a small number of subjects. Contradictory results on the effect of OCs in animals also exist in the literature. This situation arises because of differences in conditions employed by various laboratories, and partly because of differences in dose levels applied.

Since results from studies in laboratory animals and humans are conflicting, the use of the rat as a model for the effects of OCs in women should be re-assessed. Concurrent studies employing human subjects and rats on dosages of OCs sufficient to give rise to an anti-fertility effect in each

should be conducted and the results compared. An explanation for the variation in animal studies and human studies may be that an absolute difference in the mode of action of OCs in rats and humans exist. It is clear that, before an animal model for the effect of OCs in humans can be reliably used, the correct drug doses, species, sex and age of the animals need to be established.

Effects of Oral Contraceptive Steroids on the Adrenal Cortex

The results reported indicate that norethynodrel at 1 mg/kg and both dose levels of ethinyl estradiol have the same effect on the adrenal cortex, namely that of lipid depletion. A similar effect was noted, following administration of 20 mg/kg norethynodrel, up to the sixteenth day. After 30 days of administration with 20 mg/kg of norethynodrel however, electron dense, spherical bodies of variable size appeared in the cells, but the significance of this finding is uncertain. These bodies appear to be lipid droplets since they do not display a limiting membrane. It is unlikely to be an artifact arising from the fixation or staining process since such bodies were absent in the preparations from control adrenals which were fixed and stained simultaneously. If this body consists of lipids, these lipids probably contains a high degree of unsatuaration. Fawcett (1967) states that unsaturated lipid droplets are exceedingly osmophilic, while lipid droplets that are saturated take up much less osmium, with the result that they are lighter staining (as in control animals). This behaviour suggests that the lipidic material present after 30 days of norethynodrel treatment is of a different chemical composition to that found in normal adrenal cortex and in the other experimental groups.

Lipid droplets serve as a local store of energy and as a potential source of short, carbon chains that can be utilized by the cell for the synthesis

of lipid-containing structural components, such as membrane, or in the elaboration of specific secretory products, such as, in the case of the adrenal cortex, the corticosteroids.

According to Moses et al. (1969), in the rat adrenal gland only about 8% of total cholesterol occurs in the microsomal fraction, whereas 75% is found in the lipid droplets. If this is the case, then a depleted lipid content in the form of droplets could be interpreted as meaning that an increase in synthesis of one, or perhaps all, of the corticosteroids is occurring, since the number and size of the lipid droplet in the adrenal cortex may be an indication of the balance between the need for corticosteroids and, hence, the mobilization of the precursor, cholesterol, and the storage of cholesterol in the lipid droplets. If OCs increase corticosteroidogenèsis, the hormone that is most likely to be increasing is aldosterone, since Beckerhoff et al. (1973) found an increase in aldosterone in OCs users (for discussion see Section 1.5.2.). However in drawing this parallel, one should be aware that species differences do exist, and what occurs in humans may not occur in rats, since, after all, the OCs could have very different effects in these two species. An increased steroidogenesis would be associated with an increase in the number of mitochondria and SER, since these are the principle steroidogenic organelles of the adrenal cortex. A depletion of lipid droplets on the other hand could simply mean that the OCs cause a decrease in the ability of the adrenal to retain lipid.

The observation that norethynodrel and ethinyl estradiol appear to have the same effect on the adrenal might be explained by the fact that norethynodrel possesses some estrogenic activity, and changes in the metabolism of adrenocorticoids in women have been explained on this basis (Layne 1962). The estrogenic activity of norethynodrel is equivalent to 3 to 7% of that of estrone (Saunders 1964). Thus, it is quite reasonable to expect that the adrenal cortex would respond to norethynodrel as it does to ethinyl

estradiol. However without supporting evidence, one cannot conclude with certainty that this is the case since the response elicited by various estrogens on the adrenal cortex may be different, and it is possible that the progestins could exert their own influence on this organ, especially since the appearance of the electron dense bodies following 30 days of treatment with norethynodrel (20 mg/kg) cannot be explained by the estrogenic nature of norethynodrel since these organelles, were absent in estrogen treated rats.

The Effects of Oral Contraceptives on Vaginal Cytology and the Ovary

Administration of norethynodrel and ethinyl estradiol abolished normal and regular oestrus cycle.

No study was undertaken however to correlate the changes in cellular pattern observed in the vaginal smears of the individual rats to events occurring in their respective ovaries. Any such comparison would have to consider both histological changes and circulating hormone levels. Not much additional information can be gleaned from the electron microscopy of the ovary at this stage. To glean more information, the author suggests that experiments having the sophistication required by the complexity of the subject be designed and this will involve morphological, histological, histochemical and biochemical studies with regard to steroidogenesis.

The significance of the observed change in chemical composition of lipid from the droplets is unclear. Lipid droplets of cells which secrete steroids probably contain a mixture of different lipids. It is possible then, that the images seen in the electron micrographs of cells from rats to which steroids have been administered give some indication of the composition of the droplets and the size of the droplets. However, knowledge of the differential extraction which occurs during the preparation of tissues for electron microscopy and of differences in density of image is not at present in a stage which makes reasonable a detailed analysis of these droplets from observations in electron micrographs.

SECTION 9

CONCLUSION

Evidence from this study has shown norethynodrel to be a moderately potent inducer of the hepatic mixed function oxidase system in female rats. Its inductive profile places it in the same group of inducers as phenobarbital. This conclusion is based on the evidence that norethynodrel

(1) increases the relative liver mass

(2) modifies the nature and the extent of the hepatic endoplasmic reticulum

(3) augments the protein content of the liver

(4) increases the hepatic cytochrome P-450 levels

(5) reduces the sleeping time after pentobarbital administration

(6) increases the biotransformation of

  (i) aniline

  (ii) aminopyrine.

In contrast, ethinyl estradiol when administered alone has been shown by this work to have little or no inductive capacity as judged by the same criteria.

In oral contraceptive practice, these two types of agents are generally administered simultaneously. A synergistic effect on the inductive potential of each of these agents on each other could possibly result. This possibility was, however, not investigated in this study but would be an important extension of it.

At high dose levels, both the progestin and the estrogen caused an inhibition of the growth of the animals and a net loss of mass over a period of time. This loss in mass was associated with alterations in glycogen content of the liver and apparent alterations in the chemical composition of the lipidic material in the adrenal cortex and ovary as evidenced by the study

of electron micrographs of these tissues. The lipidic material in the adrenal cortex and ovary had become more osmophilic (susceptible to osmium tetroxide staining) an indication of an increase in unsaturation. A further investigation into the biochemical nature of the changes in carbohydrate and lipid metabolism is indicated.

Alterations in the shape of the organelles in ovarian and adrenal cells are also apparent but the results do not permit specific and definitive conclusions to be drawn and further studies are required. Unfortunately, attempts to extrapolate these findings to man would not be justified, but the necessity and significance of extensive studies into these aspects of the effects of these agents in man become evident.

It is apparent that physiological changes of a general nature occur during prolonged use of these contraceptive agents. In addition, oral contraceptives cause changes in the hepatic mixed function oxidase system. These changes may alter the effectiveness of other medicaments that women taking oral contraceptives might be using. It is evident that such changes in the metabolizing system may lead to considerable enhancement of the rate of biotransformation of such medicaments, thereby shortening their duration of action and reducing their effectiveness. There is added danger, of course, that enhanced biotransformation may result in increased levels of metabolites, compounds which may be toxic. The importance of findings of this nature cannot be overemphasized when millions of women throughout the world are involved.

REFERENCES

1. ACHTARI, H., LEMARCHAND-BERAUD, T. and CHURCHOD, A. : Acta. Endocr. Suppl. 155, 169 (1971).

2. ADAMS, P.W., WYNN, V., ROSE, D.P., SEED, M., FOLKARD, J. and STRONG, R.: Lancet 1, 897 (1973).

3. AFTERGOOD, L. and AFLIN-SLATER, R.B. : in Metabolic Effects of Gonadal Hormones and Contraceptive Steroids, p.265; Eds. H.A. Salhanick, D.M. Kipnis and R.L. Vande Wiele, Plenum Press, N.Y., 1969.

4. AFTERGOOD, L. and AFLIN-SLATER, R.B. : Lipids 9, 91 (1974).

5. AHMED, F. and BAMJI, M. : Contraception 14, 309 (1976).

6. ALVARES, A.P., SCHILLING, G., LEVIN, W. and KUNTZMAN, R. : Biochem. Biophys. Res. Commmun. 24, 521 (1967).

7. ALVARES, A.P. : Biochem. Pharmac. 22, 1037 (1973).

8. ALVAREZ DE, R.R., JAHED, F.M. and SPITALNYK, J. : Am. J. Obstet. Gynecol. 116, 727 (1973).

9. ANDERSON, J.E., CLARK, J.H. and PECK, E.J. : Biochem. J. 126, 561 (1972).

10. ANONYMOUS EDITORIAL : Nutr. Rev. 31, 49 (1973).

11. ANONYMOUS EDITORIAL : Nutr. Rev. 31, 5 (1973).

12. ASBECK, F., BOLTE, A., HENNING, H.D., HÖRDEMANN, K., LATZ, R., LECHLER, E. and VAN DE LOO, J. : Haemostasis 1, 108 (1973).

13. BARRY, J. and GORSKI, J. : Biochemistry 10, 2384 (1971).

14. BAULIEU, E.E., ALBERGA, A., JUNY, I., LEBAN, M., MERCIER-BODARD, C., MILGROM, E., RAYNAND, J.P., RAYNAND JANNET, C., ROCHEFORT, H., TRUONY, H. and ROBEL, P. : Recent Prog. Horm. Res. 27, 351 (1971).

15. BAULIEU, E.E. : Biochem. Pharmacol. 24, 1743 (1975).

16. BECK, P. : in Metabolic Effects of Gonadal Hormones and Contraceptive Steroids, p.97; Eds. H.A. Salhanick, D.M. Kipnis and R.L. Vande Wiele, Plenum Press, N.Y., 1969.

17. BECK, W.S. and GOULIAN, M. : in Drills Pharmacology 4th edition, p.1061; Ed. J.R. Di Palma, Mc Graw-Hill Book Company, 1971.

18. BECKENRIDGE, A. and ORUSE, M. : Ann. N.Y. Acad. Sci. 179, 421 (1970).

19. BECKENRIDGE, A. and ROBERTS, J. : Pharmacol. Res. Commun. 8, 229 (1976).

20. BECKERHOFF, R., VETTER, W., ARMBRUSTER, H., LUETSCHER, J.A. and SIEGENTHALER, W. : Lancet 1, 1218 (1973).

21. BELINA, H., COOPER, S., FARKAS, R. and FEUER, G. : Biochem. Pharmacol. 24, 301 (1975).

22. BERGSJO, P., FAYERHOL, M.K. and ABILDGAARD, U. : Am. J. Obstets. Gynecol. 112, 938 (1972).

23. BIERMAN, E.L. : in Metabolic Effects of Gonadal Hormones and Contraceptive Steroids, p.207; Eds. H.A. Salhanick, D.M. Kipnis and R.L. Vande Wiele, Plenum Press, N.Y., 1969.

24. BILLIAR, R.B., JASSANI, M. and LITTLE, B. : Am. J. Obstets. Gynecol. 121, 7 (1975).

25. BLACKHAM, A. and SPENCER, P.S.J. : Br. J. Pharmacol. 37, 129 (1969).

26. BOGANDOVE, E.M., NOUN, J.M. and CAMBELL, G.T. : Recent Prog. Horm. Res. 31, 567 (1975).

27. BOOTH, J. and GILLETTE, J.R. : J. Pharmacol. Exp. Ther. 137, 374 (1962).

28. BRIATICO, G., GUISO, G., JORI, A. and RAVAZZANI, C. : Br. J. of Pharmacol. 58, 173 (1976).

29. BRIGGS, M.H., PITCHFORD, A.G., STANFORD, M., BARKER, H.M. and TAYLOR, D. : Ad. Steroid Biochem. Pharmacol. 2, 111 (1970).

30. BRIGGS, M.H. and BRIGGS, M. : Nature (London) 238, 277 (1972).

31. BRIGGS, M. : Ad. Steroid Biochem. Pharmacol. 5, 65 (1976).

32. BROOKS, S.C. : in Steroid Hormone Action and Cancer, p.36; Eds. K.M.J. Menon and J.R. Reel, Plenum Press, N.Y. and Lon., 1976.

33. BROWN, J.B., FOTHERBY, K. and LORRAINE, J.A. : Endocrinology 25, 331 (1962).

34. BROWN, R.R., ROSE, D.P., LEKLEM, J.E. and LINKSWILER, H.M. : Acta Vitaminol. Enzymol. 29, 151 (1975).

35. BURKE, C.W. : J. Clin. Path. 23, suppl. (Ass. Clin. Path.) 3, 11 (1970).

36. CAPPON, I.D. and NICHOLLS, D.M. : Pest. Biochem. Physiol. 5, 109 (1975).

37. CARDELL, R., Jr. : Am. J. Anat. 139, 49 (1974).

38. CARGILLE, C.M., VAITUKAITIS, J.L., BERMUDEZ, J.A. and ROSS, G.T. : J. Clin. Endocr. Metab. 36, 87 (1973).

39. CARTER, D.E., GOLDMAN, J.M., BRESLER, R., HUXTABLE, R., CHRISTIAN, D. and WAYNE HEINE, M. : Clin. Pharmacol. and Ther. 15, 22 (1974).

40. CINTI, D.L., MOLDEUS, P. and SCHENKMAN, J.B. : Biochem. Pharmacol. 21, 3249 (1972).

41. CHAMNESS, G.C. and McGUIRE, W.L. : Biochemistry 11, 2466 (1972).

42. CHAPLIN, M.D. and MANNERING, G.J. : Mol. Pharmacol. 6, 631 (1970).

43. CHURCH, R.E. and McCARTHY, B.J. : Biochem. Biophys. Acta. 199, 103 (1970).

44. CLAUDE, A. : in Microsomes and Drug Oxidation, p.3; Eds. J.R. Gillette, A.H. Conney, G.J. Cosmides, R.W. Estabrook, J.K. Fouts and G.J. Mannering, Academic Press, N.Y., 1969.

45. COMAI, K. and GAYLOR, J.L. : J. Biol. Chem. 248, 4947 (1973).

46. CONNEY, A.H. and GILMAN, A.G. : J. Biol.Chem. 238, 3682 (1963).

47. CONNEY, A.H. : Pharmacol. Rev. 19, 317 (1967).

48. CONNEY, A.H. : in Fundamentals of Drug Metabolism and Disposition, p.253; Eds. B.N. La Du, H.G. Mandel and E.L. Way. The Willaems and Wilkins Company, Baltimore, 1972.

49. COOPER, D.Y., LEVIN, S., NARASIMULU, S., ROSENTHAL, O. and ESTERBROOK, R.W. : Science 147, 400 (1965).

50. CRANE, M.G., HEITSCH, J., HARRIS, J.J. and JOHNS, V.J. : J. Clin. Endocr. Metab. 26, 1403 (1966).

51. CRAWFORD, J.S. and RUDOFSKY, S. : Br. J. Anaesth. 38, 446 (1966).

52. CROMBIE, D.L., PINSEL, R.J.F.H., SLATER, B.C., FLEMING, D. and CROW, K.W. : Br. Med. J. 4, 178 (1970).

53. CROSSLAND, J. : in Lewis's Pharmacology 4th edition, chapter 38; Revised by J. Crossland, E and S Livingstone, Edinburgh and London, 1970.

54. DALLNER, G. and ERICSSON, J.L.E. : Prog. in Liver Dis. 5, 35 (1976).

55. DAVIS, L.E. and SMITH, R.K. : Lancet 1, 1245 (1973).

56. DAVSON, H. : in Textbook of General Physiology 4th edition, p.154; J and A Churchill, London, 1970.

57. DEHLINGER, P.J. and SCHIMKE, R.T. : J. Biol. Chem. 244, 2017 (1972).

58. DEHLINGER, P.J. and SCHIMKE, R.T. : J. Biol. Chem. 247, 1257 (1972).

59. DELVOYE, P., HASAN, S.H., L'HERMITE, M., NEUMANN, F. and ROBYN, C. : Acta. Endocr. Suppl. 117, 133 (1973).

60. DE MATTEIS, F. : Pharmacol. Rev. 19, 523, (1967).

61. DEPIERRE, J.W. and ERNSTER, L. : FEBS Letters 38, 219 (1976).

62. DHALL, K., KUMAR, M., RASTOGI, C. and PUTHUSSERI, K. : Fert. and Steril. 28, 156 (1977).

63. DICZFALUSY, E. : Am. J. Obstets. Gynecol. 100, 136 (1968).

64. DOAR, J.W.H., WYNN, V. and CRAMP, D.G. : in Metabolic Effects of Gonadal Hormones and Contraceptive Steroids, p.178; Eds. H.A. Salhanick, D.M. Kipnis and R.L. Vande Wiele, Plenum Press, N.Y., 1969.

65. DOAR, J.W.H. and WYNN, V. : Br. Med. J. 1, 149 (1970).

66. DODDS, M.I. : J. Am. Diet. Assoc. 54, 32 (1969).

67. DRILLS, V.A. : in Oral Contraceptives, McGraw-Hill Book Company, N.Y., 1966.

68. ECKSTEIN, P., WATERHOUSE, J.A.H., BOND, G.M., MILLS, W.G., SANCHLANDS, D.M. and SHOTTON, B.M. : Br. Med. J. 2, 1172 (1961).

69. EISENFELD, A.J., ATEN, R., WEINBERGER, M., HASELBACHER, G. and HALPERN, K. : Science 191, 862 (1976).

70. ELING, T.E. and DLANGUSTINE, R.P. : Biochemistry 123, 539 (1971).

71. ESTABROOK, R.W., COOPER, D.Y. and ROSENTHAL, O. : Biochem. Z. 338, 741 (1963).

72. ESTABROOK, R.W. and COHEN, B. : in Microsomes and Drug Oxidation, p.95; Eds. J.R. Gillette, H.H. Conney, G.J. Cosmides, R.W. Esterbrook, J.R. Fouts and G.J. Mannering, Academic Press, N.Y., 1969.

73. ESTABROOK, R.W. : Biochem. Biophys. Res. Commun. 42, 132 (1971).

74. EVERETTE, J.W. : Ann. Rev. Physiol. 31, 383 (1969).

75. FABER, L.E., SANDMANN, M.L. and STAVELY, H.E. : J. Biol. Chem. 247, 8000 (1972a).

76. FABER, L.E., SANDMANN, M.L. and STAVELY, H.E. : J. Biol. Chem. 247, 5648 (1972b).

77. FANG, S., ANDERSON, K.M. and LIAO, S. : J. Biochem. 224, 6584 (1969).

78. FAWCETT, D.W. : in The Cell. Its Organelles and Inclusions, W.B. Saunders Company, Philadelphia and London, 1967.

79. FEHERTY, P., ROBERTSON, D.M., WAYNSFORTH, H.B. and KELLIE, A.E. : Biochem. J. 120, 837 (1970).

80. FEUER, G., KARDISH, R. and FARKES, R. : Biochem. Pharmacol. 26, 1495 (1977).

81. FOUTS, J.R. and ROGERS, L.A. : J. Pharm. Exp. Ther. 147, 112 (1965).

82. FREGLY, M. : Toxicol. App. Pharmacol. 25, 560 (1973).

83. FREUDENTHAL, R.I. and AMERSON, A.E. : Biochem. Pharmacol. 23, 2651 (1974a)

84. FREUDENTHAL, R.I., AMERSON, A.E., MARTIN, J. and WALL, M.E. : Pharmacol. Res. Commun. 6, 457 (1974b).

85. FUJITA, T., SHOEMAN, D.W. and MANNERING, G.J. : J. Biol. Chem. 248, 2192 (1973).

86. FURMAN, R.H. : in Metabolic Effects of Gonadal Hormones and Contraceptive Steroids, p.246; Eds. H.A. Salhanick, D.M. Kipnis and R.L. Vande Wiele, Plenum Press, N.Y., 1969.

87. GAFAAR, A., TOPPOZADA, H.K., HOZAYEN, A., ABDELL-MALER, A.T., MOGHAZY, M. and YOUSEF, M. : Contraception 8, 43 (1973).

88. GAL, I., PARKINSON, C. and CRAFT, I. : Br. Med. J. 2, 436 (1971).

89. GAL, I. : Nature (London) 240, 241 (1972).

90. GAL, I. and PARKINSON, C.E. : Contraception 8, 13 (1973).

91. GARFINKEL, D. : Arch. Biochem. Biophys. 77, 493 (1958).

92. GARG, B.D., BLASCHECK, J.A. and KOVACS, K. : Tohoku J. Exp. Med. 104, 205 (1972).

93. GARMENDIA, F., KESSURU, E. and LLERENA, L.A. : Horm. Metab. Res. 5, 134 (1973).

94. GELBOIN, H.V. and SOKOLOFF, L. : Science 134, 611 (1961).

95. GELBOIN, H.V. : in Fundamentals of Drug Metabolism and Disposition, Chapt.14 Eds. B.N. La Du, H.G. Mandel and E.L. Way, The Willaems and Wilkens Company, Baltimore, 1972.

96. GERLEHRTER, T.D. : Metabolism 22, 85 (1973).

97. GIANNOPOULOS, G. and GORSKI, J. : J. Biol. Chem. 246, 2530 (1971).

98. GILBERT, D. and GOLDBERG, L. : Fd. Cosmet. Toxicol. 3, 417 (1965).

99. GILLETTE, J.R., DAVIS, D.C. and SASAME, H.A. : Ann. Rev. Pharmacol. 12, 57 (1972).

100. GLASSER, S.R., CHYTIL, F.C. and SPELSBERG, T.C. : Biochem. J. 130, 947 (1972).

101. GLUECKSOHN-WAELSCH, S., GREENGARD, P., QUINN, G.P. and TEICHER, L.S. : J. Biol. Chem. 242, 1271 (1967).

102. GORSKI, J. : J. Biol. Chem. 239, 889 (1964).

103. GORSKI, J., TOFT, D., SHYAMALA, G., SMITH, D. and NOTIDES, A. : Recent Prog. Horm. Res. 24, 45 (1968).

104. GORSKI, J. and GANNON, F. : Ann. Rev. Physiol. 38, 425 (1976).

105. GREIM, H. : Biochem. Biophys. Acta. 201, 20 (1970).

106. GRANICK, D. : J. Biol. Chem. 241, 1359 (1966).

107. GRANICK, S. and KAPPUS, A. : J. Biol. Chem. 242, 4857 (1967).

108. GSCHWENDT, M. and HAMILTON, T.H. : Biochem. J. 128, 611 (1972).

109. GÜNZEL, P. : in Methods in Steroid Toxicology, p.1; Eds. E.J. Plotz and J. Haller, Geron-X, Inc., Los Altos, California, 1972.

110. HAMILTON, T.H., WIDNELL, C.C. and TATA, J.R. : J. Biol. Chem. 243, 408 (1968).

111. HAMRICK, M.E., ZAMPAGLIONE, N.G., STRIPP, B. and GILLETTE, J.R. : Biochem. Pharmacol. 22, 293 (1973).

112. HAUGEN, D.A., VAN DER HOEVEN, T.A. and COON, M.J. : J. Biol. Chem. 250, 3567 (1975).

113. HAYAT, M.A. : in Principles and Techniques of Electron Microscopy, Van Nostrand Reinhold Company, N.Y./Cincinnati/Toronto/London/ Melbourne 1970.

114. HAZZARD, W.R., SPICER, M.J. and BIERMAN, E.L. : in Metabolic Effects of Gonadal Hormones and Contraceptive Studies, p.232; Eds. H.A. Salhanick, D.M. Kipnis and R.L. Vande Wiele, Plenum Press, N.Y. 1969.

115. HAZZARD, W.R., NOTTER, D.T., SPIGER, M.J. and BIERMAN, E.L. : J. Clin.Endocr. Met. 35, 425 (1972).

116. HILDEBRANDT, A., REMMER, A. and ESTABROOK, R.W. : Biophys. Res. Comm. 30, 607 (1968).

117. HILDEBRANDT, A., and ESTABROOK, R.W. : Arch. Biochem. Biophys. 143, 66 (1971).

118. HILLIARD, J., CROXATTO, H.B. HAYWARD, J.N. and SAWYER, C.H. : Endocrinology 79, 411 (1966).

119. HOPKINS, G.J. and WEST, C.E. : Lipids 11, 736 (1976).

120. HUANG, J.J. and KIMURA, T. : Biochem. Biophys. Res. Commun. 44, 1065, (1971).

121. IDLEMAN, S. : Int. Rev. Cytol. 27, 181 (1970).

122. INMAN, W.H.W. : Br. Med. Bull. 26, 248 (1970).

123. ISHIMUAE, Y. : Biochem. Biophys. Res. Commun. 42, 140 (1971).

124. IYANAGI, T. and MASON, H.S. : Biochemistry 12, 2297 (1973).

125. JACKSON, V. and CHALKLEY, G.R. : J. Biol. Chem. 249, 1615 (1974).

126. JACOB, F. and MONOD, J. : J. Molec. Biol. 3, 318 (1961).

127. JEFCOATE, C.R.E. and GAYLOR, J. : Biochemistry 8, 3464 (1969).

128. JELLINCK, P.H. and LUCIEER, I. : Endocrinology 32, 91 (1965).

129. JENSEN, E.V. and JACOBSON, H.J. : Recent Prog. Horm. Res. 18, 387 (1962).

130. JENSEN, E.V. and DE SOMBRE, E.R. : Ann. Rev. Biochem. 41, 203 (1972).

131. JICK, H. and SHUSTER, L. : J. Biochem. 241, 5366 (1966).

132. JICK, H., SLONE, P., WESTERHOLM, W., INMAN, M.P., VESSEY, S., SHAPIRO, G., LEWIS, P. and WORCESTER, J. : Lancet 1, 534 (1969).

133. JOHNSON, K., GEENEN, J.E., HEUSLEY, G.T. and SOERGEL, K.H. : Digest. Dis. 18, 185 (1973).

134. JORI, A., BIANCHETTI, A. and PRESTINI, P.E. : Europ. J. Pharmacol. 7, 196 (1969).

135. JOSS, U., BASSAND, C. and DIERKS-VENTLING, C. : FEBS Letters 66, 293 (1976).

136. JUCHAU, M.R. and FOUTS, J.R. : Biochem. Pharmacol. 15, 891 (1966).

137. KATO, R. and TAKANAKA, A. : Jap. J. Pharmacol. 17, 208 (1967).

138. KATZENELLENBOGEN, B.S. and GORSKI, J. : J. Biol. Chem. 247, 1299 (1972).

139. KATZENELLENBOGEN, J., JOHNSON, H.J. Jr. and CARLSON, K.E. : Biochemistry 12, 4092 (1973).

140. KATZENELLENBOGEN, B.S. and GORSKI, J. : in Biochemical Action of Hormones Vol. 3, p.187; Ed. G. Litwack, Academic Press, N.Y., 1975.

141. KATZENELLENBOGEN, J.A., RUH, T.S., CARLSON, K.E., IWAMOTO, H.S. and GORSKI, J. : Biochemistry 4, 2310 (1975).

142. KAULLA, E., DROEGEMUELLER, W., AOKI, N. and KAULLA VON, K.N. : Am. J. Obstets. Gynecol. 109, 868 (1971).

143. KAWALEK, J.S., LEVIN, W., RYAN, D., THOMAS, P.E., WEST, S.B. and LU, A.Y.H. : Mol. Pharmacol. 11, 874 (1975).

144. KEYE, W.R. Jr., HO YUEN, B. and JAFFE, R.B. : J. Clin. Endocr. Metab. 2, 3 (1973).

145. KIMURA, T. : in Biogenesis and Action of Steroid Hormones, p126; Eds. R.I. Dorfman, K. Yamasaki and M. Dorfman, Geron-X, Inc., Los Altos, 1968.

146. KING, R.J. and GORDON, J. : Nature (London) 240, 185 (1972).

147. KING, R.J.B. and MAINWARING, W.I.P. : in Steroid-Cell Interactions, Chapter 8; Butterworth, London, 1974.

148. KLINGENBERG, M. : Arch. Biochem. Biophys. 75, 376 (1958).

149. KNOWLES, J.T. and SMELLIE, R.M.S. : Biochem. J. 125, 605 (1971).

150. KRITCHEVSKY, D., TEPPER, T.A. and STORY, J.A. : Lipids 8, 8 (1973).

151. KUBIK, M.M. and BHOWMICK, B.K. : Br. Heart J. 35, 1271 (1973).

152. KUNTZMAN, R., JACOBSON, M. SCHEIDMEN, K. and CONNEY, A.H. : J. Pharmacol. Exp. Ther. 146, 280 (1964).

153. KUNTZMAN, R., LEVIN, W., SCHILLING, G. and ALVARES, A. : in Microsomes and Drug Oxidation, p.349; Eds. J.R. Gillette, A.H. Conney, G.S. Cosmides, E.W. Estabrook, J.R. Fouts and G.S. Mannering, Academic Press, N.Y. 1969.

154. KURIMAYA, Y., OMURA, T., SIEKEVITZ, P. and PALADE, G.E. : J. Biol. Chem. 244, 2017 (1969).

155. LAEGNER, A. and DETERING, K. : Lancet 2, 600 (1974).

156. LARSSON-COHN, U., JOHANSSON, E.D.B. and GEMZELL, C. : Acta. Endocr. 66, 702 (1971).

157. LARSON-COHN, U. : Acta Soc. Med. Uppsal. 76. 223 (1970).

158. LAYNE, D.S., MEYER, C.J., VAISHWANER, P.S. and PINCUS, G. : J. Endocr. 22, 107 (1962).

159. LECOCQ, F.R., BRADLEY, E.M. and GOLDZIENER, J.W. : Am. J. Obstets. Gynecol. 99, 374 (1967).

160. LECOMPTE, F. and RENAUD, S. : Thrombos. Diath. Haemorrh. (Stuttg.) 29, 510 (1973).

161. LEKLEM, J.E. and BROWN, R. : J. Natl. Can. Instit. 56, 1101 (1976).

162. LEVIN, W.D., RYAN, D., WEST, S.B. and LU, A.Y.H. : J. Biol. Chem. 249, 1747 (1974).

163. LIAO, S., LIANG, T., TYMOCZKO, J.L. : Nature New Biol. 241, 211 (1973).

164. LINDEBAUM, J., WHITEHEAD, N. and REYNER, F. : Am. J. Clin. Nutr. 28, 346 (1975).

165. LOH, H.S. : Int. Inc. Vit. Res. 42, 86 (1972).

166. LOWENSTEIN, J. : Ann. Rev. Med. 23, 333 (1972).

167. LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. : J. Biol. Chem. 193, 265 (1951).

168. LU, A.Y.H. and COON, M.J. : J. Biol. Chem. 243, 1331 (1968).

169. LU, A.Y.H., JUNK, K.W. and COON, J. : J. Biol. Chem. 244, 3714 (1969).

170. LU, A.Y.H., KUNTZMAN, R., WEST, S., JACOBSON, M. and CONNEY, A.H. : J. Biol. Chem. 247, 1727 (1972).

171. LU, A.Y.H., WEST, S.B., VORE, M., RYAN, D. and LEVIN, W. : J. Biol. Chem. 249, 6701 (1974).

172. LU, A.Y.H. : Fed. Proc. 35, 13 (1976).

173. MADSEN, J.A. : in Serotonin - New Vistas, p.249; Eds. E.A. Costa, G. Gena and M. Sader, Raven Press, N.Y. 1974.

174. MAINWARING, W.I.P. and PETERKEN, B.M. : Biochem. J. 125, 285 (1971).

175. MALAMED, S. : in Handbook of Physiology Section 7, Endocrinology, Vol. VI. Adrenal Gland, p.25; Eds. H.Blaschka, A. David Smith, G. Sayers, American Physiological Society, Washington, D.C., 1975.

176. MANNERING, G.J. : in Selected Pharmacological Testing Methods, p.51; Ed. A. Burger, Marcel Dekker, N.Y., 1968.

177. MANNERING, G.J. : in Drug Metabolism and Disposition, Chapter 12; Eds. B.N. La Du, H.G. Mandel and E.L. Way, The Williams and Wilkins Company, Baltimore, 1972.

178. MARSHALL, W.J. and McLEAN, A.E.M. : Biochem. J. 122, 569 (1971).

179. MARTINEZ-MANAUTOU, J., GINER-VELASQUEZ, J., CORTES-GELLEGOS, V., AZNAR, R., ROJAS, R., GUITTEREZ-NAJAR, A. and RUDEL, H.W. : Br. Med. J. 2, 730 (1967).

180. MARVER, H.S. : in Microsomes and Drug Oxidations, p.495; Eds. J.R. Gillette, A.H. Conney, G.S. Cosmides, E.W. Estabrook, J.R. Fouts and G.S. Mannering, Academic Press, N.Y., 1969.

181. MASON, B., OATLEY, N. and WYNN, V. : Br. Med. J. 3, 317 (1973).

182. MAYOL, R.F. and THAYER, S.A. : Biochemistry 9, 2484 (1970).

183. MAZEL, P. : in Fundamentals of Drug Metabolism and Drug Disposition, Chapter 26; Eds. B.N. La Du, H.G. Mandel and E.L. Way, The Williams and Wilkins Company, Baltimore, 1972.

184. McCANN, S.M. and RAMIREZ, V.D. : Recent Prog. Horm. Res. 20, 131 (1964).

185. McELROY, V.G. and SCHENDEL, H.E. : Am. J. Clin. Nutr. 26, 191 (1973).

186. MILLER, G.L. : Anal. Chem. 31, 964 (1959).

187. MISHELL, D.R. and ODELL, W.D. : Am. J. Obstets. Gynecol. 109, 140 (1971).

188. MOGHISSI, K.S. and MARKS, C. : Fert. Steril. 22, 424 (1971).

189. MOSES, H., DAVIS, L.W., ROSENTHAL, A.S. and GARREN, L.D. : Science 163, 1203 (1969).

190. MUELLER, G.C., HERRANEN, A.M. and JERVELL, K.F. : Recent Prog. Horm. Res. 14, 95 (1958).

191. MUELLER, G.C., VONDELAAR, B., KIM, U.H. and LE MAHIEU, M. : Recent Prog. Horm. Res. 28, 1 (1972).

192. MULL, R.H., SCHGAGUTER, M. and FLEMMING, K. : Biochem. Biophys. Res. Comm. 67, 3 (1975).

193. MUSLINER, T.A., CHADER, G.V. and VILLÉE, C.A. : Biochemistry 9, 4448 (1970).

194. NASH, T. : J. Biol. Chem. 55, 416 (1953).

195. NEBERT, D.W. and GELBOIN, H.V. : J. Biol. Chem. 245, 160 (1970).

196. NEWTON, M.A., SEALEY, J.E., LEDINGHAM, J.G.G. and LARAGH, J.H. : Am. J. Obstets. Gynecol. 101, 1037 (1968).

197. NORROD, W.P. and WADE, A.E. : Biochem. Pharmacol. 21, 2887 (1972).

198. NOTIDES, A.C. and NIELSEN, S. : J. Biol. Chem. 249, 1866 (1974).

199. NILSSON, O.L. and DALLNER, G. : J. Cell Biol. 72, 568 (1977).

200. NYGREN, K.G. and JOHANSSEN, E.D.B. : Acta. Endocr. Supp. 155, 170 (1971).

201. O'MALLEY, B.W., ARONOW, A., REINOUR, A.C. and DINGMAN, C.W. : Science 162, 567 (1968).

202. O'MALLEY, B.W., TOFT, D.O. and SHERMAN, M.R. : J. Biol. Chem. 246, 1117 (1971).

203. O'MALLEY, B.W., ROSENFELD, G.C., COMSTOCK, J.P. and MEANS, A.R. : Nature New Biol. 240, 45 (1972).

204. O'MALLEY, B.W., SPELSBERG, T.C., SCHRADER, W.T., CHYRIL, F. and STEGGLES, A.W. : Nature (London) 235, 141 (1972).

205. O'MALLEY, K., STEVENSON, I.H. and CROOKS, J. : Clin. Pharmacol. Ther. 13, 552 (1972).

206. O'MALLEY, B.W. and MEANS, A.R. : Science 183, 610 (1974).

207. O'MALLEY, B.W. and SCHRADER, T.W. : Scient. Am. 234, 32 (1976).

208. OMURA, T. and SATO, R. : J. Biol. Chem. 239, 2379 (1964).

209. OMURA, T., SATO, R., COOPER, D.Y., ROSENTHAL, O. and ESTABROOK, R.W. : Fed. Proc. 24, 1181 (1965).

210. OMURA, T., SANDERS, E., ESTABROOK, R.W., COOPER, D.Y. and ROSENTHAL, O. : Arch Biochem. Biophys. 117, 660 (1966).

211. OTWAY, W. and ROBINSON, D.S. : Biochem. J. 106, 677 (1968).

212. PATON, A.L. : Lancet 1, 418 (1969).

213. PECK, E.J. Jr., BURCHER, J. and CLARK, J.H. : Biochemistry 12, 4596 (1973).

214. PEISACH, J. and BLUMBERG, W.E. : Biochem. Biophysics. 150, 725 (1972).

215. PERRY, J.S. : The Ovarian Cycle of Mammals, Chapter 3; Oliver and Boyd, Edinburgh, 1971.

216. PINCUS, G.J. : The Control of Fertility, Academic Press, N.Y. and London, 1965.

217. PINCUS, G. : Science 153, 493 (1966).

218. PRASAD, A.S., LEI, K.Y., OBERLEAS, D., MOGHISSI, K.S. and STRYKER, J.C. : Am. J. Clin. Nutr. 28, 385 (1975).

219. PUCA, G.A., NOLA, E., SICA, V. and BRESCIANI, F. : Biochemistry 10, 3769 (1971).

220. PUCA, G.A., NOLA, E., SICA, V. and BRESCIANI, F. : Biochemistry 11, 4157 (1972).

221. QUINN, G.P., AXELROD, J., BRODIE, B.B. : Biochem. Pharmacol. 1, 152 (1958).

222. RAMIREZ, V.D. and McCANN, S.M. : Endocrinology 73, 193 (1963).

223. REMMER, H. and MERKER, H.J. : Science 142, 1657 (1963).

224. REMMER, H. and MERKER, H.J. : Ann. N.Y. Acad. Sci. 123, 79 (1965).

225. REMMER, H., SCHENKMAN, J., ESTABROOK, R.W., SESAME, H., GILLETTE, J., NARASIMULU, S., COOPER, D.Y. and ROSENTHAL, O. : Mol. Pharmacol. 2, 187 (1966).

226. REMMER. R. : German Medical Monthly XIII 2, 53 (1968).

227. REMMER, H. : J. Mond. Pharm. 3, 169 (1969).

228. REMMER, H. : Am. J. Med. 49, 617 (1970).

229. RENAUD, S . and GANTHERON, P. : Thrombos. Diath. Haemorrh (Stuttg.) 30, 299 (1973).

230. RIVERS, J.M. and DEVINE, M.M. : Am. J. Clin. Nutr. 25, 684 (1972).

231. ROSE, D.P., BROWN, R.R. and PRICE, J.M. : Nature, 219, 1259 (1968).

232. ROSE, D.P. : in Metabolic Effects of Gonadal Hormones and Contraceptive Steroids, p.352; Eds. H.A. Salhanick, D.M. Kipnis and R.L. Vande Wiele, Plenum Press, N.Y., 1969.

233. RYAN, D., LU, A.Y.H., WEST, S.B. and LEVIN, W. : J. Biol. Chem. 250, 2157 (1975).

234. SACHAN, D.S. : J. Nutr. 106, 1569 (1976).

235. SACKELD, R.M., KNORR, K. and KORNER, W.F. : Clin. Chem. Acta. 49, 195 (1973).

236. SANDBORN, E.B. : Cells and Tissues by Light and Electron Microscopy Vol. II, Academic Press, N.Y. and London, 1970.

237. SANDERSON, J.H. and DELAMORE, I.W. : J. Obstets. Gynecol. Br. Comm. 80, 639 (1973).

238. SARATU, T., SAUDE, G.A. and KAPLAN, N.M. : Arch. Int. Med. 16, 621 (1970)

239. SAUBERLICK, H.E. : S. Afr. Med. J. 49, 2235 (1975).

240. SAUNDERS, F. : Recent Prog. Horm. Res. 20, 395 (1964).

241. SAUNDERS, D.M., MARCUS, S.L., SAXENA, B.B., BELING, C.G. and CONNELL, E.B. : Fert. Steril. 22, 332 (1971).

242. SARFF, M. and GORSKI, J. : Biochemistry 10, 2557 (1971).

243. SCHALLY, A.V., BOWERS, C.Y., WHITE, W.F. and COHEN, A.J. : Endocrinology 81, 77 (1967).

244. SCHALLY, A.V., CARTER, W.H., SAITO, M., ARIMURA, A. and BOWERS, C.Y. : J. Clin. Endocr. Metab. 28, 1747 (1968).

245. SCHALLY, A.V. and KASTIN, A.J. : Steroid Biochem. Pharmacol. 2, 41 (1970).

246. SCHARWZ, N.B. : Recent Prog. Horm. Res. 25, 1 (1969).

247. SCHARWZ, R.J., TSAI, M.J., TSAI, S.Y. and O'MALLEY, B.W. : J. Biol. Chem. 250, 5175 (1975).

248. SCHENKMAN, J.B., REMMER, H. and ESTABROOK, R.W. : Mol. Pharmacol. 3, 113 (1967).

249. SCHENKMAN, J.B. and SATO, R. : Mol. Pharmacol. 4, 613 (1968).

250. SCHENKMAN, J.B., GREIM, H., ZANOA, M. and REMMER, H. : Biochem. Biophys. Acta. 17, 23 (1969).

251. SCHENKMAN, J.B. : Biochemistry 9, 2081 (1970).

252. SCHENKMAN, J.B., CINTI, D.L., ORRENIUS, S., MOLDEUS, P. and KRANSCHITNZ, R. : Biochemistry 11, 4243 (1972).

253. SCHILLINGER, E. and GERHARDS, E. : Biochem. Pharmacol. 22, 1835 (1973).

254. SCHRADER, W.T., TOFT, D.O. and O'MALLEY, B.W. : J. Biol. Chem. 247, 2401 (1972a).

255. SCHRADER, W.T. and O'MALLEY, B.W. : J. Biol. Chem. 247, 51 (1972b).

256. SHERMAN, M.R., CORVOL, P.L. and O'MALLEY, B.W. : J. Biol. Chem. 245, 6085 (1970).

257. SHOEMAN, D.W., CHAPLIN, M.D. and MANNERING, G.J. : Mol. Pharmacol. 5, 412 (1969).

258. SHOJANIA, A.M., HORNADY, G.J. and BARNES, P.H. : Lancet 1, 1376 (1968).

259. SHOJANIA, A.M., HORNADY, G.J. and BARNES, P.H. : Lancet 1, 886 (1969).

260. SHOJANIA, A.M. : Lancet 2, 932 (1971).

261. SHOJANIA, A.M., HORNADY, G.J. and BARNES, P,H. : Am. J. Obstets. Gynecol. 111, 782 (1971).

262. SHOJANIA, A.M. and HORNADY, G.J. : J. Lab. Clin. Med. 82, 869 (1973).

263. SHOJANIA, A.M. : J. Lab. Clin. Med. 85, 185 (1975).

264. SHYAMALA, G. and GORSKI, J. : J. Cell Biol. 35, 125A (1967).

265. SINGH, E.J. and SWARTWOUT, J.R. : Am. J. Obstets. Gynecol. 114, 484 (1972).

266. SLADEK, N.E. and MANNERING, G.J. : Biochem. Biophys. Res. Commun. 24, 668 (1966).

267. SLADEK, N.E. and MANNERING, G.J. : Mol. Pharmacol. 5, 186 (1969).

268. SOBRERO, A.J., BRAKMAN, P. and ASTRUP, T. : Am. J. Obstets. Gynecol. 110, 122 (1971).

269. SPELLACY, W.N. : in Metabolic Effects of Gonadal Hormones and Contraceptive Steroids, p.126; Eds. H.A. Salhanick, D.M. Kipnis and R.L. Vande Wiele, Plenum Press, N.Y., 1969.

270. SPELLACY, W.N., BUHI, W.C., SPELLACY, C.E., MOSES, L.E. and GOLDZIEHER, J.W. : Am. J. Obstet. Gynecol. 106, 173 (1970).

271. SPELLACY, W.N., BUHI, W.C., BIRK, S.A. and McCREARY, S.A. : Fert. Steril. 22, 217 (1971b).

272. SPELLACY, W.N., BUHI, W.C., BIRK, S.A. and McCREARY, S.A. : Fert. Steril. 22, 224 (1971c).

273. SPELLACY, W.N., BUHI, W.C., BIRK, S.A. and CABUL, R. : Fert. Steril. 24, 178 (1973).

274. SPELSBERG, T.C., STEGGLES, A.W. and O'MALLEY, B.W. : J. Biol. Chem. 246, 4188 (1971).

275. SPELSBERG, T.C., STEGGLES, A.W., CHYRIL, F. and O'MALLEY, B.W. : J. Biol. Chem. 247, 1368 (1972).

276. SPIES, H.G., STEVENS, K.R., HILLIARD, J. and SAWYER, C.S. : Endocrinology 84, 277 (1969).

277. SPRAY, G.H. : Lancet 2, 110 (1968).

278. STANCEL, G.M., LEUNG, K.M.T. and GORSKI, J. : Biochemistry 12, 2130 (1973a)

279. STANCEL, G.M., LEUNG, K.M.T. and GORSKI, J. : Biochemistry 12, 2137 (1973b).

280. STARUP, J., DATE, J. and DECKERT, T. : Acta. Endocr. 58, 537 (1968).

281. STEPHENS, M.E., CRAFT, I., PETERS, T.J. and HOFFBRAND, A.V. : Clin. Sci. 42, 405 (1972).

282. STOKES, T. and WYNN, V. : Lancet 2, 677 (1971).

283. STROBEL, H.W., LU, A.H., HEIDEMAN, J. and COON, M.J. : J. Biol. Chem. 245, 4851 (1970).

284. SVANBORG, A. : in Metabolic Effects of Gonadal Hormones and Contraceptive Steroids, p.242; Eds. H.A. Salhanick, D.M. Kipnis and R.L. Vanda Wiele, Plenum Press, N.Y., 1969.

285. SWANECK, G.E., CHU, L.L.H. and EDELMAN, I.S. : J. Biol. Chem. 245, 5382 (1970).

286. SWEAT, M.L., DUTCHER, J.S. YOUNG, R.B. and BRISON, M.J. : Biochemistry 8, 4956 (1969).

287. SWERDLOFF, R.S. and ODELL, W.D. : J. Clin. Endocr. Metab. 29, 157 (1969).

288. TABACCHI, M.H. and KIRKSLEY, A. : J. Nutr. 103, 1270 (1973).

289. TEPHLY, T.R. and MANNERING, G.J. : Mol. Pharmacol. 4, 10 (1968).

290. TOMKINS, G.M., GELEHRTER, T.D., GRANNER, D., MARTIN, D.W., SAMUELS, H.S. and THOMPSON, E.B. : Science 166, 1474 (1969).

291. TSAI, M.J., SCHARWZ, R.J., TSAI, S.Y. and O'MALLEY, B.W. : J. Biol. Chem. 250, 5165 (1975).

292. TÜTTENBERG, K.H., HÜTHWOHL, B., KAHL, R. and KAHL, G.F. : Biochem. Pharmacol. 23, 2037 (1974).

293. TYSON, J.E.A. : Am. J. Obstets. Gynecol. 100, 875 (1968).

294. VAN DER HOEVEN, T.A. and COON, M.J. : J. Biol. Chem. 249, 6302 (1974).

295. VAN NIEKERK, I.N.A. : Lancet 1, 1277 (1962).

296. VESSEY, M.P. and DOLL, R. : Br. Med. J. 2, 199 (1969).

297. VESSEY, M.P. and INMAN, W.H.W. : J. Obstets. Gynecol Br. Comm. 80, 562 (1973).

298. VORE, M., LU, A.Y.H., KUNTZMAN, R. and CONNEY, A.H. : Mol. Pharmacol. 10, 963 (1974).

299. VORYS, N., ALLERY, J. and STEVEN, V. : Am. J. Obstets. Gynecol. 93, 641 (1965).

300. WEINSTEIN, I., BENTSKY, R., SEEDMAN, S., FREGHLY, M. and TURDSHER, T. : Biochem. Pharmacol. 24, 1583 (1975).

301. WEIR, R.H., TREE, M. and FRASER, R. : Clin. Path. 23 Supp. 3, 49 (1970).

302. WEISZ, J., LLOYD, C.W., LOBOTSKY, J., PUPKINS, M., ZANARTU, J. and PUGA, J. : J. Clin. Endocr. Metab. 37, 254 (1973).

303. WERRINGLOER, J. and ESTABROOK, R.W. : Arch. Biochem. Biophys. 167, 270 (1975).

304. WILSON, B.J. and ORRENIUS, S. : Biochem. Biophys. Acta. 261, 94 (1972).

305. WIRA, C. and MUNCK, A. : J. Biol. Chem. 245, 3436 (1970).

306. WINIKOFF, A. : Med. J. Austr. 2, 13 (1968).

307. WOLD, F. : Macromolecules: Structure and Function, p.245; Prentice Hall, Inc. New Jersey. (1971).

308. WYNN, V. and DOAR, J.WH. : in Metabolic Effects of Gonadal Hormones and Contraceptive Steroids, p.219; Eds. H.A. Salhanick, D.M. Kipnis and R.L. Vande Wiele, Plenum Press, N.Y., 1969.

309. WYNN, V. : in Metabolic Effects of Gonadal Hormones and Contraceptive Steroids, p.219; Eds. H.A. Salhanick, D.M. Kipnis and R.L. Vande Wiele, Plenum Press, N.Y., 1969.

310. YGGE, J., BRODY, S.R., KORSAN-BEGSTEN, R. and WILSON, W. : Am. J. Obstets. Gynecol. 104, 87 (1969).

311. ZAHAVI, J., DREYFUSS, F., KALEF, M. and SOFERMAN, J. : Am. J. Obstets. Gynecol. 117, 107 (1973).

312. ZANATU, J., DABACENS, A., RODRIGUEZ-BRAVO, R. and SCHALLY, A.V. : Br. Med. J. 1, 605 (1974).