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'IN VITRO' METABOLISM OF CONTRACEPTIVE STEROIDS.

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A B S T R A C T.

A comparative 'in vitro' investigation of the effect of various substituent groups on the structurally related synthetic 19-norprogestogens (norethisterone norgestrel, lynestrenol and their esterified derivatives) in the rabbit hepatic and extrahepatic tissues is described.

Using total tissue homogenates, the study indicated that various structural modifications of the progestogens resulted in compounds with varying degrees of resistance to metabolism. (The esterified derivatives were metabolised at a comparatively slower rate than the non-esterified progestogens). However, the route of metabolism was not affected by these modifications as compared to natural steroids. Amongst the extrahepatic tissues small intestine, lung and skeletal muscle tissue metabolised the progestogens but at a slower rate than in the liver. Mainly ring-A reduced metabolites were identified.

In contrast to the study with total liver homogenates, in the microsomal fraction of rabbit liver ring-A reduced and hydroxylated products were identified from d-, dl- and l-norgestrel. d-Norgestrel followed the reductive and oxidative pathways equally, whereas dl- and l-norgestrel were mainly hydroxylated. In comparison to testosterone d-, dl- and l-norgestrel were metabolised at a slower rate.

As compared to dehydroepiandrosterone and ethynyloestradiol, the rate of sulphate conjugation of the 19-norprogestogens was relatively slower in the liver. Gastrointestinal and lung tissues also sulphated these compounds. The 19-norprogestogens were conjugated at C-17, dehydroepiandrosterone at C-3 and ethynyloestradiol at both C-3 and C-17 in the liver.

The 'in vivo' investigation of the metabolism of (4-^{14}C) norethisterone oxime (racemate, 'anti'- and 'syn'-isomers) indicated that after intra-peritoneal administration these compounds were excreted mainly in the urine, in the glucuronide fraction. Small amounts of radioactivity were excreted in the faeces. In plasma, peak levels of norethisterone oxime were seen at two hours and the levels of radioactivity declined gradually. The major metabolite identified from the urine and faeces was unchanged norethisterone oxime.

In the 'in vitro' study the oxime derivatives of norethisterone and d-norgestrel were metabolised more slowly than the parent compounds. Gastrointestinal and lung tissues also metabolised these compounds at a similar rate to that in the liver. The metabolites identified were hydrolysed and ring-A reduced products.

TRIVIAL AND SYSTEMATIC NAMES OF STEROIDS.

TRIVIAL NAME	SYSTEMATIC NAME.(IUPAC 1971)
Cholestenone	4-Cholesten-3-one.
Dehydroepiandrosterone	3β-Hydroxy-5-androsten-17-one.
Dehydroepiandrosterone Sulphate	3β-Sulphato-androst-5-en-17-one.
Dihydronorgestrel	17α-Ethynyl-17β-hydroxy-18-methyl-5α -estrane-3-one.
6ε -Hydroxynorgestrel	17α-Ethynyl-6ε ,17β-dihydroxy-18-methyl-4-estren-3-one.
16α-Hydroxynorgestrel	17α-Ethynyl-16α,17β-dihydroxy-18-methyl-4-estren-3-one.
16β-Hydroxynorgestrel	17α-Ethynyl-16β,17β-dihydroxy-18-methyl-4-estren-3-one.
Ethynyloestradiol	17α-Ethynyl-1,3,5-estratriene-3, 17β-diol.
Ethynyloestradiol-3-sulphate	17α-Ethynyl-1,3,5-estratriene-17β-ol-3-sulphate.
Ethynyloestradiol-17-sulphate	17α-Ethynyl-1,3,5-estratriene-17β-sulphato-3β-ol.
Ethynyloestradiol-3,17-sulphate	17α-Ethynyl-1,3,5-estratriene-3, 17-sulphate.
Lynestrenol	17α-Ethynyl-4-estren-17β-ol.
Lynestrenol acetate	17α-Ethynyl-4-esten-17β-acetate.
Lynestrenol sulphate	17α-Ethynyl-4-estren-17β-sulphate.
Norethisterone	17α-Ethynyl-17β-hydroxy-4-estren-3-one.
Norethisterone acetate	17α-Ethynyl-17β-acetoxy-4-estren-3-one.
Norethisterone oenanthate	17α-Ethynyl-17β-heptonate-4-estren-3-one.
Norethisterone oxime	3-Hydroxyimino-17α-Ethynyl-17β-hydroxy-4-estrene.
Norethisterone sulphate	17α-Ethynyl-17β-sulphato-4-estren-3-one.
d-Norgestrel	D-17α-Ethynyl-17β-hydroxy-18-methyl-4-estren-3-one.
dl-Norgestrel	DL-17α-Ethynyl-17β-hydroxy-18-methyl-4-estren-3-one.
l-Norgestrel	L-17α-Ethynyl-17β-hydroxy-18-methyl-4-estren-3-one.
dl-Norgestrel acetate	DL-17α-Ethynyl-17β-acetoxy-18-methyl-4-estren-3-one.
Norgestrel oxime	3-Hydroxyimino-17α-Ethynyl-17β-hydroxy-18-methyl-4-estren-3-one.

Norgestrel sulphate	17α-Ethynyl-17β-sulphato-18-methyl-4-estren-3-one.
19-Nortestosterone	17β-Hydroxy-4-estren-3-one.
Testosterone	17β-Hydroxy-4-androsten-3-one.
Tetrahydronorethisterone	17α-Ethynyl-17β-hydroxy-estrane.
Tetrahydronorgestrel	17α-Ethynyl-17β-hydroxy-18-methyl-estrane.

A B B R E V I A T I O N S

ABBREVIATIONS FOR STEROIDS.

19-nort.	19-nortestosterone
DHA	dehydroepiandrosterone
DHA-SO_4	dehydroepiandrosterone sulphate
E_2	oestradiol
E_2 -3-SO_4	oestradiol-3-sulphate
E_2-17-SO_4	oestradiol-17-sulphate
E_2-3-17-SO_4	oestradiol-3-17-sulphate
EE	ethynyloestradiol
EE-3-SO_4	ethynyloestradiol-3-sulphate
EE-17-SO_4	ethynyloestradiol-17-sulphate
EE-3-17-SO_4	ethynyloestradiol-3-17-sulphate
Lyn	lynestrenol
Lyn-AC	lynestrenol acetate
Lyn-SO_4	lynestrenol sulphate
NET	norethisterone
NET-AC	norethisterone acetate
NET-OX	norethisterone oxime
NET-EN	norethisterone oenanthate
NET-SO_4	norethisterone sulphate
d-,dl-, and l-Ng.	d-, dl-, and l-norgestrel
d, dl-, and l-Ng-AC	d-, dl-, and l-norgestrel acetate
d-, dl-, and l-Ng-SO_4	d-, dl-, and l-norgestrel sulphate
d-Ng-OX	d-norgestrel oxime
T	testosterone
THNET	tetrahydronorethisterone
THNg	tetrahydronorgestrel
DHNET	dihydronorethisterone

OTHER ABBREVIATIONS

ATP	adenosine 5'-triphosphate
A	ampere
A.R.	analytical reagent
cm	centimeter
CV	coefficient of variation
c.p.m.	counts per minute
Ci	Curie
^oC	degrees celcius
EDTA	ethylenediaminetetra acetate

g.l.c.	gas-liquid chromatography
g	gram
g	unit of the gravitational field ($9.81\ m.s^{-2}$)
h	hour
I.D.	internal diameter
KCl	potassium chloride
K_2SO_4	potassium sulphate
KH_2PO_4	potassium dihydrogen orthophosphate
l	litre (dm^3)
μ	micro ($10^{-6}x$)
μg	microgram
μm	micron ($10^{-6}m$)
mCi	milli Curie
mμ	millimicron
m	milli ($10^{-3}m$)
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
$MnCl_2$	manganese chloride
ml	millilitre
mM	millimolar
min	minute (60 s)
M	molar
mol	mole
mol. wt.	molecular weight
n	nano ($10^{-9}x$)
NaCl	sodium chloride
ng	nannogram
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced
%	per cent
p	probability
PAPS	adenosine 3'-phosphate 5'-phosphosulphate
p.c.	paper chromatography
psi	pounds per square inch
r.p.m.	rev per minute
R_T	retention time
RR_T	relative retention time
s	seconds (time)
S.D.	standard deviation

sp. act.	specific activity
t.l.c.	thin-layer chromatography
TMS ethers	trimethylsilyl ethers
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol.
vol.	volume
$\frac{v}{v}$	volume to volume
by vol.	by volume
λ	wavelength
wt.	weight
$\frac{w}{v}$	weight to volume
$\sum$	sum of
$<$	is less than
$>$	is greater than

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CHAPTER 2.

I N T R O D U C T I O N.

The synthetic progestogens related to 19-nortestosterone, in combination with a synthetic oestrogen, are constituents of various contraceptive pills in widespread use. Although, several 'in vivo' and 'in vitro' investigations on the metabolism of these compounds in man and rabbit have been reported; no detailed examination of the effect of molecular modification of the 19-norprogestogens on the rate of metabolism appears to have been done. We have chosen the rabbit (Oryctolagus cuniculus) as the experimental model on the basis that various studies have indicated certain similarities in the metabolism of progestational steroids between the rabbit and man. However, it has not been fully established as to how far these similarities extend.

In Section one of this thesis, the three main pathways of metabolism of synthetic 19-norprogestogens have been investigated under 'in vitro' conditions. In Chapter one, the rate of ring-A reduction of some of the 19-norprogestogens is compared to the parent compound 19-nortestosterone, by the hepatic and extrahepatic tissues of the male and female rabbit. The progestogens examined are norethisterone, d-, dl- and l-norgestrel, lynestrenol, norethisterone acetate, norethisterone oenanthate, dl-norgestrel acetate and lynestranol acetate. The 'in vitro' system consisted of total tissue homogenates fortified with NADPH.

In Chapter two, the metabolism of the natural steroid testosterone is compared to the synthetic progestogens d-, dl- and l-norgestrel. In this study the microsomal fraction of female rabbit liver was used as the enzyme source.

In Chapter three, the rate of sulpho-conjugation of the natural steroid dehydroepiandrosterone is compared to the synthetic oestrogen ethynyloestradiol and the synthetic 19-norprogestogens. The 700g fraction from hepatic and extrahepatic tissue homogenates of the female rabbit was employed.

In the search for better and more active contraceptive agents it has been suggested that the oxime derivatives of existing progestogens are highly effective as antifertility agents. In Section two of this thesis, we describe the 'in vivo' metabolism of norethisterone oxime. In Chapter two the 'in vitro' metabolism of norethisterone oxime and d-norgestrel oxime is described.

REVIEW OF THE LITERATURE

The discovery of orally active progestogens by the pharmaceutical houses of Europe and the United States made possible the development of the "contraceptive pill" which was first approved by the Food and Drug Administration in 1960 (Petrow, 1970). The progestational steroids used in "the pill", with a few exceptions, fall into two broad classes, derivatives of progesterone and derivatives of testosterone substituted at carbon-17 by a saturated or unsaturated hydrocarbon (Petrow, 1966). Initially, all progestogens used in "the pill" were derivatives of testosterone and are being widely used today (Briggs and Brotherton, 1970; Bingel and Benoit, 1973; Bennett, 1974).

Some of the testosterone related progestogens that are commonly being used in the "contraceptive pill" are norethisterone, dl-norgestrel, d-norgestrel, lynestrenol and norethisterone acetate in combination with the synthetic oestrogens mestranol or ethynyloestradiol. Pharmacological investigations of lynestrenol acetate have shown that this compound has biological activities similar to those of lynestrenol (Coert, et al., 1975). Norethisterone oenanthate is being used as a long-acting contraceptive administered by intramuscular injection (Zanartu and Navarro, 1968 and Jurgensen, et al., 1971). The chemical structures of these progestogens and three other progestogens investigated in this study, dl-norgestrel acetate, norethisterone oxime and d-norgestrel oxime are shown in Fig. 1.

Development of the synthetic progestational steroids related to testosterone

The synthetic chemical development of the testosterone related progestogens can be viewed in terms of increasing attempts to inhibit metabolic inactivation while maintaining progestational activity, by the modification of structure of the parent steroid,

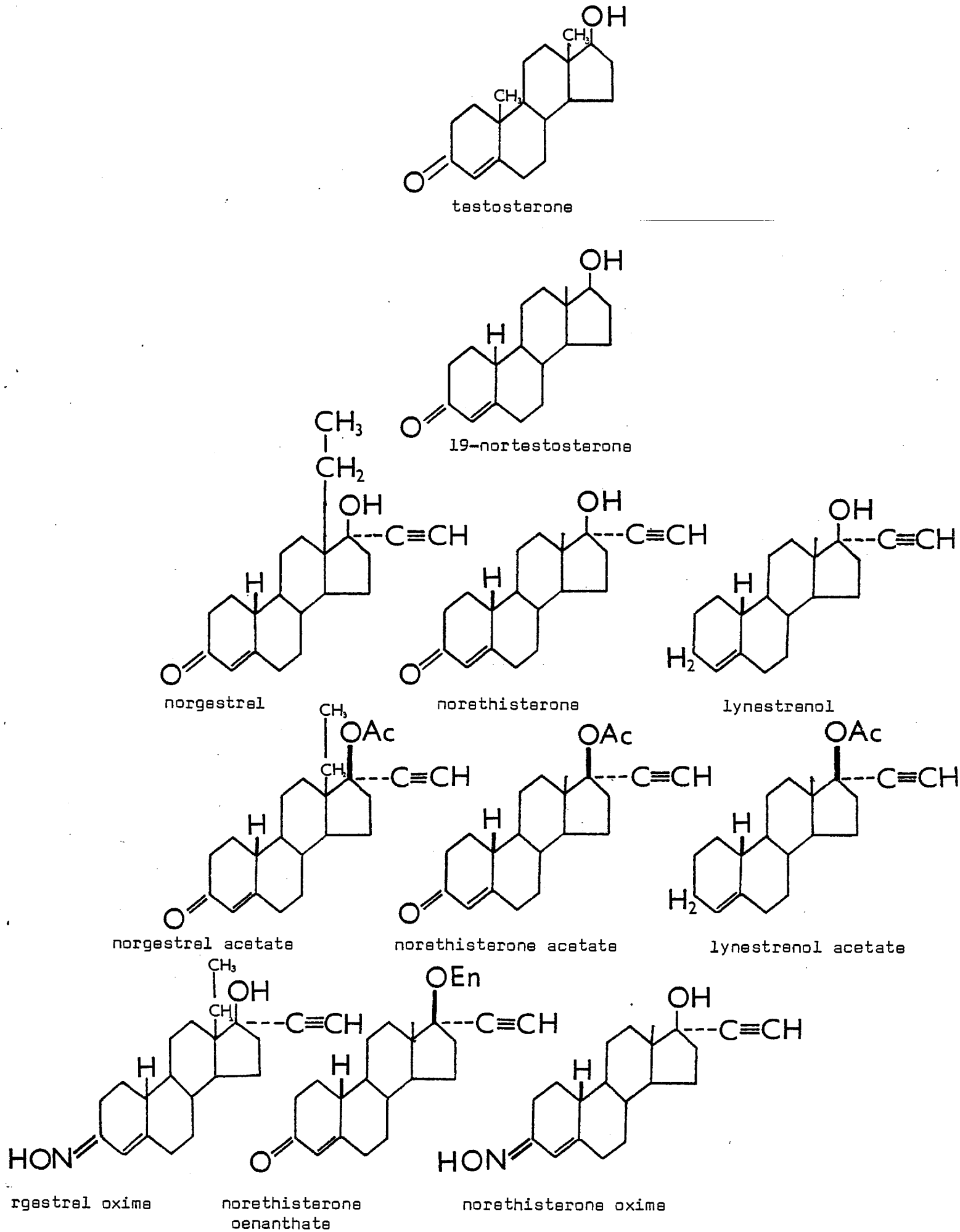


Fig. 1. Structural relationships of various synthetic progestogens derived from testosterone.

testosterone (Diczfalusy, 1971). Removal of the C-19-methyl group from testosterone yielding 19-nortestosterone, markedly reduces the androgenicity of the molecule (Saunders, 1970). It is actually the latter compound to which these progestogens are related. The addition of the 17α-ethynyl group to 19-nortestosterone results in the orally effective progestational hormone, norethisterone (McGinty and Djerassi, 1958). This was the first 19-norsteroid on which extensive clinical work was done (Hertz, et al., 1954; Jadrijevic, et al, 1956; Tyler, 1955; Greenblatt, 1956). Esterification of the 17β-hydroxy group of norethisterone gave rise to norethisterone acetate (Engelfield, et al., 1957) and norethisterone oenanthate (Junkmann, 1954). Norethisterone acetate was found to be a highly effective oral progestational agent (Engelfield, et al., 1957). Norethisterone oenanthate, parenterally administered was reported to be a long-acting steroid more potent than progesterone (Wied, et al., 1958).

Removal of the 3-oxo group from norethisterone yielded a highly active progestational agent, lynestranol (De Winter, et al., 1959). More recently, the formation of a homosteroid, in which the angular methyl group is substituted by an ethyl group as in norgestrel (Smith, et al., 1963) was found to be 80 times more potent than norethisterone. This is the most potent progestational agent synthesised (Ferin, 1970). During the synthesis of norgestrel two sterioisomers are formed (Smith, et al., 1964) due to the presence of the asymmetric carbon atom at position 13. Biological studies have shown that only the d-form has biological activity (Edgren, et al., 1963 a, b; De Johng, et al., 1968). In 1973, Shroff, et al., indicated that the oxime derivative of norethisterone acetate was more potent progestationally than the parent compound, norethisterone acetate.

Metabolic alterations ofanaturally occurring steroid hormone, testosterone

The main reactions responsible for the metabolic inactivation of testosterone are:

i) Reduction of ring-A,
ii) Oxidation of the secondary hydroxyl group at C-17,
iii) Hydroxylation,
iv) Aromatisation,
v) Conjugation of the hydroxyl group.

The metabolism of testosterone has been examined in several studies (Baulieu and Robel, 1970; Sandberg and Slaunwhite, 1956; Vida, 1969). Testosterone is primarily attacked in ring-A by the 4-ene-5α and 4-ene-5β-reductases, which are substrate specific. In the reduction of the C_4 - C_5 double bond, the hydrogen at C_5 can be introduced in either the α or β orientation. Thus, both 5α-androstanolone (androsterone) and 5β-androstanolone (aetiocholanolone) are formed and equal amounts of these two metabolites are excreted in the urine of man (Baulieu, 1964; Kirschner and Lipsett, 1964; Korenman and Wilson, 1966). Some 5α- and 5β-androstone-3 , 17β-diols and 3, 17 diones are also formed (Stylianou, et al., 1961; Baulieu and Mauvais-Jarvis, 1964a, b; El Attar, et al., 1964). After the reduction of the C_4-C_5 double bond, the 3-ketone function is also reduced, giving rise to a new asymmetric center at carbon-3. The major proportion of the secondary alcohols formed on the reduction of the 3-ketone have the 3α-configuration in man (Samuels and Eik-Nes, 1968).

The order of appearance of the metabolites in liver suggests that ring-A reduction precedes C-17 oxidation (El Attar, et al., 1964). Some of the diols formed are excreted immediately rather than equilibrating with the 17-ketone, since 17α-H^3-testosterone is

excreted in part as 5α-androstane-diol 17α-^{3}H (Baulieu and Robel, 1963). After the administration of radioactive testosterone the percentage conversion to various metabolites was as follows: androsterone 20%, aetio cholanolone 26%, 3β-hydroxy-5α-androstan-17-one, 1%, 5α-androstanediol 1% and 5β-androstanediol, 1.5% (Fotherby and James, 1972).

Hydroxylation reactions lead to metabolites which are usually less active than the precursors (Vida, 1969). A small amount of testosterone may undergo hydroxylation at C-1, C-6 or C-16 in the liver and is excreted into the urine in this form, although the amounts normally formed are small (Schubert, et al., 1964).

The conversion of radioactive testosterone to oestradiol has been demonstrated with mammalian tissue (Bagett, et al., 1955). This transformation has been demonstrated in the castrated, adrenalectomised women after intramuscular injection of testosterone (West, et al., 1956), but could not be shown following oral administration of the hormone to the male (Dimick, et al., 1961). It has been calculated that 0.5% of circulating testosterone is converted to oestradial in the adult male and 0.2 % in the adult female (MacDonald and Siiteri, 1966). Metabolism of testosterone is shown in Fig. 2.

After the administration of (^{14}C) testosterone to humans about 90% of the dose is excreted in the urine in 48h and small amounts (about 6%) appear in the faeces. Only about 50% of the radioactivity in urine could be extracted after the application of various hydrolytic and extraction procedures (Fotherby and James, 1972). The major part of the radioactivity extracted is conjugated to glucuronic acid. Most of the 3β-hydroxy-5α and 5β-metabolites are predominantly conjugated with sulphuric acid. The 3α-hydroxysteroids appear mainly as glucuronides although some sulphate formation takes place. Conjugation of the 17β-hydroxy group of testosterone with glucuronic acid also occurs to a small extent.

The synthetic testosterone related progestogens and testosterone

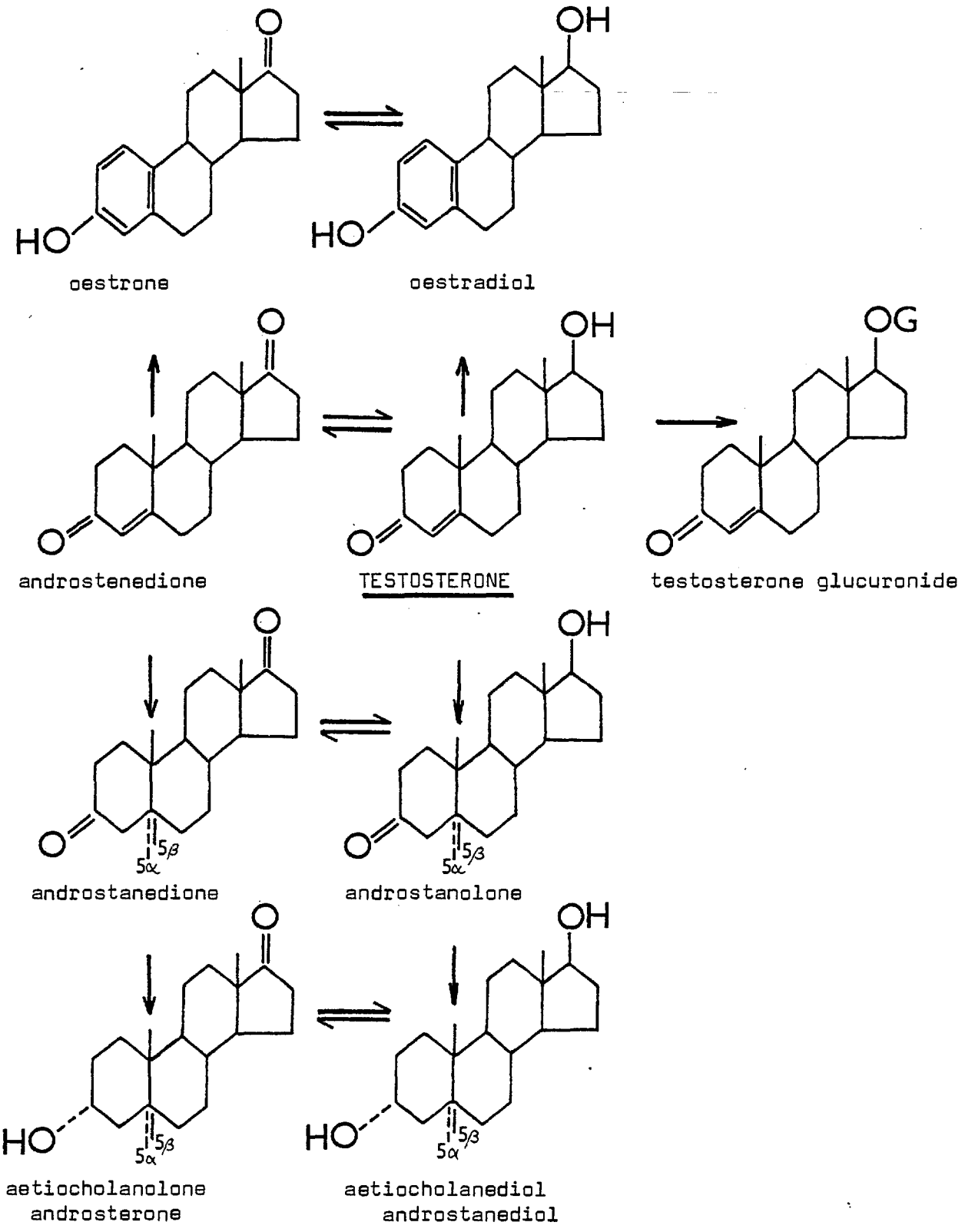


Fig. 2. Metabolism of testosterone in man.

have two main structural features in common. These are the double bond at C_4-C_5 and the hydroxyl group at C-17. The progestogens differ from testosterone in the following ways:

i) the absence of the 19-methyl group,
ii) the absence of the 3-oxo-group,
iii) the presence of the 3-oxime group,
iv) the presence of the ethyl group at C-13,
v) the presence of the 17α-ethynyl group,
vi) the presence of ester groups at C-17,

Thus, the question arises as to whether the synthetic progestogens are metabolised by pathways similar to the parent compound testosterone. In Fig. 3 are shown the structure of testosterone and a composite molecule of the progestogens discussed. As shown there are several possible points of metabolic alteration in the synthetic progestogens.

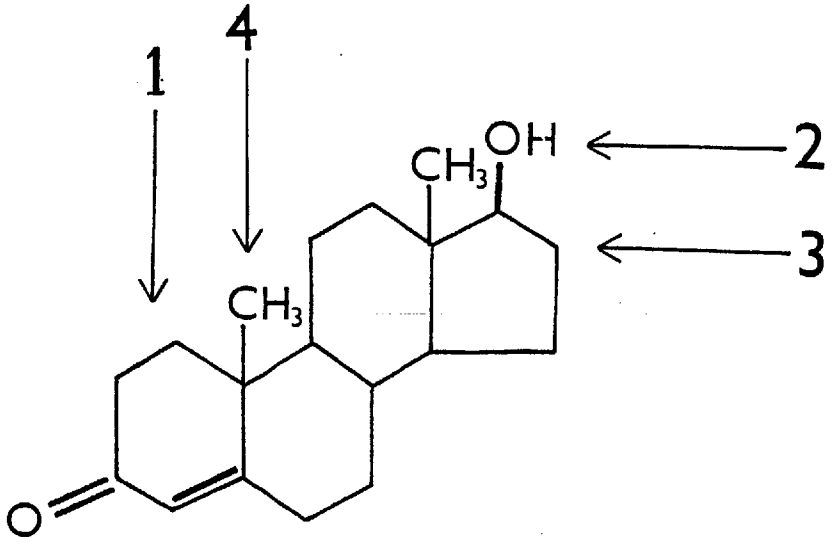
i) Ring-A reduction,
ii) Oxidation of the 17β-hydroxyl group,
iii) Hydroxylation reactions,
iv) Reduction of the ethynyl side chain,
v) Removal of the ethynyl side chain,
vi) De-esterification at C-17,
vii) Hydrolysis of the oxime group at C-3,
viii) Aromatisation of ring-A,
ix) The resulting products may then undergo conjugation. with glucuronic or sulphuric acid.

Metabolic alterations of 19-nortestosterone

19-Nortestosterone differs from testosterone only in the absence of the 19-methyl group.

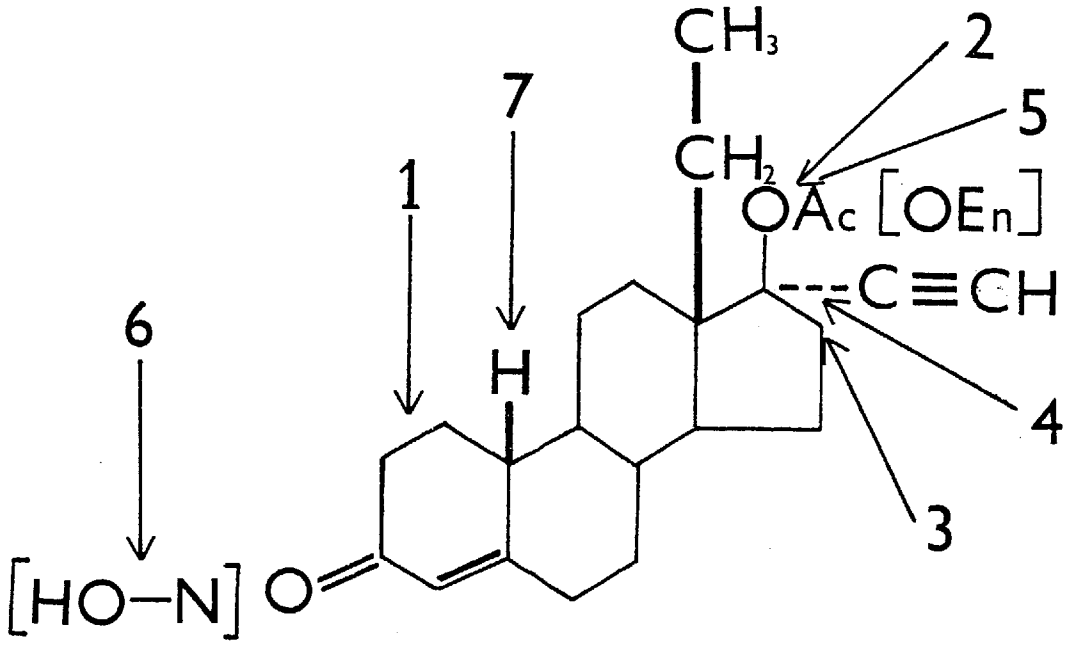
a) "In vivo" and "in vitro" metabolism in man

It has been shown that 19-nortestosterone is metabolised in



POINTS OF METABOLIC ALTERATION.

1. Ring-A reduction.
2. Oxidation of the 17 β-hydroxyl group.
3. Hydroxylation reaction.
4. Aromatisation.



POINTS OF METABOLIC ALTERATION OF 19-NORPROGESTOGENS.

1. Ring-A reduction.
2. Oxidation of the 17β-hydroxyl group.
3. Hydroxylation reactions.
4. Reduction of the ethynyl side chain.
   Removal of the ethynyl side chain.
5. De-esterification at C-17.
6. Hydrolysis of the oxime group at C-3.
7. Aromatisation.

Fig. 3. Structure of testosterone and a composite molecule of the 19-norprogestogens showing the various possible points of metabolic alteration.

a similar way to testosterone (Engel, et al., 1958). Thus, Engel, et al., investigated the "in vivo" metabolism of 19-nortestosterone in man and isolated 19-norandrosterone (3α-hydroxy-5α-estran-17-one) and 19-noraetiocholan-3α-ol-17-one (3α-hydroxy-5β-estran-17-one). The ratio of 5α- to 5β, 17-ketosteroid metabolites of 19-nortestosterone was similar to the ratio of 5α- to 5β, 17-ketosteroid metabolites of testosterone. The authors concluded that the absence of the angular methyl group exerts little or no influence upon either the oxidation of the 17β-hydroxyl group or the stereochemical course of the reduction of the Δ4-3-ketone group. The increased excretion of oestrone after administration of 19-nortestosterone was of the same order of magnitude as that observed after administration of testosterone.

Ryan (1960) indicated that the relative substrate activity in steroid aromatisation, measured by conversion of androgens to oestrogens by the human placenta, was 20% for 19-nortestosterone as compared to 100% for testosterone.

b) "In vitro" metabolism in animals

In contrast to the "in vivo" study in man the following metabolites were obtained from the incubation of 19-nortesterone with rat liver; 5α-estrane, 3α, 17β diol,
5α-estrane, 3β, 17β diol,
3α-hydroxy-5α-estrane-17-one and
3β-hydroxy-5α-estrane-17-one
(Kupfer, et al., 1960)

These studies indicate that in man both 5α- and 5β-ring-A reduced products are formed, whereas in the rat only the 5α-reduced metabolites appear to occur. In both species the oxidation of the 17β-hydroxy group was also observed. The possible aromatisation of 19-nortestosterone in man is also indicated. These studies bring two points to attention:

i) that there are considerable specie differences in the metabolism of steroids (Breuer, 1972),

ii) there are differences in the "in vivo" and "in vitro" metabolism of 19-nortestosterone.

However, in both species the pathways of metabolism of 19-nortestosterone are similar to that of testosterone. The removal of the 19-methyl group does not influence the metabolic pathways.

Metabolic alterations of the synthetic progestogens structurally related to testosterone

The synthetic testosterone related compounds are similar in that they have all been shown to be effective as progestogens. However, as indicated previously (Fig. 1) they differ structurally.

The metabolism of the commonly used synthetic 19-norprogestogens, norethisterone, norgestrel and lynestrenol has been mainly investigated "in vivo" in man and rabbit, and "in vitro" in rabbit tissues (Fotherby and James, 1972; Thijssen, 1972; Fotherby, 1974). Fotherby (1964) has indicated that there are certain similarities in the metabolism ofprogesterone in man and rabbit, and in the metabolism of the synthetic 19-norprogestogens (Fotherby, 1974). However, it is not known as to how far these similarities extend.

Metabolism of norethisterone

a) "In vivo" metabolism in man

Norethisterone is closely related to testosterone and differs only in the absence of the 19-methyl group and the presence of the 17α-ethynyl group. Numerous "in vivo" studies in man have been reported and the metabolism of the three progestogens,norethisterone, norgestrel and lynestrenol, has been most extensively examined

(Fotherby and James, 1972; Thijssen, 1972).

After the administration of norethisterone to man, mainly ring-A reduced products are present in the urine. An extensive study of Gerhards, et al. (1971) indicates that after the administration of (^{14}C) norethisterone to male subjects, 25-28% of the glucuronide fraction consisted of the ring-A reduced product 17α-ethynyl-5β-estrane-3α-17β diol and 17α-ethynyl-5β-estrane-3β diol was identified in the sulphate fraction. Other metabolites identified in the glucuronide fraction were;

17α-ethynyl-5α-estrane-3α, 17β diol,

17α-ethynyl-5α-estrane-3β, 17β diol

and 17α-ethynyl-5β-estrane-3β, 17β diol.

Stillwell, et al. (1972) identified 17α-ethynyl-5β-estrane-3α, 17β diol and 17α-ethynyl-5α-estrane-3α, 17β diol from the urine of a women after the oral administration of noresthisterone. In postmenopausal women (Murata, 1967) 17α-ethynyl-17β-hydroxy-5β-estrane-3-one and 17α-ethynyl-3β, 17β-dihydroxy-5α-estrane were identified.

Layne, et al. (1963) described the occurrence of the C-10β-hydroxynorethisterone in human urine. Little or no metabolism of the ethynyl group was found to occur (Kamyab, et al., 1968a and Fotherby, et al., 1966). Norethisterone does not appear to be converted to phenolic compounds in man.

In man, 90% of the administered radioactive testosterone was recovered in urine in 48h (Fotherby and James, 1972); in contrast after the oral administration of (^{3}H) norethisterone to a woman, 50% of the dose was excreted in 5 days. However, after intravenous administration, 70% of the dose was recovered in the same period of time (Layne, et al., 1963). Similarly, Kamyab, et al. (1968a) recovered 54% of the administered (4-^{14}C) norethisterone in the urine in 5 days.

Of the radioactivity excreted 2% was unconjugated, 45% was present in a β-glucuronidase enzyme hydrolysable form and 19-24% was obtained after solvolysis (Layne, et al., 1963). Similar results to these were obtained by Kamyab, et al. (1968a). Thus, 3.1% of the recovered dose was present unconjugated, 49% was obtained after enzyme hydrolysis and 16% after solvolysis.

The excretion of norethisterone in human faeces is not well documented. Gerhards et al. (1971) recovered 35-43% of administered (^{3}H) norethisterone in 10 days in human faeces. These figures are much higher than those obtained for testosterone (Fotherby and James, 1972).

b) <u>"In vivo" and "in vitro" metabolism in rabbit</u>

Two main "in vivo" studies in rabbit are that of Kamyab et al. (1967) and Orino (1969). From the glucuronide fraction of rabbit urine, Orino (1969) identified 17α-ethynyl-17β-hydroxy-5β-estrane-3-one and 17α-ethynyl-3β, 17β-dihydroxy-5β-estrane and its 3α-5β-isomer, which are different from the isomers obtained in human urine.

The removal of the 17α-ethynyl side chain does not appear to occur "in vivo" in the rabbit either, since nearly all the radioactive metabolites excreted in the urine still contained the ethynyl group (Kamyab, et al., 1967).

After the intravenous administration of (4-^{14}C)norethisterone to five rabbits, Kamyab et al. (1967) found that during a seven-day period, 45% of the dose was excreted in the urine, a value similar to their human studies (Kamyab, et al., 1968a). 31% of the urinary radioactivity was extractable after enzymic hydrolysis and 45% after acid hydrolysis, suggesting that the main form of conjugation was as sulphates. Smaller amounts of radioactive norethisterone were excreted in the faeces (1.4 - 4% of the dose). This value is lower

than that obtained in the human (Gerhards, et al., 1971).

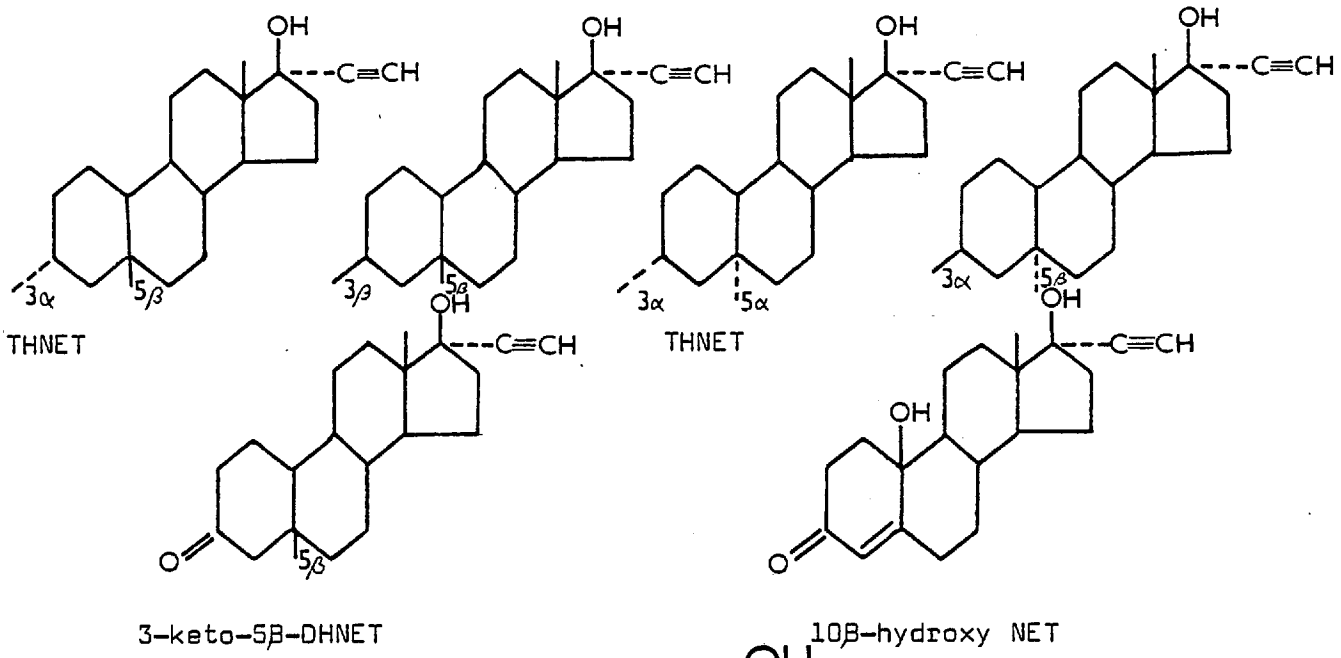
In contrast to the "in vivo" studies, the metabolites obtained "in vitro" in rabbit liver are quite different. Thus, the removal of the ethynyl side chain was demonstrated in rabbit liver by Palmer, et al., 1969, and the formation of phenalic compounds (Palmer, et al., 1970). Ethynyloestradiol, D-homo-estra-4-ene-3, 17 dione as well as the hydroxylated metabolites - 10β-hydroxynorethisterone and 10β-hydroxy-17α -ethynyl-5β-estrane-3 α, 17β diol were identified in rabbit liver incubations.

Therefore it is seen that in man and rabbit the metabolism of norethisterone is similar to that of testoserone. Thus, mainly ring-A reduced metabolites are obtained in the two species but different isomeric forms of these metabolites are present in the urine. The removal of the ethynyl side chain and conversion to ethynyloestradiol is demonstrated "in vitro" in rabbit liver. However, these reactions have not been shown "in vivo" in man or in the rabbit; whereas, hydroxylation at C-10 is demonstrated "in vivo" in man and "in vitro" in the rabbit. The excretion of norethisterone in urine in the two species is similar, whereas, the amount excreted in the faeces differs. In comparison with testosterone the amounts excreted in urine are much lower in both species. The pathways of conjugation also differ in the two species. Thus, norethisterone is conjugated mainly to glucuronic acid in man and to sulphuric acid in the rabbit. The metabolism of norethisterone is shown in Fig. 4.

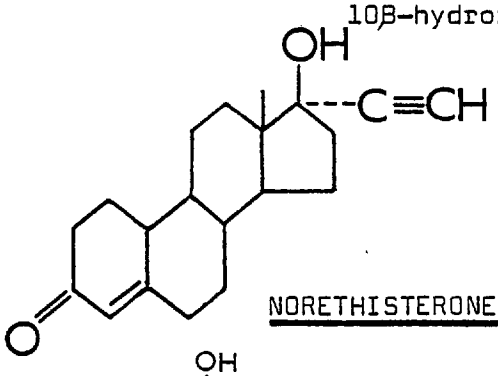
Metabolism of Norgestrel

The structure of norgestrel is shown in Fig. 1. This compound differs from testosterone in the following structural features; absence of the 19-methyl group, presence of the 17α -ethynyl side chain and the ethyl group at C-13.

'IN VIVO' METABOLISM IN MAN.



'IN VIVO' AND 'IN VITRO' METABOLISM IN RABBITS.



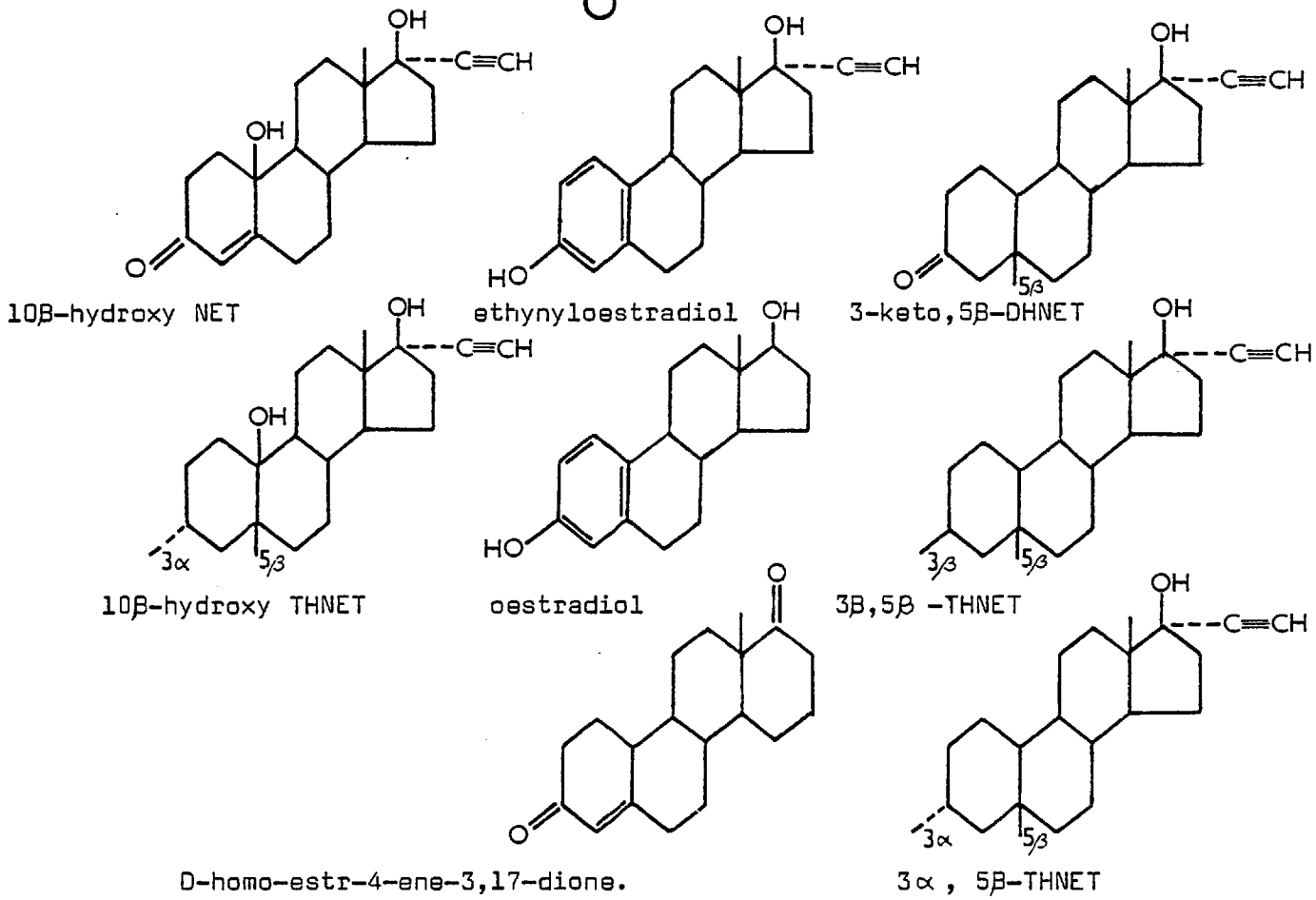


Fig. 4. 'In vivo' and 'in vitro' metabolites of norethisterone identified in man and rabbit.

a) "In vivo" metabolism in man

After the administration of dl-norgestrel to humans (Littleton, et al., 1968; De Jongh, et al., 1968; Gerhards, et al., 1971; Sisenwine, et al., 1973), the main metabolite in the glucuronide fraction of urine was shown to be identical to 3α-hydroxy, 5β-isomer of tetrahydronorgestrel; whereas the main metabolite isolated from the sulphate fraction was the 3β-hydroxy, 5β-isomer. Sisenwine, et al. (1973) also isolated the 3β, 5β- and 3α, 5α-isomers of tetrahydronorgestrel. The 3α, 5β-tetrahydronorgestrel accounted for 23% (Fotherby and Keenan, 1969) and 15-50% (Gerhards, et al., 1971) of the glucuronide fraction. The configuration of the isolated tetrahydrometabolite of dl-norgestrel was identified to be d-3α, 5β-tetrahydronorgestrel (Fotherby and Keenan, 1969). In a recent study of Sisenwine, et al.(1975), it was shown that the d-, dl- and l-forms of norgestrel may follow different metabolic pathways. Thus, l-norgestrel was found to be hydroxylated at 16β, 16α and 1β positions, whereas, d-norgestrel was metabolised mainly in ring-A. The pattern for the racemate appeared to be an approximate composite of the metabolite pattern of the two enantiomers. The occurrence of 2α and 6ε -hydroxylated metabolites was shown in urine of women receiving dl-norgestrel (Sisenwine, et al., 1973).

The formation of phenolic derivatives occurred to a very minor degree from the biologically inactive l-enantiomer. With, d-norgestrel, this transformation occurred to an even lesser extent (Sisenwine et al., 1975).

After administration of norgestrel labelled with ^{14}C in the ethynyl side chain, 43% of the dose was excreted in urine in 5 days (Littleton, et al., 1968). In a further series of experiments, the d- and l-isomers of norgestrel were studied separately, the mean recoveries were 35% and36% respectively and were not significantly different from dl-norgestrel (Fotherby and Keenan, 1969). Sisenwine, et al. (1975) recovered 58% of dl-norgestrel, 45% of d-norgestrel and

64% of l-norgestrel in urine of women after the administration of ^{14}C-norgestrel (d-, dl- and l-form).

Littleton, et al. (1968) obtained about 6% of the urinary radioactivity in a non-conjugated form, 32% following enzyme hydrolysis and 24.6% of the urinary radioactivity was extracted after solvolysis. That norgestrel appears to be mainly conjugated to sulphuric acid in urine in man was also shown by Gerhards et al. (1971). Thus, 50-60% of the excreted dose was in the form of sulphate conjugates.

Both Gerhards, et al. (1971) and Sisenwine, et al. (1975) have shown that the faecal route of excretion of norgestrel appears to be quite important. Thus, 20-40% of the administered dose of dl-norgestrel was recovered in the faeces of man in three days (Gerhards, et al., 1971). Similarly 23.4% of dl-norgestrel, 32% of d-norgestrel and 25% of l-norgestrel was recovered in the faeces of women (Sisenwine, et al., 1975). These values are much higher than those obtained for testosterone.

b) "In vivo" metabolism in rabbit

Few "in vivo" studies on the metabolism of norgestrel have been reported in the rabbit. Kamyab, et al. (1967) found that 60% of the urinary radioactivity had a chromatographic behaviour similar to norgestrel.

After administration of (^{14}C) dl-norgrestrel to rabbits, 57% of the dose was recovered in the urine in 7 days, and less than 5% of the dose in the faeces. 21% of the urinary radioactivity was extracted after enzyme hydrolysis and 59% after acid hydrolysis in rabbit.

Thus, norgestrel in man is also reduced to ring-A metabolites and hydroxylated metabolites have also been identified. The three forms of norgestrel appear to follow different metabolic pathways

in man. However, the pathways are qualitatively similar to testosterone. In contrast to norethisterone, norgestrel both in man and rabbit is mainly conjugated to sulphuric acid. Some formation of phenalic metabolites is indicated in man, however, the removal of the ethynyl group does not appear to occur. The metabolism of norgestrel is shown in Figure 5.

Metabolism of lynestrenol

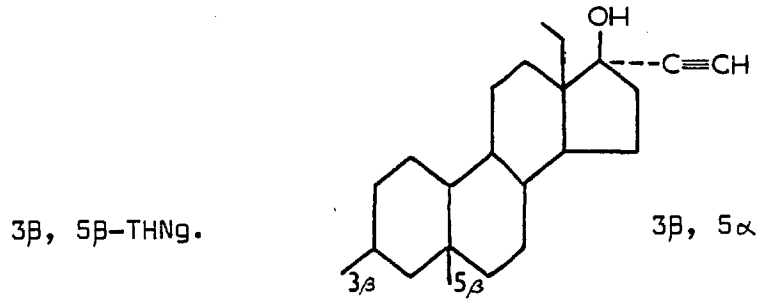
Lynestrenol differs from testosterone not only because it lacks a 19-methyl group but it also does not have a 3-oxo group. Therefore it is likely to undergo different pathways of metabolism.

a) "In vivo" metabolism in man

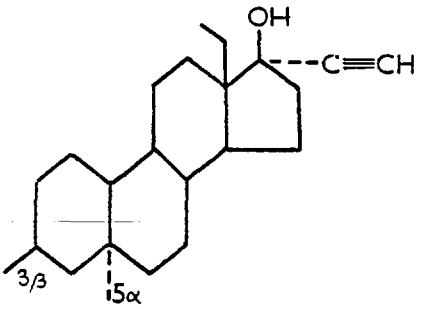
In man, Murata (1967) described the presence of 17α-ethynyl-17β-hydroxy-5β-estrane-3-one and 17α-ethynyl-3β, 17β-dihydroxy-5β-estrane - the same metabolites as were found in the urine of subjects receiving norethisterone. Also identified in the urine was norethisterone.

As has been found in most studies with steroids containing an ethynyl side chain, so with lynestrenol this group is hardly metabolised (Kamyab, et al., 1968b;Fotherby, et al., 1966). Okada, et al. (1964b) reported on the transformation of lynestrenol to 17α-ethynyloestradiol. However, this may have been an artifact produced during the isolation procedures (Breuer, 1970a).

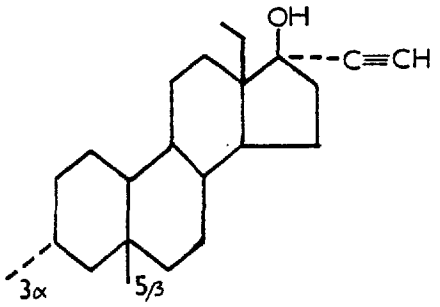
After the intravenous administration of (4-^{14}C) lynestrenol to human subjects, 44% of the radioactivity was excreted in the urine (Kamyab, et al., 1967a and 1968b) within 5 days. Van der Molen et al. (1969) found that the route of administration appeared to affect the rate of excretion of lynestrenol in women. Thus, when the compound was administered in tablet form orally, 59% was excreted in 4 days. Oral administration in gelatine capsules resulted in the



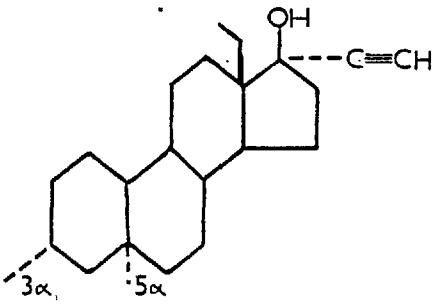
3β, 5β-THNg.



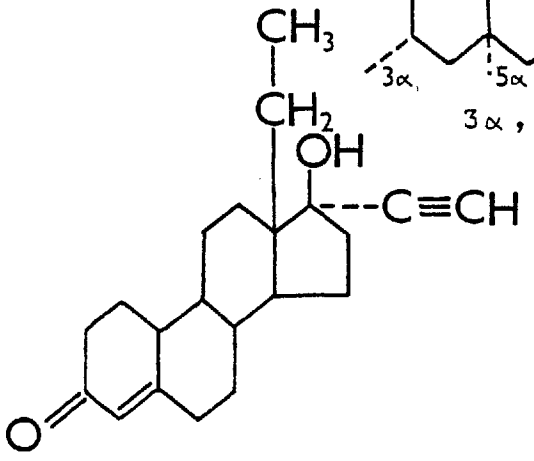
3β, 5α-THNg.



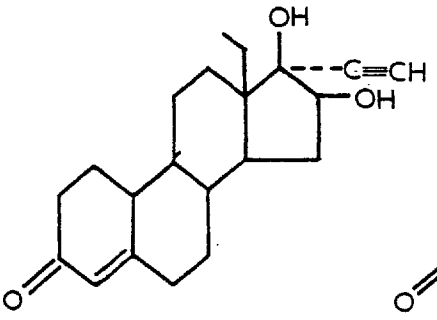
3α, 5β-THNg.



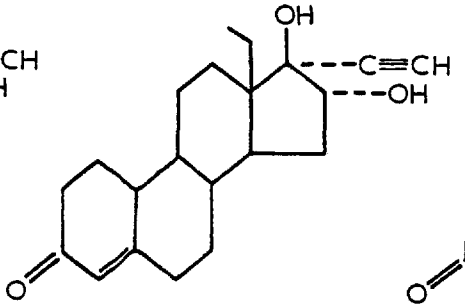
3α, 5α-THNg.



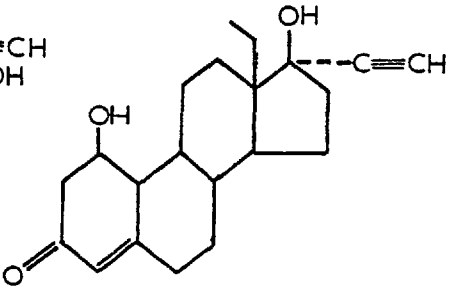
NORGESTREL



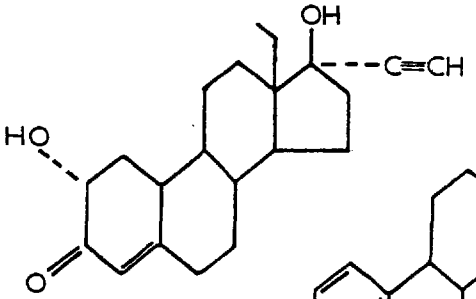
16β-hydroxy norgestrel.



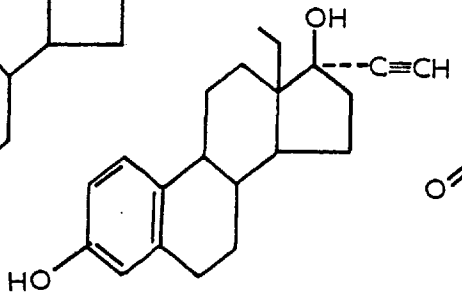
16α-hydroxy norgestrel.



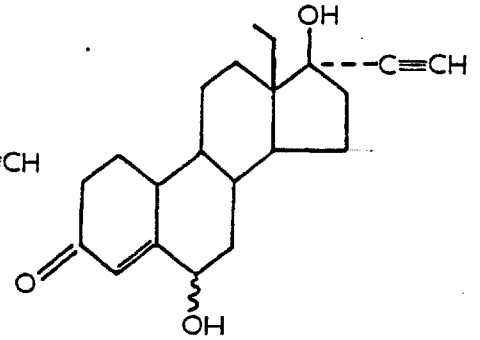
1β-hydroxy norgestrel.



2α-hydroxy norgestrel.



18-homo-ethynyloestradiol.



6ε-hydroxy norgestrel.

Fig. 5. Some 'in vivo' metabolites of norgestrel identified in man.

excretion of 12-14% of the dose in 5 days.

Kamyab, et al. (1968b) have shown that of the excreted dose in urine, 1.2% is present in a non-conjugated form, 36% in the glucuronide fraction and 12.2% in the sulphate fraction. These values are lower than those obtained for norethisterone and norgestrel in man.

b) "In vivo" and "in vitro" metabolism in rabbit

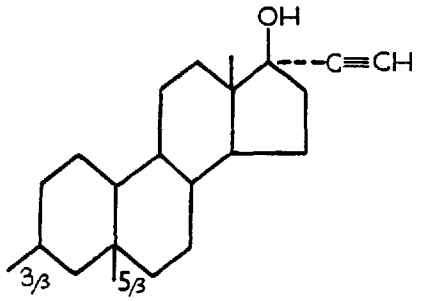
Although ring-A reduced metabolites were identified in the urine of rabbits, two different isomers were isolated (Yamamoto, 1968). Thus, 17α-ethynyl-17β-hydroxy-5α-estrane-3-one and 17α-ethynyl-3α, 17β-dihydroxy-5β-extrane were identified.

After the intravenous administration of (H^3) lynestrenol, 35% of the dose was recovered in urine in 5 days, 40% of this was present in a solvolysable form and 43% in an enzyme hydrolysable form.

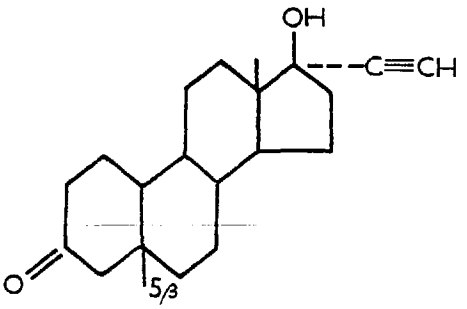
The conversion of lynestrenol to norethisterone has been shown "in vitro" with rabbit liver by Okada, et al. (1964a) and Mazaheri, et al. (1970). Also identified was a ring-A reduced metabolite - 17α-ethynyl-5β-estrane-3α, 17β diol or an isomer of this compound (Mazaheri, et al., 1970).

Therefore, both " in vivo" in man and in rabbits, and "in vitro" in rabbit, lynestrenol is converted to norethisterone. As with norethisterone, ring-A reduced metabolites were identified; however, the isomers present in the two species were different. The removal of the ethynyl group was not shown and the conversion to phenolic steroids is doubtful. Conjugation reactions occur to different extents in the two species. The metabolism of lynestrenol is shown in Figure 6 and does not differ greatly from norethisterone, although the rate of excretion in urine is slightly lower than norethisterone.

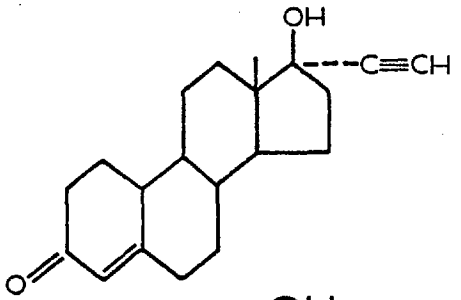
'IN VIVO' METABOLITES IN MAN.



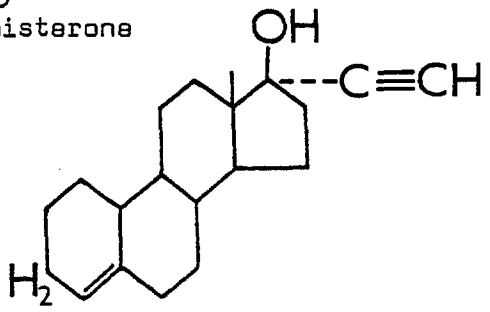
3β, 5β-THNET



3-keto-5β-DHNET

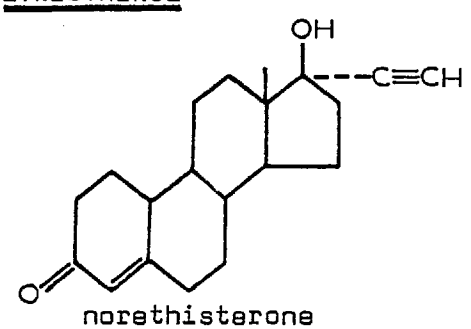


norethisterone

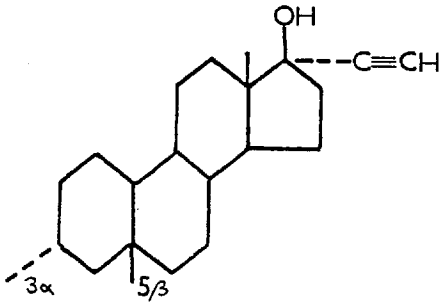


LYNESTRENOL

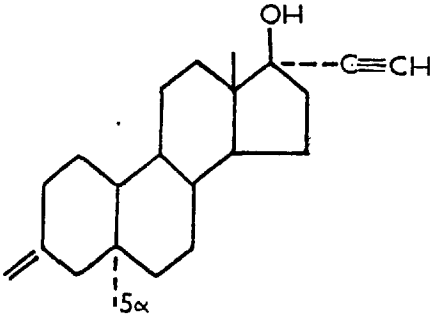
'IN VIVO' AND 'IN VITRO' METABOLITES IN RABBIT.



norethisterone



3α, 5β-THNET



3-keto-5α-DHNET

Fig. 6. 'In vivo' and 'in vitro' metabolites of lynestrenol identified in man and rabbit.

Metabolism of esterified derivatives of synthetic 19-norprogestogens

Little information is available on the "in vivo" metabolism of the esterified derivatives of 19-norprogestogens. Khan, et al. (1975) have shown the "in vivo" conversion of norethisterone oenanthate to norethisterone in women receiving 200 mg of this compound.

The hydrolysis of norethisterone oenanthate and norethisterone acetate has been compared "in vitro" by Bellman, et al. (1976) in human tissues. Thus, in female subcutaneous tissue, the rate of enzymatic cleavage of the oenanthate derivative was considerably faster than the acetate derivatives. Similar results were shown in human myometrium and endometrium. However, in the gastric mucosa, rectus muscle, placenta and vaginal mucosa the acetates were split off more rapidly than the oenanthates. The hydrolysis of norethisterone acetate has also been shown in human foetal tissues. Thus, norethisterone acetate was hydrolysed to norethisterone in placenta, foetal lung and liver. The hydrolysis of norethisterone oenanthate has also been demonstrated in the tissues of the rabbit (Khan, et al., 1975).

Summary of the comparison of "in vivo" and"in vitro" metabolism of synthetic testosterone related progestogens in man and rabbit.

In summary, the pathways of metabolism of the testosterone related progestogens appear to be qualitatively similar to testosterone (Fotherby and James, 1972; Thijssen, 1972; Fotherby, 1974). Thus:

a) Ring-A reduction has been shown in rabbit and man both "in vivo" and "in vitro",
b) Hydroxylation reactions have been shown "in vivo" in man,
c) Aromatisation reactions have been demonstrated "in vitro" in the rabbit, these reactions are similar to that of testosterone,
d) The removal of the ethynyl group has been demonstrated "in vitro" in the rabbit, however, metabolic removal of

the ethynyl side chain does not appear to occur in man or the rabbit 'in vivo',

e) De-esterification of the esterified progestogens is shown "in vivo" and "in vitro" in man,

f) As with testosterone, the metabolic products of the synthetic progestogens are conjugated with glucuronic or sulphuric acid. However, the oxidation of the 17β-hydroxyl group has not been demonstrated in the progestogens. This pathway is fairly important in the metabolism of testosterone, thus, the ethynyl group at C-17 appears to prevent oxidation reactions since oxidation of this group has been demonstrated in 19-nortestosterone. Quantitative comparisons are not possible in these studies, however, the rate of excretion in urine and faeces in man and rabbit is slower for the progestogens as compared to the excretion of testosterone. In these studies as in the study with 19-nortestosterone, specie differences are apparent, as well as differences in the types of metabolites obtained "in vivo" and "in vitro".

Comparison of the "in vivo" and "in vitro" metabolic studies of synthetic 19-norprogestogens

Additional comments

The literature reviewed indicates that most of the studies carried out are "in vivo" in man, and therefore have been restricted, by the limited ability to sample tissues, with the exception of blood, urine and faeces and occasionally bile (Arai, et al., 1962). Although "in vivo" studies provide a large amount of information concerning the rate of clearance from the body, and the route of metabolism, "in vitro" studies in parallel with "in vivo" studies would provide further relevant information with reference to the metabolism of these steroids. For example, until recently, the liver was held to be the main site in the transformation of steroids, but it is becoming

generally accepted that extrahepatic tissues may also be involved in the overall metabolism of synthetic steroids. "In vivo" studies cannot provide the type of information that can be obtained by "in vitro" studies. Thus, a study of the type carried out in this thesis where the effect of various substituent groups on the rate of metabolism of these compounds is directly compared, is not possible "in vivo".

However, "in vitro" studies must be interpreted with caution since they are performed under "artificial" conditions. Several factors must be taken into account in the interpretation of results obtained "in vitro". It is possible when using subcellular fractions that one of the fractions may enhance or inhibit the activity of the other. The separation of homogenates into cell fractions by high speed centrifugation relies on the fact that different cell particles vary in size, although even after extensive washing of the fractions, cross contamination cannot be avoided. The isolation of a metabolite from an incubation of such a fraction indicates only the presence of the enzymes necessary for a particular steroid transformation. It does not necessarily follow that a metabolic pathway demonstrated "in vitro" can occur to the same extent, if at all "in vivo". However, the demonstration of an enzyme activity "in vitro" may be regarded as a potential capacity for "in vivo" activity. Similarly, if it is shown that the enzyme conversion takes place predominantly in one particular cell fraction, it can be taken as strong evidence that the reaction might occur in the same fraction in the intact cell.

Comparison of the metabolism of synthetic 19-norprogestogens in man and rabbit

Finally, it has been suggested (Overbeek, 1974) that the rabbit may not be a suitable animal for studying oral contraceptives and that sub-human primates may be more useful. However, in the metabolism of progesterone and synthetic 19-norprogestogens the rabbit has been shown to closely resemble man (Fotherby, 1964 and 1974) where

as substantial differences are seen in the metabolism of this compound among the various sub-human primates (Goldzieher, et al., 1974). Thus, the statement that the metabolism of sub-human primates comes closest in all respects to man and is therefore the most suitable experimental animal, needs further verification(Breuer, 1972). Furthermore, it has been pointed out by Goldzieher, et al. (1974) that in terms of synthetic steroid hormone metabolism similar data can be obtained from sub-human primates that can also be obtained in the laboratory rodent. In this study we have used the rabbit as an experimental model not only because it may be similar in metabolic activities to man, but also because it is more easily obtained than the sub-human primates and inexpensively maintained.

C H A P T E R 1

RING-A REDUCTION OF 19-NORPROGESTOGENS

INTRODUCTION

In this chapter the tissues of the New Zealand white rabbit were used to investigate the metabolism of the following 19-norprogestogens: Norethisterone (NET), d-, l-, dl-norgestrel (d-Ng, l-Ng, dl-Ng), lynestrenol (Lyn), norethisterone-17β-acetate (NET-AC), dl-norgestrel-17β-acetate (dl-Ng-AC), lynestrenol-17β-acetate (Lyn-AC) and norethisterone-17β-oenanthate (NET-En) compared to the parent steroid 19-nortestosterone (19-nort). Studies with these steroidal compounds were carried out to determine:

a) their rate of metabolism by the hepatic tissue and the types of metabolites formed,
b) their rate of metabolism and the formation of metabolites by the extrahepatic tissues,
c) whether there are any sex differences in the metabolism of these steroidal compounds by the hepatic and extrahepatic tissues.

MATERIALS AND METHODS

1. Animals

Male and female New Zealand white rabbits were used throughout the study. Some of the animals were pregnant (gestation period 6 days). The age of the animals ranged from three months to two years and the weight of the animals ranged from 2.5 Kg - 4.0 Kg. The animals were housed in metal cages with free access to food and water until required for experimentation.

2. Chemicals

a) Steroids

Table 1.1 indicates the source of radioactive and non-radioactive steroids used. The radioactive steroids were more than 98% pure as determined by paper chromatography using the following system - toluene: light petroleum (80^{o}-100^{o}C boiling range): methanol: water (4:1:4:1, by volume). The non-radioactive steroids were checked for purity by gas-liquid chromatography, using a 3% SE 30 (80-100 mesh Gas Chrom Q) column, in a Pye 104 chromatograph instrument with a flame-ionisation detection system, at 220^{o}C. All the steroids were used without further purification.

b) Organic solvents

Organic solvents of analytical reagent grade were redistilled before use. Toluene, acetone, hexane, chloroform (B.D.H. Ltd., Poole, Dorset) and light petroleum (80^{o}-100^{o} boiling range)(Hopkin and Williams Ltd., Chadwell Heath) were all of analytical reagent grade. Absolute ethanol (R.R. grade) and methanol (A.R. grade) (J. Burroughs Ltd., London) were refluxed with 10% potassium hydroxide for eight hours and redistilled before use. Acetic acid (A.R. grade) was obtained from B.D.H. Ltd., Poole, Dorset. Acetic anhydride and pyridine (A.R. grade) were redistilled and stored under anhydrous conditions, when not used.

c) Other chemicals

Potassium dihydrogen orthophosphate, potassium citrate, manganese sulphate, sucrose and nicotinamide were of "Analar" grade.

d) Cofactors

Nicotinamide adenine dinucleotide phosphate reduced (NADPH)

T A B L E 1.1

NON-RADIOACTIVE AND RADIOACTIVE STEROIDS USED IN THIS STUDY.

S T E R O I D	S O U R C E
NON-RADIOACTIVE	
19-nortestosterone	Sigma, London, U.K.
norethisterone	Sigma, London, U.K.
dihydro-norethisterone	Organon, London, U.K.
tetra-hydronorethisterone	Organon, London, U.K.
l-norgestrel	Wyeth Research Inc., Philadelphia USA
d-norgestrel	Wyeth Research Inc., Philadelphia USA
dl-norgestrel	Wyeth Research Inc., Philadelphia USA
lynestrenol	Organon, London, U.K.
norethisterone-17β-acetate	Schering A.G. Berlin.
dl-norgestrel-17β-acetate	Schering A.G. Berlin
lynestrenol-17β-acetate	Organon, London, U.K.
norethisterone-17β-oenanthate	
RADIOACTIVE	
(4-^{14}C) norethisterone sp.act. 3.8 μCi/mg	Schering A.G. Berlin.
(^{14}C) l-norgestrel sp.act. 1.6 mCi/mM	Tracerlab, Mass. USA
(^{14}C) d-norgestrel sp.act. 1.58 mCi/mM	Tracerlab, Mass. USA
(^{14}C) dl-norgestrel sp.act.1.6 mCi/mM	Tracerlab, Mass. USA
(4-^{14}C) norethisterone-17β-acetate	Schering A.G. Berlin.
(4-^{14}C) lynestrenol sp.act. 10.1 uCi/mg.	Organon, London, U.K.

Type 1 (95-99% pure) was obtained from Sigma, London.

e) Gas-liquid chromatographic material

The stationary phases 3% QF1 (Flurosilicone polymer), 3% XE 60 (cyanoethyl-methyl silicone gum) and 3% SE 30 (methyl-silicone) were obtained from Pye Unicam Ltd., York Street, Cambridge, U.K. or Applied Science Lab. Inc., U.K. agents, Field Instruments, Richmond, Surrey, U.K.

Glass coil columns, 150 cm. x 0.3 cm. I.D. were obtained from Pye Unicam Ltd., York Street, Cambridge, U.K.

Hydrogen, air and nitrogen were obtained from BOC (London). These were purified using activated molecular sieves (Pye Unicam, York Street, Cambridge, U.K.) immediately before entry into the gas-liquid chromatograph.

f) Scintillation fluid for estimation of radioactivity

The toluene based scintillant used for the estimation of radio activity in samples consisted of 0.1 g of 1,4-bis (2-(5-phenyloxazolyl)-benzene)(POPOP) and 4 g of 2,5-diphenyloxazole (PPO) in 1 litre of toluene. The phosphors were scintillation grade reagents (Koch-Light Ltd., London).

3. Washing of glassware

All glassware was soaked overnight in detergent (Decon 75, 2% or Diversey, Diversey Ltd., London), scrubbed and thoroughly washed in tap water, followed by ethanol and deionised water.

4. Purification of substrates

Descending partition paper chromatography (Bush, 1952) was employed both for the purification of radioactive steroids and isolation of metabolic products from the incubations. The following system - light petroleum (80^{o}-100^{o}C boiling range): toluene: methanol: water (4:1:4:1, by volume) was used as described by Bush (1952). The steroids were chromatographed on Whatman No. 42 chromatography paper; and after development, the radioactive areas were located by scanning for radioactivity using a Packard Model No. 7200 radiochromatogram scanner. Unlabelled reference steroids were detected after chromatography by spraying the

paper with a 10% ($\frac{w}{v}$) ethanolic solution of phosphomolybdic acid and warming until colour development occurred (Bush, 1954). Areas of radioactivity were eluted with ethanol. The ethanol was evaporated to dryness and radioactivity estimated by liquid scintillation counting.

5. "In Vitro" Methodology.

a) Preparation of tissues.

Each animal was weighed and killed by cervical dislocation, followed by jugular incision, and the blood was drained from the body. The required organs were removed immediately after killing the animals, and either used at once or stored at 4°C until required. Maximum period of storage was 6-8 weeks for liver, kidney, spleen, heart and lung. Organs from the gastrointestinal tract were stored not longer than 2-3 days. Earlier control experiments had shown that the storage of frozen organs, did not alter the enzymic activity, except in the organs of the alimentary canal.

All manipulations were carried out at 4°C. Each organ was washed in 0.25M nicotinamide-sucrose solution and blotted dry. The organs were trimmed of adherent fat and connective tissue, and weighed. The entire alimentary canal was removed and small sections were washed 2-3 times by forcing ice-cold saline (0.9% sodium chloride) from a pippette through the lumen until the intestines were visibly free of excrete. The intestine was cut longitudinally and kept in ice until homogenisation.

Tissue from each organ was minced into small pieces, weighed and homogenised in 2 vol. of ice-cold 0.25M nicotinamide-sucrose solution, using an Ultra-Turrax Type TP/18-2 tissue disintegrator at medium speed for 3 x 10 s periods.

b) Incubation procedure.

The incubation procedure was essentially that of Davidson and Fotherby (1965) except that the incubation medium was fortified with 0.0006M NADPH. Preliminary studies had established optimal conditions with respect to NADPH and substrate. 50 µg of each steroid (steroid:tissue ratio, 1:40,000) and 10,000 cpm of (^{14}C) radioactive tracer were added to each tube and dissolved in 0.1 ml 1-2-propane diol (A.R. grade,

B.D.H. Ltd, Poole, Dorset. To each tube the following were added:

a) 4 ml of tissue homogenate (0.5 g/ml),

b) 1 ml 0.01M potassium citrate, pH 7.4,

c) 1 ml 0.04M potassium dihydrogen orthophosphate, pH 7.4,

d) 1 ml 0.005M manganese sulphate ,

and the total incubation volume was 7.1 ml.

The incubations were always carried out in duplicate, with suitable controls. Duplicate sets of tubes with and without added enzyme were incubated at 37°C and 4°C, the latter tubes served as zero controls. In parallel with each set of incubations of 19-norprogestogens, 19-nortestosterone was also incubated under similar conditions. The incubations were performed in a shaking water bath (Baird and Tatlock, Ltd, London) in air, for the required period of time.

c) Extraction of metabolic products.

At the required time intervals, redistilled ice-cold acetone (30 ml) was added to each tube to terminate the enzymic reaction. The tubes were stoppered and shaken for a few minutes. The aqueous-acetone medium was removed by centrifugation for 10 min at 2000 rpm (500 g) at 4°C in an MSE centrifuge. The supernant was removed and the remaining tissue pellet re-suspended in a further 30 ml ice-cold acetone. After centrifugation, the combined aqueous-acetone mixture was reduced to a small volume and diluted with 25 ml glass distilled water. This aqueous phase thus obtained was extracted with 60 ml redistilled chloroform. The organic phase was dried with anhydrous sodium sulphate, and evaporated to dryness, "in vacuo". The residue was dissolved in 25 ml aqueous methanol (4 : 1, $\frac{v}{v}$) and submitted to a hexane-methanol partition (Fotherby et al. 1957). The methanol phase was dried "in vacuo" at 40°C using a rotary evaporator (Quickfit, U.K.). The residue was dissolved in 5 ml redistilled ethanol transferred to glass vials and dried under nitrogen. This procedure recovers 85-95% of the added radioactive tracer (Table 1.6). The residue was dissolved in 0.5 ml redistilled ethanol and aliquots were taken for:

i) the estimation of radioactivity,

ii) identification of metabolic products by thin-layer and paper chromatography, and

iii) for identification and quantitation by gas-liquid chromatography.

With the incubation extracts from lynestrenol and lynestrenol acetate, the lipid material was removed by employing alumina column chromatography, since these steroids are hexane soluble. After chloroform extraction, the dried residue was dissolved in 5 ml acid-washed redistilled toluene. This was passed through a column (1 cm I.D. x 10 cm with a 0.32 mm precision bore) containing 3 g neutral activity alumina (2% water content) (M. Woelm, Germany) prepared in acid-washed redistilled toluene. The steroids were eluted with 40 ml, 1% ethanol-toluene. This phase was evaporated at 40°C in a vacuum rotary evaporator, and the residue treated as described earlier. Suitable controls were simultaneously carried through the procedure. Using 10,000 cpm of (4-^{14}C) lynestrenol and (4-^{14}C) norethisterone, the mean recovery calculated from four assays was (4-^{14}C) lynestrenol 95.1 $\pm$ 1.2% and (4-^{14}C) norethisterone 92.5$\pm$1.6%.

Since norethisterone oenanthate is also hexane soluble, but unlike lynestrenol and lynestrenol acetate, is quantitatively extracted by this solvent, alumina chromatography was not carried out with this compound. The final quantitation of norethisterone oenanthate was done on the hexane fraction by gas-liquid chromatography.

6. Chromatography.

a) Paper chromatography (p.c.)

Descending partition paper chromatography was performed according to Bush (1952) as described earlier.

b) Alumina column chromatography.

Absorption alumina column chromatography was carried out as described, based on the method of Fotherby and Love (1960).

c) Thin-layer chromatography (t.l.c.).

The metabolic products were also isolated by using ascending one-dimensional thin-layer chromatography (Lisboa and Diczfalusy, 1962). Chromatography was performed on thin-layer plates, 0.25 mm thickness using either silica gel G or H (Merck, Darmstadt, Germany) and the solvent system cyclohexane:ethylacetate (1:1, $\frac{v}{v}$) (Siisenwine et al., 1973), or alumina type T, thin-layer plates (Merck, Darmstadt, Germany) and the solvent system toluene: acetone (4:1, $\frac{v}{v}$) (Simard and Lodge, 1970). Silica gel

GF_{254} was also employed when detection by ultra-violet light was required. Reference steroids were located by spraying with phosphomolybdic acid as described earlier. The required areas were eluted with ethanol. The ethanol was removed from the tubes, after sedimentation of the stationary phase by centrifugation for 10 min at 500 g.

d) Gas-liquid chromatography (g.l.c.).

Gas-liquid chromatography was employed in the quantitative estimation of steroids investigated. This analytical procedure has been regarded as simple, sensitive, fast and reliable for the estimation of steroids in biological fluids (Vanden Heuvel and Horning, 1964 and Lipsett, 1965).

i) Instrumentation and operating conditions.

The gas-liquid chromatographic system used was Pye chromatograph (series 104, Model 24) - dual hydrogen flame ionisation, temperature programmed chromatograph. 150 cm x 0.3 cm I.D. glass coil columns were used. The oven temperature was maintained at 220°C and the gas inlet pressure of the carrier gas, nitrogen, was 130 psi (50 ml/min). The flow rates of air and hydrogen were adjusted to obtain the maximum sensitivity for the steroids chromatographed.

In general 1-5 µl vol. of the incubation extracts were injected using a 10 µl Hamilton Syringe (701 SN) with a 4½ inch needle (Clark Hamilton, AG Bonaduz, Switzerland). In order to monitor the overall chromatographic system, the injection of reference steroids was repeated after every third injection of the unknown steroids and after completing the analyses of a group of extracts. The response factor for each steroid was calibrated at regular intervals. The detector response was linear for both the non-derivatised and derivatised steroids quantitatively determined in the ranges studied. The detector response was directly proportional to the weights of the steroids, thus no correction factor was needed in the calculation of the results.

In Fig. 1.1 is shown a chromatograph of a standard mixture of 200 ng of norethisterone and the internal standard cholestenone, using the flame-

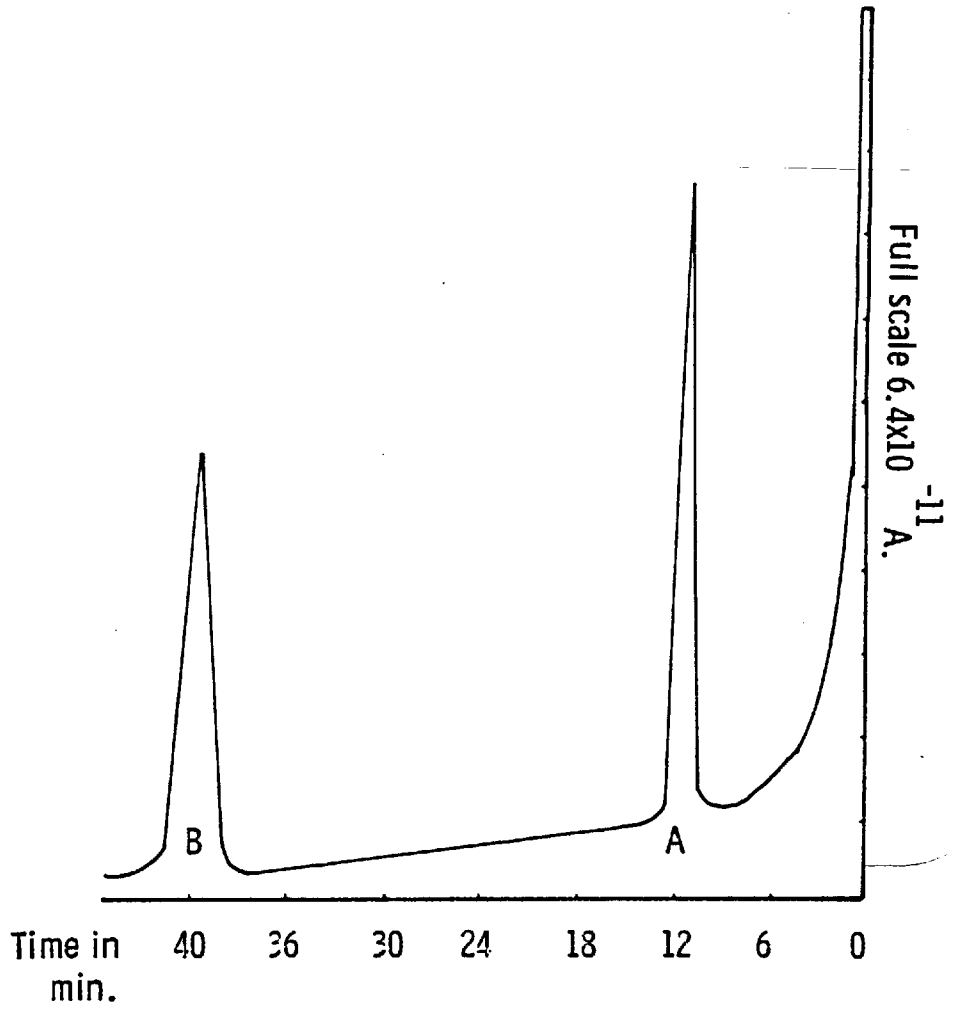


Fig.1.1 G.l.c. chromatogram of a mixture of norethisterone (A) and cholestenone (B). 200ng of each steroid was chromatographed using a flame ionisation detector. The conditions used are described in the text.

ionisation detector. A standard curve obtained for norethisterone is shown in Fig. 1.2 demonstrating the linear relationship between peak area and the amount of steroid chromatographed.

Gas liquid chromatography was carried out using the stationary phases 3% QF1, 3% XE 60 or 3%SE 30 on Gas Chrom Q (80-100 mesh). These were packed and standardised according to Szepesy (1970).

The efficiency of each column was evaluated at regular intervals by means of the "number of theoretical plates" method as described by Szepesy (1970) using the following formula:

$$N = 16\left(\frac{t_r}{w}\right)^2$$

where N = total number of theoretical plates

t_r = absolute retention time

w = base width of peak.

The maximum efficiency achieved for the columns was 380 theoretical plates per foot, and the column was discarded if this level decreased to 250 theoretical plates per foot.

ii) Quantitation

Quantitation was carried out by calculating the area of the peak from the peak height times the width measured at half peak height in the earlier part of our study, since the peaks obtained were well defined and symmetric. In the later part of the study, an electronic integrator became available and was used for the measurements of peak areas. (Infotronics Ltd., Ireland, Model CRS-208). The following equation for quantitation of each steroid was employed:

Amount of steroid in sample =

$$\frac{\text{Area produced by the unknown steroid}}{\text{Area produced by the internal standard}} \times \text{amount of internal standard added}$$

The dose response curve was linear between 300-1000ng for all steroids examined. Thus, as a double check, the amount of steroid in

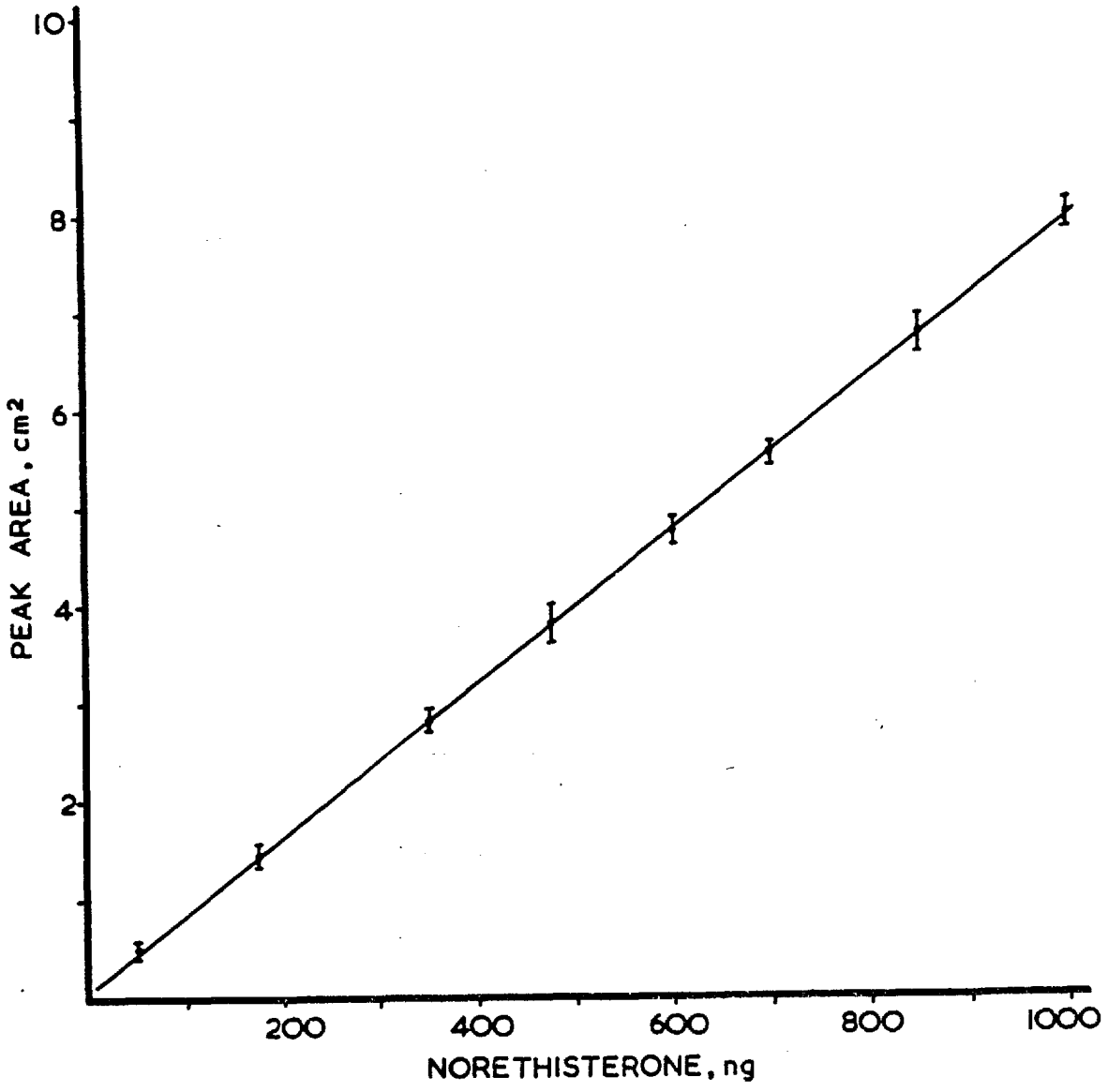


Fig. 1.2 A standard curve for norethisterone using a flame ionisation detector. Each point represents mean of 20 determinations. For chromatographic conditions see text.

the unknown sample was also obtained from the standard curve obtained for each steroid.

The third method used for checking the quantitative procedure was that of direct calibration, where the standard steroids with known compositions were injected, so that areas smaller and greater than that of the peak under observation were obtained. Linear interpolation was then performed between the areas for the two different compositions. Each sample was injected in duplicate and mean values were taken for calculating the result.

iii) Use of internal standard

Besides using (^{14}C) radioactive steroids as internal standards to monitor procedural losses, cholestenone was also used as an internal standard in gas-liquid chromatography. This was used to compensate for small variations in the volume of incubation extracts injected and for variations in the sensitivity of the detector.

iv) Reliability data for gas-liquid chromatography

a) Characterisation of steroids

Characterisation of the progestogens was performed by using relative retention times to cholestenone. The relative retention times have been given in table 1.2. Using cholestenone as the internal standard, a quantitative analysis was performed by peak height times width at half peak height with good reproducibility (Table 1.3).

b) Precision

Precision (defined as the extent to which a given set of measurements of a given sample agrees with the mean) was estimated from the coefficient of variation, determined from the standard deviation of duplicate samples.

$$\left(S = \sqrt{\frac{\sum d^2}{n - 1}} \right)$$

Where "d" is the difference between duplicates and "n" the number of

TABLE 1.2

RELATIVE RETENTION TIMES (RRT) TO CHOLESTENONE OF 19-NORPROGESTOGENS (MEAN OF 20 DETERMINATIONS). OPERATING CONDITIONS HAVE BEEN DESCRIBED IN TEXT.

PROGESTOGEN	RRT
Cholestenone	1
19-nortestosterone	0.25
norethisterone	0.27
dihydronorethisterone	0.11
tetrahydronorethisterone	0.18
l-norgestrel	0.35
d-norgestrel	0.36
dl-norgestrel	0.35
tetrahydronorgestrel	0.24
lynestrenol	0.12
norethisterone acetate	0.41
dl-norgestrel acetate	0.59
lynestrenol acetate	0.26

TABLE 1.3

QUANTITATIVE DETERMINATION OF PROGESTOGENS BY PEAK HEIGHT x WIDTH AT HALF PEAK HEIGHT, USING CHOLESTENONE AS INTERNAL STANDARD (mean of 10 determinations).

PROGESTOGEN	PR	SD	CV%
19-nortestosterone	0.97	0.012	1.23
norethisterone	0.99	0.014	0.99
dihydronorethisterone	0.88	0.016	1.8
tetrahydronorethisterone	0.78	0.018	2.3
d-norgestrel	1.36	0.020	1.4
l-norgestrel	1.27	0.013	1.0
dl-norgestrel	1.29	0.030	2.3
tetrahydronorgestrel	0.90	0.040	4.4
lynestrenol	0.86	0.020	2.3
norethisterone acetate	0.71	0.050	7.0
dl-norgestrel acetate	1.10	0.20	1.8
lynestrenol acetate	0.66	0.030	1.5

(PR = peak height ratio $\frac{\text{progestogen}}{\text{cholestenone}}$

SD = standard deviation ($\pm$)

CV = coefficient of variation)

of duplicates. Blank (zero time) incubation extracts were used for these determinations (Table 1.4).

The values for the coefficient of variation in extracts containing 10 µg / 0.1 ml and 50 µg /0.5 ml are shown in Tables 1.4 and 1.5.

Between assay variability was determined by analysing reference incubations on eight different occasions. The coefficient of variation was 8% for a concentration of 5 µg/0.5 ml and 5% for a concentration of 50 µg/0.5 ml.

c) Accuracy.

The accuracy of the method, or the extent to which the mean of an infinite number of measurements of a steroid agrees with the exact amount of the steroid which is present, was evaluated from recovery experiments of non-tissue incubation blanks and tissue incubation blanks from eight experiments.

As shown in Table 1.6, the recovery of the (^{14}C) tracer (internal standard for procedural losses) for all the steroids examined varied between 80 and 95% with a mean of 86%. After correction for procedural losses, the recovery of the steroid, estimated by g.l.c., was $94.0 \pm 1.5\%$ (SD) (overall mean) for an initial steroid content of 50 µg.

d) Sensitivity.

This is defined as the smallest amount of steroid distinguishable from zero with a signal to noise ratio of 10 : 1. This value varies with the structure of the compound and hence the retention time. Steroids with low retention times, such as tetrahydronorgestrel (R_T 3.8 cm), have a sensitivity of 100 ng under optimal conditions, when the detector current is maintained at 200×10^{-12}A,(Pye 104 series, Model 24). Generally, one-tenth of the extract was injected (larger volumes overloaded the column with impurities, thus decreasing the sensitivity and accuracy). This means that the extract from the incubations of norgestrel should contain a minimum of 900 ng in order to give a significant peak. The sensitivity of each compound was determined prior to final quantitation, in order to obtain optimal conditions.

T A B L E 1.4

VALUES OF 10 REPLICATE DETERMINATIONS OF PROGESTOGENS FROM BLANK INCUBATIONS CONTAINING 50 ug OF STEROID IN 0.5 ml OF ETHANOL.

PROGESTOGEN	MEAN µg /0.5 ml.	SD µg/0.5 ml	CV%
19-nortestosterone	48.2	2.1	4.3
norethisterone	46.5	3.2	6.8
d-norgestrel	47.2	2.4	5.0
l-norgestrel	48.1	1.9	3.9
dl-norgestrel	49.1	2.0	4.0
lynestrenol	48.4	1.8	3.7
norethisterone acetate	47.2	2.1	4.4
lynestrenol acetate	48.1	1.5	3.1
dl-norgestrel acetate	46.4	2.4	5.1

(SD= standard deviation ($\pm$), CV = coefficient of variation).

T A B L E 1.5

VALUES OF 10 REPLICATE DETERMINATIONS FOR PROGESTOGENS FROM BLANK INCUBATIONS CONTAINING 10 µg OF STEROID IN 100 µlit OF ETHANOL.

PROGESTOGEN	MEAN µgm/100µlit.	SD µg/100 µlit	CV%
19-nortestosterone	9.2	0.8	8.6
norethisterone	9.0	0.7	7.7
d-norgestrel	9.3	0.9	9.6
l-norgestrel	8.9	1.0	11.2
dl-norgestrel	8.8	0.9	10.2
lynestrenol	8.5	1.0	11.7
norethisterone acetate	9.1	0.8	8.7
lynestrenol acetate	9.3	0.8	8.6
dl-norgestrel acetate	9.1	0.8	8.7

(SD = standard deviation ($\pm$), CV = coefficient of variation).

The lower limits of detection of each steroid, under standardised conditions are shown in Table 1.7 (values are means of 30 estimations).

e) Specificity.

The specificity of a procedure using g.l.c. to analyse substances present in biological fluids depends on the purity of the sample prior to g.l.c. Thus the following criteria were used to determine the specificity of the peaks in the chromatograph scans:

a) Separation of the peak under examination from other peaks,
b) Shape of the peak,
c) homogeniaety of the progestogen peak with reference steroids available.

Tissue blanks, non-tissue blanks, and steroid blanks were run with each series of incubation extracts. If, a significant blank was obtained, the series of extracts were either purified further or discarded. A significant blank was usually due to solvent impurities.

7. Identification of Incubation Products.

Due to the stability and constant behaviour of most columns, operating conditions in gas-liquid chromatography are reproducible. Retention times of individual compounds under defined operating conditions are characteristic properties. Their correspondence with data of reference steroids, under a variety of chromatographic conditions, is good evidence of identity. Thus,the metabolic products in the incubation extracts were chromatographed on three different stationary phases (described earlier) and retention times compared to reference steroids as non-derivatised and as trimethylsilyl ether derivatives.

Each metabolite was further characterised using micro-analytical techniques involving acetylation, reduction and oxidation procedures. These products were then compared to reference steroids treated in the same way and chromatographed on thin-layer plates and gas-liquid chromatograph. When possible, the metabolites were identified by mass-spectrometry.

T A B L E 1.6

PERCENT RECOVERY OF PROGESTOGENS FROM BLANK INCUBATIONS WITH LIVER TISSUE (mean of 10 determinations).

PROGESTOGEN	STEROID ADDED (µg)	PERCENT RECOVERY OF TRACER	PERCENT RECOVERY OF STEROID (corrected for tracer recovery)
19-nortestosterone	50	88(80-92)	97.0±1.8
norethisterone	50	84(80-88)	94.4±1.2
d-norgestrel	50	87(83-91)	92.6±1.3
l-norgestrel	50	86(82-90)	92.4±1.8
dl-norgestrel	50	89(84-91)	94.1±2.8
lynestrenol	50	88(86-94)	95.2±1.2
norethisterone acetate	50	90(88-95)	93.1±1.5

T A B L E 1.7

LOWER LIMITS OF DETECTION OF STANDARD REFERENCE STEROIDS AND PROGESTOGENS IN INCUBATION EXTRACTS (mean of 30 determinations).

STEROID	REFERENCE STEROID (ng)	STEROIDS IN INCUBATION EXTRACTS(ng)
19-nortestosterone	200	320
Ring-A reduced metabolite of 19-nort.	-	300
norethisterone	200	120
tetrahydronorethisterone	120	250
dihydronorethisterone	100	140
d-norgestrel	400	450
l-norgestrel	410	480
dl-norgestrel	420	490
tetrahydronorgestrel	250	280
dihydronorgestrel	200	-
lynestrenol	300	300
lynestrenol acetate	280	300
norethisterone acetate	300	320
dl-norethisterone acetate	320	380.

8. Derivative Formation.

a) Preparation of trimethylsilyl ether derivatives.

Trimethylsilyl ether derivatives were prepared according to Chambaz and Horning (1969) except that redistilled petroleum ether (80° - 100° boiling range) was used as the solvent. After drying the sample, 50 µlit of petroleum ether and 40 µl of Powersil-prep (Applied Science Laboratories, Inc., U.K. distributors Field Instruments Company Limited, Surrey) consisting of trimethylsilyl-imidazole (TSIM), bis(trimethylsilyl) acetamide (BSA) and trimethylchlorosilane (TMCS) (3 : 3 : 2, by vol.) was added and the tube tightly closed. The reaction was usually complete in 15 min at 50°C. Excess reagent was removed, after completion of reaction, under nitrogen. The sample was then dissolved in known volumes of anhydrous petroleum ether and chromatographed on g.l.c..

b) Acetylation.

The acetylation of steroids was carried out according to Bush (1961). The sample and the authentic steroids were separately dissolved in redistilled pyridine (0.1 ml) and redistilled acetic anhydride (0.1 ml). The reaction mixture was left over night at room temperature. After completion of the reaction, excess reagents were removed under nitrogen. The acetylated compounds were dissolved in a known volume of ethanol and chromatographed.

c) Oxidation.

The method used was that of De Jongh et al. (1968). 0.45 µg of the steroid was kept at room temperature for 6 min in acetone (100 µl) containing 8N chromic acid (1.4 µl) . Water (75 µl) was added, and the mixture was extracted with redistilled chloroform (4 x 50 µl). The extracts were examined by gas-liquid chromatography or thin-layer chromatography.

d) Reduction.

The method used was that of Lisboa and Diczfalusy (1962). One to twenty µg of steroid in aqueous methanol was incubated with 5 mg/ml sodium borohydride (B.D.H. Ltd, Poole, Dorset). The contents were mixed thoroughly and left at room temperature for 30 min. The reaction was terminated by the addition of dilute acetic acid, 2 ml water was added and the reduced steroids extracted with chloroform. The extract was dried with anhydrous sodium sulphate and chromatographed with reference steroids.

9. Estimation of Radioactivity.

Radioactive (^{14}C) labelled steroids (10,000 cpm) were used as internal standard for procedural losses. Aliquots of incubation extracts in ethanol were estimated for radioactivity using a toluene-based scintillant (10 ml) as described earlier. A Beckman Model No. 1550 liquid scintillation spectrometer was used for counting the samples and sufficient counts were recorded to give a standard error of less than 5%. The counting efficiency for carbon-14 was greater than 90%. Background determinations were obtained by counting blank samples similar to that under investigation. The degree of quenching was estimated by the internal standard method (Kerr et al., 1957) using (4-^{14}C) progesterone and did not exceed 5%.

10. Statistical Treatment of Data.

The results were treated statistically to obtain means and standard deviations. The student's t test was used as a test of significance between the means of two groups of results.

RESULTS.

1. Metabolism of 19-nortestosterone and various synthetic 19-norprogestogens by rabbit hepatic tissue.

Studies were carried out to compare the rates of metabolism and formation of metabolites of 19-nortestosterone and synthetic 19-norprogestogens by rabbit hepatic tissues.

a) Rate of metabolism.

The incubation procedure has been described in the Methods section. In this part of the study only female rabbit livers were used. The results of the time course study (0 min-300 min) of the hepatic metabolism of 19-nortestosterone and the synthetic 19-norprogestogens are presented in Table 1.8 as means ($\pm$ S.D.) of percent steroid not metabolised. (Individual values are given in Appendix tables 1 - 3) The results up to 30 min of incubation time are also shown in Fig. 1.3.

Rapid metabolic transformation of the parent compound 19-nortestosterone was seen to occur within the first 10 min where only 43.7 $\pm$ 28% of the steroid remained unchanged. Relative to 19-nortestosterone the synthetic 19-norprogestogens were more slowly metabolised, but statistically only the rate of metabolism of l-norgestrel was significantly different from 19-nortestosterone ($p < 0.05$) at this time (Appendix table 1).

By 30 min of incubation time, the differences in metabolic rate became more apparent (Fig. 1.3) and by 180 min (Table 1.8) distinct differences in metabolic rate were seen. Thus, at 180 min 13.0 $\pm$ 12.0% of 19-nortestosterone remains; whereas, for the unesterified steroids 9.9 $\pm$ 0% of norethisterone, 22.3 $\pm$ 8.1% of d-norgestrel, 44.2 $\pm$ 18.1% of dl-norgestrel, 88.9 $\pm$ 3.3% of l-norgestrel, and 27.5 $\pm$ 2.9% of lynestrenol remained unchanged. The mean values of d-norgestrel, dl-norgestrel, l-norgestrel and lynestrenol are significantly different ($p < 0.05$) from the mean value of 19-nortestosterone (Appendix table 3). Whereas the mean value for norethisterone was not significantly ($p > 0.1$) different from that of the parent compound.

T A B L E 1.8 COMPARISON OF THE RATE OF METABOLISM OF 19-NORTESTOSTERONE AND VARIOUS SYNTHETIC 19-NORPROGESTOGENS IN THE HEPATIC TISSUE OF FEMALE RABBIT. THE RESULTS ARE EXPRESSED AS MEAN (±SD) OF PERCENT STEROID RECOVERED AT VARIOUS TIME INTERVALS. (Figures in parentheses indicate number of specimens examined).

	T I M E O F I N C U B A T I O N (min)							
	0	10	15	20	30	60	180	300
STEROID INCUBATED	PERCENT OF STEROID NOT METABOLISED.							
19-nortestosterone	92.0±5.5 (9)	43.7±28,0 (3)	50.1±12.3 (4)	53.6±5.3 (3)	45.1±11.3 (9)	24.7±17.0 (4)	13.0±12.0 (3)	0±0 (3)
norethisterone	90.6±10.4 (11)	67.9±9.1 (6)	56.9±19.7 (3)	62.2±1.3 (4)	42.4±15.5 (8)	29.4±21.1 (4)	9.9±0 (4)	0 (2)
d-norgestrel	94.7±2.5 (4)	63.0±10.3 (4)	68.0±7.2 (4)	45.5±3.9 (4)	53.6±17.7 (4)	27.0±9.0 (4)	22.3±8.1 (4)	11.6±5.0 (4)
dl-norgestrel	92.5±7.3 (8)	62.8±8.3 (8)	60.5±13.0 (6)	49.2±10.2 (5)	58.5±19.7 (8)	45.9±13.9 (8)	44.2±18.1 (8)	43.4±13.0 (8)
l-norgestrel	94.1±1.8 (4)	92.6±2.0 (4)	93.1±1.4 (4)	91.4±2.7 (4)	92.3±2.3 (4)	88.6±3.9 (4)	88.9±3.3 (4)	88.8±3.0 (4)
lynestrenol	93.6±2.9 (3)	63.0±4.1 (3)	55.9±2.3 (4)	52.9±1.6 (3)	40.2±1.6 (6)	35.6±6.1 (5)	27.5±2.9 (4)	20.0±9.0 (4)
lynestrenol acetate	93.0±2.1 (3)	86.0±2.4 (3)	82.5±2.2 (3)	79.5±1.7 (3)	73.2±2.0 (3)	64.6±1.5 (3)	60.0±1.8 (3)	56.0±1.8 (3)
norethisterone acetate	91.1±4.7 (3)	81.3±5.9 (3)	73.8±0.8 (3)	75.6±4.1 (3)	64.4±5.0 (3)	58.9±3.8 (3)	52.7±5.6 (3)	45.2±5.1 (3)
dl-norgestrel acetate	95.0±1.7 (3)	84.0±2.8 (3)	79.1±2.7 (3)	73.0±1.9 (3)	70.0±1.9 (3)	70.0±3.2 (3)	55.2±3.5 (3)	50.0±1.7 (3)
norethisterone oenanthate	86.5±3.6 (6)	63.3±4.9 (6)	-	43.9±3.7 (6)	35.7±3.6 (6)	39.8±7.7 (6)	47.2±3.9 (6)	-

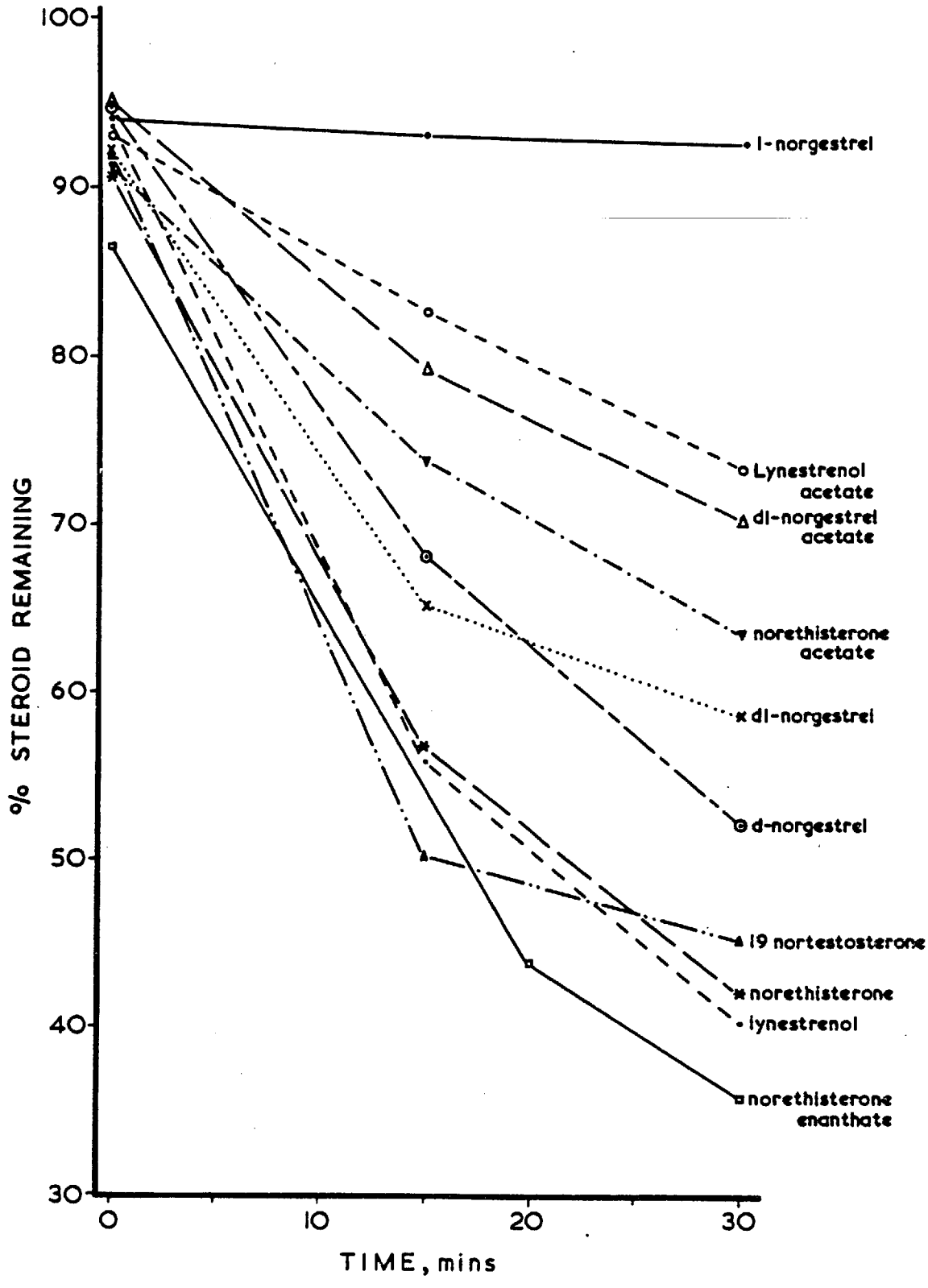


Fig. 1.3 Relative rates of metabolism of 19-nortestosterone and the synthetic 19-norprogestogens at 15 and 30 min incubation time with female rabbit liver tissue. The conditions of incubation are given in the text.

Comparing the rate of metabolism of 19-nortestosterone with the esterified derivatives, the results indicate (Table 1.8) that at 180 min of incubation time, the rate of metabolism of all the steroid esters examined were significantly different ($p < 0.05$) from 19-nortestosterone (Appendix table 5). Thus at 180 min 60.0±1.8% of lynestrenol acetate 52.7±5.6% of norethisterone acetate, 55.2±3.5% of dl-norgestrel acetate and 47.2±3.9% of norethisterone oenanthate remained unmetabolised, as compared to 13.0±12.0% of 19-nortestosterone.

b) Nature and formation of metabolites in hepatic tissue.

On Tables 1.9 - 1.10, the mean (±SD) values obtained for each substrate, expressed as percent steroid remaining, and the percent formation of metabolites are shown. The methods used in the identification of these metabolites have been described in the Methods section. (Individual values are given in Appendix tables 1 - 5).

19-nortestosterone was metabolised to one product which was shown to be a ring-A reduced compound and was not definitely identified.

Norethisterone, d-, l-, dl-norgestrel were metabolised to ring-A reduced products, thus, only tetrahydronorethisterone and tetrahydronorgestrel were identified. The nature of the metabolite of norgestrel in terms of optical-activity was not determined, however, the configuration of the metabolite was identified to be 3α, 5β-tetrahydronorgestrel for d-norgestrel. The rate of formation of these products was seen to increase with time (Fig. 1.4 compares the metabolism of d-, l-, dl-norgestrel). The mean values are given in Table 1.9.

Attempts were made to determine whether the pathway of ring-A reduction of the synthetic 19-norprogestogens is similar to that taken by the natural steroids. The metabolic pathway of d-norgestrel was examined by incubating 5α-dihydronorgestrel, 5 β-dihydronorgestrel, the ring-A saturated 3α-and 3β-hydroxynorgestrel under the same conditions used for d-norgestrel in liver. The results of this study

T A B L E 1.9 RATE OF METABOLISM AND METABOLITE FORMATION OF 19-NORTESTOSTERONE, NORETHISTERONE, dl-, d-, l-NORGESTREL AND LYNESTRENOL IN FEMALE RABBIT LIVER. THE RESULTS ARE EXPRESSED AS MEAN ±SD OF PERCENT STEROID RECOVERED. (Number in parentheses indicates number of experiments performed)

STEROID INCUBATED	19-NOR TESTOSTERONE	NORETHISTERONE		dl-NORGESTREL		d-NORGESTREL		l-NORGESTREL		LYNESTRENOL		
% STEROID RECOVERED	19-NorT	NET	Tetrahydro NET	dl-Ng	Tetrahydro Ng	d-Ng	Tetrahydro Ng	l-Ng	Tetrahydro Ng	Lyn	NET	Tetrahydro NET
Time - min												
0	92.0±5.5 (9)	90.6±10.4 (11)	0 (11)	92.5±7.3 (8)	0 (8)	94.7±2.5 (4)	0 (4)	94.1±1.8 (4)	0 (4)	93.6±2.9 (3)	0 (3)	0 (3)
10	43.7±28.0 (3)	67.9±9.1 (6)	22.9±7.6 (6)	62.8±8.3 (8)	26.4±6.1 (8)	63.0±10.3 (4)	29.3±9.1 (4)	92.6±2.0 (4)	1.0±0.5 (4)	63.0±4.1 (3)	18.0±1.4 (3)	14.2±0.7 (3)
15	50.1±12.3 (4)	56.9±19.7 (3)	19.1±1.0 (3)	60.5±13.0 (6)	27.5±6.7 (6)	68.0±7.2 (4)	23.4±5.2 (4)	93.1±1.4 (4)	0.48±0.4 (4)	55.9±2.3 (4)	24.0±3.6 (4)	16.6±2.5 (4)
20	53.6±5.3 (3)	62.6±1.3 (3)	17.3±1.3 (3)	49.2±10.2 (6)	37.9±4.4 (5)	45.5±3.9 (4)	47.7±6.6 (4)	91.4±2.7 (4)	1.9±0.8 (4)	52.9±1.6 (3)	26.3±0.9 (3)	18.2±2.4 (3)
30	45.1±11.3 (3)	42.4±15.5 (8)	47.1±14.5 (8)	58.5±19.7 (8)	30.1±16.8 (8)	52.4±18.3 (4)	38.5±19.3 (4)	92.3±2.3 (4)	1.3±0.9 (4)	40.2±1.6 (6)	33.0±1.8 (6)	22.9±2.2 (6)
60	24.7±17.0 (4)	29.4±21.1 (4)	60.7±20.7 (4)	45.9±13.8 (8)	38.4±11.0 (8)	27.0±9.0 (4)	60.5±6.6 (4)	88.6±3.9 (4)	3.9±1.5 (4)	35.6±6.1 (5)	27.8±1.8 (5)	15.1±2.2 (5)
180	13.0±12.0 (3)	9.9±0 (4)	85.8±9.9 (4)	44.2±18.1 (8)	42.8±19.2 (8)	22.3±8.1 (4)	66.4±5.9 (4)	89.0±3.4 (4)	3.6±1.3 (4)	27.5±2.9 (4)	35.8±2.7 (4)	25.0±4.4 (4)
300	0 (3)	0 (2)	85.5±1.4 (3)	43.4±13.0 (8)	46.1±12.6 (8)	11.6±5.0 (4)	78.3±9.9 (4)	88.8±3.0 (4)	4.2±1.2 (4)	20.0±9.0 (4)	39.7±3.8 (4)	38.0±1.3 (4)

70.

TABLE 1.10 RATE OF METABOLISM AND METABOLITE FORMATION OF LYNESTRENOL ACETATE, NORETHISTERONE ACETATE, dl-NORGESTREL ACETATE AND NORETHISTERONE OENANTHATE IN FEMALE RABBIT LIVER. THE RESULTS ARE EXPRESSED AS MEAN ± SD OF PERCENT STEROID RECOVERED. (number in parentheses indicates numbers of experiments performe

STEROID INCUBATED	LYNESTRENOL ACETATE				NORETHISTERONE ACETATE			dl-NORGESTREL ACETATE			NORETHISTERONE OENANTHATE		
STEROID RECOVERED	Lyn-AC	Lyn	NET	Tetra-hydro NET	NET-AC	NET	Tetra-hydro NET	dl-Ng-AC	Ng	Tetra-hydro Ng.	NET oenanthate	NET	Tetra-hydro NET
Time - hour													
0	93.0±2.1 (3)	0 (3)	0 (3)	0 (3)	91.1±4.7 (3)	0 (3)	0 (3)	95.0±1.7 (3)	0 (3)	0 (3)	86.5±3.6 (6)	0 (6)	0 (6)
10	86.0±2.4 (3)	3.0±0.6 (3)	4.1±1.0 (3)	0 (3)	81.3±5.9 (3)	7.0±1.7 (3)	3.0±1.2 (3)	84.0±2.8 (3)	4.0±1.1 (3)	3.1±0.2 (3)	63.3±4.9 (6)	15.3±1.8 (6)	6.4±08 (6)
15	82.5±2.2 (3)	4.5±1.2 (3)	5.1±1.1 (3)	2.0±0.1 (3)	73.8±0.8 (3)	7.3±1.2 (3)	4.2±0.3 (3)	79.1±2.7 (3)	6.2±1.0 (3)	5.1±0.2 (3)	-	-	-
20	79.5±1.7 (3)	6.0±0.9 (3)	10.2±1.0 (3)	4.1±0.3 (3)	75.6±4.1 (3)	10.6±1.0 (3)	3.8±0.7 (3)	73.0±1.9 (3)	8.0±1.2 (3)	7.2±0.8 (3)	43.9±3.7 (6)	19.6±2.8 (6)	17.1±1.4 (6)
30	73.1±2.0 (3)	8.4±0.7 (3)	13.0±1.3 (3)	7.5±1.9 (3)	64.4±5.0 (3)	17.9±0.7 (3)	8.1±1.8 (3)	73.0±1.9 (3)	13.2±1.0 (3)	11.1±0.8 (3)	35.7±3.6 (6)	24.7±3.9 (6)	21.3±3.0 (6)
60	64.6±1.5 (3)	10.0±1.5 (3)	16.4±1.4 (3)	9.0±1.6 (3)	58.9±3.8 (3)	14.6±1.7 (3)	17.1±2.6 (3)	70.0±3.2 (3)	17.3±1.1 (3)	13.1±1.3 (3)	39.8±7.7 (6)	8.2±2.1 (6)	26.9±1.7 (6)
120	-	-	-	-	-	-	-	-	-	-	47.2±3.9 (6)	2.7±0.7 (6)	32.6±2.1 (6)
180	60.0±1.8 (3)	12.0±2.4 (3)	18.3±1.1 (3)	11.3±0.7 (3)	52.7±5.6 (3)	15.5±2.7 (3)	22.0±1.9 (3)	55.2±3.5 (3)	18.1±1.4 (3)	15.1±1.8 (3)	-	-	-
300	56.0±1.8 (3)	14.5±1.7 (3)	20.0±2.5 (3)	13.4±1.0 (3)	45.2±5.1 (3)	12.3±3.7 (3)	24.4±2.1 (3)	50.0±1.7 (3)	20.0±1.5 (3)	18.0±1.4 (3)	-	-	-

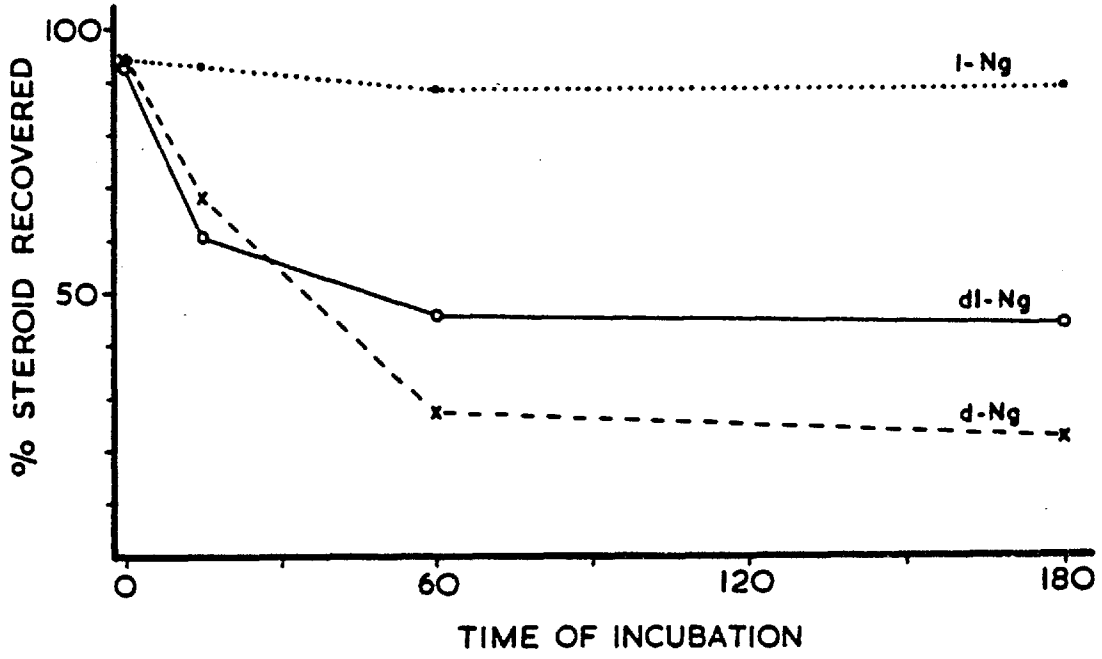


Fig. 1.4 Relative rates of metabolism of d-, dl- and l-norgestrel in female rabbit liver tissue. Incubation conditions are given in the text.

are shown in Table 1.11, and indicate that 5β-dihydronorgestrel was converted more rapidly (80.1 ± 4.1%) to 3α,5β-tetrahydronorgestrel, than 5α-dihydronorgestrel (4.1 ± 1.2%) in 10 min. Under the same conditions only 2.1 ± 1.5% of 3α-hydroxynorgestrel and 3.1 ± 2.0% of 3β-hydroxynorgestrel were converted to 3α, 5β-tetrahydronorgestrel. Thus these results show that the pathway of ring-A reduction of the synthetic 19-norprogestogen, d-norgestrel is similar to the natural steroid testosterone. Lynestronol was metabolised to norethisterone and tetrahydronorethisterone by rabbit liver (Table 1.9).

Table 1.11
INCUBATION OF d-NORGESTREL AND INTERMEDIARY METABOLITES WITH RABBIT LIVER, INCUBATION TIME 10 min.

Steroid Incubated	% Conversion to tetrahydronorgestrel.
d-norgestrel	48.1 ± 8.4%
5α-dihydronorgestrel	4.1 ± 1.2%
5β-dihydronorgestrel	80.1 ± 4.1%
3α-hydroxynorgestrel	2.1 ± 1.5%
3β-hydroxynorgestrel	3.1 ± 2.0%

The esterified derivatives of norethisterone, dl-norgestrel and lynestrenol gave rise to the free non-esterified compounds and ring-A reduced metabolites (Table 1.10). Thus norethisterone acetate and norethisterone oenanthate were metabolised to norethisterone and tetrahydronorethisterone. dl-norgestrel acetate was metabolised to norgestrel and tetrahydronorgestrel while lynestrenol acetate was hydrolysed to lynestrenol and further metabolised to norethisterone and tetrahydronorethisterone (Fig. 1.5, Table 1.10).

Thus in summary, the results of the hepatic metabolism of 19-nortestosterone and the synthetic 19-norprogestogens show that:

a) the initial rates of metabolism, as seen at 10 min of incubation time, of the synthetic 19-norprogestogens do not differ significantly from 19-nortestosterone, except for l-norgestrel;

b) by 180 min of incubation time significant differences are seen in the rates of metabolism of 19-nortestosterone and the

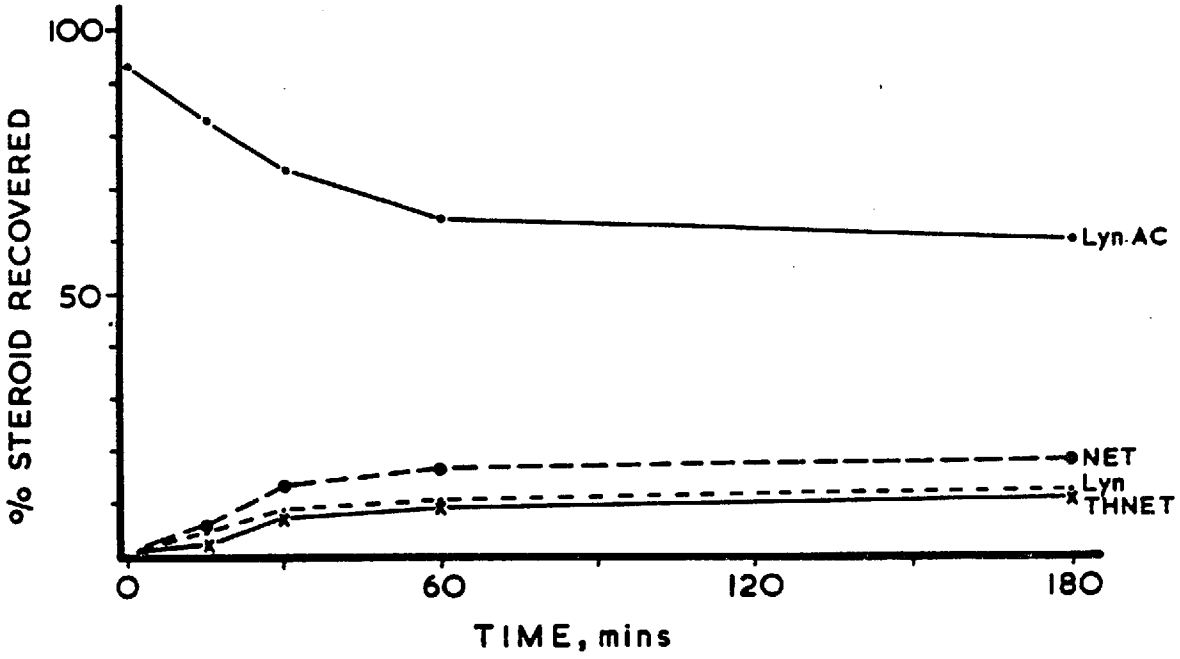


Fig. 1.5 Rate of metabolism of lynestrenol acetate and formation of metabolites in female rabbit liver tissue. Incubation conditions are given in the text.

synthetic 19-norprogestogens;

c) thus the progestogens can be put in the following order, in decreasing rates of metabolism:- norethisterone, d-norgestrel, lynestrenol, dl-norgestrel, norethisterone oenanthate, norethisterone acetate, dl-norgestrel acetate, lynestrenol acetate and l-norgestrel. Therefore, esterification of 19-norprogestogens reduces their rate of metabolism as compared to that of the non-esterified progestogens and 19-nortestosterone;

d) the rate of metabolism of norethisterone oenanthate is faster than the rate of metabolism of the acetate derivative;

e) the synthetic progestogens norethisterone, d-norgestrel, dl-norgestrel and l-norgestrel are metabolised to ring-A reduced products in rabbit liver. The major pathway of metabolism of d-norgestrel appears to be through the dehydrogenation of 5β-dihydronorgestrel resulting in 3α, 5β-tetrahydronorgestrel;

f) lynestrenol is transformed to norethisterone and ring-A reduced, tetrahydronorethisterone;

g) the esterified steroids undergo hydrolysis, since the non-esterified steroids were one of the metabolic products, and ring-A reduction.

2. Metabolism of 19-nortestosterone and various synthetic 19-norporgesto--gens by the hepatic and extrahepatic tissues.

These studies were carried out to determine:

a) the rate of metabolism of 19-nortestosterone and the synthetic 19-norprogestogens in various extrahepatic tissues,

b) to compare the metabolism of these compounds in male and female tissues,

c) to identify the metabolic products from each tissue.

In order to increase the comparative value of these experiments the steroid to tissue ratio (1 : 40,000) and the incubation time of 120 min were kept constant throughout the study. In most experiments organs fromasingle animal were examined simultaneously. The incubation procedure has been described in the Methods section and was similar to that used in the liver studies. The results expressed as percent steroid not metabolised in 120 min are given as means ($\pm$S.D.) in Table 1.12 and 1.13. Figures 1.6 - 1.13 compare the mean values obtained with male and female tissues (individual values are given in Appendix tables 6 - 13)

a) Hepatic tissue metabolism.

The mean values ($\pm$S.D.) are given in Table 1.12 and compared in Fig. 1.6 . During a period of 120 min, 20.2 $\pm$ 2.1% of 19-nortestosterone remained unmetabolised by female liver. During the same period of time l-norgestrel was the least metabolised (89.2 $\pm$ 0.8%), of the non-esterified synthetic progestogens examined and norethisterone the most (38.8 $\pm$ 6.1% remained).

The rate of metabolism of the esterified derivatives, (Table 1.13) lynestrenol acetate (66.5 $\pm$ 3.2%), norethisterone acetate (46.3 $\pm$ 8.5%), dl-norgestrel acetate (58.8 $\pm$ 5.0%) and norethisterone oenanthate (50.2 $\pm$ 2.6%) was relatively slower than the non-esterified steroids under the same conditions. The mean values of the synthetic progestogens in female liver were highly significantly different ($p < 0.01$) from the mean value of 19-nortestosterone (Appendix table 8).

The rate of metabolism of 19-nortestosterone and the synthetic 19-norprogestogens in female liver was not significantly different from the values obtained for male liver (Tables 1.12 and 1.13, Fig. 1.6).

The metabolic products identified for female liver tissue have already been described in the previous section. Similar products were obtained from male liver tissue (Table 1.12 and 1.13).

TABLE 1.12 COMPARISON OF THE RATE OF METABOLISM OF 19-NORTESTOSTERONE, dl-, d-, l-NORGESTREL AND LYNESTRENOL IN MALE AND FEMALE RABBIT TISSUES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED (MEAN ± SD) IN 120 min.

STEROID INCUBATED	19-NORTESTOSTERONE		NORETHISTERONE		dl-NORGESTREL		d-NORGESTREL		l-NORGESTREL		LYNESTRENOL		
STEROID RECOVERED	SEX	19-Nort.	NET	THNET	Ng	THNg	Ng	THNg	Ng	THNg	Lyn	NET	THNET
LIVER	male	18.9±2.0	29.5±6.4	68.8±3.2	49.8±1.5	48.3±3.1	42.1±1.5	53.6±3.6	88.8±1.9	1.0±0.2	34.7±9.5	20.6±8.4	36.0±5.2
	female	20.2±2.1	38.8±6.1	61.0±7.5	44.5±3.1	49.2±7.7	41.8±5.3	51.5±4.0	89.2±0.8	4.0±2.0	38.1±5.2	22.9±3.0	33.9±2.3
KIONEY	male	76.1±6.2	86.2±4.2	3.2±1.2	80.3±8.2	0	85.6±2.6	0	93.5±4.2	0	86.8±3.0	0	0
	female	81.9±2.0	91.8±2.3	1.2±0.4	84.9±5.9	0	78.1±5.3	0	87.8±1.5	0	83.2±3.1	0	0
SKELETAL MUSCLE	male	58.9±2.5	64.1±5.9	28.0±2.6	88.9±2.1	10.3±1.5	81.2±2.5	12.4±2.7	95.7±3.0	0	89.2±9.4	0	0
	female	61.5±2.9	69.1±3.5	30.0±4.0	88.1±5.2	8.0±2.7	75.8±2.4	17.3±3.0	88.2±1.0	0	88.1±2.5	0	0
LUNG	male	38.4±1.6	41.5±1.2	40.8±5.9	84.8±5.8	8.4±2.4	84.2±3.5	12.4±2.7	93.9±2.6	0	95.8±2.3	0	0
	female	39.5±2.3	43.7±5.6	49.0±4.5	89.5±0.5	9.1±1.8	81.9±7.0	14.3±1.6	88.0±1.5	0	93.2±1.0	0	0
SPLEEN	male	91.6±3.1	88.5±4.5	0	85.4±4.9	0	88.1±4.2	0	94.6±1.5	0	86.5±2.5	0	0
	female	88.9±0.4	87.8±2.0	0	88.5±1.9	0	85.9±5.7	0	85.0±3.3	0	79.9±8.6	0	0
SMALL INTESTINE	male	42.5±2.0	67.1±6.9	30.1±1.9	87.4±1.2	7.0±2.6	83.8±4.2	12.1±1.9	96.5±3.7	0	92.0±1.7	0	5.1±0.9
	female	45.2±0.9	56.2±4.0	35.1±2.8	88.5±1.9	8.4±1.8	81.9±1.9	13.8±1.5	87.9±1.5	0	95.5±1.5	0	0
ADIPOSE	male	74.8±6.3	84.6±3.8	10.3±1.1	80.5±1.2	0	80.5±9.2	0	91.3±2.9	0	90.5±3.2	0	0
	female	88.2±4.4	83.5±2.0	11.0±1.9	82.8±7.5	0	86.8±3.0	0	87.8±1.5	0	82.1±9.8	0	0
HEART	male	78.1±2.7	79.9±8.4	0	81.8±15.2	0	85.9±4.1	0	91.5±2.0	0	85.7±2.4	0	0
	female	84.9±2.3	84.6±1.7	0	85.8±3.5	0	74.6±12.5	0	89.2±2.5	0	86.2±3.0	0	0

TABLE 1.13 COMPARISON OF THE RATE OF METABOLISM OF LYNESTRENOL ACETATE, NORETHISTERONE ACETATE, dl-NORGESTREL ACETATE AND NORETHISTERONE OENANTHATE IN MALE AND FEMALE RABBIT TISSUES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED (MEAN±SD) in 120 min.

STEROID INCUBATED		LYNESTRENOL ACETATE				NORETHISTERONE ACETATE			dl-NORGESTREL ACETATE			NORETHISTERONE OENANTHATE		
STEROID RECOVERED		Lyn-AC	Lyn	NET	THNET	NET-AC	NET	THNET	dl-Ng-AC	Ng	THNg	NET-AC	NET	THNET
TISSUE	SEX													
LIVER	male	64.8±2.5	20.1±1.9	15.5±1.5	5.5±1.5	48.2±5.9	19.5±2.3	21.9±3.6	59.8±2.0	15.1±1.1	15.8±2.5	-	-	-
	female	66.5±3.2	19.9±1.5	13.2±1.0	6.9±1.2	46.3±8.5	20.9±3.9	23.1±9.5	58.8±5.0	15.5±1.5	16.5±1.6	50.2±2.6	8.4±2.7	27.8±3.0
KIDNEY	male	86.5±1.4	10.4±1.5	0	0	81.6±15.3	9.6±0.6	0	84.8±3.7	6.7±1.0	0	-	-	-
	female	85.4±1.5	12.1±2.0	0	0	90.2±8.7	0	0	87.1±1.7	7.5±1.4	0	74.9±3.9	7.5±0.7	0
SKELETAL MUSCLE	male	68.5±3.7	26.2±4.0	0	0	61.8±4.1	28.0±3.2	7.9±1.3	59.4±2.0	22.5±3.1	7.4±1.5	-	-	-
	female	71.8±1.5	23.2±2.0	0	0	77.7±1.9	20.0±2.0	2.4±1.4	63.2±2.1	17.1±0.9	6.9±2.2	74.4±6.4	3.6±0.1	0
LUNG	male	83.9±3.2	12.5±2.5	3.5±1.4	0	80.8±4.3	7.8±3.6	3.3±0.6	82.1±2.0	7.7±0.5	2.8±0.7	-	-	-
	female	84.8±4.1	11.9±1.3	3.8±1.5	0	84.3±7.2	5.3±4.1	3.7±3.1	82.2±0.9	9.1±1.0	3.5±1.1	56.2±4.3	27.1±5.1	2.0±0.5
SPLEEN	male	-	-	-	-	-	-	-	-	-	-	-	-	-
	female	-	-	-	-	94.9±4.3	0	0	-	-	-	-	-	-
SMALL INTESTINE	male	19.2±1.0	64.8±5.0	8.7±0.4	2.2±1.1	10.5±1.3	80.8±5.9	4.4±2.4	17.5±1.9	72.9±3.2	2.4±0.6	-	-	-
	female	21.1±1.0	66.9±6.4	10.1±1.0	3.2±0.8	8.8±0.6	82.1±3.8	3.3±1.1	17.8±2.0	69.1±0.1	4.1±1.0	41.3±1.7	22.9±2,3	18.5±0.5
ADIPOSE	male	94.3±2.3	4.4±1.5	0	0	97.6±0.5	1.1±0.2	0	96.2±1.9	0	0	-	-	-
	female	93.8±2.4	4.8±0.3	0	0	99.9±0.1	0	0	97.5±1.4	0	0	74.1±6.1	2.0±0.01	0
HEART	male	76.9±1.9	16.5±1.5	1.8±0.4	0	80.1±8.8	15.7±4.6	0	83.8±3.0	12.5±1.6	0	-	-	-
	female	80.8±5.0	17.9±1.6	4.1±1.0	0	88.8±2.8	5.9±3.1	0	86.9±1.9	13.9±1.9	0	65.2±5.5	12.6±4.1	0

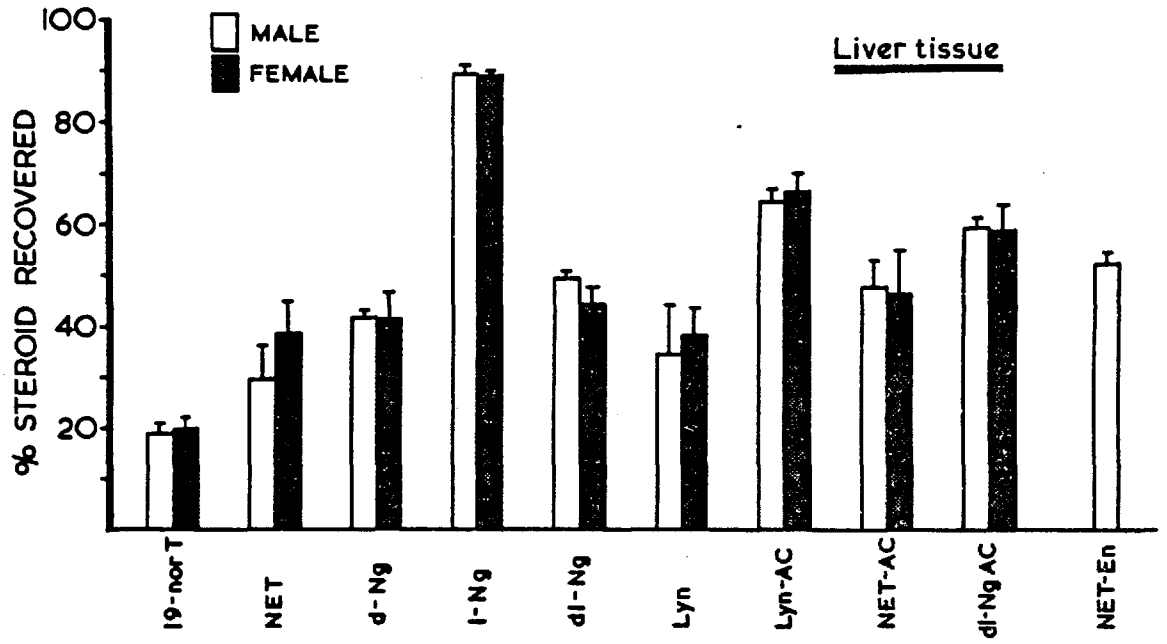


Fig. 1.6 Comparative rates of metabolism of 19-nortestosterone and the synthetic progestogens in male and female rabbit liver tissue.

b) Kidney tissue metabolism.

In female kidney tissue (Table 1.12, Fig 1.7), little metabolism of 19-nortestosterone was seen. Thus, at 120 min of incubation time 81.9 $\pm$ 2.0% of 19-nortestosterone remained unchanged. Of the non-esterified 19-norprogestogens examined, norethisterone was least metabolised (91.8 $\pm$ 2.3%). This value and the mean value for l-norgestrel (87.8 $\pm$ 1.5%) were significantly higher from the mean value for 19-nortestosterone ($p < 0.05$) (Appendix table 6).

Examination of the esterified 19-norprogestogens (Table 1.13) also indicated that little metabolism of these steroids occurred in female kidney tissue. The mean values were not significantly different from those obtained for the non-esterified progestogens (Appendix table 6 and 8).

Comparison of female and male tissues shows that the rate of metabolism of 19-nortestosterone, non-esterified progestogens and esterified progestogens does not differ significantly in the two sexes (Tables 1.12 and 1.13, Fig. 1.7).

As shown in Table 1.12, in both female and male tissues only norethisterone was metabolised to tetrahydronorethisterone. Thus 1.2 $\pm$ 0.4% of norethisterone in female kidney and 3.2 $\pm$ 1.2% in male kidney was metabolised to the ring-A reduced metabolite. The other non-esterified progestogens, d-, dl-, l-norgestrel and lynestrenol were not metabolised to ring-A reduced products and no other metabolic products were detected.

Of the esterified progestogens (Table 1.13) in both female and male tissue, 12.1 $\pm$ 2.0% in female and 10.4 $\pm$ 1.5% in male, of lynestrenol acetate was metabolised to lynestrenol. No further metabolism of this compound was seen in this tissue. Only in male kidney tissue, norethisterone was identified as a metabolic product of norethisterone acetate. Whereas, in both male and female kidney tissue dl-norgestrel acetate was metabolised to norgestrel (7.5 $\pm$ 1.4% in female and 6.7 $\pm$ 1.0% in male). During the same period of time 7.5 $\pm$ 0.7% of norethisterone oenanthate was hydrolysed to norethisterone.

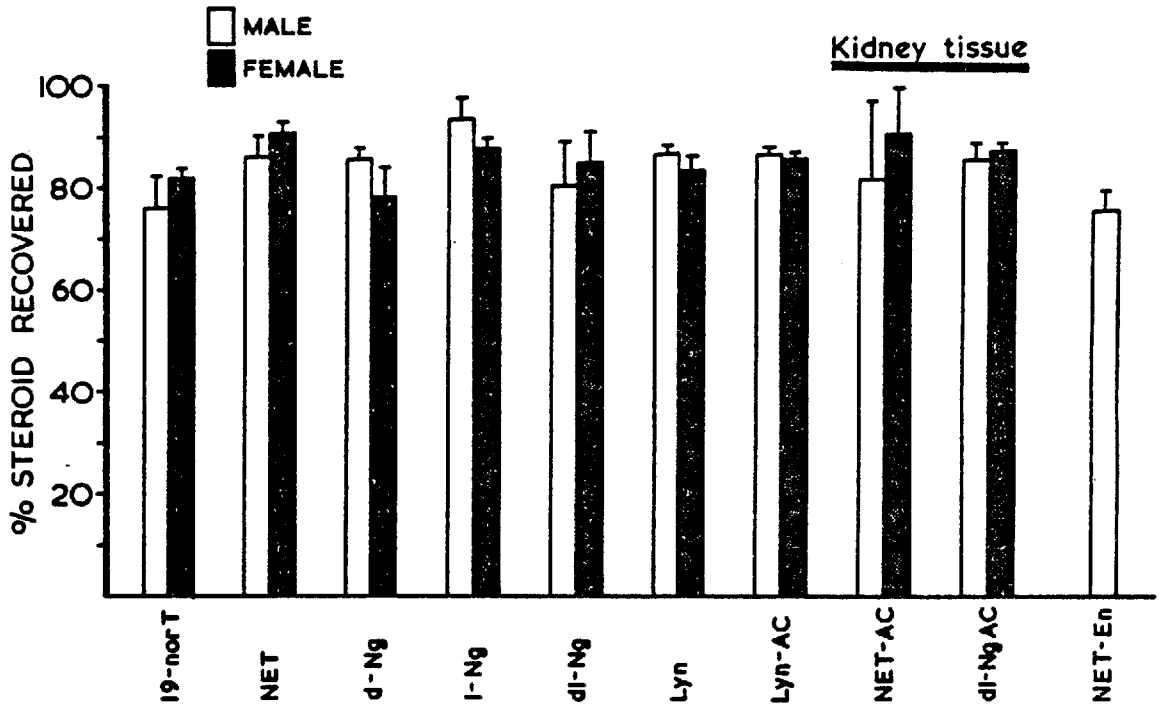


Fig. 1.7 Comparative rates of metabolism of 19-nortestosterone and the synthetic progestogens in male and female rabbit kidney tissue.

c) Skeletal muscle tissue metabolism.

Examination of skeletal muscle tissue indicated (Table 1.12, Fig. 1.8), that some metabolism of 19-nortestosterone occurred with female tissue. Thus, in 120 min of incubation time 61.5 $\pm$ 2.9% of 19-nortestosterone remained unchanged in female tissue. During the same period of time 69.1 $\pm$ 3.5% of norethisterone and 75.8 $\pm$ 2.4% of d-norgestrel remained. Comparatively, little metabolism of dl-norgestrel (88.1 $\pm$ 5.2%), l-norgestrel (88.2 $\pm$ 1.0%) and lynestrenol (88.1 $\pm$ 2.5%) occurred in this tissue. All the mean values for the 19-norprogestogens were significantly different from the mean value of 19-nortestosterone (Appendix table 6).

The investigation of esterified derivatives indicated (Table 1.13, Fig. 1.8), that 71.8 $\pm$ 1.5% of lynestrenol acetate, 77.7 $\pm$ 1.9% of norethisterone acetate, 63.2 $\pm$ 2.1% of dl-norgestrel acetate and 74.4 $\pm$ 6.4% of norethisterone oenanthate remained unchanged in female skeletal muscle, whereas 61.5 $\pm$ 2.9% of 19-nortestosterone remained unmetabolised. The mean values of lynestrenol acetate, norethisterone acetate and norethisterone oenanthate are significantly higher ($p < 0.05$) from the mean of 19-nortestosterone (Appendix table 8).

Comparing the rates of metabolism of non-esterified progestogens with the esterified derivatives (Table 1.12 and 1.13), the results indicate that lynestrenol acetate and dl-norgestrel acetate were metabolised relatively faster than the non-esterified lynestrenol and dl-norgestrel in female skeletal muscle tissue. Whereas norethisterone is metabolised faster than norethisterone acetate and norethisterone oenanthate.

No sex differences were noted in the rates of metabolism of these steroids in skeletal muscle tissue (Table 1.12 and 1.13).

Examination of the metabolic products (Table 1.12) shows that norethisterone, dl-norgestrel and d-norgestrel gave rise to the ring-A reduced products, tetrahydronorethisterone and tetrahydronorgestrel The configuration of tetrahydronorgestrel was identified to be 3α , 5β-tetrahydronorgestrel arising from d-norgestrel. Both l-norgestrel and lynestrenol were not metabolised to any detectable metabolites, both by male and female tissue.

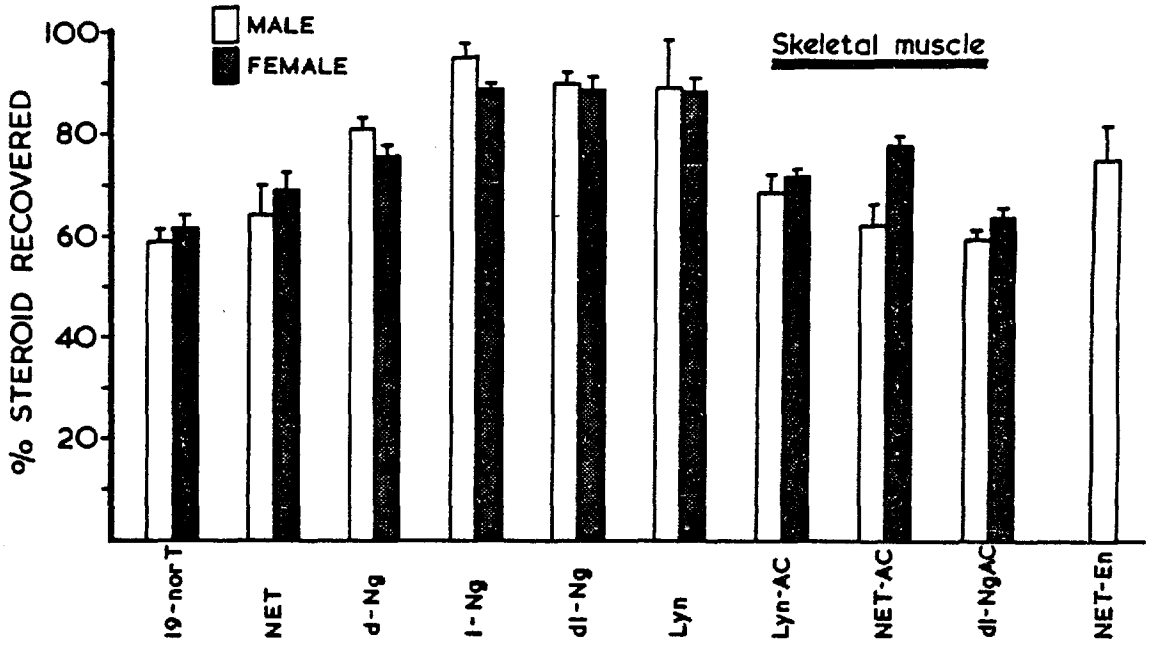


Fig. 1.8 Comparative rates of metabolism of 19-nortestosterone and the synthetic progestogens in male and female rabbit skeletal muscle tissue.

With the esterified derivatives the results show (Table 1.13) that lynestrenol acetate, in both female and male tissue was hydrolysed to lynestrenol. No further metabolites were detected. However, with norethisterone acetate and dl-norgestrel acetate, both the hydrolysed products, norethisterone and norgestrel were identified as well as the ring-A reduced products tetrahydronorethisterone and tetrahydronorgestrel. Norethisterone oenanthate was metabolised to a small percent of norethisterone ($3.6\pm0.1\%$).

d) Lung tissue metabolism.

Besides the liver, lung tissue was one of the extrahepatic tissues seen to fairly actively metabolise 19-nortestosterone and some of the 19-norprogestogens (Table 1.12 and 1.13, Fig. 1.9). Thus, in 120 min $39.5\pm2.3\%$ of 19-nortestosterone remained unchanged in female rabbit lung tissue. In the same period, $43.7\pm5.6\%$ of norethisterone remained unchanged. The mean values for d-norgestrel, l-norgestrel, dl-norgestrel and lynestrenol were significantly higher from the mean value of 19-nortestosterone ($p < 0.05$, Appendix table 6) and comparatively little metabolism of these progestogens occurred (Table 1.12).

Similarly, little metabolism of the acetate derivatives occurred in female lung tissue (Table 1.13). Thus, $84.8\pm4.1\%$ of lynestrenol acetate, $84.3\pm7.2\%$ of norethisterone acetate, $82.2\pm0.9\%$ of dl-norgestrel acetate, remained unchanged. Whereas, $56.2\pm4.3\%$ of norethisterone oenanthate was recovered unchanged. The mean values of these progestogens were significantly higher ($p < 0.05$, Appendix table 8) than the mean value of 19-nortestosterone. The rate of metabolism of both the non-esterified and esterified progestogens was not significantly different in male and female lung tissue (Tables 1.12 and 1.13).

When the metabolic products were examined, the results indicated that lung tissue could reduce norethisterone, dl-norgestrel and d-norgestrel in ring-A giving rise to tetrahydro-metabolites (Table 1.12). But neither l-norgestrel nor lynestrenol were metabolised by this tissue. However, examination of the metabolism of lynestrenol acetate

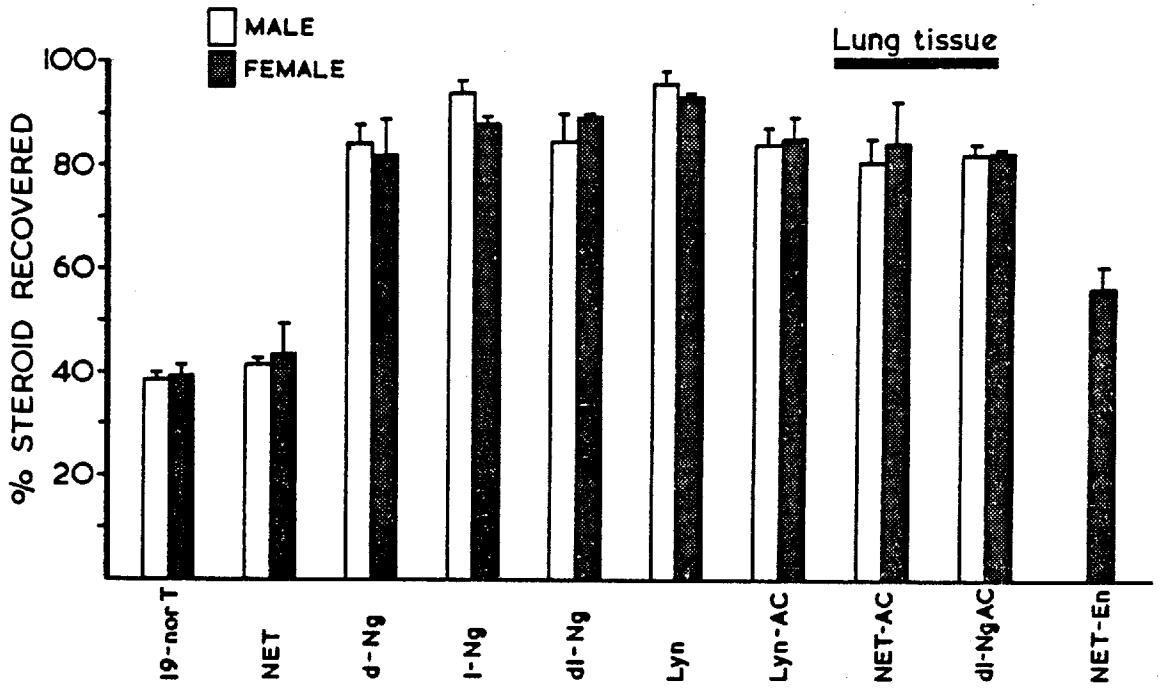


Fig. 1.9 Comparative rates of metabolism of 19-nortestosterone and the synthetic progestogens in male and female rabbit lung tissue.

(Table 1.13) indicated that 11.9 ± 1.3% of lynestrenol and 3.8 ± 1.5% of norethisterone were formed, but the tetrahydro-metabolites were not detectable. Whereas, norethisterone acetate, dl-norgestrel acetate and norethisterone oenanthate gave rise to the hydrolysed metabolites norethisterone and norgestrel, as well as the ring-A reduced products, tetrahydronorethisterone and tetrahydronorgestrel.

e) Spleen tissue metabolism.

In both female and male spleen tissue (Table 1.12, Fig. 1.10) little metabolism of 19-nortestosterone and the synthetic 19-norprogestogens was seen. Thus 88.9 ± 0.4% of 19-nortestosterone was recovered unchanged in female spleen tissue, and the values obtained for the 19-norprogestogens were similar and not significantly different from 19-nortestosterone ($p > 0.1$, Appendix table 7).

f) Small intestinal tissue metabolism.

Small intestinal tissue was seen to be fairly active in the metabolism of the non-esterified progestogens (Table 1.12, Fig. 1.11). Thus 45.2 ± 0.9% of 19-nortestosterone remained unmetabolised after 120 min of incubation time in female tissue. The metabolism of norethisterone was not significantly different (56.2 ± 4.0%) ($p > 0.1$), Appendix table 7) from the mean value obtained for 19-nor-testosterone. Whereas the rates of metabolism of d-norgestrel, dl-norgestrel, l-norgestrel and lynestrenol were slower (Table 1.12) and the mean values significantly higher ($p < 0.05$, Appendix table 7) than 19-nortestosterone.

In contrast the metabolism of the esterified progestogens was found to be extensive by this tissue (Table 1.13, Fig. 1.11). Thus 21.1 ± 1.0% of lynestrenol acetate, 8.8 ± 0.6% of norethisterone acetate, 17.8 ± 2.0% of dl-norgestrel acetate and 41.3 ± 1.7% of norethisterone oenanthate remained unchanged. The mean values for the acetate derivatives were significantly different ($p < 0.05$, Appendix table 9). from the mean value of 19-nortestosterone.

As in the other tissues examined there was no sex differences in the metabolism of 19-nortestosterone and the synthetic 19-norprogestogens by this tissue (Tables 1.12 and 1.13, Fig. 1.11).

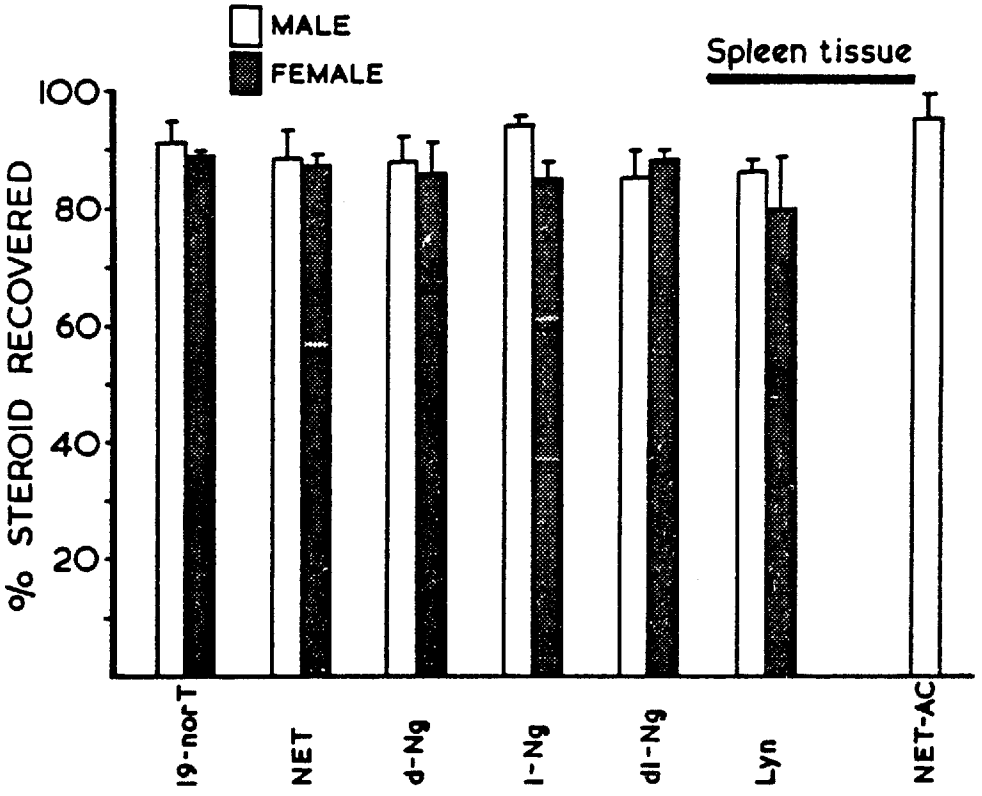


Fig. 1.10 Comparative rates of metabolism of 19-nortestosterone and the synthetic progestogens in male and female rabbit spleen tissue.

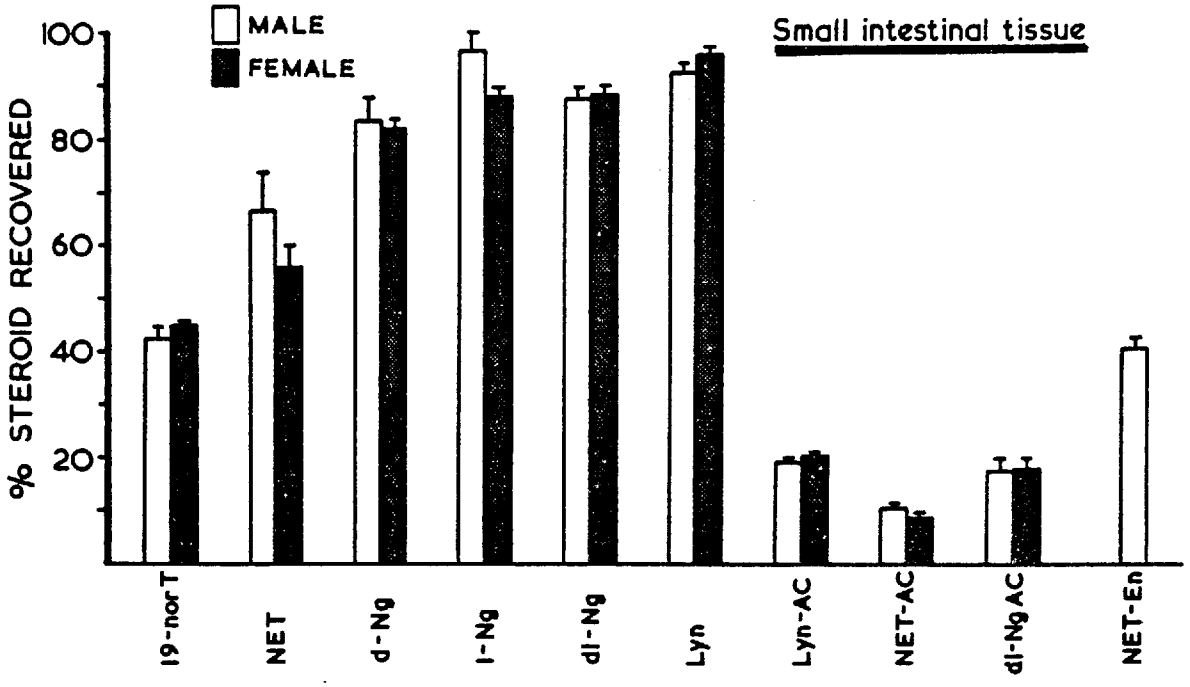


Fig. 1.11 Comparative rates of metabolism of 19-nortestosterone and the synthetic progestogens in male and female small intestinal tissue.

Examination of the metabolic products formed by small intestinal tissue of the rabbit (Table 1.12) indicated that the non-esterified 19-norprogestogens were metabolised to ring-A reduced products, tetrahydronorethisterone and tetrahydronorgestrel. However, l-norgestrel and lynestrenol were not metabolised in detectable amounts, except in male tissue, where a small percentage ($5.1 \pm 0.9\%$) of lynestrenol was seen to be metabolised to tetrahydronorethisterone.

The esterified derivatives were found to be extensively metabolised to the hydrolysed products (Table 1.13). Thus in female intestinal tissue $66.9 \pm 6.4\%$ of lynestrenol acetate, $82.1 \pm 3.8\%$ of norethisterone acetate, $69.1 \pm 0.1\%$ of dl-norgestrel acetate were hydrolysed to lynestrenol, norethisterone and norgestrel respectively. Whereas, only $22.9 \pm 2.3\%$ of norethisterone oenanthate was converted to norethisterone by this tissue. Lynestrenol acetate was also seen to be metabolised to norethisterone ($10.1 \pm 1.0\%$) and tetrahydronorethisterone ($3.2 \pm 0.8\%$). $3.3 \pm 1.1\%$ of norethisterone acetate and $18.5 \pm 0.5\%$ of norethisterone oenanthate were reduced to tetrahydronorethisterone. Similarly, $4.1 \pm 1.0\%$ of dl-Ng-AC was metabolised to tetrahydronorgestrel by this tissue from female rabbits.

g) Adipose tissue metabolism.

As in rabbit kidney and spleen tissue, little metabolism of 19-nortestosterone occurred in adipose tissue (Table 1.12, Fig. 1.12). Thus, during the incubation period of 120 min, $88.2 \pm 4.4\%$ of 19-nortestosterone remained unmetabolised in female tissue. No metabolism of the non-esterified 19-norprogestogens was found to occur in this tissue except for the metabolism of norethisterone ($83.5 \pm 2.0\%$). The mean values for all the non-esterified progestogens were not significantly different from the mean of 19-nortestosterone ($p > 0.1$, Appendix table 7).

Similarly, little metabolism of the esterified progestogens occurred in this tissue (Table 1.13, Fig. 1.12). Comparison of male and female tissues again indicated that there was no sex difference in the metabolism of these steroids in adipose tissue (Table 1.12 and 1.13).

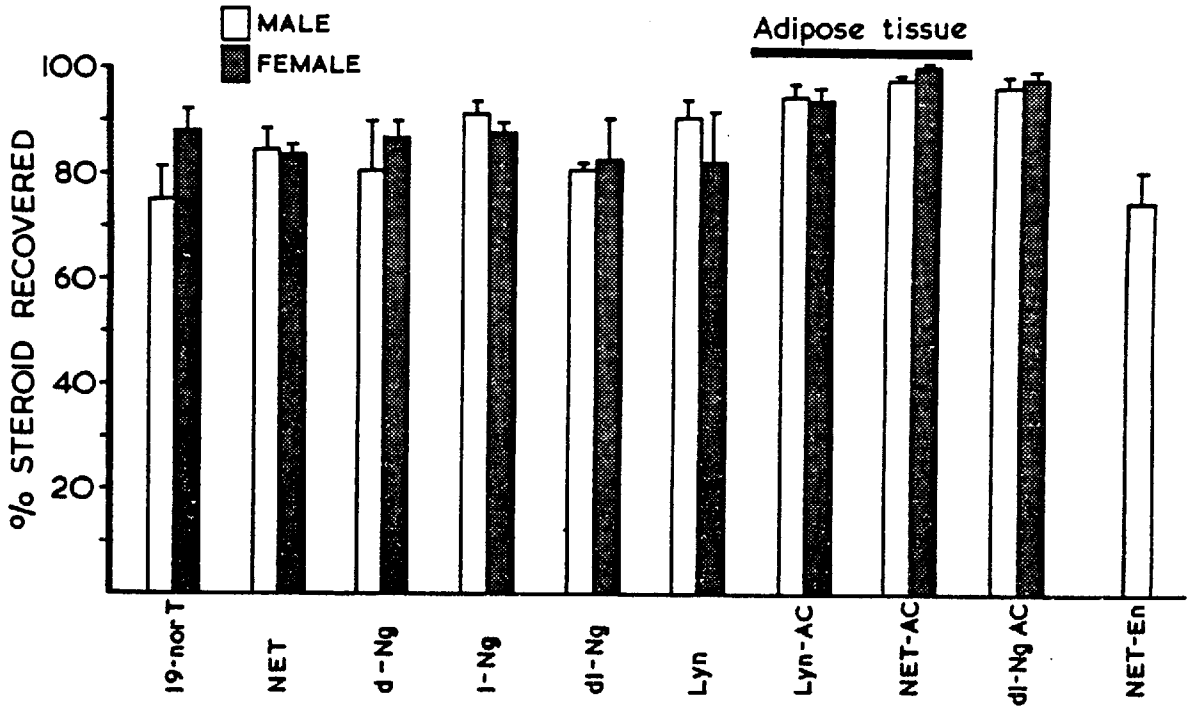


Fig. 1.12 Comparative rates of metabolism of 19-nortestosterone and the synthetic progestogens in male and female adipose tissue.

Examination of the metabolic products indicated that 11.0±1.9% of norethisterone was converted to tetrahydronorethisterone (Table 1.12) but no ring-A reduced metabolites were identified with the other non-esterified 19-norprogestogens. However, lynestrenol acetate was hydrolysed to lynestrenol (4.8±0.3%) and norethisterone oenanthate to norethisterone (2.0±0.0 %) (Table 1.13). No metabolic products were detected with norethisterone acetate and d-norgestrel acetate.

h) Heart tissue metabolism.

During the incubation period of 120 min, 84.9±2.3% of 19-nortestosterone remained unmetabolised in female heart tissue (Table 1.12, Fig. 1.13). The mean values obtained for the non-esterified and esterified progestogens (Table 1.13) were similar and not significantly different ($p > 0.1$, Appendix table 7) from the mean value of 19-nortestosterone. No sex differences were seen in the metabolism of 19-nortestosterone and the synthetic 19-norprogestogens (Tables 1.12 and 1.13) in this tissue.

Examination of the metabolites indicated that no ring-A reduced metabolites were formed either from the non-esterified (Table 1.12) or the esterified (Table 1.13) 19-norprogestogens. However, the esterified derivatives were hydrolysed to the free steroid. Thus, 17.9±1.6% of lynestrenol acetate, 5.9±3.1% of norethisterone acetate, 13.9±1.9% of dl-norgestrel acetate and 12.6±4.1% of norethisterone oenanthate were recovered as lynestrenol, norethisterone, and norgestrel. Some (4.1±1.0%) of lynestrenol was also converted to norethisterone.

In summary, the results of the metabolism of 19-nortestosterone and 19-norprogestogens in rabbit hepatic and extrahepatic tissues shows that:

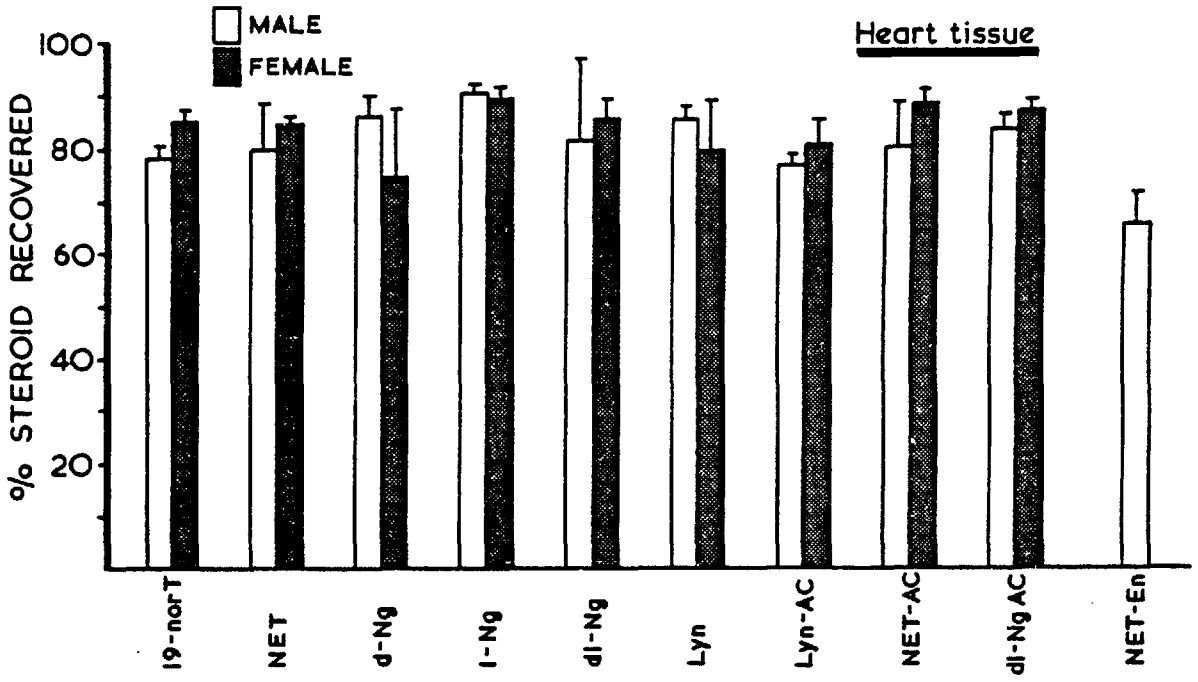


Fig. 1.13 Comparative rates of metabolism of 19-nortestosterone and the synthetic progestogens in male and female heart tissue.

a) 19-norestosterone, the parent steroid is metabolised at a faster rate than the synthetic 19-norprogestogens in all tissues, except for small intestinal tissue where the esterified progestogens are metabolised faster.

b) The liver is the main organ involved in the metabolism of these compounds.

c) The small intestine appears to be of considerable importance in the metabolism of the esterified progestogens.

d) Besides the small intestine, other extrahepatic tissues that appear to be actively involved in progestogen metabolism are lung tissue and skeletal muscle tissue.

e) Adipose, heart and spleen tissue are relatively inactive in relation to the metabolism of both the non-esterified and esterified progestogens.

f) The rates of metabolism of the esterified steroids differ from tissue to tissue. Thus, the oenanthate group is hydrolysed faster in small intestinal tissue, whereas the hydrolyses of the acetate group is more dominant in kidney, lung and heart tissue.

g) The metabolism of the 19-norprogestogens is not significantly different in male and female tissues.

h) The metabolites identified from each tissue were mainly ring-A reduced products of the non-esterified progestogens, and hydrolysed and ring-A reduced products of the esterified progestogens under conditions of incubation described.

DISCUSSION.

Although metabolism of naturally occurring steroids has been extensively investigated both 'in vivo' and 'in vitro' (Dorfman and Ungar, 1965, Samuels and Eik-Nes, 1968), relatively little is known about the metabolism of synthetic progestational compounds (Fotherby and James, 1972, Thijssen 1972 and Fotherby, 1974). In this study, we have investigated the metabolism of various progestational steroids related to 19-nortestosterone by the rabbit hepatic and extrahepatic tissues. These studies were undertaken to examine the effects of various substituent groups (Diczfalusy, 1971) on the rate of metabolism of these steroidal compounds, as well as the formation of metabolites.

Our data suggests that the introduction of various functional groups to 19-nortestosterone appears to result in a decrease in the rate of ring-A reduction of the synthetic 19-norprogestogens in rabbit liver. The differences become apparent at 30 min of incubation time (Table 1.8) and these differences become statistically significant after incubation for longer periods of time, as seen at 180 min (Table 1.8). Thus, the 19-norprogestogens examined can be tabulated in decreasing rates of metabolism (as shown by the values obtained at 180 min) as norethisterone, d-norgestrel, lynestrenol, dl-norgestrel, norethisterone oenanthate, norethisterone acetate, dl-norgestrel acetate, lynestrenol acetate and l-norgestrel. These results suggest that alteration in the rates of metabolism of these steroidal compounds may probably be due to the structural modifications caused by different substituent groups. The degree of resistance to metabolic alteration seems to depend on the type of substituent present. When the 17α-ethynyl group is added to 19-nortestosterone, as in norethisterone, the rate of metabolism is not significantly different from 19-nortestosterone; however, when the angular methyl group at C-13 is substituted by an ethyl group (norgestrel) a distinct decrease in metabolism is seen (Table 1.8). This finding is in agreement with that of Gerhards et al. (1971) who compared the rates of metabolism of norethisterone and norgestrel in rat liver. Their results indicated that the introduction of the ethyl group in norgestrel inhibited ring-A reduction of this steroid as compared to norethisterone.

The introduction of the ethyl group also gives rise to an asymmetric carbon atom at position 13, resulting in optical isomers (Smith et al., 1964). Thus, d-norgestrel, the biologically active isomer (Edgren et al., 1963a and 1963b) was metabolised faster than the racemate, dl-norgestrel. The rate of metabolism of dl-norgestrel was half that of the active isomer. Under similar experimental conditions, l-norgestrel, the biologically inactive isomer, was metabolised at a much slower rate. The elimination of the 3-oxo group as in lynestrenol, effectively decreases the metabolic rate as compared to norethisterone and d-norgestrel. These results are comparable to the studies of Glenn et al. (1959) and Breuer (1970b). Both investigators have shown that structural alteration of progestational steroids results in the modification of the rate of metabolism of these compounds. Glenn et al.(1959) using rat liver preparations found that medroxyprogesterone was metabolised half as rapidly as progesterone. Breuer (1970) indicated that during the incubation of retroprogesterone with human liver, no reduction of ring-A occurred whereas under the same conditions, the Δ^4-3-oxo group of progesterone was rapidly reduced.

The data from the present study also indicates that esterification of the 17β-hydroxyl group also affects the rate of metabolism of 19-norprogestogens, (Table 1.8) in rabbit liver. Thus, norethisterone acetate, norethisterone oenanthate, dl-norgestrel acetate and lynestrenol acetate were seen to be metabolised at a much slower rate than 19-nortestosterone and the non-esterified progestogens. That esterification of the progestational steroids and introduction of other substituents results in resistance to metabolism is supported by the study of Cooke and Vallance (1965). These investigators compared the rates of metabolism of norgestrel acetate, 17α-acetoxy-6α-methyl progesterone, 6α-methylprogesterone, 17α-acetoxyprogesterone and progesterone in rat and rabbit liver preparations. They observed that introduction of the 17α-acetoxy group diminished the rate of metabolism in the rabbit but not in rat. Introduction of a 6α-methyl group into the progesterone molecule markedly decreased the metabolism by liver preparations in rat but not in the rabbit. However, introduction of both the 6α-methyl and 17α-acetoxy group into the progesterone molecule markedly decreased the rate of metabolism in both species. More recently Dericks-Tan et al. (1975) have also shown that ester-substitution of 4-pregnene-3, 20-dione in the 17α-position reduced the rate of hydrogenation as compared to unesterified compounds in rat liver microsomes.

When the rates of metabolism of the esterified derivatives were compared (Table 1.8), there were no marked differences in the metabolism of the three acetate derivatives, lynestrenol acetate, dl-norgestrel acetate and norethisterone acetate. However, the length of the esterified chain at C-17 appears to be important, since our results indicate that the heptonate (norethisterone oenanthate) was metabolised at a comparatively faster rate than the acetate (norethisterone acetate) in rabbit liver (Table 1.8). These findings are in contrast to the earlier study of Dirschel and Dardenne (1954) in human and rabbit liver, who have shown a reduction in cleavage rate of the ester with increasing chain length of the monocarboxyclic acids. Similar findings were observed by Schenk and Junkmann (1955) on the chemical hydrolysis of steroid esters. In these studies crystalline suspensions were used instead of true solutions thus,it is quite likely that with the use of suspensions, varying dissolution rates of the long-and short-chain esters become rate limiting for the cleavage rate.

In comparing the rates of metabolism of 19-nortestosterone, the non-esterfied 19-norprogestogens in male and female rabbit liver tissue, no sex differences were found (Tables 1.12 and 1.13). This is in agreement with the study of Taylor (1955) who also found no sex difference in the metabolism of progesterone by rabbit liver homogenates.

In most species the naturally secreted steroids are 'inactivated' by reduction in ring-A in the liver (Samuels and Eik-Nes, 1968). The vast majority of the known metabolites of steroid hormones lack the C4-C5 double bond (Bush, 1962), and its reduction in the liver probably represents the rate-limiting step in the metabolic inactivation of these hormones (Tomkins 1956, 1957). The present study shows that the total homogenates of rabbit liver can reduce the Δ^4-3-oxo group of progestational compounds. The data also shows that the additional substituents on 19-nortestosterone can alter the rate of metabolism, but not the route of metabolism (Tables 1.9 and 1.10) in rabbit liver. Thus, the main metabolic products obtained with the non-esterfied 19-norprogestogens were ring-A reduced metabolites. Norethisterone was metabolised to tetrahydronorethisterone. This is consistant with the studies of Matsuyoshi (1967) and Breuer (1964). Matsuyoshi (1967) isolated 17α -ethynyl-19-norandrostane-17β-ol-3one and 17α -ethynyl-19-norandrostane-3α, 17β diol from incubations of norethisterone with rat liver homogenates. Breuer (1964) indicated that norethisterone follows the same metabolic pathways as 19-nortestosterone and

testosterone, when norethisterone was incubated with rat liver homogenates. The products isolated corresponded to 5α-compounds including the saturated 3-ketone as well as 3α- and 3β-hydroxy compounds. Several other metabolites of norethisterone have been identified in rat, rabbit and beagle liver under 'in vitro' conditions. Thus, Palmer et al. (1970) identified 17α-ethynyloestradiol, D-homo-estr-4-ene-3, 17-dione, 10β-hydroxy-norethisterone and 10β-hydroxy-17α-ethynyl-5β-estrane-3α, 17β-diol in both rat and rabbit liver. Cook et al. (1974) have described the formation of norethisterone epoxide (17β-hydroxy-19-nor-4β, 5β-epoxy-17α-pregn-20-yn-3-one) from norethisterone in the 10,000g fraction of beagle liver.

No 'in vitro' study has been reported in the literature on the rate of metabolism of norgestrel, where the metabolites have also been identified. However, several 'in vivo' studies (Fotherby and James, 1972 and Thijssen, 1972) have indicated that the ring-A reduced metabolites are the predominant products isolated from urine of man and animal species. Sisenwine et al. (1975) isolated d-3α, 5β-tetrahydro-norgestrel from the urine of women receiving d-norgestrel. In the present 'in vitro' study all three forms of norgestrel (d-, dl- and l-norgestrel) were seen to be metabolised to the tetrahydro-metabolite (Table 1.9) which was identified as the 3α, 5β isomer.

The pathway of ring-A reduction was examined in detail by Tomkins (1956 and 1957). In rat liver two types of products result from the reduction of the C4-C5 double bond in steroidal hormones: the 5α-(H)-3-ketone steroids with a trans-fusion of the A and B rings, and the 5β-(H)-3-ketone steroids with a cis-fusion . These are further reduced to the 3α- and 3β-hydroxysteroids. The reduction of the 3-ketone group does not occur 'in vivo' in the Δ^4-3-ketosteroids (Bush, 1962); it proceeds very rapidly and completely after the reduction of the C4-C5 double bond has occurred (Tomkins 1956 and 1957). By examining possible intermediary metabolites, our results also suggest that the pathway of metabolism of d-norgestrel 'in vitro' follows a similar route to that of the natural steroids (Table 1.11). d-Norgestrel appears to be reduced by the Δ^4-hydrogenases, giving rise to 5β-dihydro-norgestrel, which is then reduced by the hydroxysteroid

dehydrogenases resulting in the formation of 3α, 5β-tetrahydronorgestrel. That the reduction of 5β-dihydronorgestrel proceeds rapidly is indicated, since this metabolite was not present in detectable amounts in the incubation extracts from d-norgestrel. Similar sequence of events have been reported for natural steroids, such as testosterone (Fotherby and James, 1972, Samuels and Eik-Nes, 1968).

In the present study lynestrenol was found to be metabolised to norethisterone and tetrahydronorethisterone by rabbit liver homogenates (Table 1.9). This is consistant with observations of Mazaheri et al. (1970) who also isolated norethisterone and tetrahydronorethisterone from incubations of lynestrenol with rabbit liver homogenates. It seems likely that the formation of norethisterone was due to hydroxylation at C-3 followed by dehydrogenation; since steroids containing the 3-hydroxy-4-ene group can be converted by liver tissue to the 4-ene-3-one structure (Ungar et al., 1957). That this probably does occur has been further supported by the study of Dericks-Tan (1975) who have shown that the 3-oxo group is necessary for the hydrogenation of the double bond at C4-C5.

In rabbit liver incubations the metabolic products of the esterified derivatives, lynestrenol acetate, norethisterone acetate, dl-norgestrel acetate and norethisterone oenanthate (Table 1.10) were the hydrolysed and ring-A reduced products. Thus, the addition of the ester group at C-17 does not appear to alter the route of metabolism but affects the rate of metabolism of these synthetic progestogens.

The presence of non-specific esterase has been demonstrated in rat liver by Hayase and Tappell(1969). Thus, hydrolysis of the acetates has been shown by rat liver (Tokuda et al., 1967) and by rabbit liver (Orino, 1969). Rat liver was able to convert ethynodiol diacetate to 17α-ethynyl-19-nortestosterone (NET). In 1963, Betz and Warren reported that the human foetal liver at 14, 16 and 18 weeks of gestation was able to hydrolyse norethisterone acetate to norethisterone.

Thus, in rabbit liver under 'in vitro' conditions, it seems that ring-A reduction forms a major pathway in the metabolism of 19-nor-progestational steroids. Both esterified and non-esterified progestogens follow this route, the esterified derivatives also being hydrolysed to the free compounds.

It has become increasingly apparent from investigations of steroid metabolism that extrahepatic tissues can carry out some of the oxidation and reduction reactions performed by the liver (Berliner and Dougherty, 1961). Berliner and Weist (1956) found that extrahepatic metabolism of cortisol was extensive in hepatectomised rats, although no significant amounts of water soluble conjugates were formed. In the human also, there is considerable extrahepatic metabolism, estimated by Tait (1963) to be approximately 30%.

The kidney has been shown to metabolise steroid hormones in various animal species (Dorfman and Ungar, 1965). In the present study both 19-nortestosterone and norethisterone were seen to be metabolised by the rabbit kidney to tetrahydro-metabolites to a minor extent. However, no ring-A reduced products were identified with dl-, d-, l-norgestrel and lynestrenol (Table 1.12). Although hydrolysis products of the esterified progestogens were seen, no ring-A reduced metabolites of lynestrenol acetate, norethisterone acetate, dl-norgestrel acetate and norethisterone oenanthate were detected (Table 1.13).

That the kidney can carry out ring-A reduction of natural steroids has been demonstrated by Chatterton et al. (1969) and Arimasa and Kochakian (1973) in rabbit and mouse kidney. Thus, after two hours of incubation of (4-^{14}C) progesterone with rabbit kidney 30% of the labelled progesterone was recovered unchanged; whereas, in contrast, progesterone-4-^{14}C remaining after incubation with rabbit liver under the same conditions was 10%. The metabolites isolated from the kidney tissue were mainly ring-A reduced products. In the present study only 1.2 ± 0.4% of norethisterone was found to be metabolised to tetrahydronorethisterone after two hours of incubation, whereas 10% of the non-substituted parent compound, 19-nortestosterone was metabolised during the same period of time by female rabbit kidney (Table 1.12). The formation of ring-A reduced products has been shown in mouse kidney (Arimasa and Kochakian, 1973). Thus, (4-^{14}C) androstenedione or testosterone were converted to epitestosterone and 5α-androstane-3α, 17β-diol by the mouse kidney. In addition, several minor metabolic products such as, androsterone, epi-androsterone, 5α-androsterone-3, 17-dione and 5α - androsterone-3β, 17β diol were also identified. That the kidney is involved in the metabolism of natural steroids has also been demonstrated 'in vivo'. Zarrow et al. (1954) found that the biological activity of exogenous progesterone remaining ten minutes after administration was 10, 30 and 50% of the dose in intact, nephrectomized and hepatectomized rats, respectively.

A number of studies have indicated the presence of esterase activity in the kidney of various animal species and man. Thus, Oirsherl and Dardenne (1954) demonstrated the hydrolysis of testosterone propionate, desoxycorticosterone acetate and cortisone acetate in human, rabbit and rat kidney (Oirsherl and Krüskemper, 1953) as well as mouse kidney. In the present study (Table 1.13) 7-12% of the esterified progestogens were hydrolysed to the free steroids by the rabbit kidney in 120 min. But no further metabolism to ring-A reduced metabolites occurred.

Little information is available regarding the steroid metabolic activity of skeletal muscle. Our study has shown that both reductase and esterase activities are present in this tissue, since both the non-esterified and esterified synthetic 19-norprogestogens were metabolised. Thus, norethisterone, dl-norgestrel and d-norgestrel as well as the parent compound 19-nortestosterone were reduced to the tetrahydro metabolites. However, l-norgestrel and lynestrenol were not metabolised by this tissue (Table 1.12). Thomas and Dorfman (1964) identified 5α-androstane-3, 17 dione, 3β-hydroxy-5α-androstan-17-one and testosterone from the incubation of (4-^{14}C) androstenedione with rabbit skeletal muscle tissue. Sweat and Bryson (1960) reported the following changes in the cortisol molecule by mouse muscle tissue, bovine muscle tissue, or both: cleavage of the side chain at C-17, oxidation at C-11, removal of the C-17 hydroxyl, reduction at C4 and C5, and reduction at C-20 to the 20α and 20β stereoisomers. Both the 20α and 20 β reduced metabolites of 3β, 17α-dihydroxypregn-5-ene-20-one (17α-hydroxyprogesterone) were identified when this substrate was incubated with rabbit skeletal muscle strips (Thomas et al., 1960). Kochakian and Stidworthy (1954) incubated androstenedione with rabbit skeletal muscle and identified testosterone and androstane-3, 17 dione. Thus, steroid metabolism does seem to occur in skeletal muscle tissue and this is in agreement with the data obtained in this study.

Lynestrenol acetate was seen to be hydrolysed to lynestrenol, but no further metabolism of this compound was seen in rabbit skeletal muscle (Table 1.13). However, norethisterone acetate, dl-norgestrel acetate and norethisterone oenanthate were seen to be hydrolysed almost as extensively as by the liver tissue of the rabbit and ring-A reduced products were also identified with these substrates. In contrast,

however, Dirscherl and Dardenne (1954) found no esterase activity in human skeletal muscle tissue, when testosterone propionate and desoxycorticosterone acetate were examined.

The results of the present study also indicated that rabbit lung tissue was metabolically active towards the synthetic non-esterified and esterified 19-norprogestogens, as well as 19-nortestosterone (Tables 1.12 and 1.13). This tissue has been shown to change many circulating substances, some becoming biologically active, some inactive and some may be stored and released later (Tierney, 1974). Thus, our study has shown that extensive metabolism of 19-nortestosterone and norethisterone can occur in rabbit lung tissue. Although dl- and d-norgestrel were also metabolised by this tissue, little metabolism of these progestogens occurred when compared to 19-nortestosterone and norethisterone (9 - 14% of norgestrel was metabolised as compared to 49.0% of norethisterone). Under the same experimental conditions, l-norgestrel and lynestrenol were not metabolised. The metabolites identified were tetrahydronorethisterone and tetrahydronorgestrel of norethisterone and dl-, d-norgestrel. This is comparable to other studies reported, where ring-A reduced metabolites of steroids were identified from lung tissue incubations. Thus, Hartiala (1974) reported that rabbit lung can metabolise (4-^{14}C) testosterone to androstenedione, 3α-hydroxy-5α-androstan-17-one and 17β-hydroxy-5α-androstan-3-one and the presence of 16α-steroid hydroxylase was also indicated. Similar metabolic products were obtained from rat and dog lung tissue 'in vitro' (Hartiala et al., 1972 and 1973). The metabolism of androstenedione and testosterone has also been shown in human foetal lungs (Mancuso et al., 1968 and Benagiano et al., 1968). The metabolites identified in the perfusate of previable human foeti , after the perfusion of testosterone-1, 2-^{3}H, were androstenedione, 3α-hydroxy-5α-androstan-17-one, 5α-androstanedione, and 3β-hydroxy-5α-androstan-3-one (Benagiano et al. 1968).

In addition the present study also shows significant metabolism of the esterified progestogens by rabbit lung (Table 1.13). All the esterified derivatives were seen to be metabolised by this tissue, particularly norethisterone oenanthate. The data of Dirscherl and Dardenne (1954) also indicates that steroid esters (testosterone propionate, desoxycorticosterone and cortisone acetate) are hydrolysed,

by human lung tissue. That human foetal lung tissue can hydrolyse norethisterone acetate has been indicated by Betz and Warren (1963). Thus, the lung tissue of man and various animal species appears to be of some importance in the metabolism of steroidal compounds.

In contrast, spleen tissue has been found to be metabolically inactive towards naturally occurring steroids. Thus, Schneider and Horstmann (1952) indicated that rat spleen did not metabolise 17-hydroxy-11-dehydrocorticosterone under conditions where liver and kidney tissues both metabolised this compound. More recently, Miyabo et al. (1975) found that the dog spleen was inactive towards 20β-dihydrocortisol. The data from our present study indicates that the spleen of the rabbit also does not appear to metabolise both 19-nortestosterone and the synthetic 19-norprogestogens (Tables 1.12 and 1.13).

The role of the intestine in the conjugation of steroids has been known for several years (Kreek et al., 1963 and Lehtinen et al., 1958) but only recently has the ability of the intestinal wall to metabolise neutral steroids by mechanisms other than conjugation been recognised (Nienstedt and Hartiala, 1969). The small intestine of the rabbit, in our study, was seen to metabolise both 19-nortestosterone and some of the synthetic 19-norprogestogens examined, to ring A reduced products. Thus, norethisterone was metabolised almost at the same rate as 19-nortestosterone, however, dl-norgestrel and d-norgestrel were metabolised comparatively slowly. As also observed in the other tissues, l-norgestrel and lynestrenol were not metabolised by this tissue in rabbit.

That ring-A reduction occurs in the intestinal wall was indicated by Kreek et al. (1963) who incubated (4-^{14}C) testosterone or androstenedione with everted sacks of rat intestine and found several ring-A reduced metabolites in the serosol media. Nienstedt (1967) and Nienstedt et al. (1970) have shown that when labelled androgens were placed in isolated segments of canine ileum, the effluent venous blood contained thirty-five to forty metabolites. In addition to the unchanged testosterone - androstenedione, androstanedione, androsterone and 5α-androstane-3β, 17β-diol were also identified (Harri et al., 1970a, 1970b and 1970c). Similar metabolism could also be demonstrated with 'in vitro' techniques where mucosal homogenates were incubated with the substrate steroid.

Besides considerable ring-A reduction in rabbit intestinal tissue, our study also indicates that the esterified progestogens are extensively hydrolysed. Thus, the main products identified were the hydrolysed free steroids of lynestrenol acetate, dl-norgestrel acetate, norethisterone acetate and norethisterone oenanthate (Table 1.13). Ring-A reduction of these steroids was also found to occur but not as rapidly as the hydrolysis reaction. Non-specific esterase activity has been demonstrated histochemically and biochemically in the small intestine of human foeti (Koldovsky 1970). The presence of specific and non-specific esterases in the various parts of the gastrointestinal tract and various levels of the intestinal mucosa has also been reported in animal species (Floch et al,1967, Nachlas and Seligman,1949, and Pelichova et al., 1967). Thus, Janssen et al.(1962) indicated that in the rat small intestine 'in vitro' steroid mono- and di-acetates, as well as a propionate, dioenanthate, and phenylacetate were hydrolysed. Esters of steroid hydroxyl groups at the 3α, 3β, 5β, 16α and 21 positions were hydrolysed, however, acylated steroids of 11α, and tert-17α and -β hydroxyl groups were stable. This is in contrast to our study where the tertiary-17β-hydroxyl acetates were seen to be hydrolysed by rabbit intestinal tissue 'in vitro'.

In contrast to the small intestinal tissue, adipose tissue of the rabbit was observed to metabolise 19-nortestosterone and norethisterone only to a minor extent (Table 1.12). Similarly, the esterified progestogens were also not metabolised by this tissue, although some hydrolysis of lynestrenol acetate and norethisterone oenanthate did occur (Table 1.13). This finding may be of significance and clinically useful since these steroids are lipid soluble and thus may be stored in the adipose tissue. This property could be the basis of the prolonged contraceptive protection provided by norethisterone oenanthate, a recently introduced injectable contraceptive agent.

Finally, the heart tissue of the rabbit seems to be metabolically inactive towards both 19-nortestosterone and the non-esterified progestogens examined; since little, if any, metabolism of these steroids took place in this study (Table 1.12). This finding is in agreement with that of Schneider and Horstmann (1952), who found the rat heart tissue to be inactive towards 17-hydroxy-11-dehydro-corticosterone. However, some esterase activity appears to be present

in the heart tissue of the rabbit since it was seen to hydrolyse the acetate derivatives as well as norethisterone oenanthate, albeit to a minor extent (Table 1.13).

Thus, as shown for naturally occurring steroids (Dorfman and Ungar, 1965 and Samuels and Eik-Nes, 1968) the metabolism of the 19-nortestosterone related synthetic progestogens in the rabbit liver and some extrahepatic tissues follows the ring-A reductive pathway, since only ring-A reduced metabolites were identified. 'In vivo'studies show that these progestational steroids have almost invariably been isolated from the urine of man and other animal species with a reduced ring-A (Fotherby and James, 1972; Thijssen,1972 and Fotherby,1974). However, various structural modifications of the 19-nortestosterone related synthetic progestogens influence the extent to which ring-A reduction occurs, not only in the liver, as discussed previously but also in the extrahepatic tissues. Thus, norethisterone, in which only the 17α-ethynyl group has been added, is metabolised by most of the rabbit tissues examined in this study. d-Norgestrel in which the methyl group at C-13 has been substituted by an angular ethyl group, decreases the rate of metabolism further, as compared to norethisterone and is not metabolised by most tissues examined. In contrast to d-norgestrel, l-norgestrel is not metabolised by any of the tissues examined except by the liver, where the metabolic rate was very slow; thus indicating that the biologically inactive l-orientated norgestrel can not be metabolised by the rabbit tissue under the conditions used. Lynestrenol is metabolised only by the liver, lung, small intestine and heart tissues,and no metabolism occurred in the other extrahepatic tissues examined. This may indicate that the enzymes responsible for hydroxylation at C-3 followed by dehydrogenation which have been suggested to be responsible for the conversion of lynestrenol to norethisterone (Fotherby and James, 1972) do not occur in extrahepatic tissues of the rabbit.

Esterase activity was demonstrated in all the tissues examined, although this activity was comparatively low in adipose tissue. The literature indicates that the number of enzymes able to hydrolyse the ester linkage are numerous; Dixon and Webb (1958) listed 49 of them and up to 16 different esterases have been observed in the various tissues of the rat.

In comparison to the non-esterified progestogens, the esterified derivatives were metabolised at a slower rate in most tissues except the small intestine, where the rate of hydrolysis was found to be greater than that observed in rabbit liver.

Although the liver was seen to be the main organ active in the metabolism of the synthetic progestogens examined, and it is also known that the compounds given orally pass through the liver before entering the general systemic circulation; the role of the small intestine should also be considered and as shown in the present study it appears to be almost as important as the liver. Lung tissue, skeletal muscle tissue and to some extent the kidney tissue were also seen to be involved in the metabolism of the 19-norprogestogens examined. The relative importance of extrahepatic tissues in controlling the general metabolism of these steroids, as compared to the liver, can not be evaluated from the present 'in vitro' study. However, since the enzymic reactions occurring in the liver have also been demonstrated in other tissues 'in vitro', it can be postulated that these reactions may also occur 'in vivo'. It is also important to consider the relative mass as well as the blood supply of the various tissues - such as the small intestine, lung tissue and skeletal muscle tissue in evaluating the role of extrahepatic tissues in the overall metabolism of these progestational compounds.

Many qualitative similarities have been reported in the metabolism of natural steroid hormones (Fotherby, 1964) and the synthetic 19-norprogestogens (Fotherby, 1974). 'However, insufficient information is available to know how far these similarities extend'. From our discussion of the metabolism of steroids in the various tissues of the rabbit and other animal species the conclusion can be drawn that each animal specie possesses an individuality of its own, consequently a direct application of results obtained in one specie to another should not be made. However, with careful selection and observance of criteria the results in animals may provide guidelines for the clinical application of the contraceptive steroids to humans. Ideally, human tissues should be used in an investigation of this type, however, it is not possible to obtain these tissues in a reasonably 'active' state.

Finally, in this study we have investigated the effect of various substituent groups on the rate of metabolism of 19-nortestosterone related compounds some of which are in common use. Similar studies with newly developed formulations may be of advantage prior to clinical testing in the human.

CHAPTER 2

HYDROXYLATION OF 19-NORPROGESTOGENS.

INTRODUCTION

In this chapter the metabolism of the three forms of norgestrel (d-, l-, and dl-Ng) is investigated using the microsomal fraction from female rabbit_livers. The rates of metabolism and the formation of metabolites of d-, l- and dl-Ng are compared to that of testosterone (T).

MATERIALS AND METHODS.

1. Animals.

Female New Zealand white rabbits were used as described in Chapter 1.

2. Chemicals.

a) Steroids

(4-^{14}C) Testosterone (sp. act 50 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks. (^{14}C) d-, l-, and dl- Ng were obtained as described in Chapter 1. The procedure for the purification of radioactive steroids has been described previously. All radioactive steroids were 98% pure. Other steroids used for reference purposes were obtained from Wyeth Research Inc., Philadelphia, U.S.A.

b) Organic Solvents.

In addition to the organic solvents used as mentioned in Chapter 1, dichloromethane (A.R. grade) (B.D.H. Ltd., Poole, Dorset) was washed with concentrated sulphuric acid, N-sodium hydroxide and water. The washed solvent was dried with anhydrous sodium sulphate and redistilled.

c) Other chemicals.

Manganese chloride ($MnCl_2$), magnesium chloride ($MgCl_2$), nicotinamide, sucrose, Tris (2-amino-2-hydroxymethyl propane 1, 3 diol) used in the incubation medium were analytical reagent grade (B.D.H. Ltd., Poole, Dorset). Bovine serum albumin was obtained from Armour Pharmaceutical Co., Ltd. U.K.).

d) Cofactors

NADP (Nicotinamide-adenine dinucleotide phosphate) was obtained from Sigma, London. Glucose-6-phosphate, glucose-6-phosphatase and glucose-6-phosphate dehydrogenase were obtained from C.F. Boehringer, Mannheim, Germany.

e) Gas-liquid chromatographic material.

G.l.c. material was obtained as described in Chapter 1.

f) Scintillation fluids.

These were also obtained and prepared as described in Chapter 1.

3. Centrifugation.

A M.S.E. Superspeed 75 ultracentrifuge was used for the preparation of rabbit liver microsomes.

4. "In Vitro" methodology

a) Preparation of liver microsomes.

The method used for the preparation of rabbit liver microsomes was similar to that described by Lisboa et al. (1968) for rat liver microsomes. Livers from adult female rabbits were removed immediately after killing the animals, washed in ice-cold 0.25 M sucrose solution, blotted and weighed. Connective tissue was removed and the liver cut into small pieces weighing approximately 2 g. 20% ($\frac{w}{v}$) homogenates were prepared in 0.25 M sucrose solution at 4°C using a Potter-Elvejhem homogeniser equipped with a loose fitting teflon pestle. The homogenate was submitted to differential centrifugation (M.S.E. Superspeed 75, Ultracentrifuge) for 10 min at 2 °C and 10,000 g, and at 105,000 g for 60 min. The resulting microsomal pellet was resuspended in 0.25 M sucrose and centrifugation was repeated at 105,000 g for 60 min.

The microsomal fraction thus obtained was adjusted to a concentration equivalent to 0.5 g of fresh liver per ml. The method of Lowry et al, (1951) was used for protein estimation of this fraction and gave a value of 10 - 12 mg protein per ml.

b) Purity of the microsomal fraction.

The purity of the microsomal fraction was checked by two methods:

i) Estimation of glucose-6-phosphatase.

The microsomal marker, glucose-6-phosphatase, was measured

spectrophotometrically (Unicam SP.600 spectrophotometer) essentially as described by Bergemeyer (1963).

ii) Electron microscopy.

The samples were processed by fixation in buffered 3% glutaraldehyde for 2 hours and, post fixation, in 1% osmium tetroxide for one hour at 4°C. The samples were washed in 0.1 M-phosphate buffer and dehydrated in progressive concentrations of ethanol, embedded in Araldite II and ultra thin sections prepared. Sections were stained with 15% uranyl acetate followed by Reynolds lead citrate and viewed in an AEI-EM-6B electron microscope. Fig. 2.1 shows a typical electron micrograph of a micorsomal preparation from rabbit liver.

c) Incubation procedure.

The incubation procedure used was essentially that described by Lisboa et al, (1968) except that NADPH was used instead of a NADPH-regenerating system consisting of NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase. (4-^{14}C) Testosterone was incubated in parallel with (^{14}C) d-, l-, dl-norgestrel in order to compare the rates of metabolism of the synthetic 19-norprogestogens with testosterone. Each steroid was incubated in separate experiments with the microsomal fraction from eight rabbit livers. Fifty-four µmol of radioactive steroid was dissolved in 0.05 ml 1-2-propanediol in ice-cold tubes. To each tube 12 µmol NADPH, 0.1 ml 1M nicotinamide solution, 0.1 ml 0.1M $MgCl_2$ solution, 0.1 ml 0.1mM $MnCl_2$ solution and 4 ml of the microsome suspension (equivalent to 40 mg protein) were added. 0.5 M Tris buffer (pH 7.4) was added to give a final volume of 6 ml. The ratio of steroid to tissue was 1 : 400. With all experiements suitable controls were included. Thus, tubes containing boiled tissue, no tissue and no substrate were incubated in parallel with each steroid. All incubations were carried out in duplicate under aerobic conditions, at 37°C for 10, 20 and 30 min in a shaking water bath (Baird and Tatlock Ltd., London).

Incubation reactions were terminated by the addition of 4 vol. of redistilled dichloromethane. Each tube was shaken for 30 min and the organic phase removed after centrifugation at 700 g and 4°C for 15 min.

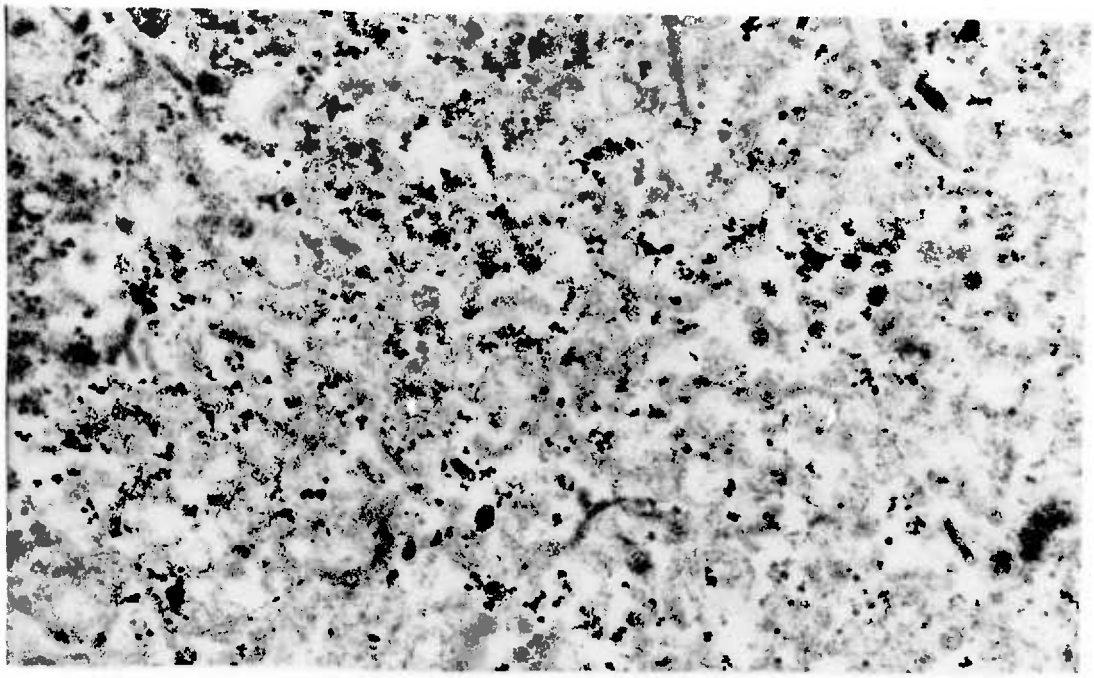


Fig. 2.1 Electron micrograph of a microsomal fraction (45, 000 x) from female rabbit liver. The procedure used to obtain the micrographs is described in the text.

The aqueous phase was extracted five times with dichloromethane, and the combined aqueous phase evaporated 'in vacuo' at 30°C. This procedure extracts 95 ± 2% (S.D.) of the steroids. The residue was dissolved in redistilled ethanol and submitted to chromatographic analyses. When larger quantities of metabolic products were required for chromatographic identification, the constituents of the incubation medium were increased tenfold.

5. Chromatographic analyses of incubation products.

a) Thin-layer chromatography.

The extracts of each incubation were submitted to ascending one-dimensional thin-layer chromatography (Lisboa and Diczfalusy, 1962) as described in Chapter 1. Suitable aliquots of the incubation extracts were applied to pre-coated Silica gel G or Silica gel GF_{254} -layers (20 x 20 cm, 250 mμ Silica gel layers, obtained from Merck, Darmstadt, Germany). These were developed in the solvent system, cyclohexane: ethylacetate (1 : 1, $\frac{v}{v}$) (Sisenwine et al, 1973). Unidimensional multiple chromatography was found to give more precise bands than a single run. Therefore each plate was rechromatographed three times in the same solvent system. Reference steroids were run in parallel with each compound and were revealed as described in Chapter 1. Chromatography of standard steroids mixed with the entire incubation extract demonstrated that the presence of endogenous lipid did not affect the mobilities of the steroids. The data of relative mobilities of reference steroids to norgestrel are given in Table 2.1

b) Evaluation of thin-layer chromatograms.

i) Detection of radioactivity by a radioactivity scanner.

The thin-layer plates were scanned for radioactivity using a Packard (Model No. 7200) radiochromatogram scanner.

ii) Detection of radioactivity by autoradiography.

Radioautographs were made from the thin-layer plates with an exposure time of 12 days (Berg and Gustafsson, 1973) on Kodirex-Autoprocess X-ray film (Kodak, London). The radioactive zones on the thin-layer plates

T A B L E 2.1 CHROMATOGRAPHIC DATA OF REFERENCE COMPOUNDS EMPLOYED FOR METABOLITE IDENTIFICATION.

	THIN-LAYER CHROMATOGRAPHY R_F values.				GAS-LIQUID CHROMATOGRAPHY R_F values	
	Free	Acetates	Reduced	Oxidised	Free	TMS Ethers
Norgestrel	0.73	0.73	0.61	0.73	16.2	20.1
3α,5β-tetrahydronorgestrel	0.61	0.90	0.61	0.83	11.0	15.0
3β,5β-tetrahydronorgestrel	0.84	0.99	0.84	0.95	10.8	14.9
16β-hydroxynorgestrel	0.49	0.62	0.37	0.40	28.8	34.2
16α-hydroxynorgestrel	0.20	0.55	0.24	0.58	16.2	22.0
6ε-hydroxynorgestrel	0.39	0.50	0.21	0.30	21.0	27.0

were determined exactly from the X-ray film. These zones were eluted with ethanol and aliquots were taken for radioactivity estimations and gas-liquid chromatography, as described in Chapter 1.

iii) Examination by short wave ultraviolet light.

Each plate was examined under short wave ultraviolet light (λ = 254 mμ) to obtain information on unsaturated (C4 - C5)(double bond retaining) steroids.

c) Gas-liquid chromatography.

Quantitation and identification of metabolic products was carried out as described in Chapter 1. The data for relative retention times of standard steroids to norgestrel are given in Table 2.1.

6. Derivative preparation .

The preparation of trimethylsilyl ether derivatives and acetate derivatives has been described in Chapter 1. The procedures used to reduce and oxidise the steroids have also been described in Chapter 1.

7. Statistical treatment of data.

The data was statistically treated as described in Chapter 1.

RESULTS.

1. Comparison of the rates of metabolism of d-, dl-, l-norgestrel (d-,dl-,l-Ng) to testosterone in rabbit liver microsomal fraction.

The conditions of incubation have been described in the Methods section. The mean ($\pm$SD) of the percent steroid recovered at 10, 20 and 30 min of incubation time are given in Table 2.2 for testosterone and Tables 2.3 - 2.5 for d-, dl-, and l-norgestrel. The mean values obtained at 30 min are compared in Fig. 2.3. (individual values are given in Appendix tables 14 - 17).

The rate of metabolism of the natural steroid testosterone in the rabbit liver microsomal fraction was seen to be significantly faster ($p < 0.01$) than the synthetic progestogens d-, dl- and l-norgestrel. Thus, at 10 min of incubation time $20.0 \pm 2.4\%$ of testosterone was recovered unchanged. During the same period of time $45.0 \pm 2.2\%$ of d-norgestrel, $62.0 \pm 3.1\%$ of dl-norgestrel and $70.0 \pm 1.7\%$ of l-norgestrel were recovered unchanged. By 30 min $6.5 \pm 1.1\%$ of testosterone, $18.0 \pm 1.4\%$ of d-norgestrel, $40.0 \pm 2.0\%$ of dl-norgestrel and $50.0 \pm 1.8\%$ of l-norgestrel were observed to be unchanged. The rate of metabolism of the three forms of norgestrel was also significantly different ($p < 0.01$) thus the biologically active d-norgestrel was metabolised at a faster rate than the racemate and the biologically inactive isomer, l-norgestrel.

2. Metabolic products obtained from the incubations of testosterone d-, dl-, and l-norgestrel in the rabbit liver microsomal fraction.

Fig.2.2. shows the autoradiograms of the thin-layer chromatographic analyses of the extracts of incubations of testosterone, d-, dl- and l-norgestrel with microsomes from female rabbit liver. The areas indicated were eluted and each product was identified as described in the Methods section. Thus, each area was acetylated, oxidised, reduced as described, and chromatographed on thin-layer plates. The metabolites were compared to reference steroids treated in the same way. These products were then analysed by gas-liquid chromatography as non-derivatised steroids and as trimethylsilyl ether derivatives. Table 2.1 summarises the pertinent thin-layer and gas-liquid chromatographic data obtained for reference steroids.

T A B L E 2.2

INCUBATION OF (4-^{14}C) TESTOSTERONE WITH FEMALE RABBIT LIVER MICROSOMES. THE RESULTS OF EIGHT EXPERIMENTS ARE EXPRESSED AS MEAN (±SD) OF PERCENT STEROID RECOVERED.

RECOVERED STEROIDS	PERCENT RECOVERED STEROID (mean ± SD)			
		TIME OF INCUBATION		
	0 min	10 min	20 min	30 min
testosterone	96.0±2.0	20.0±2.4	10.2±1.4	6.5±1.1
dihydrotestosterone	-	9.0±0.8	8.0±0.3	3.0±0.4
tetrahydrotestosterone	-	12.0±1.2	13.0±2.0	14.1±1.1
'polar' metabolite	-	53.0±3.1	62.0±5.0	70.9±2.0
Total steroids recovered	96.0	94.0	93.2	94.5

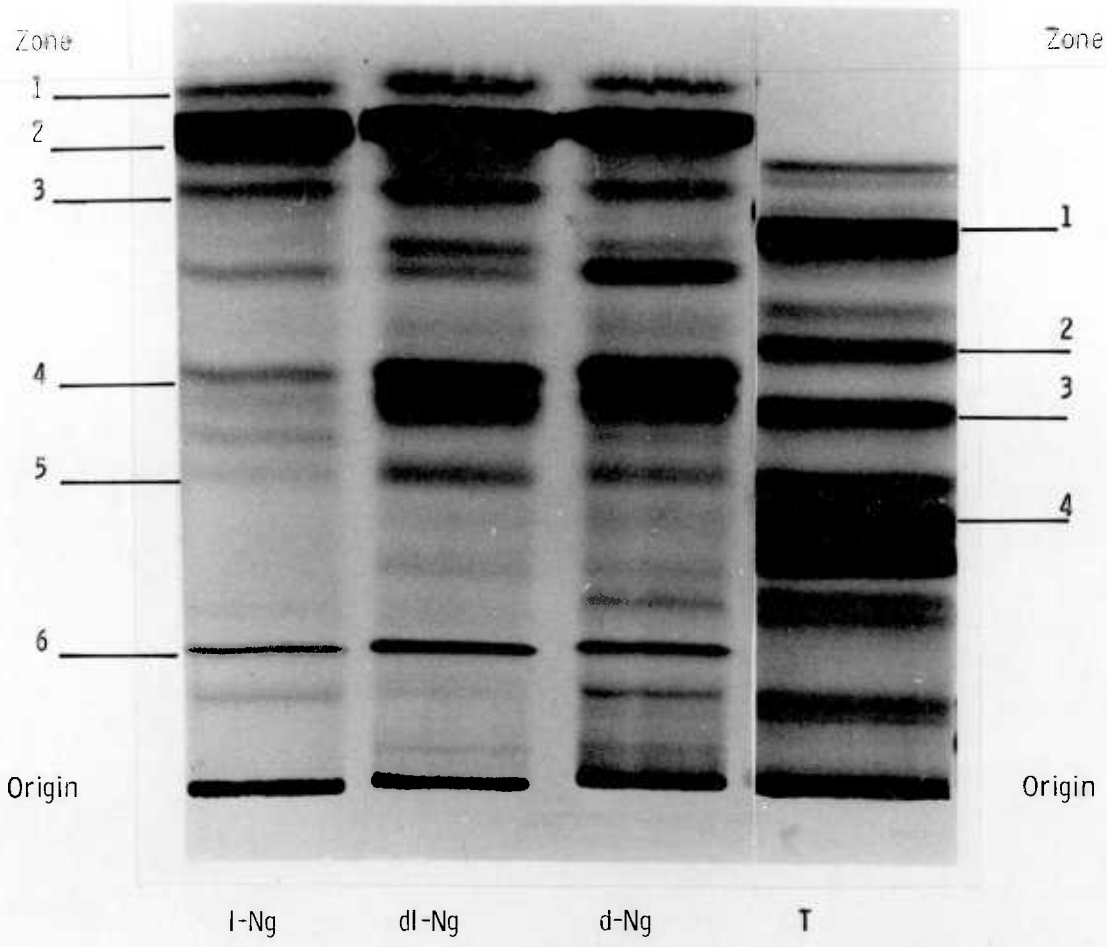


Fig. 2.2 Thin-layer chromatograms of extracts of incubations of $[^{14}C]$d-, dl-, l-, norgestrel and $[4\text{-}^{14}C]$ testosterone with the microsomal fraction of liver homogenates from female rabbits. The following compounds were identified from incubations of norgestrel Zone 1, 3ß, 5ß-tetrahydronorgestrel, Zone 2, norgestrel, Zone 3, 3α, 5ß-tetrahydronorgestrel, Zone 4, 16ß-hydroxynorgestrel, Zone 5, 6ε-hydroxynorgestrel and Zone 6, 16α-hydroxynorgestrel. The compounds identified from incubation of testosterone were Zone 1, testosterone, Zone 2, dihydrotestosterone and Zone 3, tetrahydrotestosterone. Zone 4 was estimated as the 'polar metabolite'. The amounts of extracts chromatographed for each compound were not similar.

T A B L E 2.3

INCUBATION OF (^{14}C) d-NORGESTREL WITH FEMALE RABBIT LIVER MICROSOMES. THE RESULTS OF EIGHT EXPERIMENTS ARE EXPRESSED AS MEAN (±SD) OF PERCENT STEROID RECOVERED.

RECOVERED STEROIDS	PERCENT RECOVERED STEROIDS (mean ±SD)			
		TIME OF INCUBATION		
	0 min	10 min	20 min	30 min
d-norgestrel	98.5±2.1	45.0±2.2	31.5±1.9	18.0±1.4
3β,5β-tetrahydronorgestrel	-	8.0±1.1	10.2±1.3	12.0±1.1
3α,5β-tetrahydronorgestrel	-	12.0±1.0	16.0±2.2	20.0±3.4
16α-hydroxynorgestrel	-	3.0±0.8	6.0±1.1	8.0±1.1
16β-hydroxynorgestrel	-	13.0±1.4	17.0±1.0	23.0±2.9
6ε-hydroxynorgestrel	-	7.0±1.1	8.0±0.5	10.0±1.5
Total Steroids recovered	98.5	88.0	88.7	91.0
Sum of ring-A reduced metabolites	-	20.0	26.2	32.0
Sum of hydroxylated metabolites	-	23.0	30.0	41.0
Ratio of ring-A reduced to hydroxylated metabolites	-	0.86	0.87	0.78

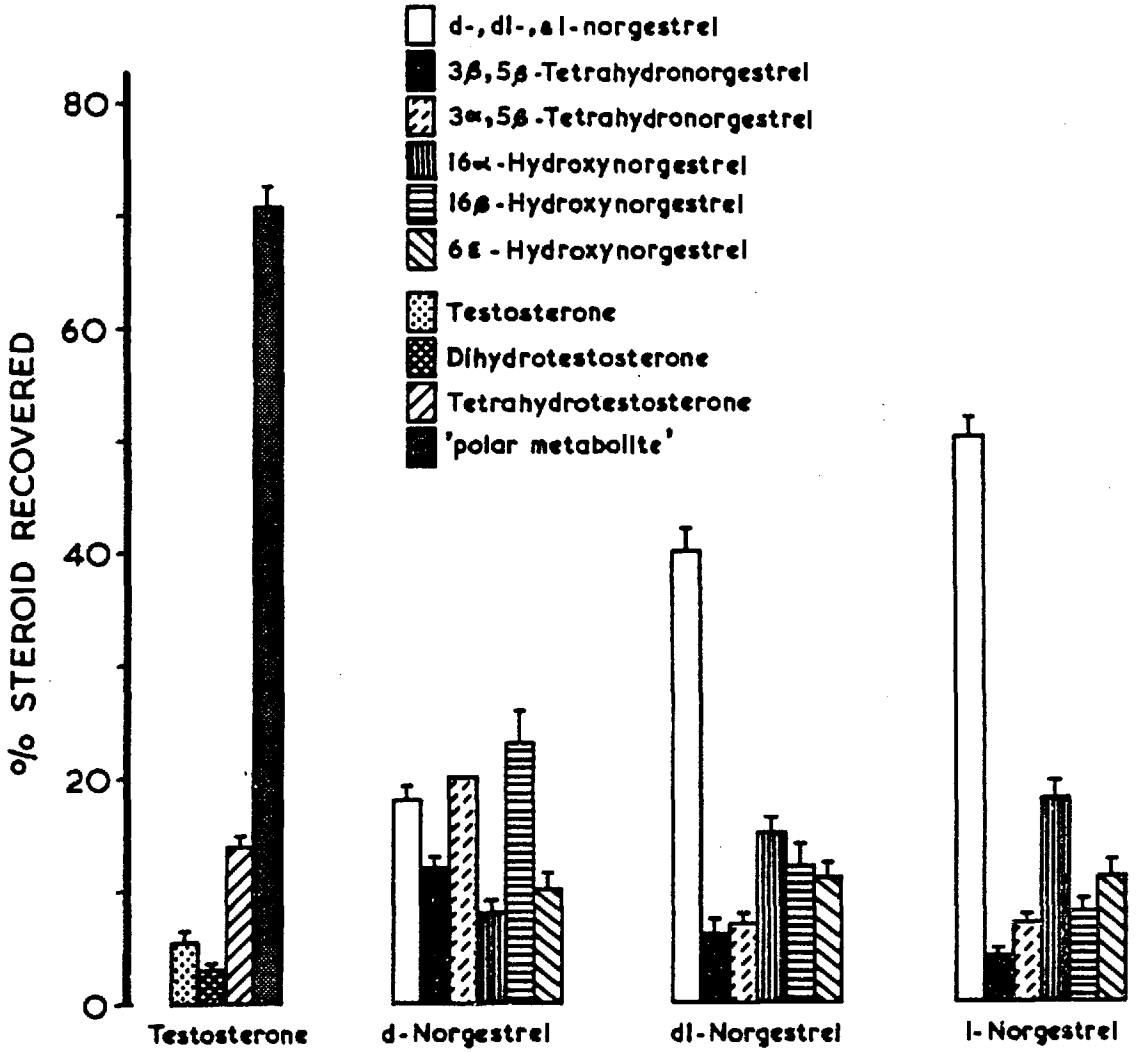


Fig. 2.3 Percent steroids recovered from the incubation of testosterone, d-, dl- and l-norgestrel with female rabbit liver microsomes at 30 min. The figure is based on the mean (± SD) in Tables 2.2 - 2.5.

TABLE 2.4

INCUBATION OF (^{14}C) dl-NORGESTREL WITH FEMALE RABBIT LIVER MICROSOMES. THE RESULTS OF EIGHT EXPERIMENTS ARE EXPRESSED AS MEAN (±SD) OF PERCENT STEROID RECOVERED.

RECOVERED STEROIDS	PERCENT RECOVERED STEROIDS (mean ± SD)			
	TIME OF INCUBATION			
	0 min	10 min	20 min	30 min
dl-norgestrel	98.0±1.1	62.0±3.1	48.1±2.6	40.0±2.0
3β,5β-tetrahydronorgestrel	-	2.0±0.6	4.0±1.1	6.0±1.3
3α,5β-tetrahydronorgestrel	-	5.0±1.2	6.0±1.6	7.0±0.8
16α-hydroxynorgestrel	-	11.0±1.6	14.0±2.1	15.0±1.5
16β-hydroxynorgestrel	-	9.0±0.7	11.0±1.5	12.0±2.0
6ε-hydroxynorgestrel	-	7.0±1.1	9.0±0.6	11.0±1.4
Total steroids recovered	98.0	96.0	92.0	91.0
Sum of ring-A reduced metabolites	-	7.0	10.0	13.0
Sum of hydroxylated metabolites	-	27.0	34.0	38.0
Ratio of ring-A reduced to hydroxylated metabolites	-	0.25	0.29	0.34

As shown in Table 2.2 and Fig. 2.2 testosterone was metabolised to dihydrotestosterone, tetrahydrotestosterone, a polar metabolite and other minor metabolites, which were not identified. These may have been hydroxylated products, however, since the relevant reference steroids were not available these products were not examined in detail.

In contrast to the metabolism of d-, dl- and l-norgestrel in total liver homogenates where tetrahydronorgestrel was the only metabolic product obtained, (Chapter 1) in the microsomal fraction of rabbit liver these compounds were seen to be metabolised to several products (Tables 2.3 - 2.5, Fig. 2.2). As shown in Fig. 2.2, the pattern of metabolites from d-, dl- and l-norgestrel was qualitatively similar but quantitatively different (Tables 2.3 - 2.5). The products identified from the incubation extracts of these three forms of norgestrel were 3β, 5β-tetrahydronorgestrel, 3α, 5β-tetrahydronorgestrel, 16α, 16β-and 6ε-hydroxynorgestrel and the rate of formation of these metabolites was examined.

Thus, at 30 min of incubation time (Tables 2.3 - 2.5 and Fig. 2.3) $12.0\pm1.1\%$ of d-norgestrel, $6.0\pm1.3\%$ of dl-norgestrel and $4.0\pm0.8\%$ of l-norgestrel were converted to 3β, 5β-tetrahydronorgestrel. The mean values at all time intervals examined were statistically significantly different for each form of norgestrel ($p < 0.01$, Appendix table 15 - 17)

However, when the mean values obtained for 3α, 5β-tetrahydronorgestrel were examined (Tables 2.3 - 2.5), the rate of formation of this metabolite from dl-norgestrel and l-norgestrel was not significantly different ($p > 0.01$). Whereas the mean value obtained for d-norgestrel was significantly different (p 0.01, Appendix table 15) from l- and dl-norgestrel. Thus, $20.0\pm3.4\%$ of d-norgestrel, $7.0\pm0.9\%$ of l-norgestrel were metabolised to 3α, 5β-tetrahydronorgestrel in 30 min.

Besides the two ring-A reduced products, 3α, 5β- and 3β, 5β-tetrahydronorgestrel several other metabolic products were observed (Fig. 2.2). However, only 16α, 16β- and 6ε hydroxynorgestrel were definitely identified. All three products were present in the extracts of d-, dl-, and l-norgestrel but in different amounts. Thus, at 30 min (Tables 2.3 - 2.5, Fig. 2.3) $8.0\pm1.1\%$ of d-norgestrel, $15.0\pm1.5\%$ of dl-norgestrel were metabolised to 16α-hydroxynorgestrel. The mean values obtained from each form of norgestrel were significantly different from each other ($p < 0.01$, Appendix table 15). In comparison,

T A B L E 2.5

INCUBATION OF (^{14}C) 1-NORGESTREL WITH FEMALE RABBIT LIVER MICROSOMES. THE RESULTS OF EIGHT EXPERIMENTS ARE EXPRESSED AS MEAN (±SD) OF PERCENT STEROID RECOVERED.

RECOVERED STEROIDS	PERCENT RECOVERED STEROID (mean ± SD)			
	TIME OF INCUBATION			
	0 min	10 min	20 min	30 min
1-norgestrel	98.0±1.2	70.0±1.7	60.0±2.0	50.0±1.8
3β,5β-tetrahydronorgestrel	-	1.0±0.4	2.0±0.6	4.0±0.8
3α,5β-tetrahydronorgestrel	-	2.0±0.5	3.0±0.4	7.0±0.9
16α-hydroxynorgestrel	-	10.0±1.2	14.0±1.6	18.0±1.0
16β-hydroxynorgestrel	-	3.0±0.7	6.0±0.6	8.0±1.0
6ε-hydroxynorgestrel	-	7.0±0.5	10.0±1.1	11.0±1.5
Total steroids recovered	98.0	93.0	95.0	98.0
Sum of ring-A reduced metabolites	-	3.0	5,0	11.0
Sum of hydroxylated metabolites	-	20.0	30.0	37.0
Ratio of ring-A reduced to hydroxylated metabolites	-	0.15	0.16	0.29

23.0±2.9% of d-norgestrel, 12.0±2.0% of dl-norgestrel and 8.0±1.0% of l-norgestrel were converted to 16β-hydroxynorgestrel. These values were also significantly different from each other ($p < 0.01$, Appendix table 15). The figures obtained show that the rate of formation of 16β-hydroxynorgestrel from d-norgestrel was faster than from l-norgestrel. Whereas, l-norgestrel was mainly metabolised to 16α-hydroxynorgestrel. The two hydroxylated products 16α- and 16β- were formed equally from dl-norgestrel.

The figures in Tables 2.3 - 2.5 show that the rate of formation of 6ε-hydroxynorgestrel was not significantly different from d-, dl- and l-norgestrel. Thus, in 30 min 10.0±1.5% of d-norgestrel, 11.0±1.4% of dl-norgestrel and 11.0±1.5% of l-norgestrel were converted to 6ε-hydroxynorgestrel.

Several other bands of radioactivity were observed on the radioautograms of thin-layer chromatograms (Fig. 2.2), however, these were not identified definately since they appeared to form only a minor fraction of the total metabolites obtained. The results of tentative identification indicated that two of the metabolites may be 2ε and 10ε-hydroxynorgestrel.

Examination of the total amounts of ring-A reduced metabolites and hydroxylated metabolites (Tables 2.3 - 2.5) of d-, dl- and l-norgestrel indicated that the total amounts of hydroxylated metabolites obtained were higher than the ring-A reduced products. The data also indicated that dl- and l-norgestrel were mainly converted to the hydroxylated products whereas, d-norgestrel was equally converted to the ring-A reduced and hydroxylated metabolites. When the ratio of ring-A reduced to hydroxylated metabolites was calculated from the mean values, the results show (Table 2.3 - 2.5) that the ratio increases with time for dl- and l-norgestrel, whereas, for d-norgestrel it remains constant. Thus, possibly indicating that the hydroxylation reactions became more dominant with time, in dl- and l-norgestrel metabolism.

Thus, in summary, the results indicate that in the rabbit liver microsomal fraction the natural steroid testosterone is metabolised at a faster rate than the synthetic 19-norprogestogens, d-, dl- and l-norgestrel. Of the three forms of norgestrel the biologically active isomer d-norgestrel is metabolised at a comparatively faster rate than the racemate and the biologically inactive isomer, l-norgestrel.

Testosterone is metabolised to 'more polar metabolites' which were not identified but may have been hydroxylated products.

The metabolism of the three forms of norgestrel is qualitatively similar but quantitatively different. Thus, 3α, 5β- and 3β, 5β-tetrahydronorgestrel, 16α, 16β- and 6ε-hydroxynorgestrel were identified as products of d-, dl- and l-norgestrel.

dl- and l-Norgestrel are metabolised mainly to hydroxylated products, whereas d-norgestrel is metabolised to both ring-A reduced and hydroxylated products in equal portions . Of the ring-A reduced metabolites formed from d-norgestrel, the amount of 3α, 5β- tetrahydronorgestrel was greater than the 3β, 5β-isomer. However, these two products were formed in equal portions from dl- and l-norgestrel. Examination of the hydroxylated products indicates that l-norgestrel is converted mainly to 16α-hydroxynorgestrel, whereas d-norgestrel is metabolised to 16β-hydroxynorgestrel. The two products are formed equally from dl-norgestrel . However, 6ε-hydroxynorgestrel is formed at the same rate from d-, dl- and l-norgestrel in female rabbit liver microsomes. The ratio of ring-A to hydroxylated products from d-norgestrel remained steady with time, whereas, with l- and dl-norgestrel the ratio was seen to increase with time.

DISCUSSION.

In the chemical synthesis of the 19-nortestosterone related progestogen, norgestrel, two stereoisomers are formed thus resulting in the racemate dl-norgestrel, d-norgestrel and l-norgestrel (Smith et al., 1964). Experimental studies have shown that only the d-form has biological activity (Edgren et al., 1963, De Johng et al., 1968). Terenius (1972) and McGuire et al. (1974) have indicated that the d- and l-forms also have different binding affinities for the rat uterine progestogen receptor. Although several 'in vivo' studies on the metabolism of the three forms of norgestrel, both in animal species and man (Kamyab et al., 1967, Littleton et al., 1968, Gerhards et al., 1971, Zaldivar and Gallegos, 1971, Hendeles et al, 1972, and Sisenwine et al., 1973, 1974 and 1975) have been reported, no 'in vitro' investigation has been reported in which the rates of metabolism of dl-norgestrel and the two enantiomers, d- and l-norgestrel, are compared.

In this study, we have compared the rates of metabolism of d-, dl- and l-norgestrel using the microsomal fraction of female rabbit liver. The rate of metabolism of the three forms of norgestrel has also been compared with the rate of metabolism of the natural steroid testosterone. The results show (Tables 2.2 - 2.5) that the natural steroid is metabolised more rapidly than the three forms of the synthetic progestogen, norgestrel, under the incubation conditions described in the Methods section. A comparison of the rate of metabolism of d-, dl- and l-norgestrel indicates that the biologically active d-form is metabolised faster than the biologically inactive l-enantiomer and the racemate dl-norgestrel. l-Norgestrel was observed to be least metabolised. Differences in the rate of urinary excretion of d-, dl- and l-norgestrel have been shown both in the African Green Monkey (Sisenwine et al., 1974) and in women (Sisenwine et al., 1975). Thus, after a single dose of (^{14}-C) dl-norgestrel the radioactivity excreted was significantly higher (51.4$\pm$5.0%) than that observed after the administration of the d-enantiomer (37.5$\pm$5.4%) but not the l-enantiomer (44.2$\pm$8.9%), in the urine of African Green Monkeys. Similarly in women, Sisenwine et al. (1975) have shown that after a single dose of ^{14}C- labelled norgestrel, the average percentage of administered radioactivity recovered in urine after seven days was 58.1$\pm$7.9%

for dl-norgestrel, 44.8±8.9% for d-norgestrel, and 63.6±15.1% for l-norgestrel. Thus, the excretion of the d-form in urine appears to be slower than the dl- and l-forms of norgestrel, both in African Green Monkeys and women. This data also shows that there are some differences in the rates of excretion of d-, dl- and l-norgestrel between the human and African Green Monkey, which may probably be due to a specie difference. However, in general, the results of these 'in vivo' studies (Sisenwine et al., 1974 and 1975) indicate that d-norgestrel is excreted more slowly than the dl-form and l-form. Whether this is related to its rate of metabolism cannot be ascertained from these studies. Our 'in vitro' data in rabbit liver indicates that the d-form is metabolised more rapidly than the dl- and l-form of norgestrel.

Examination of the metabolic products obtained from the liver incubations of d-, dl- and l-norgestrel indicates that metabolism of the three forms is qualitatively similar (Fig. 2.2) but quantitatively different (Fig. 2.3). Thus, all three forms gave rise to 3β, 5β-and 3α, 5β-tetrahydronorgestrel, 16α, 16β- and 6ε-hydroxynorgestrel. A compound similar to 2ε -hydroxynorgestrel in chromatographic mobility was also observed, however, this was not characterised further.

The biologically inactive enantiomer, l-norgestrel (Edgren et al., 1963) and the racemate dl-norgestrel were metabolised mainly to the hydroxylated products (Tables 2.4 and 2.5). But, the biologically active (Edgren et al., 1963) d-enantiomer was metabolised to the ring-A reduced and hydroxylated metabolites in almost equal proportions. However, comparatively more ring-A reduced products were obtained from d-norgestrel than dl- and l-norgestrel. These findings are comparable with the 'in vivo' studies in the African Green Monkey (Sisenwine et al., 1974) and man (Sisenwine et al., 1975). Thus, in both species the oxidative pathway predominates in the metabolism of l-norgestrel, whereas in d-norgestrel metabolism, reductive transformations seem to be more important. The pattern of identified urinary metabolites observed after the administration of the racemate dl-norgestrel reflects the pathways operative in the two enantiomers. Thus, in the African Green Monkey stereoselective pathways for l-norgestrel were demonstrated, where 16β- hydroxnorgestrel, 16α-hydroxynorgestrel, and 16-hydroxytetrahydronorgestrel were detected only in the urine samples obtained from (^{14}C) dl- and l-norgestrel. Whereas, following (^{14}C) d-norgestrel administration,

3α, 5β-tetrahydronorgestrel was found to be the major matabolite. These differences in the metabolite pattern due to stereoselective pathways were also seen in man (Sisenwine et al., 1975) where l-norgestrel was metabolised mainly to 16β-hydroxynorgestrel and d-norgestrel to ring-A reduced products. In contrast to the African Green Monkey, minor amounts of l-, 3α, 5β-tetrahydronorgestrel were also identified in human urine.

In the rabbit liver, two ring-A reduced metabolites, 3α, 5β- and 3β, 5β-tetrahydronorgestrel were identified from the incubations of d-, dl- and l-norgestrel. However, d-norgestrel was predominantly converted to the 3α, 5β-isomer whereas dl- and l-norgestrel were seen to be metabolised to both the 3α, 5B- and 3β, 5β-isomers in equal proportions. Similar to our findings, the 3α, 5β-tetrahydro metabolite of d-norgestrel and dl-norgestrel was also found to be the major metabolite excreted in the urine of man (Littleton et al., 1968, Gerhards et al, 1971, Sisenwine et al., 1973 and 1975), and the African Green Monkey (Sisenwine et al., 1974). Small amounts of the 3β,5β-isomer were also found in the urine of man after the administration of dl-norgestrel and d-norgestrel (Gerhards et al., 1971) and dl-norgestrel (Littleton et al., 1968, and Sisenwine et al., 1973). As shown in our 'in vitro' study (Table 2.5) with rabbit liver, small amounts of l-norgestrel were also converted to 3α, 5β-tetrahydronorgestrel in man (Sisenwine et al., 1975).

Examination of the hydroxylated metabolites obtained from rabbit liver 'in vitro', indicates that although all three forms of norgestrel give rise to these products, the quantities of 16α- and 16β-hydroxy-norgestrel formed differ from d-, dl- and l-norgestrel. Thus, d-norgestrel is mainly hydroxylated at the 16β-position whereas, l-norgestrel mainly follows the 16α-hydroxypathway. The formation of these two products was similar for dl-norgestrel (Tables 2.3 - 2.5). In contrast, in the African Green Monkey (Sisenwine et al., 1974) the major metabolite of l-norgestrel was observed to be the 16β-hydroxylated product and only minor amounts of 16α-hydroxynorgestrel were obtained. However, in man (Sisenwine et al., 1975) only the 16β-hydroxy metabolite was detected in the urine after the administration of d-norgestrel. The differences in the oxidative pathways seen in man, the African Green Monkey ('in vivo') and the rabbit ('in vitro') may be either due to species differences or the methods of investigation.

6ε -Hydroxynorgestrel was also identified from the liver incubations of d-, dl- and l-norgestrel (Tables 2.3 - 2.5); however, there were no differences in the rate of formation of this metabolite from the three forms of norgestrel. This metabolite has also been identified in the urine of women receiving dl-norgestrel (Sisenwine et al., 1973).

In this study, when the ratio of ring-A reduced products to hydroxylated products is examined (Tables 2.3 - 2.5) the results indicate that for d-norgestrel the ratio remains constant over time (30 min). However, with dl- and l-norgestrel the ratio appears to increase with time, possibly indicating that the oxidative pathways become more dominant than the reductive pathways for the metabolism of these two forms of norgestrel.

Thus, this 'in vitro' comparison of the metabolism of d-, dl- and l-norgestrel with the natural steroid testosterone in the rabbit liver microsomal fraction indicates that the synthetic progestogens are metabolised more slowly. This was also seen in Chapter 1, where 19-nortestosterone, the parent compound, was metabolised comparatively faster than the three forms of norgestrel. However, in contrast to the study in Chapter 1, where total liver homogenates were used for incubations, and only tetrahydronorgestrel was identified as the metabolite, in this study it is seen that d-, dl- and l-norgestrel undergo metabolism to several metabolic products. Thus, both ring-A reduced and hydroxylated products were identified from the three forms of norgestrel.

It is also seen that in the microsomal fraction of rabbit liver, not only the rate but the route of metabolism of d-, dl- and l-norgestrel is different. Thus, dl- and l-norgestrel appear to mainly follow the oxidative pathways, whereas d-norgestrel follows both the reductive and oxidative pathways under the conditions used. That the stereo-selective pathways demonstrated 'in vivo' (Sisenwine et al., 1974 and 1975) also occur to a certain extent 'in vitro' is also demonstrated in this study. However, specie differences in the metabolism of d-, dl- and l-norgestrel are apparent, as discussed.

CHAPTER 3

SULPHO-CONJUGATION OF 19-NORPROGESTOGENS.

INTRODUCTION

In this chapter the rate of sulphate conjugation of d-, l-, dl-norgestrel, (d-Ng, l-Ng, dl-Ng), norethisterone (NET), lynestrenol (Lyn) and ethynolestradiol (EE), is compared to dehydroepiandrosterone (DHA) by female rabbit liver and extra-hepatic tissues. In addition, the formation of metabolic products of these compounds by various rabbit tissues has also been investigated.

MATERIALS AND METHODS.

1. Animals.

Female New Zealand white rabbits were used throughout the study as described in Chapter 1.

2. Chemicals.

a) Steroids.

Dehydroepiandrosterone (Sigma, London) was checked for purity by gas-liquid chromatography and was 98% pure. Norethisterone-17-sulphate, dl-norgestrel-17-sulphate (Wyeth Research Inc., Philadelphia, U.S.A.), dehydroepiandrosterone-3-sulphate, oestradiol-17-sulphate and oestradiol-3-17-sulphate (Aktiebaloget, Leo Halsinborg, Sweden) used as reference compounds, were also 98% pure as checked by thin-layer chromatography.

b) Other Steroids.

Radioactive and unlabelled steroids used in the metabolic studies were obtained as described in Chapter 1. ($4-^{14}C$) ethynyloestradiol (sp. act. 12 m Ci/mM obtained as a gift from B.D.H. Ltd., London) was shown to be 96% pure by paper chromatography as described in Chapter 1.

c) Organic Solvents.

In addition to the organic solvents as described in Chapter 1, diethyl-ether and dioxane (A.R. grade) (B.D.H., Ltd, Poole, Dorset) were redistilled before use.

d) Other chemicals.

Sodium chloride (NaCl), potassium chloride (KCl), magnesium sulphate ($MgSO_4$), EDTA (ethylenediamine tetra-acetic acid - B.D.H. Ltd, Poole, Dorset) and Tris (2-amino-2-hydroxymethylpropane 1,3-diol) (Koch-Light Ltd, London) were of analytical reagent grade. Other chemicals were obtained as described in Chapter 1.

e) Cofactors.

Natural baker's yeast was used for the preparation of PAPS (Adenosine 3'-phosphate 5' phosphosulphate). NAD (Nicotinamide-adenine dinucleotide) and ATP (Adenosine 5'-triphosphate) were obtained from Sigma, London.

3. Gas-liquid chromatographic material.

These were used as described in Chapter 1.

4. Scintillation fluid.

The preparation of toluene-based scintillator was described in Chapter 1.

5. Composition of Krebs-Ringer solution.

This was prepared as described by Wallace and Lieberman (1963).

105 ml 0.1M phosphate buffer, pH 7.4.
100 ml 0.9 % NaCl.
4 ml 1.15 % ($\frac{w}{v}$) KCl.
1 ml 2.11 % ($\frac{w}{v}$) KH_2PO_4
1 ml 1.86 % ($\frac{w}{v}$) $MgSO_4$

6. Methylene blue reagent.

Methylene blue (Methylthionine Chloride, Sigma, London) reagent was prepared according to Roy (1956a). The reagent contained 250 mg methylene blue, 50 gm anhydrous sodium sulphate and 10 ml concentrated sulphuric acid (A.R. grade) in one litre of water.

7. Pyridinium sulphate.

Pyridinium sulphate was prepared by reacting one part of concentrated sulphuric acid with 30 parts of chloroform: pyridine (5 : 1, $\frac{v}{v}$) mixture at $0^{o}C$. The resulting pyridinium sulphate was obtained by filtration and stored at $-20^{o}C$.

8. Preparation of PAPS (adenosine 3'-phosphate 5'-phosphosulphate.

PAPS was prepared according to Nose and Lipmann (1958). 2 kg of baker's yeast was frozen with solid CO_2 and ether and extracted overnight with 800 ml, 0.05 M KH_2PO_4, at 4 ^{o}C. The suspension was centrifuged at 3000 g for 30 min. at 2^{o}C in a Sorvall Superspeed RC2-B centrifuge. The supernatant was decanted and adjusted to pH 5.9 by addition of IN-acetic acid at 4 ^{o}C. The precipitate was discarded after centrifugation at 2000 g for 15 min at 2^{o}C; and the supernatant solution, still acidic and containing 0.02 M EDTA, was brought to 0.6 ammonium sulphate saturation by addition of a saturated solution of ammonium sulphate. The resultant precipitate containing the activating system , was centrifuged at 8,500 g for 30 min at 2^{o}C, and the residue was dissolved in 0.02 M-Tris buffer, pH 7.6. The protein content was estimated according to the method of Lowry et al, (1951) and was always adjusted to a concentration of 5 mg/ml. The preparation was 'active' for four weeks when stored at 2^{o}C.

9. 'In vitro' methodology.

a) Preparation of tissue.

The method used for preparation of tissues was similar to that described by Wallace and Lieberman (1963). Each rabbit tissue was homogenised in ice-cold Krebs-Ringer solution, pH 7.4, using a ratio of tissue to buffer of 1 gm/ml, with an Ultra-Turrux Tissue disintegrator (Type TP/18-2) run at half speed for 3 x 10s periods. After centrifugation at 700 g for 10 min at 4^{o}C, the resulting supernatant was adjusted to contain 1 gm tissue/5 ml, as determined by the method of Lowry et al. (1951).

b) Incubation procedure.

200 µg of non-radioactive steroid (steroid to tissue ratio 1 : 2500) and 10,000 cpm of (4-^{14}C) radioactive tracer were added to each incubation tube, in ethanol. The solvent was removed under a stream of nitrogen and the steroids were dissolved in 0.1 ml 1-2-propanediol. To each tube, the following were added:

i) 0.1 ml 0.03M-K_2SO_4.

ii) 0.1 ml 0.005M-$MgCl_2$

iii) 0.1 ml 0.08M-ATP.

iv) 0.1 ml prepared PAPS

v) 2.5 ml of the tissue enzyme preparation equivalent to 500 mg of tissue, in 0.1M-phosphate buffer, pH 7.4.

Before the time curves for the sulphation of the synthetic 19-norprogestogens were attempted, substrate saturation curves were run on representstive tissues to determine the appropriate concentration of PAPS. A typical PAPS saturation curve for dehydroepiandrosterone sulphation in liver is shown in Fig. 3.1. PAPS was saturating at 0.1 ml, equivalent to 0.5 mg protein, for an incubation period of 60 min.

Incubations were performed in air at 37°C for the required time intervals. Blank incubations were identical to the above, except that boiled enzyme was added. Controls where enzyme or ATP were omitted were also run in parallel with each incubation. Each incubation was performed in duplicate. When extrahepatic tissues were examined, different tissues from the same animal were incubated simultaneously.

Enzyme reactions were arrested by the addition of 20 ml redistilled ethanol. The tissue precipitate was centrifuged at 500 g for 10 min at 4°C and the supernatant removed. After re-washing the tissue in ethanol, the combined ethanol fraction was evaporated 'in vacuo' at 30°C with the addition of 0.5 g of ammonium sulphate in order to prevent the breakdown of the steroid sulphates.

c) Isolation of conjugated and non-conjugated steroids.

Separation of the conjugated and non-conjugated steroids present in the incubation extracts, was accomplished by alumina-column chromatography (Edwards et al, 1953). The residues from the incubations were dissolved in 30 ml of ether-ethanol (3 : 1, $\frac{v}{v}$), and 15 g of ammonium sulphate was added to obtain 50% saturation. The ether-ethanol was poured directly on to neutral alumina (Merck, Darmstadt, Germany) columns (3 g; moisture content 2%) made up in toluene (height of column 10 cm, I.D. 1 cm). The columns were washed consecutively with 30 ml, absolute ethanol, which eluted the non-

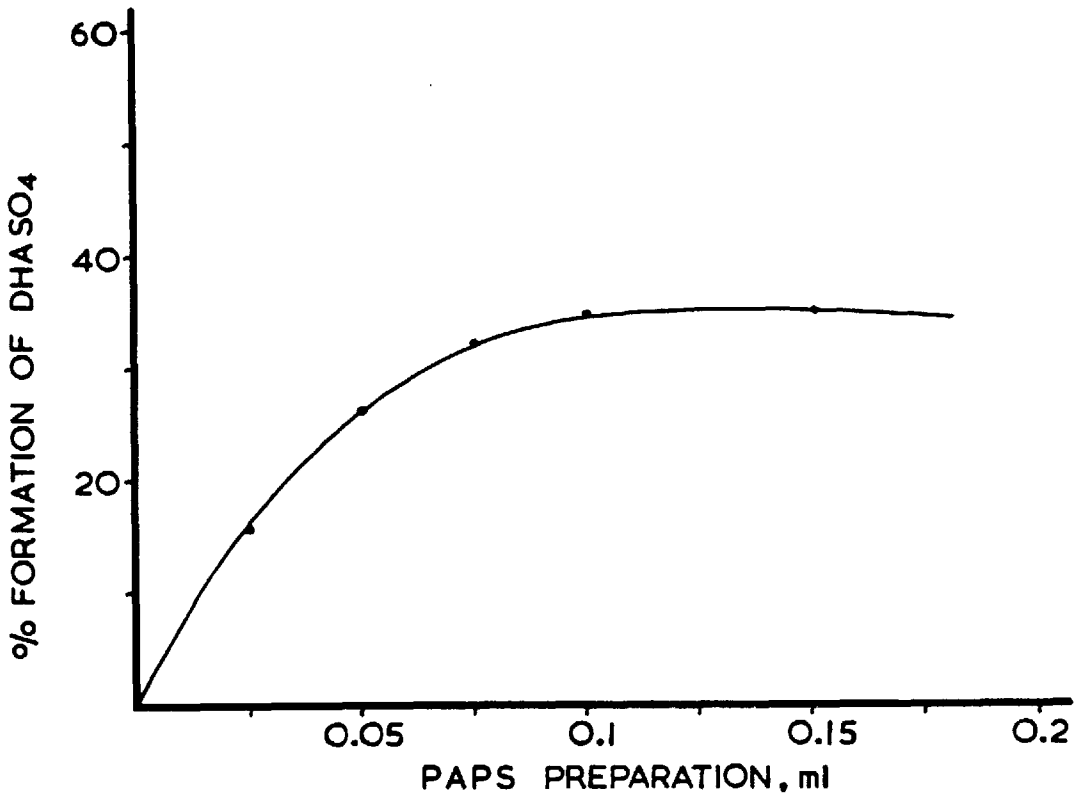


Fig. 3.1 PAPS saturation curve for DHA sulphating activity in the 700 g fraction of rabbit liver. Each sample was incubated for 120 min at 37°C containing 200 µg of DHA and 500 mg of protein.

conjugated steroids, and 50% ($\frac{v}{v}$) aqueous-ethanol (60 ml) which eluted the sulphate conjugates. The ether-ethanol and absolute ethanol fractions were combined. This fraction contained 98.2 $\pm$ 1% (40 experiments) of radioactive tracer added to blank incubations. The absence of significant protein binding of the steroids was indicated by the fact that 90-95% of the radioactive tracer could be extracted with ether-ethanol (3 : 1, $\frac{v}{v}$) from the combined supernatants after ethanol precipitation of proteins. Standard dehydroepiandrosterone sulphate added to the blank incubations, was almost completely recovered (95 $\pm$ 2%(10)) in the 50% ($\frac{v}{v}$) aqueous-ethanol phase, after alumina column chromatography.

10. Estimation of steroid conjugates.

The 50% ($\frac{v}{v}$) aqueous-ethanol phase containing the conjugated steroids, was dried 'in vacuo' at 35°C using a rotary evaporator. The residue was dissolved in 0.5 ml of redistilled ethanol. The sulphate conjugates were estimated by two methods:

a) Colorimetric procedure using methylene blue.

Estimation of the steroid conjugates was carreid out as described by Roy (1956a) using methylene blue reagent. To 1 ml of the aqueous colution of the steroid sulphate, 1 ml of methylene blue reagent was added. The mixture was extracted with 5 ml chloroform The colour intensity of the chloroform solution was determined, without dilution, using a Unicam SP 600 Spectrophotometor at 700 mμ, against a reagent blank. Non-conjugated steroids do not interfere with the colour reaction.

The sensitivity of the method for norethisterone sulphate, dl-norgestrel sulphate and dehydroepiandrosterone sulphate was between 2-50 μg. The precision of the method determined (from ten duplicate experiments) as the coefficient of variation, was 3.1%. For all the steroid conjugates, a dehydroepiandrosterone sulphate calibration curve was employed for quantitation, since reference dl-norgestrel sulphate and norethisterone sulphate were found to have the same extinction coefficients as dehydroepiandrosterone sulphate. Beer's Law was obeyed over a range of 2-50 μg/ml of steroid sulphate, for the three reference steroid sulphates.

The results were calculated as percent steroid sulphurylated according to the following equation:

$$\text{Percent of steroid sulphated} = \frac{\text{Weight of steroid sulphate} \times 100}{\text{Weight of original steroid-weight recovered.}}$$

b) Gas-liquid chromatographic estimation of conjugated steroids.

Gas-liquid chromatographic estimation of the steroid conjugates was carried out after dioxane solvolyses of the conjugates (Grant and Beall, 1950). Quantitation by this procedure has been described in Chapter 1. Aliquots of the incubation extracts were made 0.3M with respect to pyridinium sulphate and partitioned into chloroform (McKenna and Norymberski, 1960). The organic phase was evaporated to dryness. The residue was dissolved in 3 ml redistilled dioxane and kept at 38° for 18 hours. Chloroform was added to the solvolyses mixture and the solution was washed twice with redistilled water and dried with anhydrous sodium sulphate. After evaporation of the chloroform 'in vacuo', the residue was dissolved in redistilled ethanol and submitted to gas-liquid chromatography.

11. Estimation of non-conjugated steroids.

The non-conjugated steroids were estimated by gas-liquid chromatography as described in Chapter 1.

12. Estimation of radioactivity.

The procedure used has been described in Chapter 1.

13. Isolation of metabolic products.

The metabolic products were isolated by thin-layer chromatography as described in Chapter 1. The solvent system n-propanol: chloroform: methanol : IO N-ammonia (10 : 10 : 5 : 2, by vol.) was used for the conjugated steroids (Wusteman et al, 1964), and the solvent system cyclohexane : ethylacetate (1 : 1, $\frac{v}{v}$) for the

non-conjugated steroids. After development of the thin-layer plates, relevant areas were eluted with 30% ($\frac{v}{v}$) ethanolic-ether (conjugated steroids) or ethanol (non-conjugated steroids), and submitted to gas-liquid chromatography and radioactivity estimations.

14. Derivative formation.

The procedures used for acetylation, oxidation, reduction and trimethylsilyl ether formation have been described in Chapter 1.

15. Identification of metabolites.

The methods used for the identification of metabolites have been described in Chapter 1, and involved thin-layer and gas-liquid chromatography.

16. Identification of the position of the sulphate group in steroid sulphates formed.

The methodology employed was as described by Cronholm (1969). The sulphate conjugated steroids were acetylated as described in Chapter 1. Excess reagents were removed, and the steroids were solvolysed using dioxane (McKenna and Norymberski, 1960). The solvolysed steroids were then analysed by gas-liquid chromatography as non-derivatised steroids as well as trimethylsilyl ether derivatives.

17. Statistical treatment of data.

The data was statistically treated as described in Chapter 1.

RESULTS

Studies were carried out to compare the rate of sulphoconjugation of dehydroepiandrosterone (DHA) with the rate of sulphation of the synthetic oestrogen, ethynyloestradiol (EE) and the synthetic 19-norprogestogens, in the hepatic and extrahepatic tissues of the female rabbit. The position of the sulphate group on the conjugated steroids was also investigated.

1. Rate of sulphoconjugation in hepatic tissue.

The conditions of incubation have been described in the Methods section. The results of the time course study (0 - 120 min) of the rate of sulphoconjugation of the steroids examined in liver tissue are presented in Table 3.1, as percent (mean $\pm$ SD) of steroid recovered in the conjugated and non-conjugated forms. These values are compared in Fig. 3.2 (individual values are given in Appendix table 18).

The results show that under the conditions of incubation described, the sulphoconjugation of the steroids examined was low, although each steroid was conjugated by rabbit liver. The naturally occurring steroid dehydroepiandrosterone, was sulphated at a significantly faster ($p < 0.05$) rate than the synthetic 19-norprogestogens. Thus, at 30 min of incubation time, $10.0 \pm 1.4\%$ of dehydroepiendrosterone was sulphated; whereas, $7.0 \pm 0.8\%$ of norethisterone, $3.5 \pm 1.1\%$ of d-norgestrel, $2.8 \pm 1.2\%$ of dl-norgestrel, $2.0 \pm 0.6\%$ of l-norgestrel and $1.2 \pm 0.2\%$ of lynestrenol were conjugated. However, during the same period of time, $13.0 \pm 1.0\%$ of ethynyloestradiol was conjugated, which is more rapid than that observed for DHA ($10.0 \pm 1.4\%$) and the 19-norprogestogens.

The rate of sulphation was seen to increase with time. Thus, by 120 min of incubation time, $14.3 \pm 2.4\%$ of dehydroepiandrosterone was recovered as the sulphate. In comparison, $11.8 \pm 1.7\%$ of norethisterone, $9.4 \pm 1.3\%$ of d-norgestrel, $6.8 \pm 0.4\%$ of dl-norgestrel, $5.2 \pm 1.7\%$ of l-norgestrel, and $4.2 \pm 1.2\%$ of lynestrenol were recovered in the conjugated form. As seen at 30 min, the formation of the sulphate conjugate of ethynyloestradiol at 120 min was also more rapid ($17.3 \pm 2.3\%$) than that of DHA and 19-norprogestogens.

T A B L E 3.1 COMPARISON OF THE RATE OF SULPHO CONJUGATION OF DEHYDROEPIANDROSTERONE AND VARIOUS SYNTHETIC STEROIDS IN THE HEPATIC TISSUE OF THE FEMALE RABBIT. THE RESULTS ARE EXPRESSED AS MEAN (±SD) OF PERCENT STEROID RECOVERED. (number in parentheses denotes the number of specimens examined).

STEROID INCUBATED	PERCENT STEROID RECOVERED	TIME OF INCUBATION			
		0 min	30 min	60 min	120 min
Dehydroepiandrosterone	DHA	98.0±1.0 (5)	88.0±2.1 (5)	86.2±2.1 (5)	82.1±1.8 (8)
	DHA-SO_4	-	10.0±1.4 (5)	11.3±1.1 (5)	14.3±2.4 (8)
Ethynyloestradiol	EE	95.0±2.0 (5)	85.0±1.1 (5)	82.0±1.9 (5)	79.1±4.1 (22)
	EE-SO_4	-	13.0±1.0 (5)	14.8±0.8 (5)	17.3±2.3 (22)
Norethisterone	NET	96.0±1.0 (5)	89.0±2.4 (5)	86.2±2.4 (5)	84.1±5.1 (22)
	NET-SO_4	-	7.0±0.8 (5)	8.5±0.7 (5)	11.8±1.7 (22)
d-Norgestrel	d-Ng	95.0±1.2 (5)	90.0±1.4 (5)	88.0±2.9 (5)	84.0±4.5 (12)
	d-Ng-SO_4	-	3.5±1.1 (5)	7.0±1.4 (5)	9.4±1.3 (12)
dl-Norgestrel	dl-Ng	96.0±1.4 (5)	91.0±2.1 (5)	88.0±3.1 (5)	86.2±2.4 (4)
	dl-Ng-SO_4	-	2.8±1.2 (5)	5.0±1.1 (5)	6.8±0.4 (4)
l-Norgestrel	l-Ng	95.0±1.2 (5)	90.0±3.1 (5)	87.0±1.8 (5)	84.0±1.1 (11)
	l-Ng-SO_4	-	2.0±0.6 (5)	3.9±1.3 (5)	5.2±1.7 (11)
Lynestrenol	Lyn	96.0±1.3 (5)	92.0±1.4 (5)	90.1±1.2 (5)	88.4±2.1 (10)
	Lyn-SO_4	-	1.2±0.2 (5)	2.2±0.5 (5)	4.2±1.2 (10)

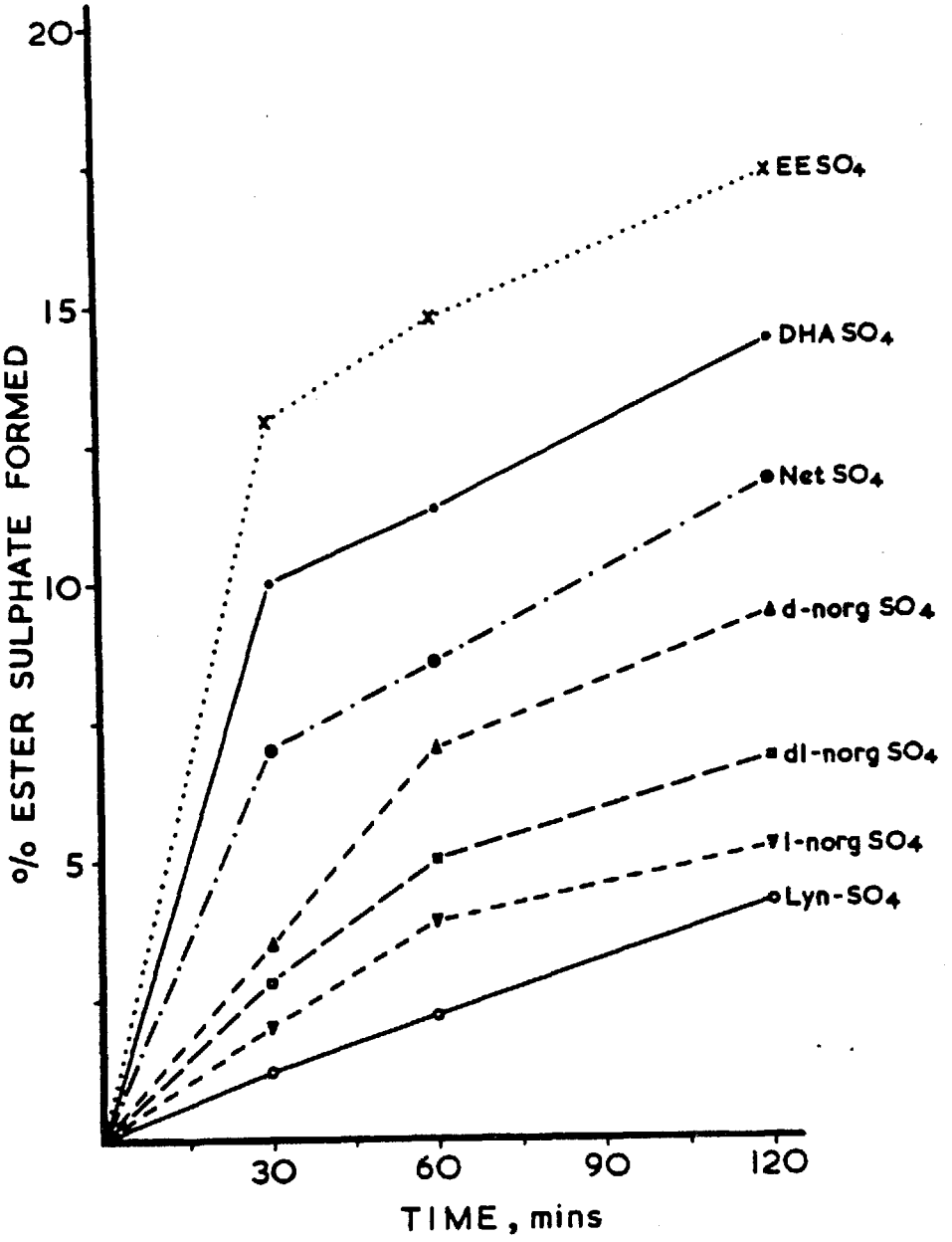


Fig. 3.2 Rate of formation of steroid sulphates with female rabbit liver 700 g fraction. Incubation conditions are described in the text.

The rate of sulphoconjugation at each time interval examined (30, 60 and 120 min) for dehydroepiandrosterone, was significantly different ($p < 0.05$, Appendix table 18), from that of ethynyloestradiol and the 19-norprogestogens examined.

In summary, the rate of sulphoconjugation of the synthetic oestrogen, ethynyloestradiol, is faster than that of the natural steroid dehydroepiandrosterone in female rabbit liver. However, the rate of sulphation of the 19-norprogestogens is slower than that observed for DHA.

2. Position of the sulphate group on the steroids conjugated by the liver of female rabbits.

The method employed for the identification of the position of the sulphate group on the steroids conjugated, was that of Cronholm (1969) and has been described in the Methods section.

Analysis of the sulphated steroids indicated that the natural steroid, dehydroepiandrosterone, was sulphoconjugated at the hydroxyl group present at C-3. From the incubations of the synthetic oestrogen, ethynyloestradiol, both mono- and di-sulphates were identified; and the results indicated that both the hydroxyl groups at C-3 and C-17 were conjugated. However, various 19-norprogestogens examined, were found to give rise to only mono-sulphates and were sulphoconjugated at the hydroxyl group at C-17.

In the incubation extracts from each of the steroids incubated with rabbit liver, no other metabolic products were recovered except the sulphate conjugated steroids and the non-conjugated steroids dehydroepiandrosterone, ethynyloestradiol, norethisterone, norgestrel and lynestrenol, the incubated steroids.

3. Rate of sulphoconjugation in extrahepatic tissues.

The rate of sulphoconjugation of dehydroepiandrosterone, ethynyloestradiol and the 19-norprogestogens was also investigated in the extrahepatic tissues of the female rabbit. As described in the Methods section, to keep the comparative values of these experiments, the steroid to tissue ratio (1 : 2500) and the incubation time (120 min) were kept constant. In all experiments, different organs from a single animal were examined simultaneously. The procedure used for the incubations was similar to that used for liver tissue, and has been described in the Methods section. The results expressed as percent steroid (mean $\pm$ SD) recovered in the forms of sulphates at 120 min, are shown in Table 3.2 and are also shown graphically in Fig. 3.3 (individual values are given in Appendix table 19).

Besides the liver, as has been discussed previously, the sulphoconjugation of dehydroepiandrosterone, ethynyloestradiol and various synthetic 19-norprogestogens was seen to occur only in the intestinal and lung tissues. Whereas, heart, spleen, adipose, skeletal muscle and kidney tissue were found to be devoid of sulphoconjugating activity under the described experimental conditions. Thus, after 120 min of incubation time, in stomach tissue $12.1\pm0.9\%$ of dehydroepiandrosterone was sulphated and $6.4\pm0.5\%$ of ethynyloestradiol. This is in contrast to the liver where ethynyloestradiol was seen to be conjugated more rapidly than dehydroepiandrosterone. In comparison, the percent conjugation of d-norgestrel was $10.8\pm1.3\%$, dl-norgestrel $4.1\pm0.9\%$, l-norgestrel $3.3\pm0.7\%$ and norethisterone $5.0\pm1.0\%$. Lynestrenol was not sulphated by this tissue. The mean values of sulphoconjugation obtained for the synthetic steroids were significantly different from that of dehydroepiandrosterone ($p < 0.05$, Appendix table 19) except the mean value of d-norgestrel.

The ileal tissue of rabbit was seen to conjugate only the natural steroid, dehydroepiandrosterone and the synthetic oestrogen, ethynyloestradiol. The percent steroids conjugated in this tissue was lower than that seen in the liver and stomach tissue. Thus, at 120 min of incubation time $5.1\pm0.9\%$ of dehydroepiandrosterone and $2.1\pm0.9\%$ of ethynyloestradiol were sulphated (Table 3.1). The mean value of dehydroepiandrosterone was significantly different from that of ethynyloestradiol ($p < 0.05$, Appendix table 19).

T A B L E 3.2 SYNTHESIS OF STEROID SULPHATES BY THE 700g FRACTION OF VARIOUS TISSUE HOMOGENATES OF THE FEMALE RABBIT. THE RESULTS OF THREE EXPERIMENTS ARE EXPRESSED AS MEAN (±SD) OF PERCENT STEROIDS RECOVERED AS SULPHOCONJUGATES.

PERCENT STEROID RECOVERED AS SULPHATE.	TISSUES EXAMINED.									
	LIVER	STOMACH	ILEUM	CAECUM	LUNG	HEART	SPLEEN	ADIPOSE	SKELETAL MUSCLE	KIDNEY
Dehydroepiandrosterone sulphate	14.3±2.4	12.1±0.9	5.1±0.9	3.2±0.7	12.2±1.0	0	0	0	0	0
Ethynyloestradiol sulphate	17.3±2.3	6.4±0.5	2.1±0.9	2.9±0.4	2.2±0.6	0	0	0	0	0
Norethisterone sulphate	11.8±1.7	5.0±1.0	0	0	1.7±0.5	0	0	0	0	0
d-Norgestrel sulphate	9.4±1.3	10.8±1.3	0	0	1.0±0.1	0	0	0	0	0
dl-Norgestrel sulphate	6.8±0.4	4.1±0.9	0	0	0	0	0	0	0	0
l-Norgestrel sulphate	5.2±1.7	3.3±0.7	0	0	0	0	0	0	0	0
Lynestrenol sulphate	4.2±1.2	0	0	0	0	0	0	0	0	0

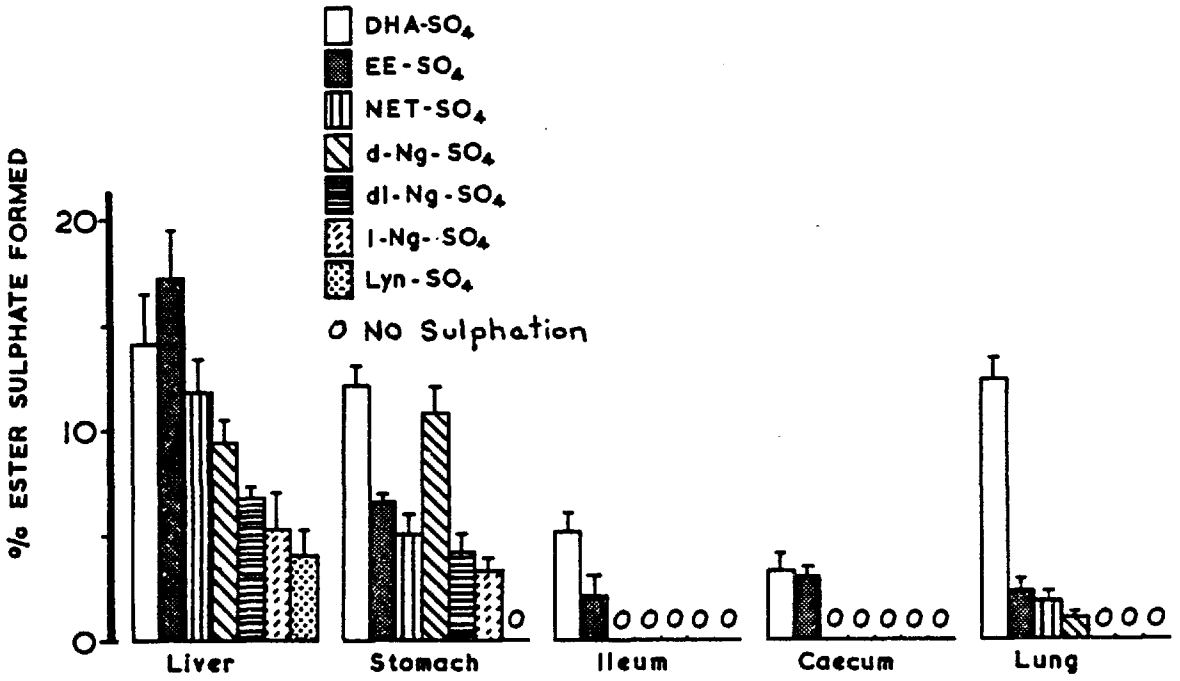


Fig. 3.3 Percent steroids recovered as sulphate conjugates from various tissues (700 g fraction) of female rabbit. The figure is based on the means ±SD given in Table 3.2.

Similarly, with the caecal tissues, the synthetic 19-norprogestogens were not sulphated, however, both dehydroepiandrosterone and ethynyl-oestradiol were found to be conjugated. Thus, $3.2\pm0.7\%$ of DHA and $2.9\pm0.4\%$ of EE were conjugated in 120 min by this tissue. In contrast to the other gastrointestinal tissues, the mean value of dehydro-epiandrosterone was not significantly different from that of ethynyl-oestradiol ($p<0.1$).

Besides the liver and stomach tissue, the only other tissue that sulphated the synthetic progestogens was lung tissue, although only norethisterone and d-norgestrel were conjugated. As in all the other tissues described previously, both dehydroepiandrosterone and ethynyl-oestadiol were also conjugated by lung tissue. The highest percentage of steroid conjugated was dehydroepiandrosterone ($12.2\pm1.0\%$, Table 3.2). This value is similar to that obtained in liver and stomach tissues. In contrast, only $2.2\pm0.6\%$ of ethynyloestradiol, $1.7\pm0.5\%$ of norethisterone and $1.0\pm0.1\%$ of d-norgestrel were seen to be conjugated by this tissue. The mean value obtained for dehydroepiandrosterone was significantly different ($p<0.05$, Appendix table 19) from the mean values obtained for ethynyloestradiol, norethisterone and d-norgestrel.

In all the other tissues examined, heart, spleen, adipose, skeletal muscle and kidney (Table 3.2) the sulphokinase activity towards dehydro-epiandrosterone, ethynyloestradiol and the synthetic 19-norprogestogens was not detectable by the method employed.

In summary, some extrahepatic tissues, particularly the intestinal tissue, appear to have sulphokinases which can conjugate the natural steroid, dehydroepiandrosterone and the synthetic oestrogen, ethynyl-oestradiol. Sulphokinases activity towards the synthetic progestogens was observed only in stomach and lung tissues. Heart, spleen, adipose, skeletal muscle and kidney tissues appeared to be devoid of activity.

In all the extrahepatic tissues that were seen to sulphoconjugate these steroids, the rate of sulphation of the natural steroid dehydro-epiandrosterone was significantly faster than that observed for the synthetic 19-norprogestogens.

4. Examination of the metabolic products of extrahepatic tissues.

The metabolic products of each steroid incubated with the extrahepatic tissues of the rabbit were examined chromatographically, as described previously in the Methods section. From the incubation extracts of dehydroepiandrosterone and synthetic 19-norprogestogens, only the mono-sulphates were detectable in stomach, ileal, caecal and lung tissues, when analysed by thin-layer chromatography. However, both the mono- and di-sulphates were seen to be formed with ethynyl-oestradiol by the stomach tissues, whereas, ileal, caecal and lung tissue formed only the mono-sulphates. No other metabolic products were identified except for the steroids incubated.

DISCUSSION

The formation of steroid sulphates 'in vitro' by crude preparations of rat liver has been known since the work of De Meio and Lewycka (1955), where the sulphation of dehydroepiandrosterone was demonstrated. In the same year, the sulphoconjugation of the phenolic hydroxyl group was reported by Segal (1955) in the 18,000 g fraction of rat liver. More recently, the ability of cell-free rat liver preparations to sulphate the steroidal 17-hydroxy group was observed by Wengle and Boström (1963).

The data from the present study show that both the phenolic hydroxyl group and the neutral hydroxyl groups of steroids can be sulphated under 'in vitro' conditions used. However, it is necessary to point out that the 'in vitro' conditions used for examining the sulphation of the synthetic oestrogen, ethynyloestradiol and the synthetic 19-norprogestogens, had been established as approximately ideal for the sulphation of dehydroepiandrosterone in rabbit liver.

Investigation of the rate of sulphoconjugation in rabbit liver 700g fraction shows that the secondary hydroxyl group in position 3β- in dehydroepiandrosterone was conjugated at a significantly faster rate than that observed for the tertiary hydroxyl group at C-17 (synthetic 19-norprogestogens) in the 17α-position. However, the rate of sulphation of ethynyloestradiol was relatively faster than DHA. In this steroid, both the phenolic hydroxyl group and the tertiary 17β-hydroxyl group were observed to be conjugated, but the rate of conjugation of each hydroxyl group was not determined (Table 3.1).

When the rates of sulphate conjugation of the synthetic 19-norprogestogens was compared, d-norgestrel was seen to be conjugated faster than dl-norgestrel and l-norgestrel. Out of the three forms of norgestrel, the biologically inactive enantiomer, l-Ng (Edgren et al., 1963 and De Johng et al., 1968) was observed to be the least conjugated. Amongst the 19-norprogestogens examined (Table 3.1) lynestrenol was the least sulphurylated, whereas, norethisterone was seen to be conjugated at the fastest rate, in rabbit liver.

Thus, the 17α-ethynyl group of the synthetic oestrogen, ethynyl-oestradiol, and the 19-norprogestogens does not appear to hinder sulphation of the 17β-hydroxyl group 'in vitro'. However, the structure of these steroids appears to influence the rate of sulphoconjugation.

Our findings can be compared with the results of Wengle and Boström (1963). These investigators had compared the rates of sulphation of a series of 17-hydroxy steroids, and found that 19-nortestosterone was sulphated approximately to the same extent as testosterone. They also observed that the introduction of an ethyl group into the 17α-position of 19-nortestosterone or of a methyl group into the 17α-position of testosterone, greatly decreased sulphation of the 17β-hydroxyl group. However, 17α-ethynyl-19-nortestosterone (NET) was sulphated to the same extent as testosterone and 19-nortestosterone. 17α-hydroxy-progesterone, having an acetyl group in the 17β-position showed a low degree of sulphation when compared to cis-testosterone.

That the sulphurylation of steroids follows certain structural rules has also been shown earlier by various investigators for other natural steroids (Schneider and Lewbart, 1956, Nose and Lipmann, 1958 and Roy, 1956b). Schneider and Lewbart (1956) had carried out detailed studies with particle-free rabbit liver extracts, and found evidence for the conjugation of fourteen out of thirty-two steroids examined. Amongst the corticosteroids examined, those with the Δ^5-3β-hydroxy system were more extensively conjugated than a number of related fully saturated steroids. Sulphate conjugation occurred more rapidly when the steroid belonged to the allo-series (A/B ring fusion, trans); and conjugation was not limited to carbon-3 hydroxyl group, since testosterone and deoxycorticosterone were also sulphated. Both Roy (1956b) and Wengle and Boström (1963) have demonstrated that cis-testosterone having a hydroxyl group in the 17α-position was sulphated to a greater degree than testosterone in rat liver.

That the rate of sulphoconjugation of steroids appears to be dependant on structure was also demonstrated by Nose and Lipmann (1958) in rabbit liver. Thus, of a series of 3β, ring-A unsaturated and saturated steroids, dehydroepiandrosterone was conjugated at the fastest rate (3β-hydroxy) and 3β, 5β-aetiocholanolone at the slowest rate. This investigation also indicated, as did the later study of Wengle and Boström (1963) in rat liver, that a double bond between the fourth and fifth carbon atoms inhibited sulphation of the 3β-hydroxyl group (Wengle and Boström, 1963) and the 17β-hydroxyl group (Nose and Lipmann, 1958).

Our 'in vitro' data with rabbit liver also supports the previous studies of Schneider and Lewbart (1958), Roy (1956) and Wengle and Boström (1963) with rat liver, and Nose and Lipmann (1958) in rabbit liver, that sulphate conjugation follows certain rules. Thus, our results indicate that the absence of the 3-oxo group as in lynestrenol inhibited sulphation of the 17β-hydroxyl group, whereas, a double bond at C4-5 and the 17α-ethynyl group in norethisterone did not appear to hinder conjugation of the 17β-hydroxyl group. The presence of the asymmetric carbon atom at position 13 in norgestrel appeared to inhibit conjugation of the 17β-hydroxyl group, so that this progestogen was conjugated to a lesser extent than norethisterone in rabbit liver. In contrast, Kamyab et al, (1967) have shown that in 'in vivo' conditions a higher percent of administered norgestrel (59%) was obtained after acid hydrolysis than norethisterone (45%) in rabbit urine. The asymmetric carbon atom at position 13 also appears to affect the conjugation of the various forms of norgestrel. Thus, the biologically active, d-Ng (Edgren et al., 1963 and De Johug et al., 1968) was sulphated at a faster rate than the biologically inactive enantiomer, l-Ng. The rate of sulphation of dl-norgestrel was found to be intermediate.

Segal (1955) demonstrated the sulphate conjugation of the phenolic hydroxyl group of oestrone-16-C^{14} in rat liver. In 1958, De Meio et al presented evidence that two sulphate esters were formed when oestradiol-17β was incubated with microsome-free extracts of rat liver. The two products had markedly different electrophoretic mobilities; they were not identified further. However, Payne and Mason (1963) have shown that both the phenolic hydroxyl group at C-3 and the hydroxyl group at C-17 of oestradiol were conjugated, giving rise to both mono- and di-sulphates, in rat liver. Also shown in their study was the formation of mono- and di-sulphates of the synthetic oestrogen diethylstilbesterol. The sulphoconjugation of oestrogens was also demonstrated in rat liver, by Wengle and Boström (1963). Both 17α- and 17β-oestradiol were sulphated giving rise to detectable amounts of mono-sulphates, and 17α-oestradiol was sulphated to a greater extent than the 17β-epimer. Di-sulphate formation of both compounds was extremely weak. Similar to the observations of Payne and Mason (1963) in rat liver, our study with rabbit liver also demonstrated the formation of both mono- and di-sulphates of the synthetic oestrogen, ethynyl-oestradiol. However, in contrast to the study of Wengle and Boström in rat liver, although quantitative studies were not performed by us,

our semi-quantitative studies indicated that the rate of formation of ethynyloestradiol-3-sulphate was greater than the formation of ethynyloestradiol-17-sulphate, and the amount of the di-sulphate was significant.

It has been shown by many investigators, in both man and several species of animals, that several other tissues apart from the liver can sulphate steroids under 'in vitro' conditions. Thus, sulpho-conjugation has been demonstrated in the human adrenal (Adams, 1963, 1964 and Boström and Wengle, 1967), adrenal and jejunal mucosa of the ox (Holcenberg and Rosen, 1965) and jejunal mucosa of the adult human (Boström and Wengle, 1967). The existence of sulphotransferases has been demonstrated in the human ovary (Wallace and Silberman, 1964) and testis (Dixon et al., 1965).

In the present study, amongst the extrahepatic tissues of the rabbit investigated, the stomach, ileal, caecal and lung tissues showed a certain degree of sulphoconjugating activity towards some of the steroids investigated (Table 3.2). The stomach tissue conjugated the natural steroid dehydroepiandrosterone, the synthetic oestrogen ethynyloestradiol and various synthetic 19-norprogestogens. As seen in the liver, the rate of conjugation of DHA was faster than the sulphation of the 19-norprogestogens; however, ethynyloestradiol was conjugated at a slower rate than DHA, in contrast to the liver. Also in contrast to the results obtained in the liver; of the various synthetic 19-norprogestogens examined, d-norgestrel was conjugated at a faster rate than norethisterone; and lynestrenol was not sulphated at all. Comparing the mean values of the rate of sulphoconjugation of DHA and d-Ng in stomach and liver, the values obtained were not significantly different. Thus, as observed in the liver, the secondary hydroxyl group at C-3 (DHA), and the tertiary hydroxyl group at C-17 (19-norprogestogens) were found to be conjugated in rabbit stomach tissue. Both the mono- and di-sulphates, were formed in small amounts with ethynyloestradiol, indicating the occurrence of sulphoconjugating enzymes which can conjugate, 'in vitro' the phenolic hydroxyl group and the 17β-hydroxyl group of oestrogens.

In contrast, both the ileal and caecal tissues (Table 3.2) only conjugated DHA and EE, and their rates were lower than those seen in the liver and stomach. Only the mono-sulphates were identified. Although

the position of the sulphate group on ethynyloestradiol was not fully analysed, thin-layer chromatography indicated that this may have been conjugated at C-3. The formation of mono-sulphates only, may explain the lower conjugation of ethynyloestradiol.

Sulphate conjugating enzymes are known to occur in the mucosa of the gastrointestinal tract of several species. Our studies support the finding of Holcenberg and Rosen (1965) who have shown the presence of DHA sulphokinase activity in the bovine ileum, caecum, jejunum and the duodenum. However, in contrast to our studies where sulpho-conjugation of the 17β-hydroxyl group was demonstrated only in the stomach tissue, no sulphoconjugating activity for the 17β-hydroxyl group of testosterone was found in the intestinal tissues of the ox. In contrast, both 3β-hydroxyl and 17β-hydroxy sulphation was demonstrated in the jejunal mucosa of adult humans (Boström and Wengle, 1967).

Phenol sulphokinase have been demonstrated in the intestine of rabbits (Nose and Lipmann, 1958), in sheep colonic mucosa (Kent and Pasternak, 1958) and the bovine digestive tract (Holcenberg and Rosen, 1965). The phenol and oestrone sulphokinases have also been demonstrated in various parts of the adult human gastrointestinal tract (Boström et al., 1968), such as stomach, jejunum, ileum and colon; maximal oestrone sulphokinase activity being found in the jejunal mucosa. This activity amounted to almost 50 percent of that of the adult human liver. Our present study supports these findings in humans, since all the three rabbit intestinal tissues examined i.e. the stomach, ileal and caecal tissues, were capable of conjugating ethynyloestradiol.

Little evidence is available in the literature for sulpho-conjugating activity of lung tissue. Holcenberg and Rosen (1965) have shown that bovine lung tissue can conjugate the phenolic hydroxyl group of oestrone, but not the 3β-hydroxy group of DHA or the 17β-hydroxy group of testosterone. In contrast to the bovine tissue, the lung tissue of the rabbit (Table 3.2) can sulphate the 3β-hydroxy group of DHA. The rate of sulphation being almost as much as seen in the liver and stomach. The phenolic hydroxyl group of EE and the 17β-hydroxy group of norethisterone and d-norgestrel were also sulphated to a minor extent. It is possible that this difference in the results obtained for ox lung (Holcenberg and Rosen, 1965) and our study with rabbit lung, may be due to a specie difference. Thus, the rabbit lung tissue may have greater DHA sulphokinase activity than bovine lung tissue.

Bovine spleen and kidney tissues were also shown (Holcenberg and Rosen, 1965) to have some oestrone sulphokinase activity. Human spleen and kidney also appear to conjugate oestrone, but only to a minor extent (Boström and Wengle, 1967). Leung et al., (1972) demonstrated oestradiol conjugation in adult human spleen. Some DHA sulphokinase activity was demonstrated in bovine kidney (Holcenberg and Rosen, 1965), however, this activity was not demonstrated in adult human kidney (Boström and Wengle, 1967) or spleen. This finding is in contrast to Leung et al. (1972), who have demonstrated DHA sulphokinase in adult human spleen. In comparison to these studies, our present study supports the findings of Segal (1955) in rat spleen, kidney and heart that rabbit spleen, kidney, heart, skeletal muscle and adipose tissues also cannot sulphate oestrogenic steroids. In addition no such activity towards DHA or the synthetic 19-norprogestogens was found in these tissues of the rabbit.

Thus, we have shown that other rabbit tissues, besides the liver (Table 3.2) have sulphokinases, which transfer the sulphate group to various types of steroidal hydroxyl groups. However, the extent to which each steroid hydroxyl group (3β-hydroxy, phenolic and 17β-hydroxy) act as acceptors differs. As discussed earlier, the sulpho-conjugation may be dependant on the structure of the steroids. In addition, it may also depend on the specificity of the enzymes involved. Nose and Lipmann (1958) reported the separation of at least three different sulphokinase fractions from rabbit liver. One transferred the sulphate to DHA, androsterone and pregnenolone; the other two which were incompletely separated, transferred the sulphate to simple phenols and oestrone. Therefore, it is likely that the differences seen in the conjugating capacity of various rabbit tissues may also be dependent on the distribution of these enzymes in these tissues.

As mentioned earlier, the pH of the incubation mixture (7.4) was chosen to give optimum conditions for DHA sulphokinase. Although it was also realised that this pH may not necessarily be the optimal one for studying the sulphokinase activity toward the synthetic steroids. The practical advantage of having only one procedure for assaying phenol sulphokinase and various steroid alcohol sulphokinases was considered to outweigh the possible drawback of not being able to assay some of these enzymes under 'optimal' pH conditions. Furthermore,

the main purpose of our investigation was to make comparative studies between various tissues. Therefore, it is possible that the low sulphoconjugation of the 19-norprogestogens by these tissues, may at least in part, be due to the incubation conditions.

Our study with rabbit tissues has shown that although sulphoconjugation of the steroids examined did occur, the amount of steroid sulphated was low. Both Savu et al.(1968) and Quamme et al.(1971) have reported that the rabbit liver in 'in vitro' conditions, forms phenolic glucuronides more rapidly than the sulphates.

Finally, it should be emphasised that both the natural (Samuels and Eik-Nes, 1968) and the synthetic steroids (Fotherby and James, 1972 and Thijssen, 1972) undergo transformations to ring-A reduced products. These are then conjugated (Samuels and Eik-Nes, 1968) in liver (Berliner and Dougherty, 1961). However, such transformations evidently do not occur in unfortified cell-free extracts of liver (Berliner and Dougherty, 1961). In our study, no other metabolic products were detected, other than the steroid incubated (Table 3.1) in the absence of an NADPH generating system. This absence of metabolites may account for the low sulphation of the steroids examined. Thus, the 'in vitro' ability of tissues to form conjugates, is not a reliable indication of the types of conjugates excreted by the animal under 'in vivo' conditions.

CHAPTER 1

'IN VIVO' METABOLISM OF (4-^{14}C) NORETHISTERONE OXIME

INTRODUCTION

In this chapter, the 'in vivo' metabolism of (4-^{14}C) norethisterone oxime (NET-OX) racemate, 'anti' and 'syn' isomers in rabbits is described. After the intraperitoneal administration of NET-OX the amounts and nature of the radioactivity excreted in urine and faeces were investigated. The concentration of NET-OX in plasma was also examined.

MATERIALS AND METHODS.

1. Animals.

Female New Zealand white rabbits were housed in metal cages and transferred to metabolic cages when required for experimentation. The weights of the animals ranged from 2.5 kg to 4.0 kg. Each animal received a single dose of 1.5 μCi (4-^{14}C) NET-OX racemate, 'anti' or 'syn' isomer intraperitoneally, dissolved in 0.1 ml ethanol and 0.9 ml physiological saline. The animals were kept in the metabolic cages with free access to food and water. Total urine and faeces were collected daily over a five-day period and these samples were stored at 4°C until processed. Blood samples were also taken from the marginal ear vein of rabbits 7 ('anti' isomer) and rabbit 6 ('syn' isomer) at 2, 4, 7 and 24 hours. The syringes and needles used for steroid injection were washed thoroughly with ethanol and remaining radioactivity estimated. Thus, a value for the total dose administered was obtained and in no case did the non-administered residue amount to more than 1% of the administered dose.

2. Chemicals.

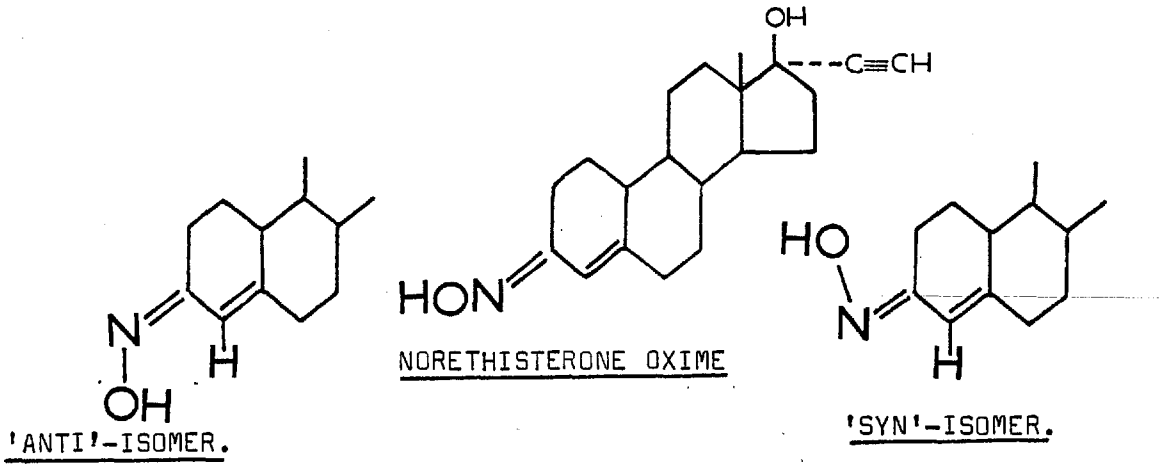
a) Steroids

(4-^{14}C) NET-OX was prepared (as a mixture of 'anti' and 'syn' isomers) from (4-^{14}C) norethisterone having the specific activity of 0.3 μCi/mg. Other steroids were obtained as described in Section 1, Chapter 1.

Preparation of (4-^{14}C) Norethisterone oxime.

29.8 mg norethisterone, 5 μCi (4-^{14}C) norethisterone (sp. act. 3.8 μCi/mg) and 7.3 mg hydroxylamine hydrochloride (mol. wt. 69) were dissolved in 0.25 ml anhydrous redistilled pyridine and warmed on a steam bath for 2.5 min. 1 N-hydrochloric acid was added to the mixture placed in an ice-bath, and the precipitate obtained was extracted with sodium carbonate (A.R. grade), acidified

T A B L E 1.1 CHARACTERISTICS OF THE PREPARED N O R E T H I S T E R O N E O X I M E



NORETHISTERONE OXIME

'ANTI'-ISOMER.

'SYN'-ISOMER.

1. Elemental Analysis: Anal. calcd. for C_{20} H_{27} NO_2: C, 76 64%; H, 8.68%; N, 4.47%. Found: NET-OX racemate: C, 76.95%; H, 8.6%; N, 4.0%. NET-OX 'anti'-isomer: C, 75.9%; H, 9.0%; N, 3.9%. NET-OX 'syn'-isomer: C, 76.4%; H, 8.7%; N, 4.1%.

2. λ Ultraviolet Max.: NET, 258 mμ; NET-OX racemate, 264 mμ; NET-OX 'anti'-isomer, 263 mμ; NET-OX 'syn'-isomer, 264 mμ. Reduced NET-OX racemate, 262 mμ; NET-OX 'anti'-isomer, 261 mμ; NET-OX 'syn'-isomer, 262 mμ.

3. ν Infrared Max.: KBr: NET, 1650 cm^{-1} (3-Ketone); NET-OX, 1630 cm^{-1} (no distinction in the three forms).

4. Nuclear Magnetic Resonance Spectrum: (δ, chemical shifts in ppm downfield from internal TMS[2]): NET-OX racemate, 10.37, 10.11, 6.39, 5.78; NET-OX 'anti'-isomer 10.37, 5.78; NET-OX 'syn'-isomer, 10.11 and 6.39. (The oxime OH-proton in the 'anti' form would be relatively deshielded by the double bond and would resonate at a lower field (10.37) than the 'syn'-isomer (10.11): conversely, the vinyl proton of 'anti'-isomer would be relatively shielded by the OH-proton and would resonate at a higher field (5.78) than 'syn' (6.39.

5. G.I.C. Retention Times as TMS Ethers: NET-OX racemate 12.6; 'anti'-siomer 13.0; 'syn'-isomer 12.8.

6. T.l.c. Data : Non-derivatised NET-OX 'anti'-isomer, R_F 0.51; NET-OX 'syn'-isomer R_F 0.43. TMS ethers, NET-OX 'anti'-siomer, R_F 0.77 and NET-OX 'syn'-isomer R_F 0.68.

7. Acid Hydrolysis: NET-OX hydrolysed to NET.

with 0.1 N-hydrochloric acid and extracted with redistilled ether. The compound was obtained in crystalline form. The 'anti' and 'syn' isomers were separated from the racemic mixture by preparative thin layer chromatography and recrystallised. After obtaining the specific activity by radioactivity estimation and quantitation by gas-liquid chromatograpny, the purity of the prepared (4-^{14}C) NET-OX, racemate, 'anti' and 'syn' isomers was established by elemental analysis, ultra-violet spectrometry, infra-red spectrometry, nuclear magnetic resonance spectrometry, mass spectrometry, gas-liquid chromatography and thin-layer chromatography. Table 1.1 shows the characteristics of the prepared (4-^{14}C) NET-OX, racemate, 'anti' and 'syn' isomers. In addition, treatment of the prepared (4-^{14}C) NET-OX with IN-HCl gave, after 30 min at 37^{o}C, a compound similar in chromatographic properties to the starting material norethisterone.

b) Organic solvents.

All organic solvents were A.R. grade and were purified by distillation as described in Section I, Chapter I.

c) Other chemicals.

All other chemicals used were analytical reagent grade.

d) Pyridinium sulphate.

The procedure for the preparation of pyridinium sulphate has been described in Section I, Chapter 3.

3. β-glucuronidase enzyme preparation.

The β-glucuronidase enzyme preparation was obtained from limpets (Patella vulgata)by the method of Fotherby and Love (1960) and was estimated to have an activity of 1 x 10^{6} units/g by the method of Fishman et al. (1948).

4. Estimation of radioactivity.

Radioactivity was estimated in the samples by liquid scintillation counting.

a) Organic samples.

Organic samples were estimated in toluene-based scintillator as described in Section 1, Chapter 1.

b) Aqueous samples.

For the estimation of radioactivity in aqueous samples, a Triton-based scintillator consisting of 6.0 g 2,5-diphenyloxazole (PPO) and 0.4 g of 1, 4-bis (2 - (5-phenyloxazolyl)-benzene) (POPOP) in one litre of toluene and 0.5 litre of Triton (Triton x-100, Koch-Light Ltd., London). With each type of sample (organic or aqueous), sufficient counts were recorded to give a standard error of less than 5%. The method for estimation of radioactivity has been described in Section 1, Chapter 1. The degree of quenching in urine samples did not exceed 10%. A higher degree of quenching was obtained for the faecal samples (40-45%).

5. Estimation of radioactivity in urine and plasma samples.

The procedure used for the estimation of total radioactivity in urine and plasma was as described by Kamyab et al. (1967) except that 10 ml of Triton-based scintillator was used. Aliquots of urine (1 ml) and plasma (0.5 ml) from each collection were assayed directly in the scintillator.

6. Processing of urine.

a) Extraction of non-conjugated radioactivity.

After aliquots had been removed for estimation of total radioactivity, 20 ml of urine from each day's collection or 80% of urine from day 1 and 2 were combined, and extracted with chloroform (2 x 2 vol). The chloroform phase was washed once with 0.1 vol.

water, and dried with anhydrous sodium sulphate. A measured aliquat was evaporated to dryness, the residue dissolved in 0.1 ml of ethanol, 10 ml of toluene-based scintillant added, and the radioactivity estimated.

b) Extraction of conjugated radioactivity.

The procedures described by Kamyab et al. (1967) and Reed et al.(1972) for hydrolysis and extraction of conjugated radioactivity, were used in our preliminary work. These procedures were compared to the amounts of radioactivity extracted after purification of urine using neutral ion-exchange resin chromatography as described by Bradlow (1968). The comparative data indicated that the purification of urine by ion-exchange chromatography resulted in 50% greater recovery of radioactivity following β-glucuronidase enzyme hydrolysis than that obtained without prior purification of urine. Ion-exchange chromatography of urine removes 90% of the urinary solids (Bradlow, 1968). The advantage of the method is that the steroids are recovered by absorption onto the neutral resin and eluted, with substantial purification ,by a volatile solvent (methanol) for easy subsequent concentration.

Thus ,in the present study, the method of Bradlow (1968) using neutral ion-exchange resin for extraction of steroid conjugates was employed for the purification of urine samples prior to the hydrolytic procedures.

i) Purification of urine using neutral ion-exchange resin chromatography.

The aqueous phase remaining (20 ml) after the removal of 'non-conjugated' radioactivity was passed through glass columns (1 cm I.D x 22 cm, with a stop-cock and a 50 ml reservoir) containing 15 g of Amberlite XAD-2 resin (B.D.H. Ltd., Poole, Dorset) (Glasswool was placed above and below the resin). The column was washed successively with 2 vol.of water and 2.5 vol. of methanol. The conjugated material was removed in the methanol phase. When larger (1 litre) volumes of urine were processed, a column (2.5 cm I.D x 60 cm, with a 100 ml reservoir) containing 50 g of resin slurried in water was employed. The flow rate was kept constant at 20 ml/min.

The recovery of the steroids from the column was measured by adding 10,000 cpm of (4-^{14}C) NET-OX to 20 ml of blank rabbit urine sample and processing it as described above, or processing 20 ml of rabbit urine sample from day 1. From five assays, the mean recovery was 88.9 $\pm$ 4% (S.D.) and there was no difference in the recovery of 'non-conjugated' and 'conjugated' radioactivity.

The influence of flow rate and resin volume was also examined. In the first series of experiments, the flow rate of urine through the column was varied, while the flow rates of water and methanol used for washing and elution, were kept constant. This was followed by variation of the flow rate of water and methanol. The results indicated that variation of flow rates of urine, water or methanol did not affect the total recovery of the steroid.

Aliquots of urine from day 1 were used to check the influence of resin volume. The results indicated that volume of resin was not critical.

c) Hydrolysis of urine.

i) β-glucuronidase enzyme hydrolysis of conjugated radioactivity.

The methanolic phase from the ion-exchange columns was concentrated 'in vacuo' at 40°C, using a rotary evaporator. The residue was dissolved in water and adjusted to pH 4.7 (Kamyab, et al., 1967). 1 ml of 5M-acetate buffer, pH 4.7 and 10,000 units / ml of a β-glucuronidase enzyme preparation was added. After incubation at 37°C for 48 hours, the urine was extracted with chloroform and treated as the freely extractable non-conjugated radioactivity fraction, described above. The water wash of the chloroform extract was combined with the residual aqueous phase and enzyme hydrolysis repeated, followed by chloroform extraction. Aliquots of the chloroform phase were taken for the estimation of radioactivity and various chromatographic procedures.

ii) Extraction and hydrolysis of non β-glucuronidase enzyme hydrolysable radioactivity.

a) Ethanolic ether:extraction.

The aqueous phase remaining after β-glucuronidase enzyme hydrolysis was treated according to the method of Edwards et al. (1953) to remove the sulphate conjugates. Ammonium sulphate was added to urine to a final concentration of 50%, $(\frac{w}{v})$. The urine was then extracted with a 3 : 1 $(\frac{v}{v})$ mixture of diethyl-ether and ethanol. The organic phase was dried and the conjugates solvolysed at 37°C for 24 hours in 5 ml dioxane (Grant and Beall, 1950). The dioxane was diluted with 20 ml chloroform, washed with water (2 ml), 2 N-sodium hydroxide (5 ml) and water (5 ml). Dried aliquots were taken for estimation of radioactivity and chromatography.

b) Pyridinium sulphate extraction.

The amount of radioactivity in urine conjugated to sulphuric acid was also estimated , using the pyridinium sulphate extraction procedure (Menini and Norymberski, 1965). The aqueous phase remaining after enzyme hydrolysis was made 0.3 M with respect to pyridinium sulphate and extracted with chloroform (0.6 vol.). The chloroform phase was dried with anhydrous sodium sulphate, evaporated to dryness, and the sulphate conjugates solvolysed with dioxane as described above.

7. Extraction and estimation of radioactivity in faeces.

a) Estimation of total radioactivity.

Faeces from each 24 hour collection were weighed, diluted with an equal volume of water and the mixture homogenised. An Ultra-turrax type TP/18-2 tissue homogeniser was used, at maximum speed for 6 x 15s periods with 10s intervals between each period of activity. The mixture was centrifuged for 20 min and 500 g at 4°C and the ethanol removed. The homogenisation and centrifugation procedures were repeated, the ethanol phases combined and evaporated to dryness. The residue was dissolved in a known volume of ethanol and aliquots taken for estimation of radioactivity.

Highly pegmented extracts were partitioned between 70% ($\frac{v}{v}$) aqueous methanol (Kamyab et al., 1967) and hexane. The methanol phase was reduced to one-tenth of its volume. Water was added to give a solution of approximately 10% methanol in water and the mixture was extracted with 2 vol. chloroform. This phase was treated as described for urine. Aliquots were taken for radioactivity estimations.

b) Extraction of non-conjugated radioactivity.

The methanol phase obtained was reduced to a small volume and the residue dissolved in water. Non-conjugated radioactivity was extracted as described for urine.

c) Extraction of conjugated radioactivity.

The remaining aqueous phase, after extraction of non-conjugated radioactivity, was hydrolysed with β-glucuronidase enzyme and treated as described for urine.

8. Extraction and estimation of radioactivity in plasma.

Plasma (2 ml) was extracted with 10 ml of chloroform to remove the non-conjugated radioactivity (Kamyab et al.,1967). The chloroform phase was treated as described previously. Conjugated radioactivity was estimated in the aqueous phase remaining as described previously.

9. Chromatographic procedures.

a) Ion-exchange column chromatography.

Purification of urine was achieved as described earlier using the method of Bradlow (1968).

b) Thin-layer chromatography.

Thin-layer chromatography on Silica gel G plates in the system toluene:acetone (4 : 1, $\frac{v}{v}$) was used to chromatograph norethisterone oxime (three forms) and metabolites in the extracts.

of urine. The procedure used for chromatography has been described in Section I, Chapter I. Autoradiographs were prepared by exposing each plate to Kodirex-Autoprocess X-ray film (Kodak, London) for 10 days, as described in Section I, Chapter 2. In addition, the thin-layer plates were scanned for radioactivity and relevant areas were eluted in ethanol. Aliquots were taken for radioactivity estimation, gas-liquid chromatography and derivative formation.

c) Paper chromatography.

Paper chromatography was carried out as described by Zaffaroni (1953) using a formamide:chloroform system, of the urine and faecal extracts. Chromatography paper (Whatman No. 42) was immersed in a solution of formamide:methanol (1 : 1, $\frac{v}{v}$) to impregnate the paper evenly with formamide. Methanol was removed by drying the paper for one hour at 37°C. The crude extract was applied at the origin in a 1 cm wide band and the paper was developed with chloroform for 7-8 hours. Reference steroids were run in parallel with each chromatogram. After development, the papers were scanned for radioactivity as described in Section I, Chapter I. The relative proportion of each metablite was calculated by triangulation and the relevant areas eluted for gas-liquid chromatography.

d) Gas-liquid chromatography.

This procedure has been described in Section I, Chapter I. An oven temperature of 230°C was used to chromatograph norethisterone oxime as trimethylsilyl ethers. Other conditions were as described previously .

10. Isolation, identification and semi-quantitation of metabolic products.

The metabolites and (4-^{14}C) NET-OX (three forms) in the urine and faecal extracts were chromatographed on thin-layer plates and paper as described above. The main metabolite from the urine fraction was treated in the following ways:

a) The extract was hydrolysed with concentrated hydrochloric acid (15%, $\frac{v}{v}$) for 30 min in a boiling water bath. After cooling, the aqueous phase was extracted with chloroform, and the remaining aqueous phase was treated again with acid. The combined chloroform phase was dried and dissolved in ethanol. The hydrolysed product was analysed by t.l.c. and g.l.c. and compared to reference steroids.

b) The acid hydrolysed product was reduced with sodium borohydride as described in Section I, Chapter 1. The reduced product was again rigourously analysed.

In both procedures the recoveries were calculated by treating a standard amount of radioactive (4-^{14}C) NET-OX in a similar manner to that of the extract.

For semi-quantitation of products in the urinary and faecal extracts, the areas of radioactivity on paper chromatograms were measured by triangulation.

11. Statistical treatment of data.

The data was statistically treated as described in chapter 1.

RESULTS.

1. Urinary excretion of radioactivity following intraperitoneal administration of (4-^{14}C) norethisterone oxime (NET-OX) to female New Zealand white rabbits.

After intraperitoneal administration of (4-^{14}C) norethisterone oxime racemate, 'anti'- and 'syn'-isomers to female rabbits, the daily excretion of radioactivity was estimated in the urine samples as described in the Methods section. The individual and mean ($\pm$SD) values obtained for each steroid are shown in Table 1.2 and the mean values are compared in Fig 1.1. Each rabbit received a single dose of 1.5 µCi (sp. act. 0.3 µCi/mg) of (4-^{14}C) norethisterone oxime. Over a period of five days about 55% of the administered dose of the three forms of NET-OX was recovered in the urine (56.3$\pm$7.7% NET-OX racemate, 56.2$\pm$1.9% 'anti'-isomer and 55.7$\pm$6.2% of the 'syn'-isomer). Of the total dose excreted in urine, 80% was excreted in the first 24 hours (Fig. 1.1). By day 5, the level of radioactivity excreted in urine had declined to less than 3% (1.1$\pm$1.5% NET-OX racemate, 1.6$\pm$1.2% 'anti'-isomer and 2.7$\pm$3.5% of the 'syn'-isomer). Thus, the pattern of daily excretion and the total radioactivity recovered for the three forms of (4-^{14}C) norethisterone oxime was seen to be similar.

2. Fractionation of urinary radioactivity.

a) Pooled urine from day 1 and 2

Since the major part of radioactivity was excreted on days 1 and 2 the urine samples were combined separately for the three administered forms of (4-^{14}C) norethisterone oxime. As described in the Methods, section, the non-conjugated (free) radioactivity was extracted with chloroform. The conjugated radioactivity was extracted after β-glucuronidase enzyme hydrolysis and dioxane solvolysis. In Table 1.3 are shown the individual and mean ($\pm$SD) values obtained for the amounts of radioactivity present. The mean values are compared in Fig. 1.2.

T A B L E 1.2

DAILY EXCRETION AND TOTAL RECOVERY OF RADIOACTIVITY IN URINE AFTER ADMINISTRATION OF (4-^{14}C) NORETHISTERONE OXIME RACEMATE, 'ANTI'-ISOMER AND 'SYN'-ISOMER, EXPRESSED AS PERCENT OF ADMINISTERED DOSE.

RABBIT NUMBER	1	2	3	mean$\pm$SD
Day After Administration of (4-^{14}C) NET-OX Racemate.				
1	45.1	47.2	40.4	44.2$\pm$3.5
2	4.6	7.5	6.9	6.3$\pm$1.5
3	5.3	0.4	1.2	2.3$\pm$2.6
4	6.3	0.4	0.3	2.3$\pm$3.4
5	3.0	0.4	0.1	1.1$\pm$1.5
Total	64.3	55.9	48.9	56.3$\pm$7.7
RABBIT NUMBER	5	7	9	mean$\pm$SD
Day After Administration of (4-^{14}C) NET-OX 'anti'-isomer				
1	41.0	46.2	43.2	43.4$\pm$2.5
2	7.8	5.4	6.2	6.5$\pm$1.2
3	4.5	1.6	3.1	3.0$\pm$1.4
4	2.1	1.4	1.5	1.6$\pm$0.3
5	3.1	0.7	1.0	1.6$\pm$1.2
Total	58.5	55.3	55.0	56.2$\pm$1.9
RABBIT NUMBER	4	6	8	mean$\pm$SD
Day After Administration of (4-^{14}C) NET-OX 'syn'-isomer				
1	37.5	41.1	43.2	40.6$\pm$2.8
2	10.8	4.4	5.2	6.8$\pm$3.4
3	2.8	2.3	3.4	2.8$\pm$0.5
4	4.6	1.9	2.0	2.8$\pm$1.5
5	6.7	0.3	1.0	2.7$\pm$3.5
Total	62.4	50.0	54.8	55.7$\pm$6.2

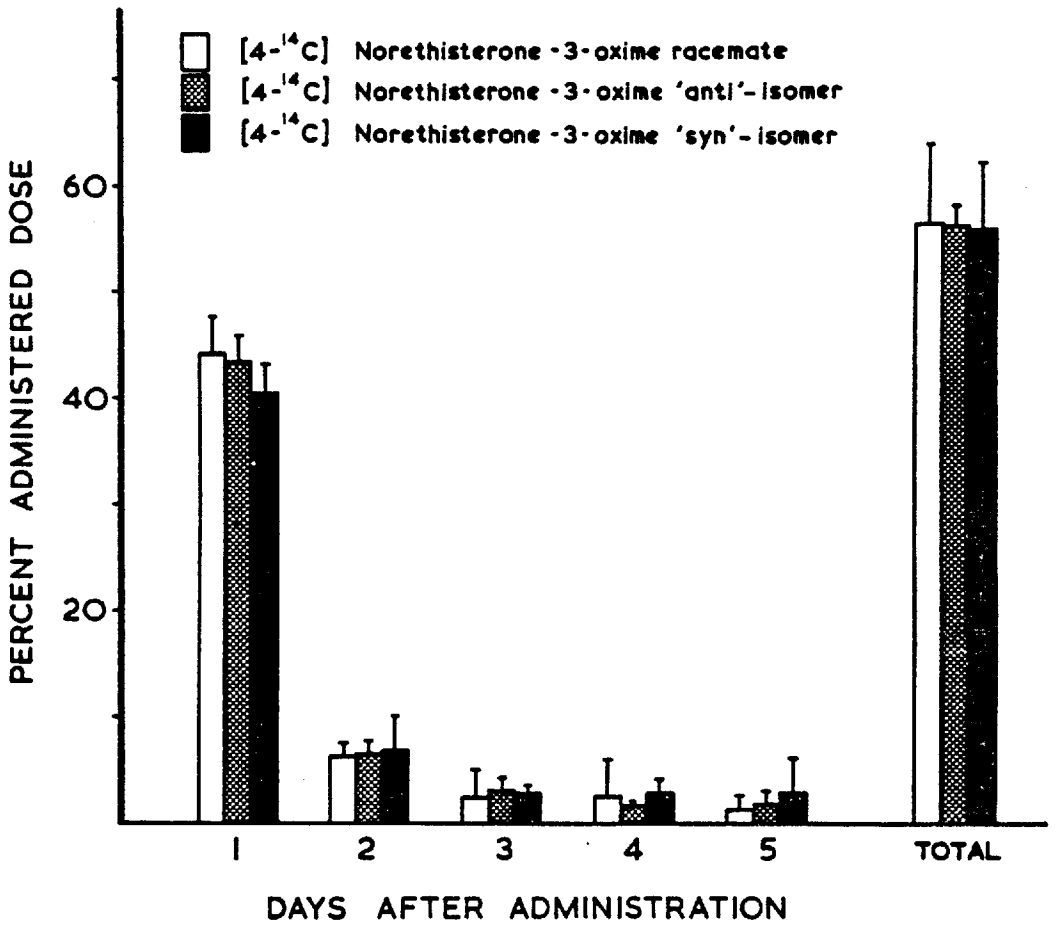


Fig. 1.1 Daily excretion and total recovery of radioactivity from urine of rabbits administered [4-^{14}C] norethisterone oxime.

T A B L E 1.3 EXTRACTION OF RADIOACTIVITY FROM POOLED URINE (DAY 1 AND 2) OF RABBITS RECEIVING (4-^{14}C) NORETHISTERONE OXIME RACEMATE, 'ANTI'- AND 'SYN'-ISOMERS, EXPRESSED AS PERCENT OF TOTAL URINARY RADIOACTIVITY PRESENT. (** denotes value significantly different from preceding mean value $p < 0.01$).

ADMINISTERED STEROID	(4-^{14}C) NET-OX RACEMATE				(4-^{14}C) NET-OX 'ANTI'-ISOMER				(4-^{14}C) NET-OX 'SYN'-ISOMER			
RABBIT NUMBER	1	2	3	mean$\pm$SD	5	7	9	mean$\pm$SD	4	6	8	mean$\pm$SD
HYDROLYSIS PROCEDURE												
NONE	1.4	4.6	5.2	3.4$\pm$1.8	17.1	12.8	10.2	13.4$\pm$3.4**	3.9	8.5	6.2	6.2$\pm$2.2
ENZYME HYDROLYSIS	75.6	49.1	60.1	65.1$\pm$13.0	41.6	52.5	54.2	49.5$\pm$6.8	79.3	70.0	71.1	73.4$\pm$5.1**
DIOXANE HYDROLYSIS AFTER ETHANOLIC-ETHER EXTRACTION	17.1	24.3	22.1	20.1$\pm$3.6	29.0	31.3	28.1	29.5$\pm$1.6**	17.4	15.4	18.2	16.9$\pm$1.4
DIOXANE HYDROLYSIS AFTER PYRIDINIUM SULPHATE EXTRACTION	-	-	17.2		-	-	27.1	-	-	-	14.2	
Total Recovery				88.6				92.4				96.5

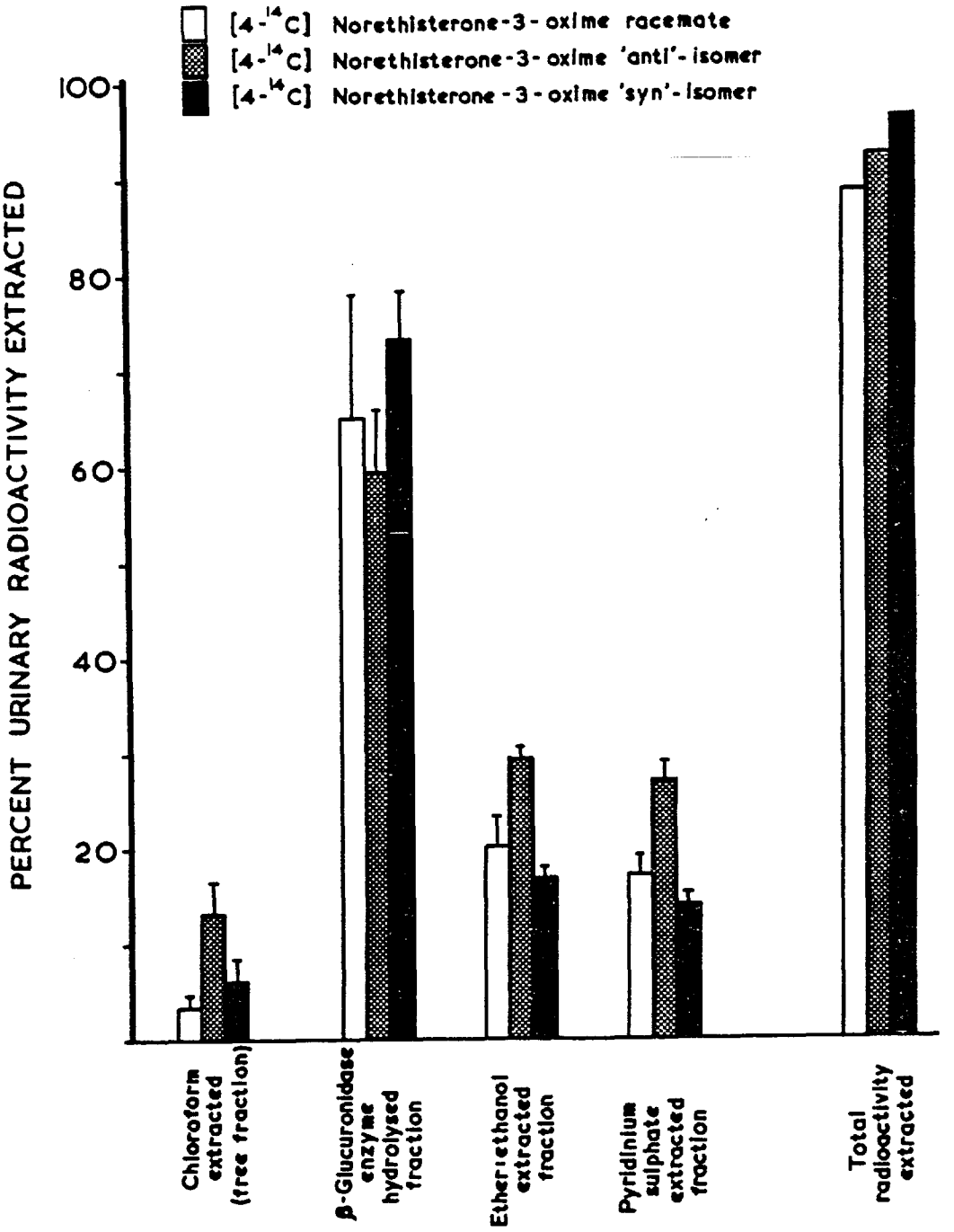


Fig. 1.2 Extraction of radioactivity from urine from Day 1 and 2 after administration of [4-^{14}C] norethisterone oxime. Columns show percent of urinary radioactivity extracted:
a) before hydrolysis (non-conjugated)
b) after β-glucuronidase enzyme hydrolysis
c) dioxane solvolyses after ethanolic:ether extraction
d) dioxane solvolyses after pyridinium sulphate extraction.

The total amount of radioactivity extracted from the pooled urine samples was 88.6% for (4-^{14}C) NET-OX racemate, 92.4% for the 'anti'-isomer and 96.5% for the 'syn'-isomer (Table 1.3). Small amounts of radioactivity were present in the non-conjugated fraction (3.4±1.8% NET-OX racemate, 13.4±3.4% 'anti'-isomer and 6.2±2.2% of the 'syn'-isomer. The mean value for the 'anti'-isomer was significantly higher ($p < 0.01$) than the mean values of the racemate and the 'anti'-isomer. The major part of the radioactivity was present in a β-glucuronidase enzyme hydrolysable form (65.1±13.0% NET-OX racemate, 49.5±6.8% 'anti'-isomer and 73.4±5.1% of the 'syn'-isomer). The mean value for the 'syn'-isomer was significantly higher than the mean values of the racemate and 'anti'-isomer ($p < 0.01$). The remainder of the radioactivity, 20.1±3.6% of NET-OX racemate, 29.5±1.6% of the 'anti'-isomer and 16.9±1.4% of the 'syn'-isomer, was obtained after ethanolic-ether extraction followed by dioxane solvolyses. The mean values for the 'anti' and 'syn'-isomer were significantly different from the mean value for the racemate ($p < 0.01$). Using the more specific method for sulphate conjugate extraction (McKenna and Norymberski, 1960) by forming pyridinium salts, of the radioactivity remaining after β-glucuronidase enzyme hydrolysis 14 - 27% of the radioactivity was recovered by this method (Table 1.3).

b) Urine from day 1 to 5.

The procedures employed for the extraction of urinary radioactivity in each 24 hour sample were as for the pooled urine and have been described in the Methods section. Table 1.4 shows the mean values obtained for the extraction of radioactivity from the urine excreted on day 1 - 5, and Fig. 1.3 compares the mean values. All estimations were carried out in duplicate. The results are expressed as percent of urinary radioactivity excreted per day.

Over the period of five days, the non-conjugated radioactivity was seen to decline. Thus, the amount of radioactivity present in the non-conjugated (free) form on day 1 was 2.2% NET-OX racemate, 8.1% 'anti'-isomer and 6.4% 'syn'-isomer. By day 5 these levels had declined to 0.5% NET-OX racemate, 1.0% 'anti'-isomer and 1.1% 'syn'-isomer. However, the radioactivity levels of the β-glucuronidase

T A B L E 1.4

EXTRACTION OF RADIOACTIVITY EXCRETED PER DAY IN URINE, FROM RABBITS RECEIVING (4-^{14}C) NORETHISTERONE OXIME RACEMATE, 'ANTI'-ISOMER AND 'SYN'-ISOMER, EXPRESSED AS PERCENT OF TOTAL RADIOACTIVITY PRESENT. THE RESULTS ARE MEANS OF DUPLICATE DETERMINATIONS FROM A SINGLE ANIMAL.

Day After Administration of (4-^{14}C) NET-OX Racemate	HYDROLYSIS PROCEDURE			TOTAL RECOVERED
	NONE	ENZYME	DIOXANE	
1	2.2	69.3	21.1	95.5
2	2.0	74.1	14.1	90.2
3	1.2	76.2	12.2	89.6
4	0.5	84.2	14.0	98.4
5	0.5	85.0	10.2	95.4
Day After Administration of (4-^{14}C) NET-OX 'anti'-isomer				
1	8.1	59.2	20.2	87.5
2	4.0	60.1	18.1	85.5
3	2.1	62.2	19.2	83.5
4	1.4	64.0	16.4	81.8
5	1.0	80.1	18.9	100.4
Day After Administration of (4-^{14}C) NET-OX 'syn'-isomer				
1	6.4	61.2	22.1	89.7
2	4.2	64.2	21.2	89.6
3	3.1	68.9	20.1	92.1
4	2.4	72.2	22.1	96.7
5	1.1	81.0	18.1	100.2

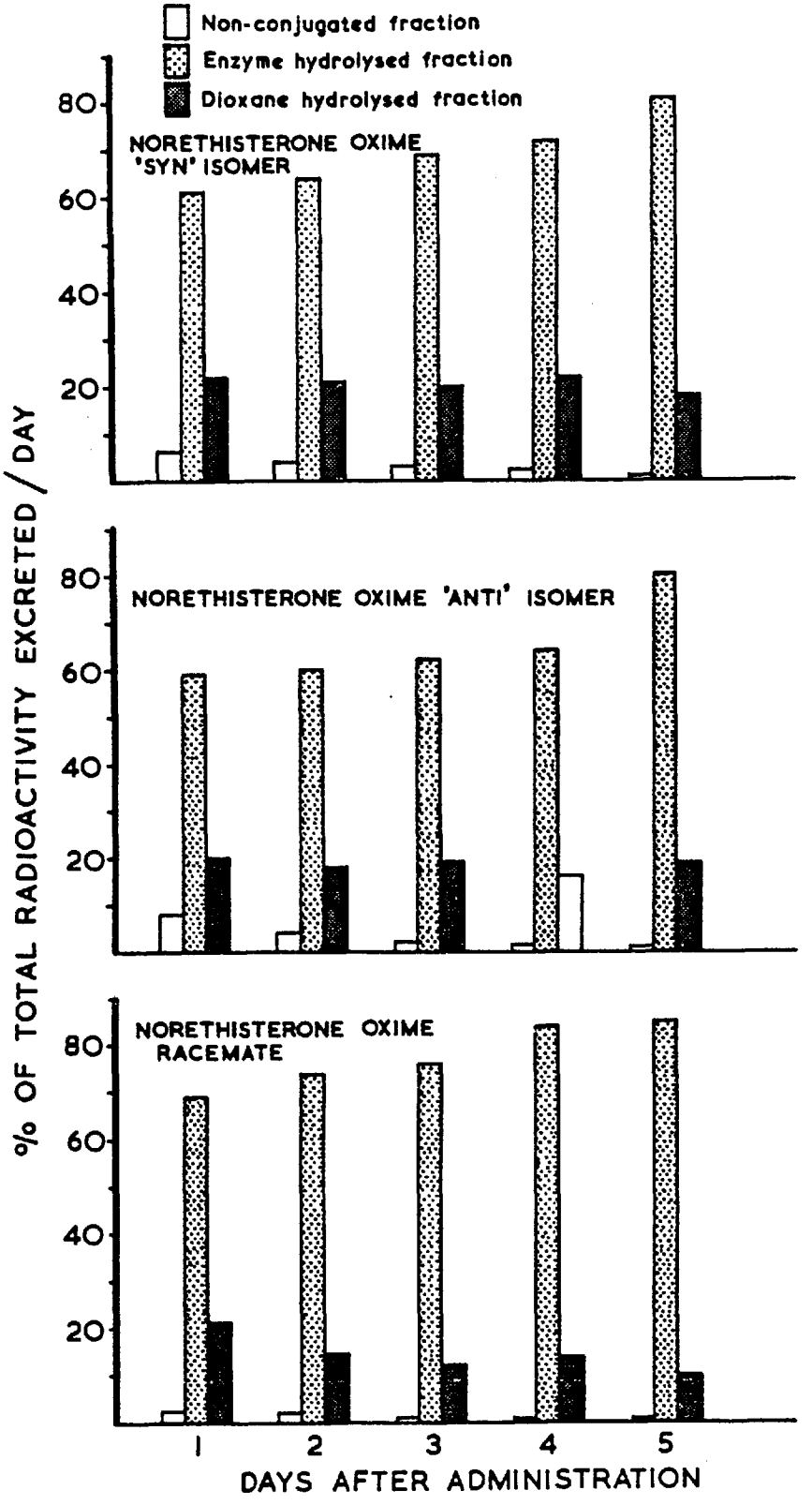


Fig 1.3 Fractionation of total urinary radioactivity excreted per day.
Columns show percent of radioactivity extracted
a) before hydrolysis (non-conjugated)
b) after enzyme hydrolysis (enzyme hydrolysed)
c) after dioxane solvolyses. Means of duplicate samples from a single animal.

enzyme hydrolysable fraction were seen to increase with time. Thus, on day 1, 69.3% of NET-OX racemate, 59.2% 'anti'-isomer and 61.2% of the 'syn'-isomer were present as β-glucuronidase enzyme hydrolysable radioactivity. The dioxane solvolysable radioactivity for the 'anti'- and 'syn'-isomers remained stable over the five day period. Thus, the radioactivity levels present on day 1 (20.2% 'anti'-isomer and 22.1% 'syn'-isomer) remained in the same range on day 5 (18.9% 'anti'-isomer and 18.1% 'syn'-isomer). In contrast, the level of radioactivity for the racemic form of (4-^{14}C) NET-OX declined from 21.1% (day 1) to 10.2% (day 5).

In summary, the pattern of radioactivity present in the non-conjugated (free) and the glucuronide conjugated fractions of urine are similar for the three forms of (4-^{14}C) norethisterone oxime (racemate, 'anti'- and 'syn'-isomers). The major part of the excreted radioactivity was present in a β-glucuronidase enzyme hydrolysable form, which increases with time; whereas the non-conjugated radioactivity decreases with time with all the three forms of (4-^{14}C) norethisterone oxime. In contrast however, for the sulphate fractions, the amount of radioactivity remains constant for the 'syn'- and 'anti'-forms, but declines for the racemate over the period of five days.

3. Faecal excretion of radioactivity following intraperitoneal administration of (4-^{14}C) norethisterone oxime (NET-OX) to female New Zealand white rabbits.

a) Faecal excretion of radioactivity on days 1 to 5.

Radioactivity in the faecal samples was estimated as described in the Methods section. The individual and mean ($\pm$SD) values obtained for the three forms of (4-^{14}C) norethisterone oxime are shown in Table 1.5, and compared in Fig. 1.4.

Over a five day period, of the administered dose 7.9$\pm$0.7% of NET-OX racemate, 7.5$\pm$2.1% of the 'anti'-isomer and 13.2$\pm$1.2% of the 'syn'-isomer were excreted via this route. The mean value for the 'syn'-isomer was significantly higher ($p < 0.05$) than the racemate and 'anti'-isomer.

T A B L E 1.5

DAILY EXCRETION AND TOTAL RECOVERY OF RADIOACTIVITY IN RABBIT FAECES AFTER ADMINISTRATION OF (4-^{14}C) NORETHISTERONE OXIME RACEMATE, 'ANTI'-ISOMER AND 'SYN'-ISOMER, EXPRESSED AS PERCENT OF ADMINISTERED DOSE. (** denotes value significantly different from preceding mean values $p < 0.05$).

RABBIT NUMBER	1	2	3	mean$\pm$SD
Day After Administration of (4-^{14}C) NET-OX racemate.				
1	5.6	4.9	7.4	5.9$\pm$1.3
2	0.7	1.2	0.7	0.8$\pm$0.2
3	0.5	2.0	0.1	0.8$\pm$0.9
4	0.2	0.1	0.1	0.1$\pm$0.04
5	0.1	0.1	0.1	0.1$\pm$0.7
Total	7.1	8.3	8.4	7.9$\pm$0.7
RABBIT NUMBER	5	7	9	mean$\pm$SD
Day After Administration of (4-^{14}C) NET-OX 'anti'-isomer				
1	4.1	5.2	4.7	4.6$\pm$0.6
2	0.7	1.1	2.1	1.3$\pm$0.7
3	0.1	1.0	1.2	0.7$\pm$0.5
4	0.1	0.7	1.1	0.6$\pm$0.4
5	0.1	0.2	0.1	0.1$\pm$0.1
Total	5.1	8.2	9.2	7.5$\pm$2.1
RABBIT NUMBER	4	6	8	mean$\pm$SD
Day After Administration of (4-^{14}C) NET-OX 'syn'-isomer				
1	12.5	10.1	9.2	10.6$\pm$0.9
2	1.2	2.1	1.9	1.7$\pm$0.2
3	0.3	0.5	0.9	0.5$\pm$0.1
4	0.0	0.2	0.1	0.1$\pm$0.1
5	0.5	0.2	0.1	0.2$\pm$0.1
Total	14.5	13.1	12.2	13.2$\pm$1.2 **

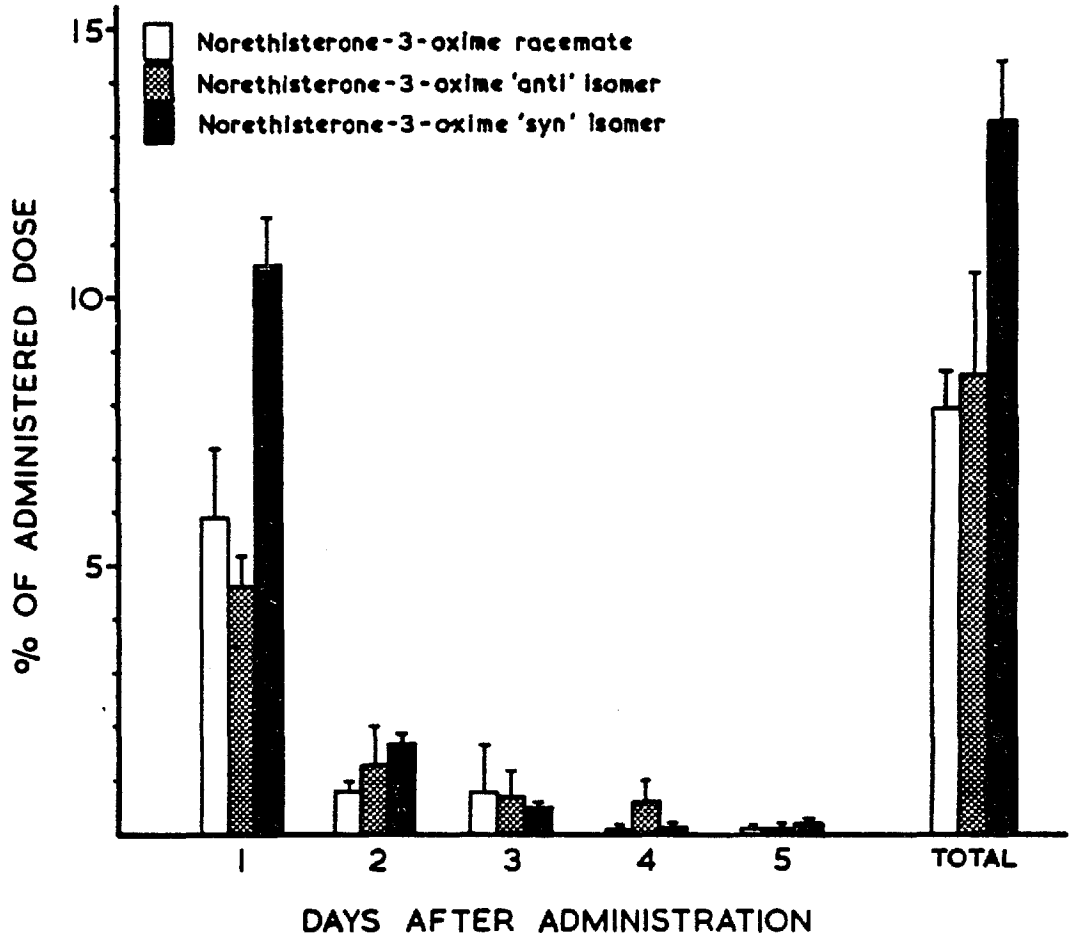


Fig. 1.4 Daily excretion and total recovery of radioactivity from rabbit faeces after administration of [4-^{14}C]norethisterone oxime.

The major part of the radioactivity was excreted on day 1 (76.6% of NET-OX racemate, 63.0% 'anti'-isomer and 80.9% 'syn'-isomer). By day 5, less than 0.5% of the adminstered dose was detected in the faecal extracts.

b) Fractionation of faecal radioactivity.

The faecal extracts from day 1 to 5 were combined and the non-conjugated and β-glucuronidase enzyme hydrolysable radioactivity was estimated as described in the Methods section. In Table 1.6, the mean ($\pm$SD) values are given. Major portion of the radioactivity excreted in the faeces over the five day period was in a non-conjugated (free) form (70.2$\pm$2.4% NET-OX racemate, 74.2$\pm$4.1% of the 'anti'-isomer and 69.1 $\pm$7.1% of the 'syn'-isomer). The remainder of the radioactivity was hydrolysable with β-glucuronidase enzyme (24.1$\pm$4.2% NET-OX racemate, 25.2 $\pm$6.4%,'anti'-isomer and 28.2$\pm$8.1%,'syn'-isomer). No radioactivity was detected in the dioxane solvolysable form for the three forms of (4-^{14}C) NET-OX.

T A B L E 1.6

EXTRACTION OF RADIOACTIVITY FROM FAECES OF RABBITS RECEIVING (4-^{14}C) NORETHISTERONE OXIME RACEMATE, 'ANTI'-ISOMER AND 'SYN'-ISOMER, EXPRESSED AS MEAN $\pm$SD OF TOTAL FAECAL RADIOACTIVITY. (numbers in parentheses indicate number of specimens examined).

STEROID ADMINISTERED	HYDROLYSIS PROCEDURE	
	NONE	ENZYME
(4-^{14}C) NET-OX racemate	70.2$\pm$2.4 (3)	24.1$\pm$4.2 (3)
(4-^{14}C) NET-OX 'anti'-isomer	74.2$\pm$4.1 (3)	25.2$\pm$6.4 (3)
(4-^{14}C) NET-OX 'syn'-isomer	69.1$\pm$7.1 (3)	28.2$\pm$8.1 (3)

In summary, the results of the faecal examination indicate that comparatively little of the intraperitoneally administerd dose of (4-^{14}C) norethisterone oxime racemate, 'anti'-isomer and 'syn'-isomer is excreted via this route. The major portion of radioactivity excreted in the faeces is present in a non-conjugated form for all three forms of (4-^{14}C) norethisterone oxime.

4. Plasma levels of radioactivity after the intraperitoneal administration of (4-^{14}C) norethisterone oxime (NET-OX) to female New Zealand white rabbits.

a) Estimation of radioactivity at 2, 4, 7 and 24 hours.

Radioactivity was estimated in the plasma samples as described in the Methods section. Table 1.7 shows the mean values ($\pm$SD) obtained for duplicate estimations on single samples, expressed as percent of administered dose per 100 ml of plasma. Fig. 1.5 compares the mean values obtained for the 'anti'- and 'syn'-isomers.

The pattern of radioactivity was similar for the two forms of (4-^{14}C)norethisterone oxime. At 2 h, 3.2$\pm$0.5% of the 'anti'-isomer and 3.8$\pm$0.8% of the 'syn'-isomer were present in plasma. The level of radioactivity declined rapidly, so that by 24 h, the levels were 0.5$\pm$0.2% for the 'anti'-isomer and 0.8$\pm$0.6% for the 'syn'-isomer. There was no significant difference in the plasma values for the two isomers of (4-^{14}C) norethisterone oxime at the times studied.

TABLE 1.7

RADIOACTIVITY IN RABBIT PLASMA AFTER ADMINISTRATION OF (4-^{14}C) NORETHISTERONE OXIME, 'ANTI'-ISOMER AND 'SYN'-ISOMER, EXPRESSED AS MEAN $\pm$SD OF PERCENT OF ADMINISTERED DOSE IN 100 ml OF PLASMA. THE RESULTS ARE OF DUPLICATE DETERMINATIONS FROM A SINGLE SAMPLE.

TIME AFTER ADMINISTRATION (h)	STEROID ADMINISTERED (4-^{14}C) NET-OX 'anti'-isomer	(4-^{14}C) NET-OX 'syn'-isomer.
2	3.2$\pm$0.5	3.8$\pm$0.8
4	2.1$\pm$0.4	2.4$\pm$0.6
7	1.0$\pm$0.3	1.6$\pm$0.2
24	0.5$\pm$0.2	0.8$\pm$0.6

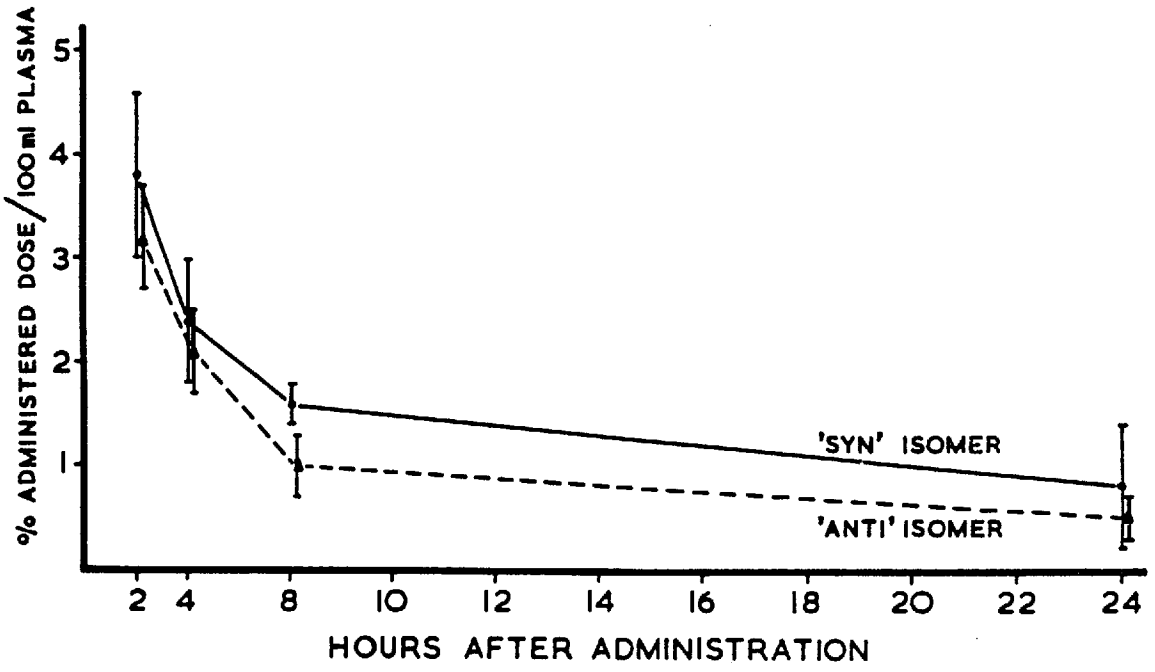


Fig. 1.5 Concentration of radioactivity in plasma after administration of [4-^{14}C]. Norethisterone oxime 'anti'- and 'syn' -isomers, expressed as percent of administered dose in 100 ml plasma.

b) Fractionation of plasma radioactivity.

Non-conjugated and conjugated radioactivity in plasma was obtained as described in the Methods section. Table 1.8 shows the mean ($\pm$SD) values obtained for dulpicate estimations from a single sample, and Fig. 1.6 compares the mean values. The values are expressed as percent of total radioactivity present in the sample.

At 2h, 65.6$\pm$0.4% of the 'anti'-isomer was in a non-conjugated form. At the same time 52.6$\pm$0.8% of the 'syn'-isomer was present in this fraction. By 24 h, the level of non-conjugated radioactivity for the 'anti'-isomer had declined to 40.0$\pm$0.6%, whereas for the 'syn'-isomer the level was 62.5$\pm$0.5%.

In the conjugated fraction 34.3$\pm$0.4% of the 'anti'-isomer and 47.3$\pm$0.3% of the 'syn'-isomer were present at 2h. By 24 h, the level of conjugated activity for the 'anti'-isomer had increased to 60.0$\pm$0.7% whereas, the conjugated radioactivity for the 'syn'-isomer had decreased to 37.6$\pm$0.6%.

In summary, as seen in Fig. 1.5, the levels of radioactivity in plasma of the 'anti'-isomer and 'syn'-isomer of (4-^{14}C) norethisterone oxime are similar. The levels of radioactivity decline rapidly for both forms. However, the pattern of conjugated radioactivity appears to differ as seen in Fig. 1.6.

T A B L E 1.8

NON-CONJUGATED AND CONJUGATED RADIOACTIVITY IN RABBIT PLASMA AFTER ADMINISTRATION OF (4-^{14}C) NORETHISTERONE OXIME 'ANTI'-ISOMER AND 'SYN'-ISOMER EXPRESSED AS PERCENT OF TOTAL RADIOACTIVITY. THE RESULTS ARE OF DUPLICATE DETERMINATIONS FROM A SINGLE SAMPLE.

TIME AFTER ADMINISTRATION	(4-^{14}C) NET-OX 'anti'-isomer.	
	NON-CONJUGATED	CONJUGATED
2	65.6$\pm$0.4	34.3$\pm$0.4
4	57.1$\pm$0.6	42.8$\pm$0.8
7	50.0$\pm$0.6	45.0$\pm$0.5
24	40.0$\pm$0.6	60.0$\pm$0.7
TIME AFTER ADMINISTRATION	(4-^{14}C) NET-OX 'syn'-isomer.	
2	52.6$\pm$0.8	47.3$\pm$0.3
4	58.3$\pm$0.7	41.6$\pm$0.7
7	75.0$\pm$0.5	25.0$\pm$0.6
24	62.5$\pm$0.5	37.6$\pm$0.6

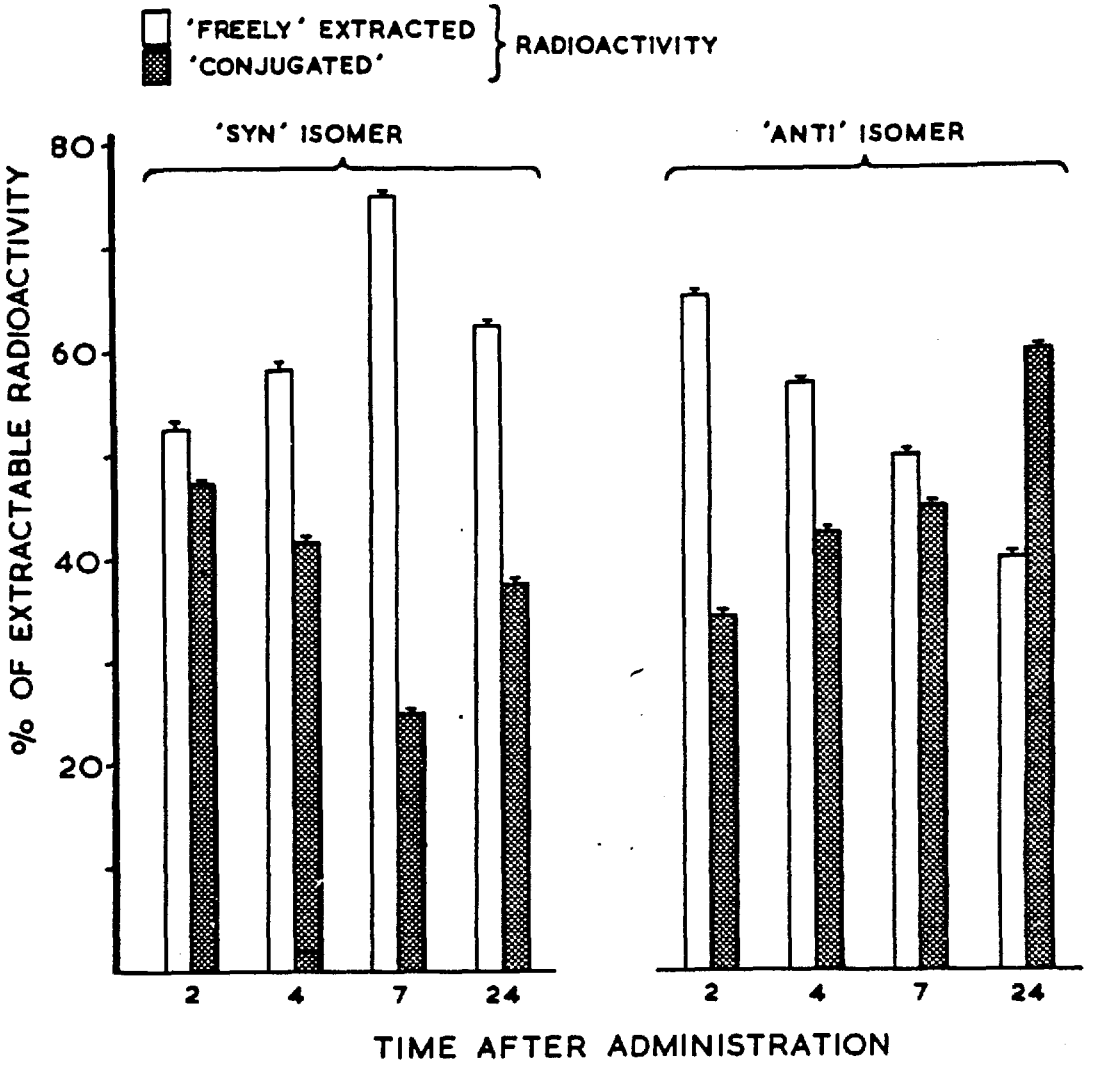


Fig. 1.6 Non-conjugated and conjugated radioactivity in plasma after administration of [4-^{14}C]norethisterone oxime 'anti'- and 'syn'-isomers.

5. Estimation of (4-^{14}C) norethisterone oxime (NET-OX) and metabolites in urine.

The urine extracts from the three fractions, the non-conjugated fraction (free), β-glucuronidase enzyme hydroysable fraction and the dioxane solvolysable fraction obtained for each form of (4-^{14}C) norethisterone oxime were analysed by thin-layer, paper and gas-liquid chromatography. The procedure has been described in the Methods section.

a) Non-conjugated fraction.

Extracts from each of the three forms of (4-^{14}C) norethisterone oxime were chromatographed on paper. The pattern of metabolites was similar for the racemate, 'anti'- and 'syn'-isomer. A tracing of a typical radiochromatogram scan obtained for the racemate is shown in Fig. 1.7. Two main areas of radioactivity were seen for the three forms of (4-^{14}C) norethisterone oxime in the non-conjugated (free) fraction. The major area (Area A) corresponded in chromatographic mobility to the administered steroid. The identity of the area was confirmed by gas-liquid chromatography. The eluted area was also hydrolysed in IN- HCl at 37°C and further analysed by thin-layer and gas-liquid chromatography. This indicated that the hydrolysed product was norethisterone.

The relative proportions of the two metabolites in this fraction, as calculated by triangulation, are shown in Table 1.9 and the mean values and ranges in Fig. 1.10. The area corresponding to norethisterone oxime (Area A) in the non-conjugated fraction accounted for 81.5$\pm$3.0% for the racemate, 79.3$\pm$4.8% for the 'anti'-isomer and 81.5$\pm$1.9% for the 'syn'-isomer. The more polar area (Area B) was not fully analysed and accounted for 18.4$\pm$1.5% for the racemate, 20.5$\pm$5.7% for the 'anti'-isomer and 12.2$\pm$3.6% for the 'syn'-isomer.

Fig. 1.9 Dioxane solvolysed fraction

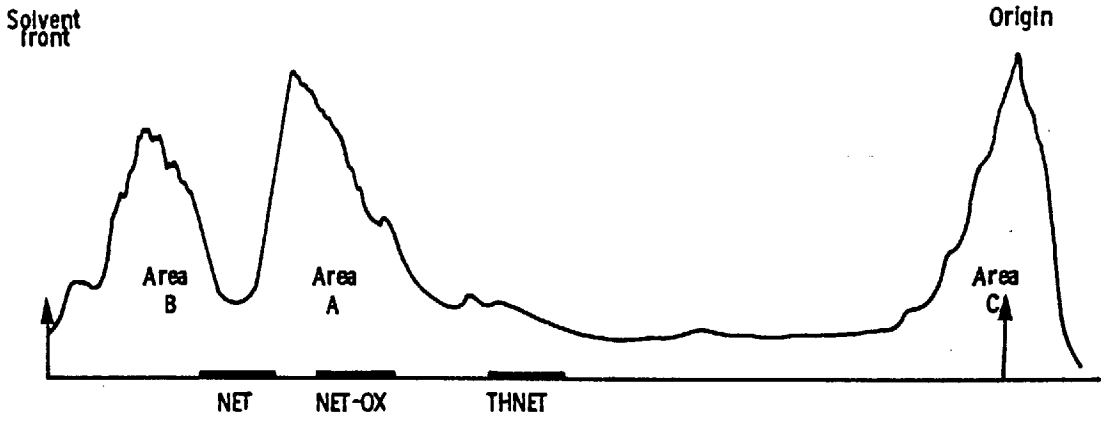


Fig. 1.8 β-Glucuronidase enzyme hydrolysed fraction

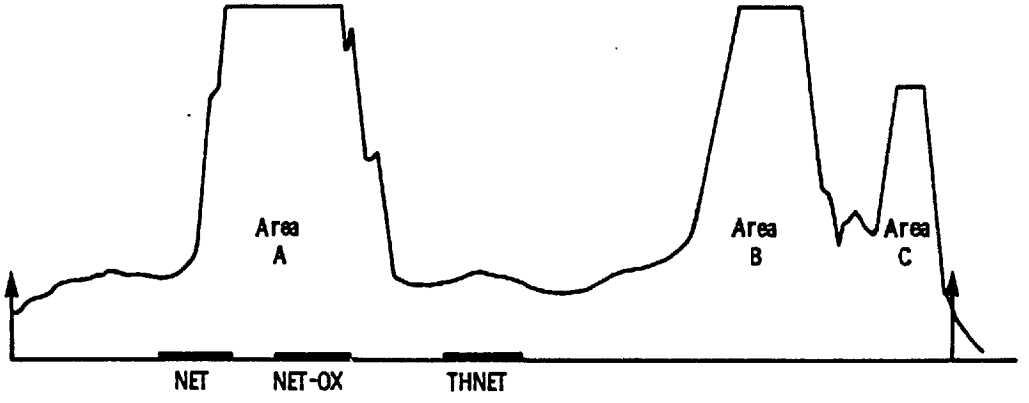
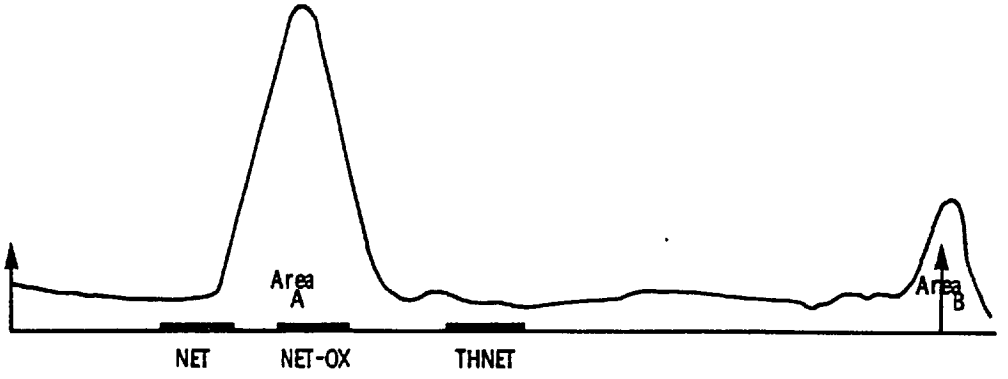


Fig. 1.7 Non-conjugated fraction



Figs. 1.7 - 1.9 Radiochromatogram scans of the non-conjugated, β-glucuronidase enzyme hydrolysed and dioxane solvolysed fraction of urine from rabbits receiving [4-^{14}C] norethisterone oxime racemate. The paper chromatograms were developed in a formamide:chloroform system.

TABLE 1.9

PROPORTION OF METABOLITES IN THE NON-CONJUGATED FRACTION OF URINE. FIGURES ARE PERCENT OF APPLIED SAMPLE IN EACH AREA, FROM THREE URINE SAMPLES.

STEROID ADMINISTERED	AREAS ON PAPER CHROMATOGRAMS.	SAMPLE NUMBER 1	2	3	MEAN±SD
(4-^{14}C) NET-OX racemate	Area A	80.4	79.2	84.9	81.5±3.0
	Area B	20.1	17.2	17.9	18.4±1.5
(4-^{14}C) NET-OX 'anti'-isomer	Area A	81.2	82.9	73.8	79.3±4.8
	Area B	27.2	16.4	18.2	20.6±5.7
(4-^{14}C) NET-OX 'syn'-isomer	Area A	90.1	86.2	87.7	81.5±1.9
	Area B	9.1	10.2	16.4	12.2±3.6

b) B-glucuronidase enzyme hydrolysed fraction.

The pattern of metabolites present as seen on paper chromatograms in this fraction were also similar for the three forms of (4-^{14}C) norethisterone oxime. A typical tracing obtained for NET-OX racemate is shown in Fig. 1.8. In this fraction three areas of radioactivity were seen in the urine extracts obtained after administration of the three forms of (4-^{14}C) norethisterone oxime. Area A (Fig. 1.8) which was identified to be norethisterone oxime, accounted for 36.9±8.5% of the total sample applied from extracts of norethisterone oxime racemate. 54.0±1.9% for the 'anti'-isomer and 57.4±6.9% for the 'syn'-isomer. (Table 1.10) shows the relative proportions of each metabolite present in this fraction and the mean values are shown in Fig. 1.11). Two other areas of radioactivity both 'more polar' than norethisterone oxime were also noted. Area B accounted for 37.5±0.9% of NET-OX racemate, 32.2±1.7% of the 'anti'-isomer and 18.9±2.1% of the 'syn'-isomer. Area C accounted for 25.5±3.2% of the NET-OX racemate, 13.7±1.4% of the 'anti'-isomer and 23.5±2.0% of the 'syn'-isomer.

Thus, the major portion of radioactivity in this fraction was identified to be norethisterone oxime, by paper chromatography. However, when this fraction was analysed by thin-layer chromatography which resolves the two forms of norethisterone oxime into the 'anti'-isomer and 'syn'-isomer, the results indicated that the configuration of the administered norethisterone oxime was different from that administered.

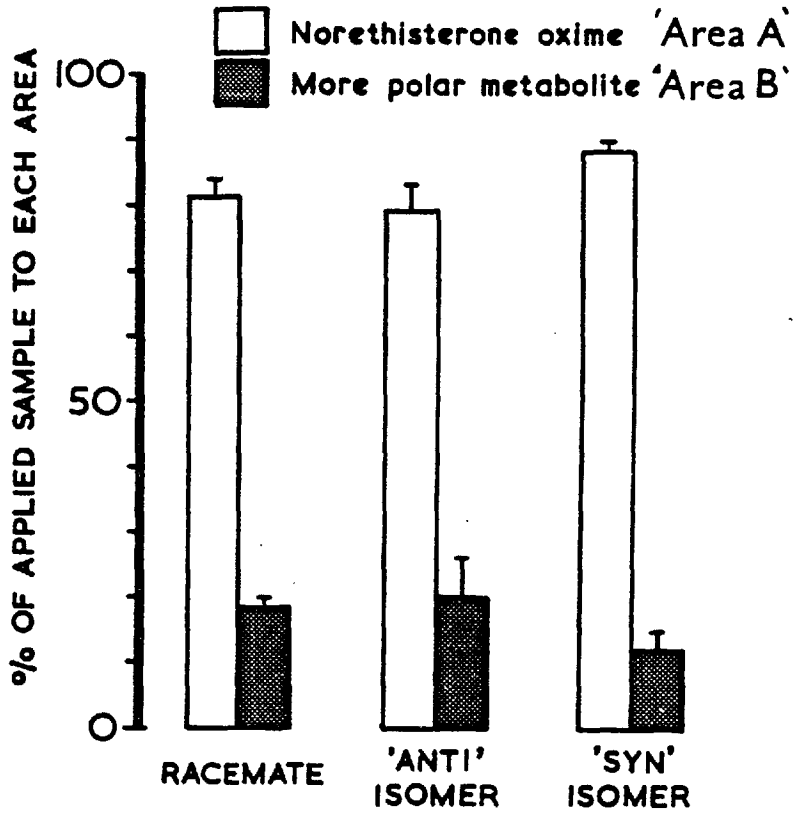


Fig. 1.10 Proportion of norethisterone oxime and metabolite in the non-conjugated fraction of urine from Day 1 and 2.

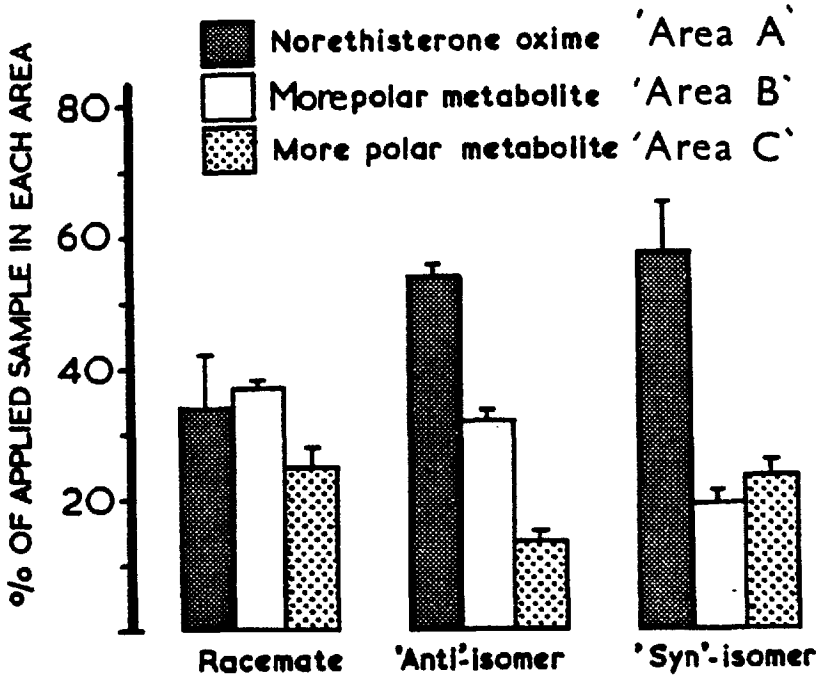


Fig. 1.11 Proportion of norethisterone oxime and metabolites in the enzyme hydrolysed fraction of urine from Day 1 and 2.

Thus, as shown in Fig. 1.12 - 1.13, the 'syn'-form in the racemic mixture and in the 'syn' administered extract had been converted to the 'anti' form. This was also confirmed by gas-liquid chromatography. The other metabolites,Area B and C in Fig. 1.8 were not fully analysed.

STEROID ADMINISTERED	AREA ON PAPER CHROMATOGRAMS	SAMPLE NUMBER 1	2	3	MEAN ±SD
(4-^{14}C) NET-OX racemate	Area A	30.1	34.2	46.6	36.9±8.5
	Area B	38.1	36.4	38.0	37.5±0.9
	Area C	24.2	23.1	29.2	25.5±3.2
(4-^{14}C) NET-OX 'anti'-isomer	Area A	56.0	52.1	53.9	54.0±1.9
	Area B	31.4	34.2	31.0	32.2±1.7
	Area C	12.0	14.5	14.6	13.7±1.4
(4-^{14}C) NET-OX 'syn'-isomer	Area A	56.1	51.2	64.9	57.4±6.9
	Area B	20.1	16.4	20.2	18.9±2.1
	Area C	21.2	24.1	25.2	23.5±2.0

T A B L E 1.10

PROPORTION OF METABOLITES IN THE β-GLUCURONIDASE ENZYME HYDROLYSED FRACTION. FIGURES ARE PERCENT OF APPLIED SAMPLE IN EACH AREA, FROM THREE URINE SAMPLES.

c) Dioxane solvolysable fraction.

Fig. 1.9 shows a typical tracing of a radiochromatogram after paper chromatography of (4-^{14}C) NET-OX racemate. As found for the other two fractions, the major area of radioactivity had a chromatographic behaviour similar to that of norethisterone oxime. This area (Area A) (Fig. 1.9) accounted for 38.2% of the total sample applied. The two other areas were also seen, one 'less polar' than norethisterone oxime (Area B, 29.3%) and one 'more polar' than norethisterone oxime (Area C, 34.8%). These two areas were not analysed further and Area A (norethisterone oxime) was not analysed by thin-layer chromatography.

Fig. 1.14 Norethisterone oxime 'syn'-isomer

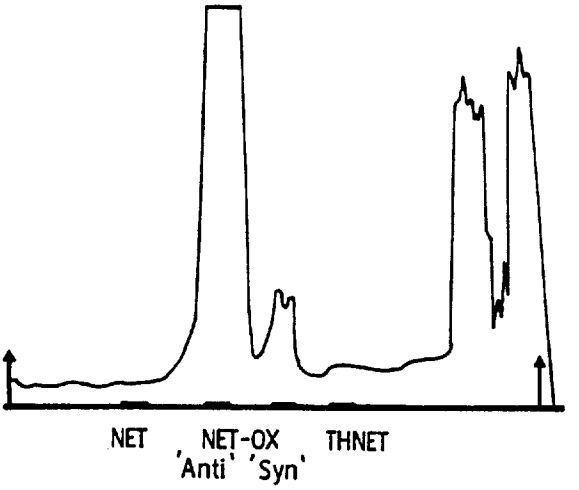


Fig. 1.12 Norethisterone oxime racemate

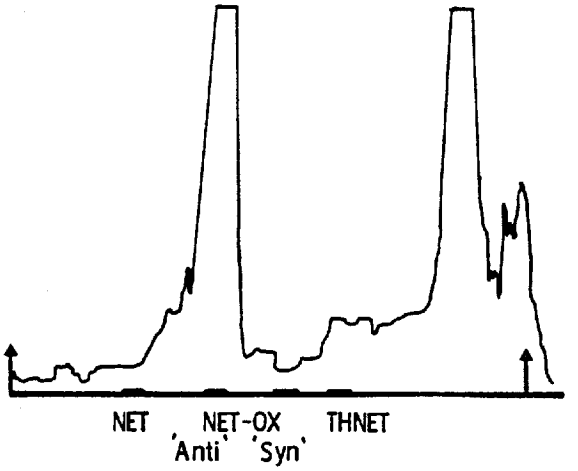
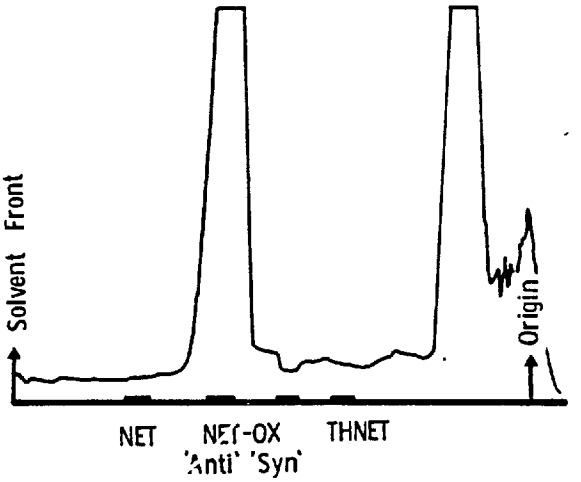


Fig. 1.13 Norethisterone oxime 'anti'-isomer



Figs. 1.12 - 1.14

Radiochromatogram scans of the β-glucuronidase enzyme hydrolysed fraction of urine from rabbits receiving [4-^{14}C] norethisterone oxime racemate, 'anti'- and 'syn'-isomers, thin-layer chromatograms were developed in the solvent system:
Toluene:Acetone 4:1 (V/V).

In summary, in all the three fractions of urine, obtained after the administration of (4-^{14}C) norethisterone oxime racemate, 'anti'-isomer and 'syn'-isomer, the main metabolite was found to be similar to the administered compound. The configuration of the racemate and 'syn'-isomer was observed to be 'anti'- form in the β-glucuronidase enzyme hydrolysed fraction. Other metabolites observed in each fraction were not analysed further.

6. Estimation of (4-^{14}C) norethisterone oxime (NET-OX) and metabolites in faeces.

Only the non-conjugated fraction of the faecal extracts, after administration of (4-^{14}C) norethisterone oxime racemate was analysed A typical tracing of a radiochromatogram obtained after paper chromatography is shown in Fig. 1.15. The procedure used was similar to that used for the urinary extracts and has been described in the Methods section.

In the non-conjugated fraction, norethisterone oxime was seen to be the major area of radioactivity (Area A, Fig. 1.15). This area accounted for 65.6% of the total sample processed. Two other 'more polar' metabolites accounting for 16.2% (area B) and 18.1% (Area C) were also present. However, these two metabolites had different chromatographic mobilities than those seen in the β-glucuronidase enzyme hydrolysed and dioxane solvolysed fraction of urine. These two metabolites were not analysed further.

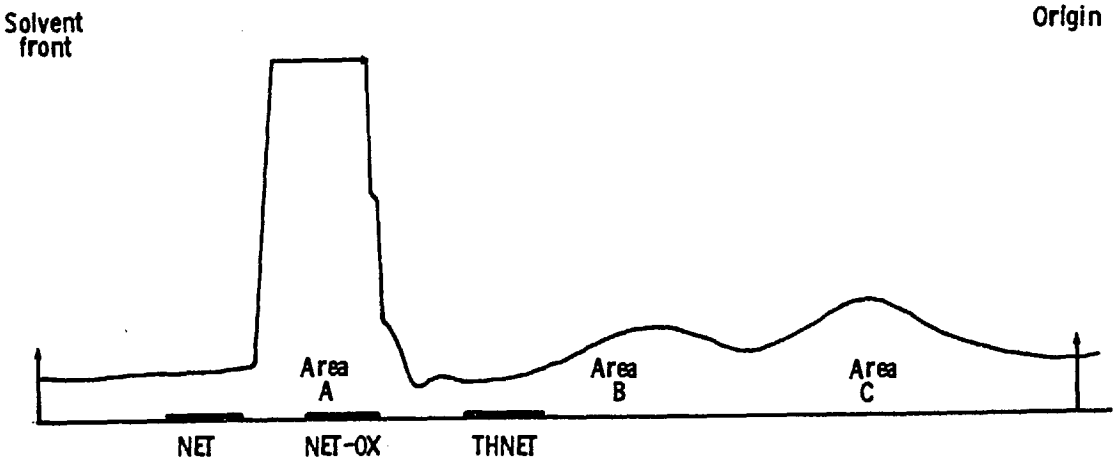


Fig. 1.15 Radiochromatogram scan of the non-conjugated fraction from the faecal extract of a rabbit receiving [4-^{14}C]norethisterone oxime racemate. Paper chromatograms were developed in a formamide:chloroform system.

DISCUSSION.

Numerous 'in vivo' investigations have been carried out during the past ten years on the metabolism of synthetic progestational compounds related to 19-nortestosterone, both in man and animals (Fotherby and James, 1972; Thijssen, 1972 and Fotherby, 1974). The 'in vivo' metabolism of norethynodrel (Arai et al., 1962) norethisterone and norgestrel (Kamyab et al., 1967) and lynestrenol (Yamamoto, 1968) has been investigated in the rabbit. Many similarities have been indicated between the rabbit and man in the metabolism of progesterone (Fotherby, 1964) and in the metabolism of 19-nortestosterone, and 17α-hydroxyprogesterone related progestational compounds (Fotherby, 1974).

In 1973, Shroff et al., indicated that 17β-acetoxy-19-norandrost-4-en-3-one oxime (norethisterone acetate-3-oxime) was a more potent antifertility agent in the rat than the parent compound norethisterone acetate. In the clinical studies of McQuarrie et al. (1972) regarding the safety and efficacy of norethisterone acetate-3-oxime, they reported this compound to be well tolerated in humans up to a dose of 100 mg/day. At the 1 mg dose, in 30 treatment cycles, no pregnancies occurred. In 1975, Kärkkäinen et al., indicated that the addition of the methoxime ($CH_3 - O - N =$) group to norethisterone increased the antifertility potency of this steroid tenfold in the female rat. In view of these observations, the present 'in vivo' investigation of the metabolism of norethisterone oxime, in female rabbit was undertaken. In the chemical synthesis of steroidal oximes, the geometric isomers, designated the 'anti'-isomer and 'syn'-isomer are formed (Table 1.1). Since the required data as to which of these isomers was biologically active was not available, the metabolism of norethisterone oxime racemate, 'anti'-isomer and 'syn'-isomer was investigated.

After the intraperitoneal administration of the three forms of (4-^{14}C) norethisterone oxime to female New Zealand white rabbits, the total amount of radioactivity excreted over a five-day period was, 56.3$\pm$7.7% of NET-OX racemate, 56.2$\pm$1.9% of the 'anti'-isomer and 55.7$\pm$6.2% of the 'syn'-isomer (Table 1.2). These values are similar to those obtained by other investigators after the administration of related 19-norprogestogens to the rabbit. Thus, Arai et al. (1962)

recovered 31 - 62% of (H^3) norethynodrel in urine of rabbits, over a seven-day period after the administration of this compound in a gelatine capsule. Kamyab et al. (1967) after the intravenous administration of (4-^{14}C) norethisterone and (^{14}C) norgestrel to rabbits, recovered 45% of NET and 57.4% of Ng in urine, in seven days. Similar values for (H^3) lynestrenol were obtained by Yamamoto (1968). Thus, after the intravenous administration of this compound 35% of lynestrenol was recovered in urine over a five day period. Thus, the rate of urinary excretion of the three forms of (4-^{14}C) norethisterone oxime does not differ from norethynodrel, norethisterone, norgestrel and lynestrenol in the rabbit.

The major part of the radioactivity excreted in urine after the administration of (4-^{14}C) NET-OX racemate, 'anti'- and 'syn'-isomers was excreted within the first 24 hours (Fig. 1.1). Thus, 78.5% of (4-^{14}C) NET-OX racemate, 77.5% of the 'anti'-isomer and 72.8% of the 'syn'-isomer, of the total dose excreted in five days was recovered on the first day. By day five, less than 3% of the total radioactivity recovered was present in the urine for all three forms of (4-^{14}C) norethisterone oxime. In contrast, only 7.7% of the administered (H^3) norethynodrel was recovered in urine on the first day (Arai et al., 1962). Maximum excretion occurred after the third day. In agreement to our study, after the intravenous administration of (4-^{14}C) norethisterone and (^{14}C) norgestrel, 80.8% and 88.5% respectively, of the total recovered dose in urine was found to be excreted on day one (Kamyab et al., 1967). With (H^3) lynestrenol, 45.7% of the total excreted dose was recovered on day one (Yamamoto, 1968). These differences in the rate of elimination of these compounds may be due to the route of administration, however, Reed et al. (1972) have reported that for the synthetic oestrogen, ethynyloestradiol, the route of administration (oral or intravenous) did not affect either the total amount of the dose recovered in urine or the rate at which the steroid was excreted in man. Therefore, it is more probable that the delay in the elimination of (H^3) norethynodrel is due to extensive biliary excretion and enterohepatic circulation in the rabbit. Thus, rabbits with biliary fistulae, excrete 20-40% of an oral dose of (H^3) norethynodrel in the bile in seven days, and 21% in the urine. Intact, animals, however, excrete over 50% of the dose in the urine, thus, much of the material eliminated in the bile is absorbed and re-excreted by the kidneys. (Arai et al., 1962).

The data from the present study indicates that the major part of the radioactivity excreted in urine on day 1 and 2, after the administration of (4-^{14}C) norethisterone oxime racemate, 'anti'-isomer and 'syn'-isomer was present in the β-glucuronidase enzyme hydrolysable fraction (Table 1.3). Thus, 65.1±13.0% of NET-OX racemate, 49.5±6.8% of the 'anti'-isomer and 73.4±5.1% of the 'syn'-isomer of the total radioactivity recovered in two days was present in this fraction. The mean value for the 'syn'-isomer was significantly higher than the values obtained for the racemate and 'anti'-isomer. In contrast, the amount of radioactivity recovered in this fraction in various studies for other related progestogens was much lower. Thus, Arai et al. (1962) found 37% of the total excreted (H^3) norethynodrel in the glucuronide fraction. Kamyab et al. (1967) found only 31% of (4-^{14}C) norethisterone and 21% of (^{14}C) norgestrel in this fraction. Similarly, Yamamoto (1968) reported that 42.6% of(H^3)lynestrer was excreted in the glucuronide fraction.

In all the above published studies with (4-^{14}C) norethisterone, (^{14}C) norgestrel and (H^3) lynestrenol the non-conjugated steroids were not removed prior to β-glucuronidase enzyme hydrolysis. Therefore, the values reported would be actually lower than those quoted above. The higher levels of radioactivity present in the β-glucuronidase enzyme hydrolysable fraction of the three forms of (4-^{14}C) norethisterone oxime in our study can be explained in two possible ways. One possibility may be that glucuronide conjugation is the major pathway for norethisterone oxime conjugation. Alternatively, it is likely that this discrepancy in the results may be due to methodological differences between the present study and those of other investigators. Bradlow (1968) has indicated that neutral ion-exchange chromatography of urine removes 90% of urinary solids, these may include a large proportion of β-glucuronidase enzyme inhibitors (Abul-Fadl, 1957). In this study we have used ion-exchange chromatography to purify the urine samples, prior to enzyme hydrolysis. As described in the Methods section, following this purification step 50% more of the urinary radioactivity was extractable after enzyme hydrolysis, in comparison to the radioactivity extracted without prior chromatographic purification. Thus, it is more likely that the relatively higher percentage of (4-^{14}C) norethisterone oxime recovered in the glucuronide fraction of urine in this study is due to methodological differences.

In the pooled samples from day 1 and 2, a small percentage of the total radioactivity present was in a non-conjugated form, which was extractable with chloroform, prior to any hydrolytic procedure (Table 1. 3). Thus, for (4-^{14}C) norethisterone oxime racemate, 3.4±1.8%, 13.4±3.4% of the 'anti'-isomer, and 6.2±2.2% of the 'syn'-isomer were in a non-conjugated form. The mean values for the 'anti'- and 'syn'-isomer were significantly higher than the racemate. In contrast, less than 2% of (H^3) norethynodrel was extractable prior to enzyme hydrolysis (Arai et al., 1962).

After the extraction of the non-conjugated radioactivity and β-glucuronidase enzyme hydrolysable radioactivity, the remainder of the radioactivity in the pooled urine from day 1 and 2 was recovered after dioxane solvolyses (Table 1.3). Thus, 20.1±3,6% of NET-OX racemate, 29.5±1.6% of the 'anti'-isomer and 16.9±1.4% of the 'syn'-isomer were recovered. In comparison, 13% of (H^3) norethynodrel was obtained in the sulphate fraction (Arai et al., 1962). However, relatively higher values have been reported in the 'sulphate' fraction for (4-^{14}C) norethisterone, (^{14}C),norgestrel and (H^3) lynestrenol. Thus, 45% of (4-^{14}C) NET was obtained after hot-acid hydrolysis and 59% of (^{14}C) norgestrel (Kamyab et al., 1967). Similarly, 39.8% of (H^3) lynestrenol was obtained after solvolyses and hot-acid hydrolysis (Yamamoto, 1968).

These studies suggest that glucuronide conjugation is dominant for the three forms of norethisterone oxime (Table 1.3) and norethynodrel (Arai et al., 1962). Whereas, sulphate conjugation appears to be more important for norethisterone and norgestrel (Kamyab et al., 1967) in the rabbit. Thus, it is suggested that the structure of the steroids may determine the route of conjugation in the rabbit.

Comparing the three urine fractions of (4-^{14}C) norethisterone oxime racemate, 'anti'-isomer and 'syn'-isomer (Table 1.3) it is seen that a significantly higher percentage of the 'anti'-isomer is present in the non-conjugated fraction and the sulphate conjugated fraction than the 'syn'-isomer. Whereas, significantly higher percentage of the 'syn'-isomer is present in the glucuronide fraction. This may be related to the physical and biological properties of the two geometric isomers and needs further investigation.

That the β-glucuronidase enzyme hydrolysable conjugates are predominant in the urine after the administration of (4-^{14}C) norethisterone oxime is also shown, when each 24 hour sample was examined (Table 1.4). This fraction is seen to increase over the five-day period; so that on day 1, 69.3% of NET-OX racemate is in this form and this value increases to 85.0% by day 5. Similar increases in this fraction are seen for the 'anti'- and 'syn'-isomers of (4-^{14}C) norethisterone oxime from day 1 to day 5. In contrast, the non-conjugated fraction decreases over the five-day period. Thus, on day 1,2.2% of (4-^{14}C) NET-OX racemate is present in the non-conjugated fraction and 0.5% on day 5. For the 'anti'-isomer, 8.1% is present on day 1 and 1.0% on day 5. Similar values are seen for the 'syn'-isomer. However, although the sulphate conjugation of the 'anti'- and 'syn'-isomers remains steady over the five day period, the sulphate conjugation of the racemate decreases with time. The reason for this is difficult to assess from this data, however, it is possible that structural differences in the three forms may account for this finding.

The data from the present study indicates that the faecal route of excretion of (4-^{14}C) norethisterone oxime forms a minor route as compared to the excretion of this compound in urine (Table 1.5). Thus, of the administered dose of (4-^{14}C) NET-OX racemate 7.9$\pm$0.7%, 7.5$\pm$2.1% of the 'anti'-isomer and 13.2$\pm$1.2% of the 'syn'-isomer was recovered in faeces over a five-day period. The mean values of the racemate and the 'anti'-isomer were significantly lower than the 'syn'-isomer. This finding is similar to that obtained for (H^3) norethynodrel in the rabbit (Arai et al.,1962). Thus, 7 - 26% of this progestogen was recovered in the faeces. However, much lower doses of (4-^{14}C) norethisterone (1.4 - 4%) and (^{14}C) norgestrel (less than 5%) were recovered in the faeces (Kamyab et al.,1967). This difference in the faecal excretion of these compounds may be dependant on the extent to which they are excreted into the bile. The bile is an important channal of excretion for the natural and synthetic sex steroids in several species including man. However, the extent to which hepatic elimination occurs varies with the compound, this is probably due to differences in chemical structure and variation in the metabolism of the various hormones (Smith, 1974).

Examination of the nature of radioactivity excreted via the faecal route after the administration of (4-^{14}C) norethisterone oxime indicates (Table 1.6) that the major part of the radioactivity is excreted in the non-conjugated fraction. Thus, 70.2±2.4% of NET-OX racemate, 74.2±4.1% of the 'anti'-isomer and 69.1±7.1% of the 'syn'-isomer was excreted in the non-conjugated form. This is in contrast to the excretion of (H^3) norethynodrel in rabbit faeces (Arai et al., 1962), where only 34% of the excreted radioactivity was extractable without hydrolysis. However, since it is known that synthetic sex hormones occur in the bile in the conjugated form (Smith, 1974) it is possible that the non-conjugated steroids present in the faeces are due to the β-glucuronidase and steroid sulphatase activities of rabbit faeces. The occurrence of such enzymes has been demonstrated in the faeces of man (Stimmel, 1954).

Estimation of radioactivity in rabbit plasma after the administration of (4-^{14}C) norethisterone oxime, 'anti'-isomer and 'syn'-isomer indicates that (Table 1.7) that at 2 hours, 3.8±0.8% of the 'syn'-isomer and 3.2±0.5% of the 'anti'-isomer were detectable. These levels had declined to less than 1% for both forms by 24 hours. These findings are in agreement with those obtained by Kamyab et al. (1967) for (4-^{14}C) norethisterone and (^{14}C) norgestrel. Thus, at 2 hours, 2.9% of the administered norethisterone was present in rabbit plasma, and 3.5% of norgestrel. By 24 hours, these levels had declined to 0.3% for (4-^{14}C) norethisterone and 0.6% for (^{14}C) norgestrel. This suggests that radioactivity associated with norethisterone, norgestrel (Kamyab et al., 1967) and norethisterone oxime is rapidly removed from circulation and that the rate of metabolism of these compounds may be similar.

Analyses of the form of radioactivity in plasma shows that at 2 hours after administration (Table 1.8) 65.6±0.4% of the 'anti'-isomer and 52.6±0.8% of the 'syn'-isomer were in a non-conjugated form. By 24 hours, 40.0±0.6% of the 'anti'-isomer and 62.5±0.5% of the 'syn'-isomer were un-conjugated. Similar studies with related 19-norprogestogens have not been reported in the rabbit. These results suggest that the 'syn'-isomer or the metabolites of this isomer remain in the 'active' state for a longer period than the 'anti' isomer in the plasma.

From an analyses of the urine and faecal extracts it is apparent from the unchanged norethisterone oxime observed, that this compound is not readily metabolised (Tables 1.9 and 1.10). Thus, norethisterone oxime formed the major metabolite in the non-conjugated fraction of urine of (4-^{14}C) norethisterone oxime racemate, 'anti'-isomer and 'syn'-isomer. Similarly, in the enzyme hydrolysed fraction, although the amounts of unchanged norethisterone oxime, apart from conjugation, varied for each form of norethisterone oxime, the main product was the administered steroid. This formed 37 - 57% of the total applied sample. In the dioxane solvolysable fraction norethisterone oxime formed 38.2% of the total extract. This finding is similar to the metabolism of norgestrel in rabbit. Thus, Kamyab et al. (1967) found that about 60% of the urinary radioactivity had a chromatographic behaviour similar to that of norgestrel. However, in contrast both norethisterone and norethynodrel are metabolised to other compounds. Thus, 75% of the urinary radioactivity after the administration of (4-^{14}C) norethisterone to rabbits was associated with metabolites 'more polar' than norethisterone (Kamyab et al.,1967). Similarly, (H^3) norethynodrel was found to be mainly hydroxylated at C-10 in the glucuronide fraction. Thus, the three forms of norethisterone oxime appear to be similar in metabolic resistance to the highly potent synthetic progestogen norgestrel in the rabbit. However, when the glucuronide extracts were analysed by thin-layer chromatography in a system which resolves the two isomers, it was found that the 'syn' form in the racemic mixture and in the extracts obtained from rabbits receiving this isomer, was mainly in the 'anti' configuration. This indicates that the 'syn'-isomer may be converted to the 'anti'-isomer in the rabbit. This finding needs further examination.

In the non-conjugated fraction a 'more polar' metabolite was also observed which formed 12-20% of the total extract of (4-^{14}C) norethisterone oxime racemate, 'anti'- and 'syn'-isomers (Table 1.9). In the enzyme hydrolysed fractions of the three forms of norethisterone oxime two other metabolic products were observed. Both were 'more polar' than the administered steroids and one of them formed 13-25% of the total extract chromatographed. The other metabolite formed 18-37% of the total sample (Table 1.10). The proportions of these two metabolites varied in the extracts obtained from urine of rabbits receiving the racemate, 'anti' and 'syn'-isomer. Similarly, in the dioxane solvolysable fraction of urine from rabbits receiving (4-^{14}C) NET-OX racemate two metabolites, other than norethisterone oxime were observed (Fig. 1.9). One of these metabolites forming 29% of the sample was 'less polar' than the administered steroid, and the other 'more polar'

metabolite formed 34.8% of the sample applied. These other metabolic products in the non-conjugated enzyme hydrolysed and dioxane solvolysed fractions of urine were not identified in detail.

As in the urine, in the faeces of rabbits receiving (4-^{14}C) norethisterone oxime racemate, 65.6% of the non-conjugated fraction was similar in chromatographic behaviour to the administered steroid (Fig. 1.15). This is comparable to the excretion of (H^3) norethynodrel in faeces where much of the extractable radioactivity in the non-conjugated fraction was unchanged norethynodrel (Arai et al., 1962).

Therefore this 'in vivo' investigation of the metabolism of norethisterone oxime indicates that the metabolism of this compound is similar in some respects to the metabolism of related 19-norprogestogens in the rabbit. Thus, the amount of radioactivity excreted in urine associated with norethisterone oxime, norethynodrel, norethisterone and lynestrenol is similar. Similarities are also seen in the rate of clearance of norethisterone oxime, norethisterone and norgestrel from the vascular system. However, the amount of radioactive norethisterone oxime and norethynodrel excreted in faeces is higher than norgestrel and norethisterone. Differences are also seen in the conjugation of these progestogens. Thus, norethisterone oxime and norethynodrel are mainly conjugated with glucuronic acid, whereas, sulphate conjugation appears to be more dominant in the metabolism of norethisterone and norgestrel. Lynestrenol is almost equally conjugated with the two acids.

Examination of the metabolic products in urine and faeces indicates that norethisterone oxime is excreted mainly unchanged, as has also been reported for norgestrel. In contrast, both norethynodrel and norethisterone undergo considerable metabolism. This may suggest that the presence of the oxime at C-3 in norethisterone, and the presence of the ethyl group at C-13 in norgestrel appear to inhibit metabolic alteration in the rabbit.

Comparison of the metabolism of the three forms of norethisterone oxime(racemate, 'anti'- and 'syn'-isomers) indicates that although the rate of elimination in urine is similar for the three forms, the

rate of excretion in faeces differs. Thus, a higher percentage of the 'syn'-isomer is eliminated via this route. Differences are also seen in the conjugation of these three forms. Thus, the racemate and the 'syn'-isomer are mainly conjugated with glucuronic acid in urine. The rate of appearance of conjugates in the plasma are also different, thus the 'syn'-isomer remains in the free state longer than the 'anti'-isomer. Preliminary investigation of the metabolic products in the urine indicates that the three forms of norethisterone oxime are excreted mainly unchanged. However, the 'syn'-isomer appears to be converted to the 'anti'-isomer. This may be of importance and may possibly indicate that the 'syn'-isomer is the 'active' component and is 'inactivated' to the 'anti'-isomer. However, this needs further investigation. Thus, it can be concluded from this study that norethisterone oxime appears to be resistant to metabolic change in the rabbit. This property of this compound may be useful in the development of long-acting contraceptive steroids.

CHAPTER 2

'IN VITRO' METABOLISM OF (4-^{14}C) NORETHISTERONE OXIME AND (^{14}C) dl-NORGESTREL OXIME.

INTRODUCTION

In this chapter, an 'in vitro' investigation is described using the liver and extrahepatic tissues of the female New Zealand white rabbit. The rates of metabolism of norethisterone oxime (NET-OX) racemate, 'anti' and 'syn' isomers and dl-norgestrel oxime (dl-Ng-OX) racemate, 'anti' and 'syn' isomers are compared to norethisterone (NET) and dl-norgestrel (dl-Ng). In addition, the formation of metabolites from these steroidal compounds was also investigated.

MATERIALS AND METHODS.

1. Animals.

Female New Zealand white rabbits were used as described in Section I, Chapter I.

2. Chemicals

a) Steroids.

The preparation of norethisterone oxime has been described in Section II, Chapter I. The procedure used for the prepartion of (^{14}C) dl-norgestrel oxime and non-radioactive dl-norgestrel oxime was similar to that used for norethisterone oxime.

b) Other chemicals and organic solvents.

All chemicals and organic solvents were used as previously described.

3. 'In vitro' methodology.

The procedures used for preparation of tissues, incubation and extraction have been described in Section I, Chapter I except that the buffer system used for incubation of the steroids in this study was potassium phosphate buffer pH 7.4.

4. Identification of incubation products.

The methods employed, thin-layer, paper and gas-liquid chromatography have been fully described in Section I, Chapter I.

RESULTS.

An 'in vitro' study was carried out to compare the rate of metabolism and formation of metabolites of norethisterone oxime (NET-OX) and d-norgestrel oxime (d-Ng-OX) with the parent compound norethisterone (NET) and d-norgestrel (d-Ng), in the tissues of the female rabbit.

1. Rate of metabolism of norethisterone oxime, d-norgestrel oxime, norethisterone and d-norgestrel in hepatic tissue.

The incubation procedure has been described in Section I, Chapter I and in the Methods section of this chapter. The results of the time course metabolism (0 - 120 min) of norethisterone oxime racemate, 'anti'-isomer 'syn'-isomer and norethisterone are given in Tables 2.1A and 2.1D as means ($\pm$SD) of percent steroid not metabolised. Tables 2.2A and 2.2D show the means ($\pm$SD) of the three forms of d-norgestrel oxime and d-norgestrel. Figures 2.1 and 2.2 compare the metabolism of NET-OX (three forms) and NET, and d-Ng-OX (three forms) and d-Ng. (Individual values are given in Appendix tables 21 - 22).

Over a period of 120 min, the three forms of norethisterone oxime were metabolised at a distinctly slower rate than the parent compound norethisterone in the liver of the female rabbit. Thus, at 120 min of incubation time, 71.6$\pm$4.6% of NET-OX racemate, 72.1$\pm$0.9% of the 'anti'-isomer and 73.2$\pm$6.9% of the 'syn'-isomer remained unchanged (Table 2.1A). In comparison during the same time period 7.8$\pm$1.4% of norethisterone was recovered unmetabolised. (Table 2.1D). At all time intervals examined, the mean values of the three forms of NET-OX were significantly different ($p < 0.01$, Appendix table 22) from the mean value of NET. However, the values obtained for the rate of metabolism for the three forms of NET-OX were not significantly different from each other in the liver.

Table 2.2A shows the mean values ($\pm$SD) obtained for the metabolism of the three forms of d-norgestrel oxime and d-Ng (Table 2.2D) by rabbit liver. Fig. 2.2 compares the metabolism of the two steroids in the liver. As with NET-OX (three forms) and NET, the substrate decreases with time. The rate of metabolism of the parent compound

T A B L E 2.1

COMPARISON OF THE RATES OF METABOLISM OF NORETHISTERONE OXIME RACEMATE, 'ANTI'- AND 'SYN'-ISOMERS WITH NORETHISTERONE IN FEMALE RABBIT LIVER. THE RESULTS OF FOUR (NET-OX) AND SEVEN (NET) EXPERIMENTS ARE EXPRESSED AS MEAN ±SD OF PERCENT STEROID RECOVERED AT VARIOUS TIME INTERVALS.

TABLE 2.1A : Percent Norethisterone Oxime not Metabolised.						
STEROID INCUBATED	TIME OF INCUBATION					
	0 min	10 min	20 min	30 min	60 min	120 min
NET-OX Racemate	87.6±1.9	80.2±2.1	76.6±3.0	75.1±1.6	72.6±2.8	71.6±4.6
NET-OX 'anti'-isomer	88.8±1.0	80.8±0.9	78.8±0.9	76.3±1.2	74.8±0.9	72.1±0.9
NET-OX 'syn'-isomer	92.6±2.4	85.1±2.6	80.1±4.3	78.1±5.1	75.0±3.7	73.2±6.9
TABLE 2.1B : Percent Formation of Norethisterone from Norethisterone Oxime						
NET-OX Racemate	0	2.6±1.0	5.6±1.2	6.2±1.6	7.2±1.0	8.8±0.6
NET-OX 'anti'-isomer	0	3.3±0.6	5.9±0.8	6.5±0.8	7.5±1.4	8.7±1.2
NET-OX 'syn'-isomer	0	2.4±0.9	5.0±0.8	5.3±0.7	5.9±0.4	6.5±0.6
TABLE 2.1C : Percent Formation of Tetrahydronorethisterone from Norethisterone Oxime.						
NET-OX Racemate	0	1.7±0.8	3.6±1.1	4.1±1.5	4.4±2.1	4.3±2.7
NET-OX 'anti'-isomer	0	2.1±0.7	3.9±0.9	4.3±1.2	4.9±1.7	5.4±1.2
NET-OX 'syn'-isomer	0	1.5±0.5	3.4±0.9	3.7±0.5	4.2±0.8	4.3±0.6
TABLE 2.1D : Metabolism of Norethisterone.						
Norethisterone	92.2±2.5	62.2±3.9	53.1±2.1	45.5±2.1	24.1±2.4	7.8±1.4
Tetrahydro-Norethisterone formed	0	35.2±2.9	44.0±2.2	53.3±2.7	72.6±2.3	86.3±4.1

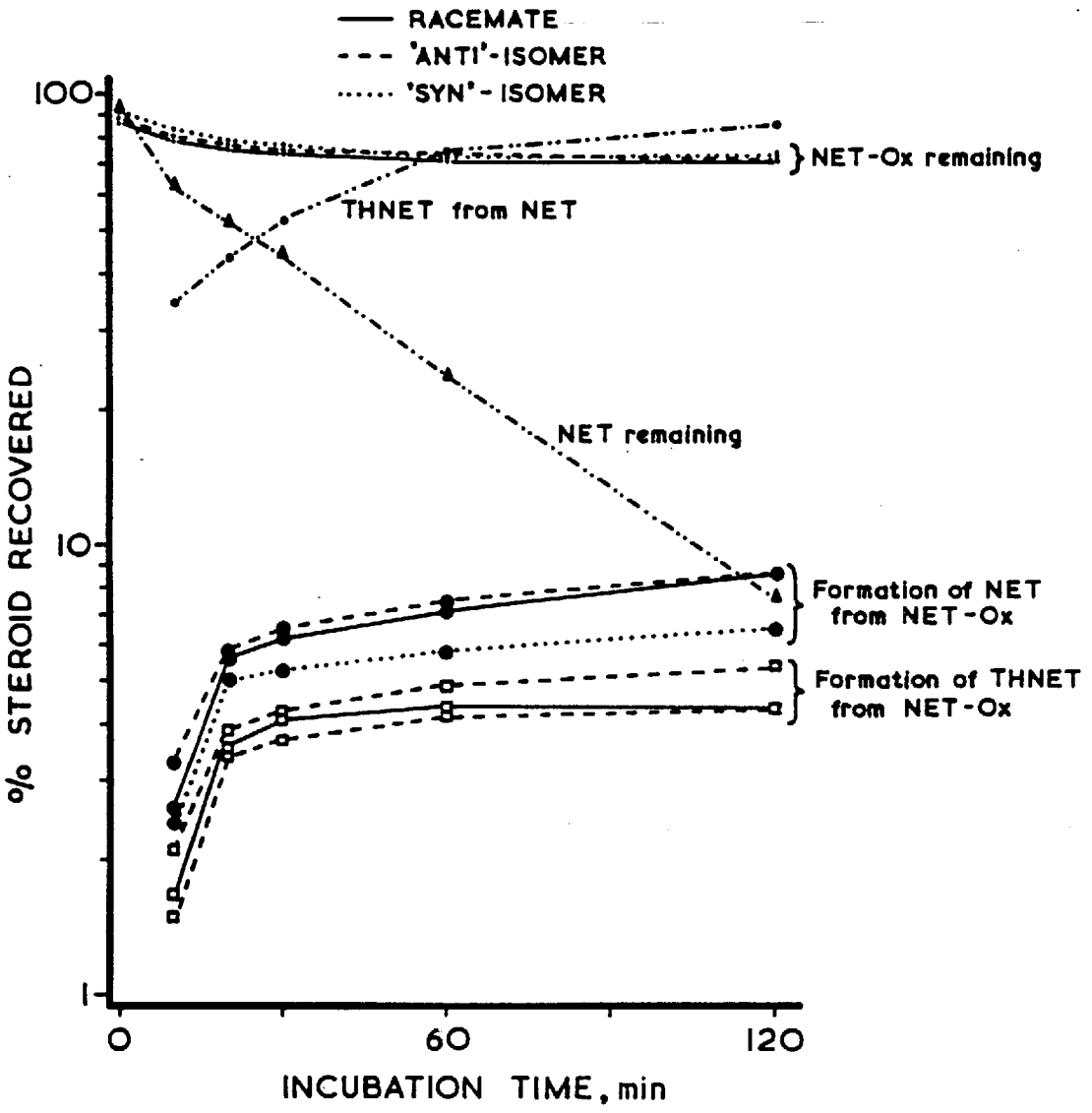


Fig. 2.1 Comparison of the rate of metabolism of norethisterone oxime racemate, 'anti'-, 'syn'-isomers and norethisterone in female rabbit liver tissue. The incubation conditions are given in the text.

T A B L E 2.2

COMPARISON OF THE RATES OF METABOLISM OF d-NORGESTREL OXIME RACEMATE, 'ANTI'-ISOMER, 'SYN'-ISOMER WITH d-NORGESTREL IN FEMALE RABBIT LIVER. THE RESULTS OF FOUR (d-Ng-OX) AND SIX (d-Ng) EXPERIMENTS ARE EXPRESSED AS MEAN ±SD OF PERCENT STEROID RECOVERED AT VARIOUS TIME INTERVALS.

TABLE 2.2A : Percent d-Norgestrel Oxime Not Metabolised.						
STEROID INCUBATED	TIME OF INCUBATION.					
	0 min	10 min	20 min	30 min	60 min	120 min
d-Ng-Ox racemate	91.4±1.5	87.2±2.2	85.1±2.1	84.1±1.6	81.6±1.5	80.1±1.4
d-Ng-Ox 'anti'-isomer	93.2±1.9	86.1±1.9	83.8±2.1	82.2±2.0	80.0±1.3	76.6±1.3
d-Ng-OX 'syn'-isomer	92.6±1.7	88.1±1.6	86.1±2.1	85.0±1.6	82.5±1.7	80.0±1.3
TABLE 2.2B : Percent Formation of Norgestrel From d-Norgestrel Oxime.						
d-Ng-OX racemate	0	2.1±0.8	4.2±0.6	6.1±1.1	8.8±1.4	10.0±1.8
d-Ng-OX 'anti'-isomer	0	3.0±0.8	6.2±1.3	7.4±1.4	8.2±1.9	9.9±1.6
d-Ng-OX 'syn'-isomer	0	3.1±0.8	4.9±0.9	6.5±1.1	7.4±0.5	8.6±1.2
TABLE 2.2C : Percent Formation of Tetrahydronorgestrel From d-Norgestrel Oxime.						
d-Ng-OX racemate	0	0	0	0	0	0
d-Ng-OX 'anti'-isomer	0	0	0	0	0	0
d-Ng-OX 'syn'-isomer	0	0	0	0	0	0
TABLE 2.2D : Metabolism of d-Norgestrel.						
d-Norgestrel	91.6±3.2	73.0±2.1	62.5±0.9	56.6±1.5	52.4±3.0	44.5±2.4
Tetrahydro-norgestrel formed.	0	21.9±2.4	31.5±1.8	36.8±1.7	44.8±1.9	53.7±1.9

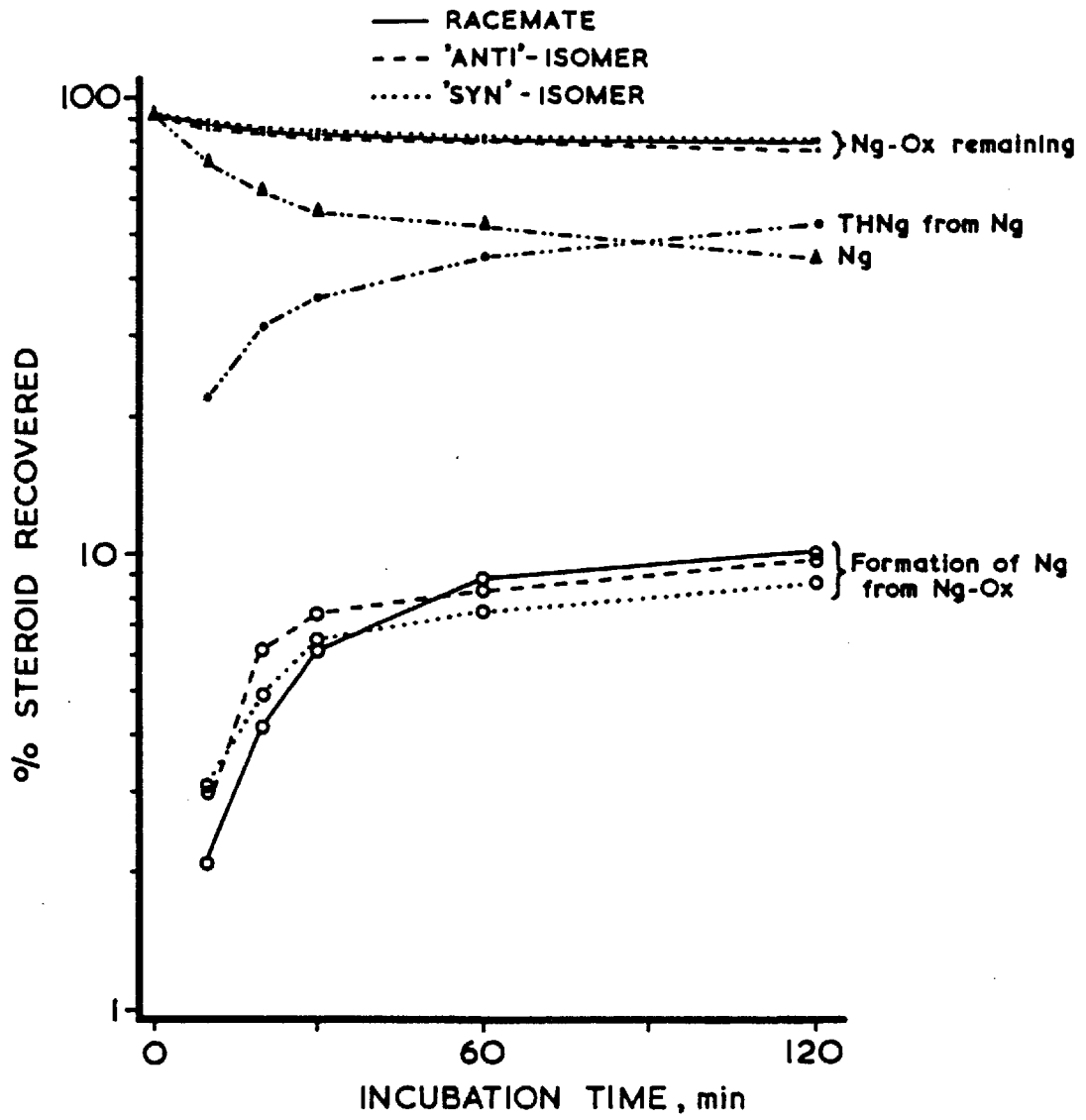


Fig. 2.2 Comparison of the rate of metabolism of d-norgestrel oxime racemate, 'anti'-, 'syn'-isomers and d-norgestrel in female rabbit liver tissue. The incubation conditions are given in the text.

d-norgestrel was distinctly faster than the rate of metabolism of the three forms of d-norgestrel oxime. Thus at 120 min of incubation time, 80.1±1.4% of d-Ng-OX racemate, 76.6±1.3% of the 'anti'-isomer and 80.0±1.3% of the 'syn'-isomer remained unchanged. In comparison 44.5±2.4% of d-Ng remained unchanged at 120 min. The mean values for the three forms of d-Ng-Ox, at all time intervals examined were significantly different ($p < 0.01$, Appendix table 22) from the mean value of d-Ng remaining. In contrast to NET-OX, the metabolism of the racemic form of d-norgestrel oxime was significantly different from the 'anti'-isomer at 120 min ($p < 0.05$, Appendix table as was the rate of metabolism of the 'anti'-isomer from the 'syn'-isomer ($p < 0.05$, Appendix table 21).

Comparing the rates of metabolism of the two oxime derivatives (Tables 2.1A and 2.2A) the results show that the rate of metabolism of the three forms of NET-OX, at all time intervals examined was significantly higher ($p < 0.05$, Appendix table 20) from the rate of metabolism of the three forms of d-Ng-OX in rabbit liver. Thus, d-Ng-OX was metabolised at a slower rate than NET-OX.

2. Nature and formation of metabolites in liver.

The metabolic products obtained from the liver incubations with the three forms of NET-OX and d-Ng-OX, were identified by thin-layer and gas-liquid chromatography, as described in Section I, Chapter I. The data on the percent formation of NET-OX metabolites (mean ±SD) are given in Tables 2.1B and 2.1C, and are shown graphically in Fig. 2.1 Fig. 2.3 - 2.5 compare the liver metabolites of NET-OX (three forms) as seen on thin-layer radiochromatograms at 120 min of incubation time.

The rate of formation of the metabolic products of the three forms of NET-OX and d-Ng-OX was seen to increase with time, indicative of an enzymic reaction. NET-OX racemate, 'anti'-isomer and 'syn'-isomer were seen to be metabolised to the parent compound norethisterone and tetrahydronorethisterone in the liver of the female rabbit. (Table 2.1). However, d-Ng-OX, (three forms) was converted only to norgestrel, and no other metabolites were detected (Table 2.2).

Fig.2.3 Norethisterone oxime 'syn'-isomer

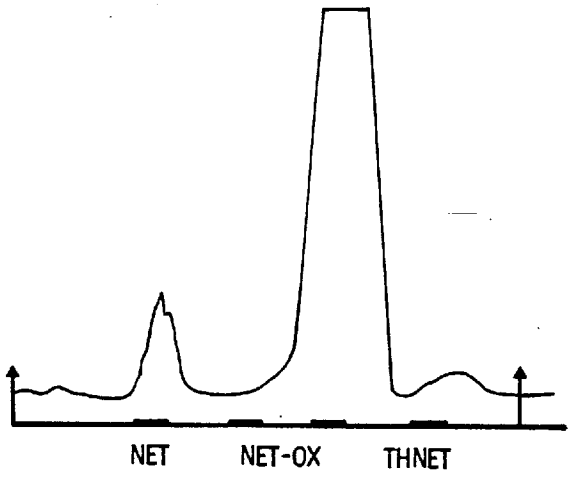


Fig.2.4 Norethisterone oxime racemate

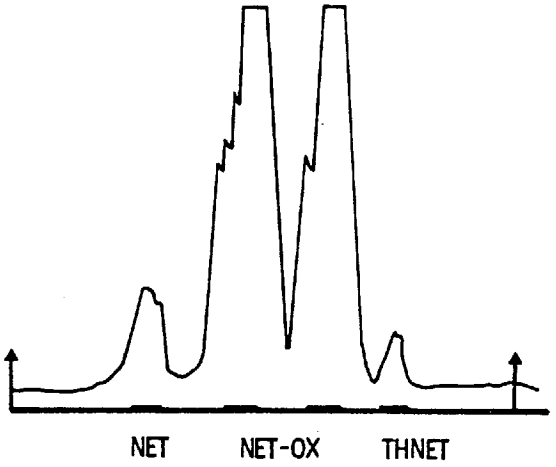
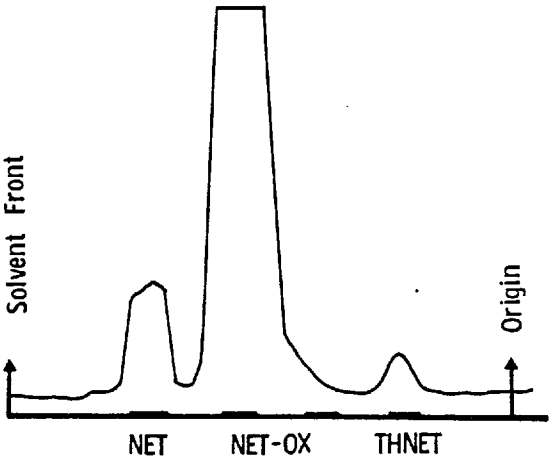


Fig.2.5 Norethisterone oxime 'anti'-isomer



Figs. 2.3 - 2.5

Radiochromatogram scans of [4-^{14}C]norethisterone oxime racemate, 'anti'- and 'syn'- isomers incubated with female rabbit liver for 120 min. Thin-layer chromatograms were developed in the solvent system. Toluene:Acetone 4:1 (V/V).

3. Metabolism of norethisterone oxime (NET-OX), d-norgestrel oxime (d-Ng-OX), norethisterone (NET) and d-norgestrel (d-Ng) in the hepatic and extrahepatic tissues of female rabbit.

These studies were carried out to investigate the rates of metabolism of the parent compound norethisterone and d-norgestrel with their oxime derivatives (NET-OX and d-Ng-OX) by the hepatic and extrahepatic tissues of the female rabbit.

The procedure used for the incubations has been described in Section I, Chapter I. With every tissue, the steroid to tissue ratio (1 : 40,000) was kept constant throughout the study. In all experiments different organs obtained from a single animal, were examined simultaneously. The results expressed as the percent of steroid not metabolised at 30 min are given in Table 2.3 (NET-OX and NET) and Table 2.4 (d-Ng-OX and d-Ng). Fig. 2.6 compares the metabolism of norethisterone in the different rabbit tissues. Fig. 2.7 compares the metabolism of norethisterone oxime racemate and Fig. 2.8 the metabolism of d-Ng-OX racemate and d-Ng (individual values are given in Appendix tables 23-26) in the various tissues examined.

a) Hepatic tissue metabolism.

The mean values ($\pm$SD) for the metabolism of NET-OX (three forms) and NET by liver tissue over a period of 30 min are shown in Table 2.3 As shown previously at 120 min of incubation time, norethisterone was metabolised faster than the three forms of norethisterone oxime. Thus, at 30 min, 48.4$\pm$7.6% of norethisterone remained unchanged, whereas 72.1$\pm$3.5% of NET-OX racemate, 68.5$\pm$1.5% of the 'anti'-isomer and 65.9$\pm$1.5% of the 'syn'-isomer remained unmetabolised. The mean value of norethisterone was significantly different ($p < 0.01$, Appendix table 23) from the mean values obtained for the three forms of norethisterone oxime.

Similarly with d-Ng-OX, the rate of metabolism of this derivative was slower than the metabolism of the parent compound, d-Ng in female rabbit liver, at 30 min (Table 2.4). Thus, at 30 min, 88.3$\pm$2.0 of d-norgestrel oxime racemate remained whereas, 52.0$\pm$5.3% of d-norgestrel

TABLE 2.3 COMPARISON OF THE RATE OF METABOLISM OF NORETHISTERONE AND NORETHISTERONE OXIME RACEMATE, 'ANTI'-ISOMER AND 'SYN'-ISOMER IN THE TISSUES OF THE FEMALE RABBIT. THE RESULTS, OF THREE EXPERIMENTS, ARE EXPRESSED AS MEANS (±SD) PERCENT STEROID RECOVERED AT 30 min.

STEROID INCUBATED	NORETHISTERONE		NORETHISTERONE OXIME RACEMATE			NORETHISTERONE OXIME 'ANTI'-ISOMER			NORETHISTERONE OXIME 'SYN'-ISOMER		
% STEROID IDENTIFIED / TISSUE	NET	THNET	NET-OX	NET	THNET	NET-OX	NET	THNET	NET-OX	NET	THNET
LIVER	48.4±7.6	50.1±3.6	72.1±3.5	8.0±0.9	3.2±1.1	68.5±1.5	11.8±1.5	2.6±0.6	65.9±1.5	11.1±0.9	4.1±1.0
STOMACH	84.2±3.6	7.2±1.0	71.1±3.9	3.2±0.9	2.1±0.9	73.5±4.1	4.0±1.1	5.0±0.9	74.0±2.1	4.4±0.4	6.5±0.6
DUODENUM	63.1±4.3	9.2±1.0	80.1±8.5	3.4±0.6	3.1±0.9	77.2±0.9	6.4±0.8	3.1±1.5	75.2±1.9	7.1±1.0	5.0±0.9
ILEUM	79.5±1.0	10.4±1.5	75.4±8.0	4.1±0.9	2.2±0.9	81.5±6.1	7.1±0.7	1.7±0.5	75.2±2.7	4.1±1.0	4.1±0.9
CAECUM	84.5±2.6	7.2±1.1	80.2±1.8	2.1±0.9	1.3±0.1	82.7±3.0	2.4±0.4	0	80.8±1.5	2.5±0.6	0
KIONEY	88.2±6.5	3.1±0.9	83.1±6.1	2.0±0.7	1.3±0.1	85.2±3.5	2.9±0.7	0	87.9±1.4	3.7±1.5	0
HEART	89.1±0.9	0	80.8±3.6	3.0±0.6	0	85.5±2.9	3.1±1.0	0	85.8±1.5	3.3±1.2	0
SKELETAL MUSCLE	63.1±9.4	3.4±0.9	86.0±4.4	2.0±0.8	1.4±0.4	84.5±3.4	3.3±0.7	3.1±0.9	81.5±1.6	2.8±0.9	1.5±0.4
LUNG	43.1±5.2	7.2±1.0	80.4±2.5	5.6±3.0	2.4±1.4	81.1±1.0	3.8±2.0	3.8±2.0	83.1±2.5	9.7±0.9	2.1±1.1
FAT	83.0±7.1	0	76.8±5.8	0	0	90.1±1.1	0	0	93.0±1.6	0	0

TABLE 2.4

COMPARISON OF THE RATES OF METABOLISM OF d-NORGESTREL AND d-NORGESTREL OXIME RACEMATE IN THE TISSUES OF THE FEMALE RABBIT. THE RESULTS OF THREE EXPERIMENTS, ARE EXPRESSED AS MEAN (±SD) OF PERCENT STEROID RECOVERED AT 30 min.

STEROID INCUBATED	d-NORGESTREL		d-NORGESTREL-OXIME RACEMATE		
PERCENT STEROID IDENTIFIED	d-Ng	THNg	d-Ng-OX	Ng	THNg
TISSUE					
LIVER	52.0±5.3	45.0±2.8	88.3±2.0	6.1±0.9	0
STOMACH MUSCLE	89.2±1.9	5.1±0.8	90.1±1.6	2.1±0 1	2.0±0.7
DUODENUM	88.7±2.0	4.1±0.5	96.0±1.4	0	0
ILEUM	84.6±2.3	6.1±1.1	92.0±0.8	0	0
CAECUM	92.0±2.8	0	93.0±1.7	0	0
KIDNEY	91.3±2.2	0	91.3±0.7	0	0
HEART	92.4±2.1	0	93.2±1.3	0	0
SKELETAL MUSCLE	82.0±3.0	8.1±1.0	95.0±1.4	0	0
LUNG	82.5±2.3	7.3±0.7	87.0±1.7	3.1±0.9	2.1±0.9
ADIPOSE	90.0±1.7	0	90.3±0.8	0	0

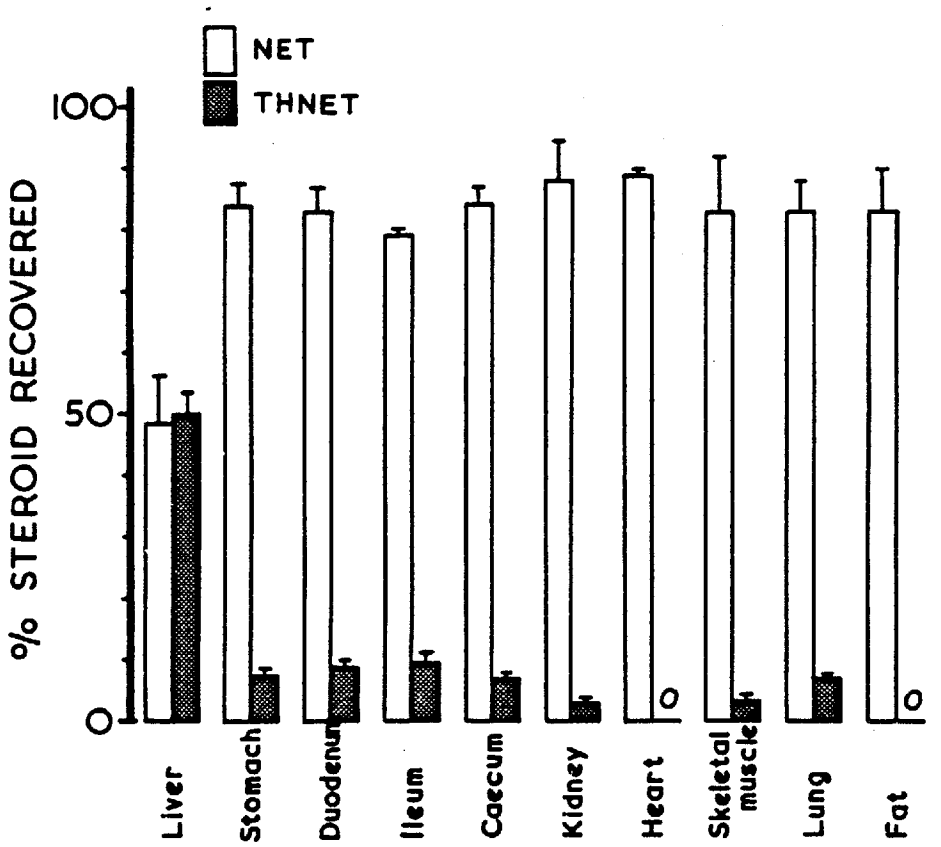


Fig. 2.6 Comparison of the metabolism of norethisterone in various tissues of the female rabbit.

remained unmetabolised. The mean value for d-Ng-OX racemate was significantly higher ($p < 0.01$, Appendix table 25) than the mean value of d-norgestrel.

Comparison of the rates of metabolism of d-norgestrel oxime racemate and norethisterone oxime racemate (Tables 2.3 and 2.4) indicates that these are significantly differently ($p < 0.05$, Appendix table 23) metabolised.

The metabolic products (Table 2.3) of NET-OX (three forms) and d-Ng-OX racemate from female liver have been described in the previous section.

b) Stomach muscle tissue metabolism.

As shown in Table 2.3, Figures 2.6 and 2.7 both norethisterone and norethisterone oxime (three forms) were metabolised by female rabbit stomach tissue. Thus, at 30 min, $84.2 \pm 3.6\%$ of NET remained unchanged. However, a lower percentage and significantly different ($p < 0.05$, Appendix table 23) of NET-OX racemate, 'anti'-isomer and 'syn'-isomer remained unmetabolised. Thus, $71.1 \pm 3.9\%$ of NET-OX racemate, $73.5 \pm 4.1\%$ of the 'anti'-isomer and $74.0 \pm 2.1\%$ of the 'syn'-isomer remained unchanged.

With d-norgestrel oxime and d-norgestrel (Table 2.4 and Fig. 2.8) little metabolism of both these progestogens was seen. Thus, $89.2 \pm 1.9\%$ of d-Ng and $90.1 \pm 1.6\%$ of d-Ng-OX racemate remained unchanged at 30 min. These values were not significantly different ($p > 0.1$) Appendix table 25).

However, when the rates of metabolism of NET-OX racemate and d-Ng-OX racemate were compared, the mean values were statistically different ($p < 0.05$, Appendix table 23).

As with the liver tissue, NET-OX (three forms) was metabolised to NET and tetrahydronorethisterone (Table 2.3) But in contrast to the liver (Table 2.4) d-Ng-OX racemate was metabolised to both norgestrel and tetrahydronorgestrel by the stomach tissue.

c) Duodenal tissue metabolism

The mean values ($\pm$SD) obtained for the metabolism of NET and NET-OX in this tissue are shown in Table 2.3 and compared to other tissues in Figs.2.6 and 2.7.

Both norethisterone and the three forms of norethisterone oxime were metabolised by this tissue. Thus, at 30 min, 63.1$\pm$4.3% of NET, 80.1$\pm$8.5%, of NET-OX racemate, 77.2$\pm$0.9% of the 'anti'-isomer and 75.2$\pm$1.9% of the 'syn'-isomer remained unmetabolised. The mean values for NET and NET-OX (three forms) were significantly different ($p < 0.05$, Appendix table 23).

Although some metabolism of d-norgestrel did occur in this tissue (88.7$\pm$2.0% remained unchanged, Table 2.4) little metabolism of d-Ng-OX racemate occurred (96.0$\pm$1.4% remained unchanged). These mean values were also significantly different ($p < 0.05$, Appendix table 25).

Comparison of the mean values of NET-OX racemate and d-Ng-OX racemate indicates that the two steroids are metabolised at significantly different rates ($p < 0.05$, Appendix table 23).

Examination of the metabolites of NET-OX racemate, 'anti'- and 'syn'-isomers indicates that as in the liver and stomach tissues, these steroids are metabolised to NET and tetrahydronorethisterone (Table 2.3 Fig. 2.7). d-Ng-OX was not metabolised to any detectable metabolites in this tissue (Table 2.4 and Fig. 2.8).

d) Ileal tissue metabolism.

In Table 2.3 are given the mean values ($\pm$SD) for the metabolism of NET and three forms of NET-OX, and these are compared with other tissues in Figs 2.6 and 2.7. As in the other tissues examined above, some metabolism of both these steroids occurred in ileal tissue. Thus, at 30 min, 79.5$\pm$1.0% of NET, 75.4$\pm$8.0% of NET-OX racemate, 81.5$\pm$6.1% of the 'anti'-isomer and 75.2$\pm$2.7% of the 'syn'-isomer remained unchanged. However, the mean values of NET and the three forms of NET-OX were not significantly different ($p > 0.1$, Appendix table 23).

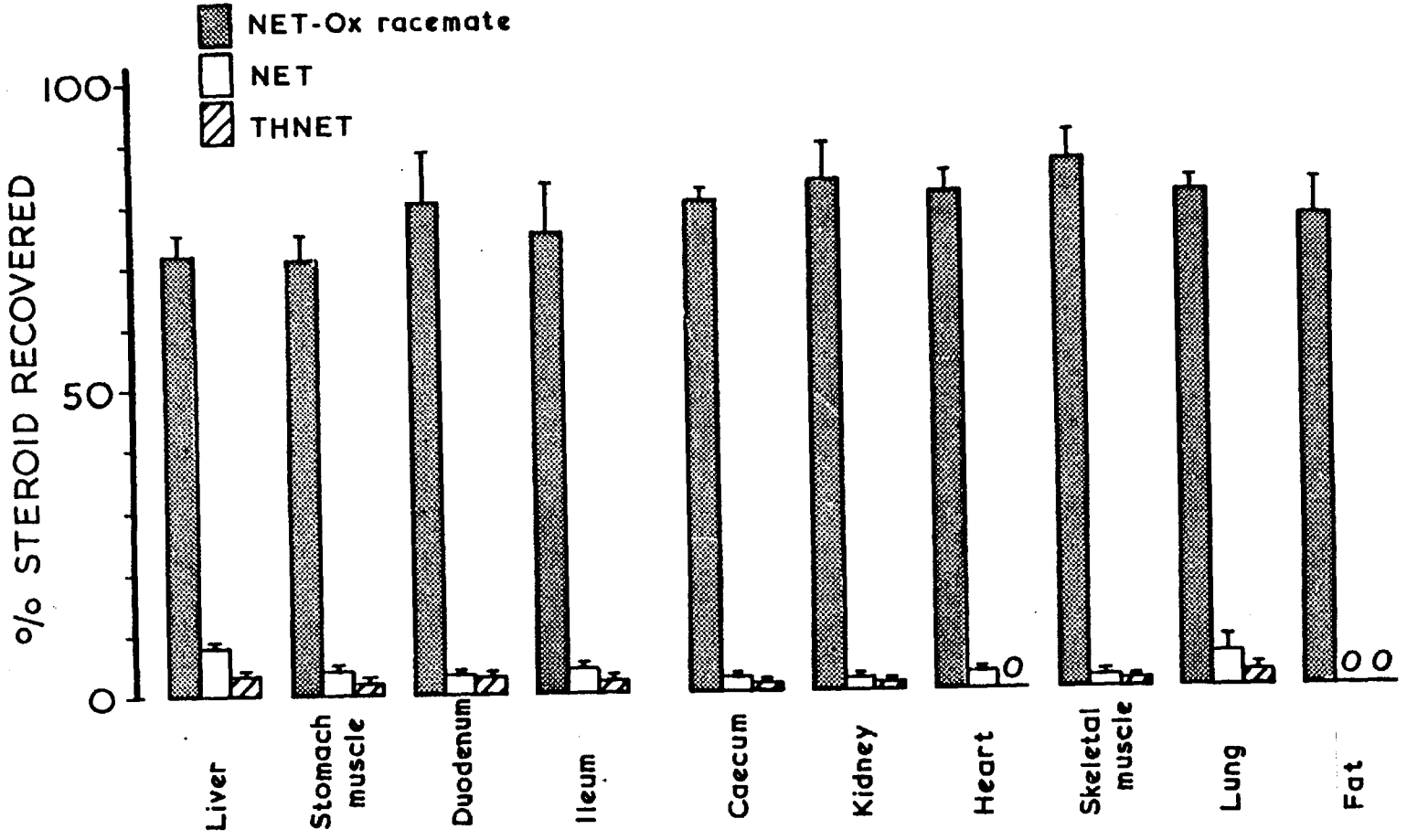


Fig. 2.7 Comparison of the metabolism of norethisterone oxime racemate in various tissues of the female rabbit.

As with the duodenal tissue, no metabolism of d-Ng-OX racemate seemed to occur in this tissue (Table 2.4) although d-Ng was metabolised. Thus, 84.6±2.3% of d-Ng remained at 30 min; whereas 92.0±0.8% of d-Ng-OX was recovered unchanged. These values were significantly different ($p < 0.05$, Appendix table 25).

When the rates of metabolism of the two oxime derivatives of norethisterone and d-norgestrel are compared, the mean values were significantly different ($p < 0.05$, Appendix table 23).

In ileal tissue, as with the other tissues discussed previously, both NET and d-Ng were metabolised to the tetrahydro metabolites (Table 2.3). NET-OX (three forms) was metabolised to NET and tetrahydronorethisterone. However, d-Ng-OX was not metabolised to any detectable products (Table 2.4).

e) Caecal tissue metabolism.

Table 2.3 gives the mean values (±SD) of NET and NET-OX not metabolised, and these values are compared with other tissues in Figs. 2.6 and 2.7. In this tissue, the metabolism of norethisterone and the three forms of norethisterone oxime was not significantly different ($p > 0.1$, Appendix table 23) but some metabolism of both steroids did occur. Thus 84.5±2.6% of NET, 80.2±1.8% of NET-OX racemate, 82.7±3.0% of the 'anti'-isomer and 80.8±1.5% of the 'syn'-isomer remained unchanged.

In comparison, both d-Ng and d-Ng-OX were not metabolised by this tissue (Table 2.4, Fig. 2.8).

The metabolism of norethisterone oxime racemate and d-norgestrel oxime racemate were significantly different ($p < 0.05$, Appendix table 23).

Examination of the metabolic products indicated that NET was metabolised to tetrahydronorethisterone in this tissue. All the three forms of NET-OX were converted to NET, but only the racemate was metabolised to tetrahydronorethisterone.

f) Kidney tissue metabolism.

Little metabolism of both NET and NET-OX (three forms) occurred in this tissue (Table 2.3, Figs 2.6 and 2.7). Thus at 30 min 88.2±6.5% of norethisterone, 83.1±6.1% of norethisterone oxime racemate, 85.2±3.5% of the 'anti'-isomer and 87.9±1.4% of the 'syn'-isomer remained unmetabolised. The mean values were not significantly different from each other ($p > 0.1$, Appendix table 23).

Neither d-norgestrel nor d-norgestrel oxime racemate (Table 2.4, Fig 2.8) were found to be metabolised by this tissue.

The rates of metabolism of d-Ng-OX racemate and NET-OX racemate were not significantly different in this tissue ($p > 0.1$, Appendix table 23).

With norethisterone, some formation of tetrahydronorethisterone (3.1±0.9%) occurred (Table 2.3, Fig. 2.6). Norethisterone oxime racemate was metabolised to norethisterone and tetrahydronorethisterone However, the 'anti'-isomer and the 'syn'-isomer were metabolised only to norethisterone.

g) Heart tissue metabolism.

Heart tissue did not metabolise norethisterone, however, some metabolism of the three forms of NET-OX did occur (Table 2.3, Figs 2.6 and 2.7). Thus, at 30 min, 89.1±0.9% of NET remained unchanged, whereas 80.8±3.6% of NET-OX racemate, 85.5±2.9% of the 'anti'-isomer and 85.8±1.5% of the 'syn'-isomer were recovered unmetabolised, The mean value for NET-OX racemate was significantly different from the mean value of NET ($p < 0.05$, Appendix table 23).

Both d-norgestrel and d-norgestrel oxime were not metabolised by this tissue (Table 2.4, Fig. 2.8).

The mean values of percent steroid remaining for NET-OX racemate and d-Ng-OX racemate were significantly different ($p < 0.05$, Appendix table 23).

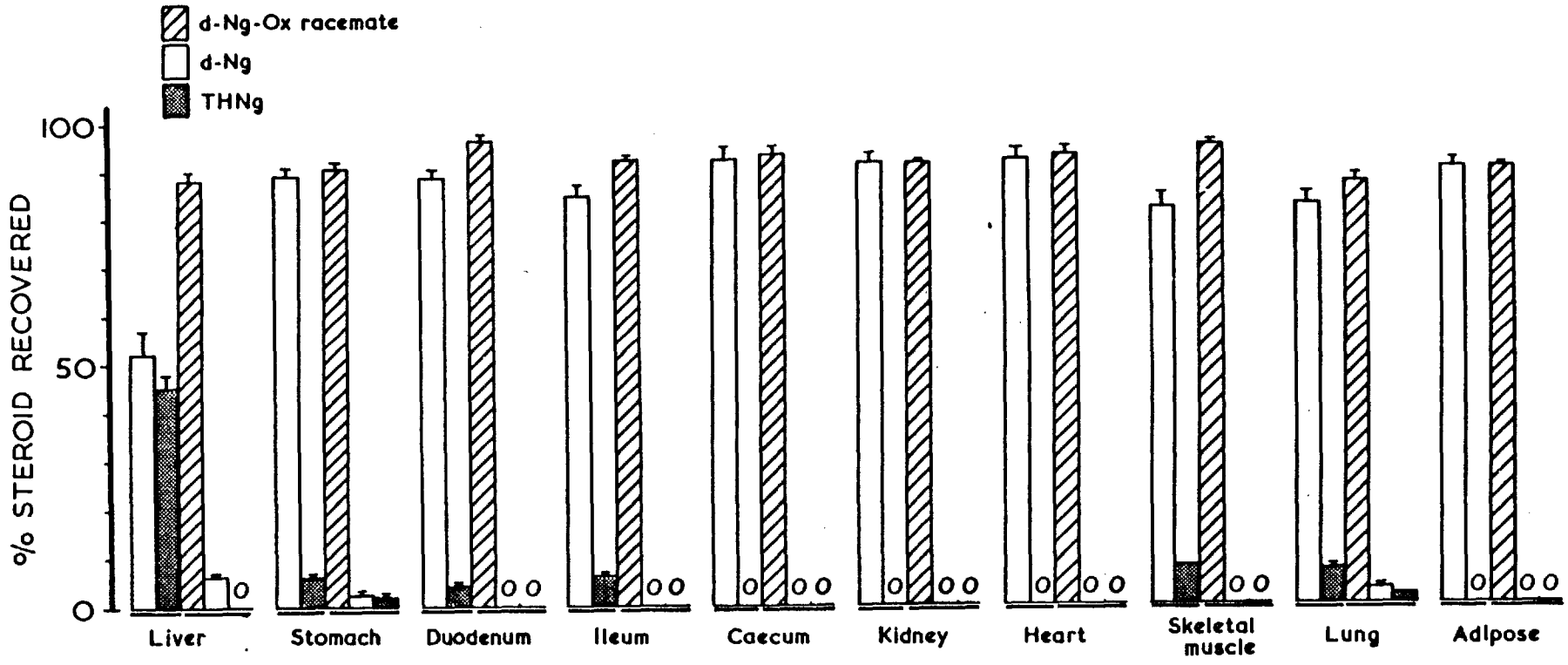


Fig. 2.8. Comparison of the metabolism of d-norgestrel oxime racemate and d-norgestrel in various tissues of the female rabbit.

Examination of the metabolic products of the three forms of NET-OX indicates (Table 2.3, Fig. 2.7) that only norethisterone was formed in heart tissue.

h) Skeletal muscle tissue metabolism.

In Table 23 are given the mean values ($\pm$SD) of norethisterone and norethisterone oxime (three forms) not metabolised by this tissue. These values are compared with the other tissues in Fig. 2.6 and 2.7. Thus, at 30 min, 63.1$\pm$9.4% of NET remained, whereas, 86.0$\pm$4.4% of NET-OX racemate, 84.5$\pm$3.4% of NET-OX 'anti'-isomer and 81.5$\pm$1.6% of the 'syn'-isomer remained unchanged. The metabolism of NET was significantly different ($p < 0.05$, Appendix table 23) from the three forms of NET-OX.

Although d-norgestrel was metabolised by this tissue (82.0$\pm$3.0% remained), d-norgestrel oxime racemate metabolism did not occur (Table 2.4, Fig. 2.8). 95.0$\pm$1.4% of d-Ng-OX racemate remained unchanged.

As in the other tissues examined, the rate of metabolism of d-Ng-OX racemate was significantly different from NET-OX racemate ($p < 0.05$, Appendix table 23).

The metabolites identified from both NET and NET-OX (three forms) were similar to those obtained in liver (Table 2.3, Fig. 2.6 and 2.7). d-norgestrel was also reduced to tetrahydronorgestrel (Table 2.4, Fig. 2.8)

i) Lung tissue metabolism.

Lung tissue was seen to metabolise both norethisterone and the three forms of norethisterone oxime (Table 2.3, Figs 2.6 and 2.7). Thus, in 30 min 43.1$\pm$5.2% of norethisterone remained unchanged, whereas 80.4$\pm$2.5% of NET-OX racemate, 81.1$\pm$1.0% of the 'anti'-isomer and 83.1$\pm$2.5% of the 'syn'-isomer were recovered unchanged. The mean value of NET was significantly different from the mean values of the three forms of NET-OX ($p < 0.05$, Appendix table 23).

This tissue was also seen to metabolise both d-norgestrel and d-norgestrel oxime racemate (Table 2.4, Fig. 2.8). However, the mean values of d-Ng were not significantly different from the mean values of d-Ng-OX racemate. Thus, $82.5 \pm 2.3\%$ of d-Ng and $87.0 \pm 1.7\%$ of d-Ng-OX racemate remained unmetabolised at 30 min.

Comparison of the rates of metabolism of NET-OX racemate and d-Ng-OX racemate by the lung tissue indicates, that the mean values were significantly different ($p < 0.05$, Appendix 23).

Examination of the metabolic products indicates that both NET and d-Ng were metabolised to tetrahydro-metabolites and the oxime derivatives were hydrolysed to NET and Ng. The oxime derivatives were also metabolised to tetrahydro-metabolites by this tissue. (Tables 2.3 and 2.4, Figs 2.6, 2.7 and 2.8).

j) Adipose tissue metabolism.

The mean values ($\pm$SD) for NET and the three forms of NET-OX are given in Table 2.3 and compared to other tissues in Figs 2.6 and 2.7 The mean values of d-Ng and d-Ng-OX racemate are given in Table 2.4 and compared to other tissues in Fig. 2.8. The results show that norethisterone and d-norgestrel and their oxime derivatives were not metabolised by this tissue.

Thus, in summary, this 'in vitro' investigation of the metabolism of norethisterone oxime and d-norgestrel oxime racemate, 'anti'-isomer and 'syn'-isomer indicates that these derivatives are metabolised at a relatively slower rate than the parent steroids, norethisterone and d-norgestrel in rabbit liver. These oxime derivatives remain unchanged over a period of time when the parent compounds have undergone substantial metabolism.

The liver was found to be the main organ of metabolism although some extrahepatic tissues particularly the stomach, duodenum, ileum and lung were also seen to be capable of metabolising the oxime derivatives. However, the rate of metabolism in these tissues was not significantly different from the liver.

The rates of metabolism of the racemate, 'anti'-isomer and 'syn'-isomer of both norethisterone and norgestrel were not significantly different in the tissues examined. But the rate of metabolism of norethisterone oxime was significantly different from d-norgestrel oxime, which was metabolised more slowly.

Norethisterone was identified as a metabolite of norethisterone oxime in all the tissues examined except adipose tissue. However, tetrahydronorethisterone was identified only in liver, stomach, duodenal, ileal, skeletal muscle and lung tissues.

Only liver, stomach and lung tissue metabolised d-norgestrel oxime to norgestrel and tetrahydronorgestrel.

DISCUSSION.

In recent years, the oxime derivatives of progesterone related synthetic progestogens (Shroff, 1970 and Shroff et al., 1971), the oxime and methoxime derivatives of 19-nortestosterone related progestogens (Shroff et al., 1973 and Kärkkäinen et al., 1975a) and the methoxime derivatives of natural steroids (Kärkkäinen et al., 1975b) have been examined for their progestational and antifertility activity. However, no study has been reported in which the rate of metabolism of these derivatives has been compared with the parent compounds.

In this study, we have investigated the rate of metabolism of norethisterone oxime and d-norgestrel oxime with the parent compounds, norethisterone and d-norgestrel in female rabbit liver and extra-hepatic tissues.

During the chemical synthesis of steroidal oximes, the geometric isomers designated the 'anti'-isomer and 'syn'-isomer (Table 1.1, Section 2) are formed. Since the required data as to which of these isomers is biologically active was not available, the metabolism of the racemate, 'anti'-isomer and 'syn'-isomer was investigated.

Comparative investigation of the parent compounds norethisterone and d-norgestrel with the oxime derivatives, norethisterone oxime and d-norgestrel oxime in the three forms, indicates that under the conditions of incubation described (see Methods section of Section 1, Chapter 1), the parent compounds were found to be extensively metabolised; whereas, the metabolism of norethisterone oxime (three forms) and d-norgestrel oxime (three forms) was significantly slower in female rabbit liver (Tables 2.1 and 2.2). When the rates of metabolism of norethisterone oxime racemate, 'anti'-isomer and 'syn'-isomer are compared to that of the three forms of d-norgestrel oxime, the d-norgestrel derivatives were seen to be metabolised at a slower rate than the norethisterone derivatives. This finding is in agreement with our previous data on the rates of metabolism of the parent compounds where d-norgestrel was metabolised at a comparatively slower rate than norethisterone, and the results obtained in this study (Section 1, Chapter 1 and this Chapter - Tables 2.1 and 2.2).

Identification of the metabolites of both norethisterone oxime (three forms) and d-norgestrel oxime (three forms) indicates that the oxime group is hydrolysed in rabbit liver, giving rise to the ketone

Thus norethisterone and norgestrel were both identified. Tetrahydronorethisterone was also observed with the incubations of norethisterone oxime, however, the ring-A reduced metabolites were not present in the incubation of d-norgestrel oxime (three forms).

In comparison to our studies with the oxime derivatives of norethisterone and d-norgestrel in rabbit liver homogenates, Hucker et al., (1971), Hucker,,1973 and Coutts et al., (1976) isolated both the hydrolysed and reduced products of phenylacetone oxime from rabbit and rat liver. Thus Hucker et al., (1971) isolated phenylacetone and phenyl-2-propanol from the 9000g fraction of rabbit liver after incubation with phenylacetone oxime. Coutts et al., (1976) also identified phenylacetone, phenyl-2-propanol and 2-nitro-1-phenylpropanone from the 12,000g fraction of rat liver.

However, in contrast to our studies where only 13 percent of norethisterone oxime and ten percent of d-norgestrel oxime were metabolised in 2 hours (Tables 2.1 and 2.2) in rabbit liver homogenates, and the study of Sternson and Hes, 1975 who found that acetophenone oxime incubated with rat and rabbit liver homogenates for one hour was recovered unchanged, the results of Hucker (1971) and Coutts et al. (1976) show extensive metabolism of phenylacetone oxime. Thus seventy percent of phenylacetone oxime incubated was metabolised in two hours in rabbit liver (Hucker, 1971). In rat liver fifty percent of phenylacetone oxime was metabolised in one hour. These differences may be due, in part, to the specificity of the oxime hydrolase enzyme in the liver.

It is also possible that the metabolism of these oximes may follow different metabolic pathways. Thus, Hes and Sternson (1974) have shown that acetophenoxime was stable to oxidative and hydrolytic transformations in rat liver. However, under anaerobic conditions acetophenoxime was reduced to the corresponding hydroxylamine, N-hydroxy-1-phenyl-1-aminomethane. It was also indicated that the oxime reductase activity was low and that this pathway was of relatively minor importance in a quantitative sense.

Thus it seems that under both anaerobic (Sternson and Hes, 1975) and aerobic conditions (Hes and Sternson, 1974) acetophenoxime oxime is stable in rat and rabbit liver. Our study with rabbit liver (Tables 2.1 and 2.2) shows that norethisterone oxime and d-norgestrel oxime are not metabolised to a great extent under aerobic conditions, in rabbit liver. However, phenylacetone oxime, both in rat and rabbit liver, is metabolised extensively (Coutts et al. 1975 and Hucker, 1971) under aerobic conditions.

The rate of hydrolysis of the oxime also appears to depend on the pH. Thus, Buhler et al. (1965) have shown that extensive hydrolysis of the steroidal oximes occurs under acidic conditions 'in vitro'. These investigators have reported that the acid hydrolysis of 11β-hydroxy-11α -methyl-5β-pregnane-3, 20-dione dioxime proceeds rapidly to the corresponding 3, 20 diketone. The half-life for the overall hydrolysis was about 21 min in gastric juice. Similarly, Huetteman and Shroff (1974) have shown the conversion of 17α -acetoxy-6α -methyl-4-pregnen-3, 20 dione-3-oxime to the corresponding diketone at pH 1.5 and 37°C to be a first order reaction, and 65% of this compound was hydrolysed in four hours. Although a complete investigation at acidic pH was not carried out in this study, three preliminary experiments were performed at pH 2.0 in buffer at 37°C. The results indicated that both norethisterone oxime racemate and d-norgestrel oxime racemate were hydrolysed rapidly to the ketone in 30 min. Thus, 70.0±2% of NET-OX and 64.2±2% of d-Ng-OX were hydrolysed to the ketone.

Examination of extrahepatic tissues of the rabbit under the conditions described in the Methods section of Section 1, Chapter 1, indicates that only stomach, duodenal, ileal and lung tissues (Tables 2.3 and 2.4) appeared to metabolise norethisterone oxime and d-norgestrel oxime. The rates of metabolism in these tissues were not significantly different from the liver.

The metabolites identified indicated that both oxime hydrolase and ring-A reductase activity was present in these tissues. Thus, the hydrolysed products norethisterone and norgestrel were identified, as well as tetrahydronorethisterone. However, tetrahydronorgestrel was identified only in stomach and lung tissue.

In our 'in vivo' study described in the previous chapter, it was observed that the 'syn'-isomer of norethisterone oxime was almost totally converted to the 'anti'-isomer as seen in the metabolites present in the β-glucuronidase enzyme hydrolysable fraction . Examination of the metabolic products from various rabbit tissues examined indicated that this did not occur 'in vitro'. The significance of this conversion 'in vivo' needs further examination.

Therefore the data from the present study, suggests that the addition of the oxime group to steroidal progestogens not only results in increased progestational potency and antifertility activity as suggested by Shroff et al., (1973) and Kärkkäinen et al., (1975 a/b). this group also renders these steroids resistant to metabolic alteration under certain 'in vitro' conditions.

Both our 'in vivo' study (previous chapter) and 'in vitro' study in rabbit shows that in this species the addition of the oxime group to 19-nortestosterone related progestogens, norethisterone and d-norgestrel, increases their resistance to metabolism, under the condition of these experiments. Thus, these compounds may be of some use in the future development of long-acting progestational steroids.

SUMMARY AND CONCLUSIONS.

The effect of structural modifications of 19-nortestosterone related progestogens on the rate of metabolism of these compounds has been investigated. The progestogens examined were norethisterone, dl-, d-norgestrel, lynestrenol and norethisterone acetate which, in combination with a synthetic oestrogen, are commonly used in the oral contraceptive pill. Norethisterone oenanthate which is being used as a long-acting progestogen, administered intramuscularly, was also studied. Additional compounds studied were lynestrenol acetate, dl-norgestrel acetate, norethisterone oxime and d-norgestrel oxime.

Various studies on the metabolism of progestational compounds have indicated that there are many similarities between the rabbit and man. However, it has not been established as to how far these similarities extend. We used the rabbit as the experimental model to investigate the 'in vitro' metabolism of 19-nortestosterone related progestogens. The metabolism of norethisterone oxime was also investigated 'in vivo' in the rabbit.

1. The rates of metabolism of norethisterone, norgestrel, lynestrenol and their esterified derivatives was compared in male and female hepatic and extrahepatic tissues of the rabbit.

i) The results of this investigation using total liver homogenates, indicated that the parent compound 19-nortestosterone was metabolised at a relatively faster rate than the synthetic progestogens. The introduction of the 17α-ethynyl group as in norethisterone resulted in a decrease in the rate of metabolism to a minor extent. Whereas, the introduction of an ethyl group at C-13 (norgestrel) and removal of the 3-oxo group (lynestrenol) substantially increased the resistance to metabolic alteration. Comparison of the rates of metabolism of the three forms of norgestrel indicated that the biologically active enantiomer, d-norgestrel was metabolised faster than the biologically inactive enantiomer, l-norgestrel. The rate of metabolism of the racemate, dl-norgestrel, was half that of d-norgestrel in liver.

Esterification of the 17β-hydroxyl group gave rise to compounds which were more resistant to metabolism than the non-esterified parent compounds. In the liver the rate of metabolism of the oenanthate derivative was faster than the acetate derivative of norethisterone.

ii) The metabolic products of the non-esterified progestogens were identified to be ring-A reduced products. The esterified derivatives gave rise to the de-esterified and ring-A reduced metabolites. Investigation of the metabolism of d-norgestrel indicated that this progestogen follows the same pathway of metabolism as the natural steroid testosterone.

Thus, the modification of the structure of 19-nortestosterone results in compounds that are resistant to metabolic alteration. However, the route of metabolism is not affected by the various structural changes.

2. i) Amongst the extrahepatic tissues examined, the small intestinal tissue was found to actively metabolise both the non-esterified and esterified progestogens. The rate of metabolism of the esterified derivatives was faster than that observed in the liver. Other tissues which were also able to metabolise these compounds were lung and skeletal muscle tissue. However, adipose, heart and spleen tissue were relatively inactive in the metabolism of these compounds.

The rates of metabolism of the esterified derivatives varied from tissue to tissue. Thus, the acetate derivative was metabolised faster in the small intestine and skeletal muscle tissue. Whereas, norethisterone oenanthate was metabolised comparatively faster in kidney, lung and heart tissues.

ii) The metabolic products obtained from each tissue were ring-A reduced and hydrolysed products, similar to the products obtained in the liver.

3. Comparison of the metabolism of the 19-norprogestogens in male and female hepatic and extrahepatic tissues, indicatrd that in the rabbit the metabolism of these compounds is not affected by sex.

4. i) When the rate of metabolism of the three forms of norgestrel (d-, dl- and l-Ng) was compared to testosterone in the microsomal fraction of rabbit liver, the results again indicated that the addition of 17α-ethynyl and 13-ethyl group resulted in compounds more resistant to metabolic change. d-Norgestrel was metabolised at a faster rate than l-norgestrel.

ii) In contrast to the study with total liver homogenates, in the microsomal fraction both ring-A reduced and hydroxylated products were identified. The metabolites obtained from each form of norgestrel were qualitatively similar but quantitatively different. Thus, 3α, 5β- and 3β, 5β-tetrahydronorgestrel, 16α-, 16β-, and 6ε-hydroxynorgestrel were identified. dl- and l-norgestrel were metabolised mainly to hydroxylated products, whereas d-norgestrel was metabolised to both ring-A reduced and hydroxylated products in equal proportions. Of the ring-A reduced metabolites from d-norgestrel, the amount of 3α, 5β-isomer was significantly greater than the 3β, 5β-isomer. However, these two products were formed equally from dl- and l-norgestrel. Of the hydroxylated products, l-norgestrel was converted mainly to 16α-hydroxynorgestrel, whereas, d-norgestrel was converted more to 16β-hydroxynorgestrel. These two products were formed equally from dl-norgestrel. However, 6ε-hydroxynorgestrel was formed at the same rate from d-, dl- and l-norgestrel. The ratio of ring-A reduced to hydroxylated products remained constant over time for d-norgestrel whereas, with dl- and l-norgestrel the ratio increased with time. This indicates that hydroxylation reactions become more dominant for dl- and l-norgestrel with time.

Thus, in conclusion, this study indicates that the three forms of norgestrel, d-, dl- and l-norgestrel are metabolised by different metabolic pathways in the microsomal fraction of rabbit liver.

5. The rate of sulpho-conjugation of the synthetic oestrogen, ethynyloestradiol and the synthetic 19-norprogestogens was compared to the natural steroid dehydroepiandrosterone in the hepatic and extra-hepatic tissues of the female rabbit.

i) Using the 700g fraction of liver, the results indicated that the rate of sulpho-conjugation of ethynyloestradiol was faster than dehydroepiandrosterone. However, the rate of sulphation of the 19-norprogestogens was slower than DHA. Of the synthetic progestogens investigated norethisterone was conjugated at a faster rate than the three forms of norgestrel (d-, dl- and l-Ng). Whereas, lynestrenol was sulphated at a comparatively slower rate.

ii) Amongst the extrahepatic tissues examined, heart, spleen adipose, skeletal muscle and kidney tissue were found to be inactive in sulphokinase activity. However, stomach and lung tissue conjugated dehydroepiandrosterone, ethynyloestradiol and the 19-norprogestogens. Ileal and caecal tissue only sulphated DHA and EE. In contrast to the liver, in stomach tissue, dehydroepiandrosterone was conjugated more rapidly than ethynyloestradiol. The 19-norprogestogens were sulphated at a significantly lower rate in stomach tissue as compared to the liver. In lung tissue, dehydroepiandrosterone was sulphated to the same extent as observed in the liver and stomach tissue. Whereas, ethynyloestradiol, norethisterone and d-norgestrel were conjugated to a minor extent.

iii) In the liver and stomach tissue ethynyloestradiol gave rise to both mono- and di-sulphates. Whereas, in ileal, caecal and lung tissue only mono-sulphates were formed. Both in hepatic and extrahepatic tissues only mono-sulphates were formed from the 19-norprogestogens. The position of the sulphate groups was identified to be at C-17 in the progestogens and C-3 in DHA, in liver. Ethynyl-oestradiol was conjugated at both C-3 and C-17.

Thus, 'in vitro' sulphation of the synthetic oestrogen ethynyl-oestradiol and 19-norprogestogens was demonstrated. The ethynyl group at C-17 does not appear to hinder sulphate conjugation. The rate of conjugation in both hepatic and extrahepatic tissues appears to depend on the structure of these compounds.

6. The 'in vivo' metabolism of (4-^{14}C) norethisterone oxime was investigated after the intraperitoneal administration of the three forms (NET-OX racemate, 'anti'- and 'syn'-isomers).

i) Over a period of five days 55% of the radioactivity was excreted in the urine. The major part of the radioactivity was excreted in the first 24 hours and was present in the β-glucuronidase enzyme hydrolysable form. A small amount of radioactivity was recovered in the sulphate fraction. Significantly higher amounts of the 'syn'-isomer were present in the glucuronide fraction, whereas, the 'anti'-isomer was in comparatively higher amounts in the non-conjugated and sulphate fraction of urine. For all the three forms of NET-OX the glucuronide fraction appeared to increase with time, whereas, the non-conjugated fraction decreased with time. The rate of appearance of the sulphate conjugates of the 'anti'- and 'syn'-isomers remained steady whereas for the racemate this fraction decreased with time.

ii) The faecal route of excretion of the three forms of NET-OX was found to be a minor route.. However, significantly higher amounts of the 'syn'-isomer were excreted via this route as compared to the 'anti'-isomer and NET-OX racemate. The major part of the radioactivity for the three forms was present in the non-conjugated fraction.

iii) In plasma, the major part of the radioactivity was seen in the non-conjugated fraction for both 'anti'- and 'syn'-isomers at 2 hours. By 24 hours, the amount of radioactivity had declined. However, the rate of decrease for the 'syn'-isomer was comparatively slower than the 'anti'-isomer.

iv) Examination of the metabolites in the various fractions of urine and faeces indicated that the three forms of norethisterone oxime are excreted unchanged. Other products were also observed in the urine and faeces but were not identified in detail. The results also suggested that the 'syn'-isomer was converted to the 'anti'-isomer 'in vivo'.

7. The 'in vitro' metabolism of the oxime derivatives of norethisterone and d-norgestrel by the hepatic and extrahepatic tissues of the rabbit was also investigated.

i) As compared to the parent compounds, norethisterone and d-norgestrel, the oxime derivatives were metabolised at a distinctly slower rate in the liver. There was no difference in the rates of metabolism of the three forms of NET-OX and d-Ng-OX. However, d-Ng-OX was metabolised relatively slowly as compared to NET-OX.

The metabolites identified from NET-OX (three forms) were the hydrolysed and ring-A reduced products. However, d-Ng-OX did not give rise to ring-A reduced metabolites.

ii) Amongst the extrahepatic tissues examined, all tissues metabolised the three forms of NET-OX except adipose tissue. Whereas, d-Ng-OX was metabolised only by stomach and lung tissue. The rate of metabolism of NET-OX and d-Ng-OX was similar to the rate observed in the liver.

In stomach, duodenum, ileum, skeletal muscle and lung tissue both hydrolysed and ring-A reduced products of NET-OX were identified. However, in caecal, kidney and heart tissue only norethisterone was identified. d-Ng-OX was metabolised to norgestrel and tetrahydronorgestrel in stomach and lung tissue.

Thus, both the 'in vivo' study and 'in vitro' study indicate that the addition of the oxime group to norethisterone and d-norgestrel results in compounds that are resistant to metabolic alteration in the rabbit.

APPENDIX TABLE 1.

COMPARISON OF THE RATE OF METABOLISM AND METABOLITE FORMATION OF 19-NORTESTOSTERONE, NORETHISTERONE, dl-, d-, l-NORGESTREL AND LYNESTRENOL IN FEMALE RABBIT LIVER. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT VARIOUS TIME INTERVALS.
(* denotes value significantly different from 19-nortestosterone $p < 0.05$).

STEROID INCUBATED	19-NORTESTOSTERONE	NORETHISTERONE		dl-NORGESTREL		d-NORGESTREL		l-NORGESTREL		LYNESTRENOL		
% STEROID RECOVERED	19-NORT	NET	THNET	dl-Ng	THNg	d-Ng	THNg	l-Ng	THNg	Lyn	NET	THNET
TIME (min)	90.2	94.4	0	76.0	0	92.9	0	92.0	0	90.4	0	0
0	80.4	96.2	0	95.9	0	92.8	0	93.0	0	94.2	0	0
	96.2	88.4	0	97.9	0	94.9	0	95.4	0	96.2	0	0
	88.4	90.2	0	92.7	0	98.3	0	95.8	0			
	96.2	92.8	0	95.2	0							
	98.4	96.4	0	100.0	0							
	89.6	94.4	0	91.0								
	92,4	96.0	0	91.7								
	96.2	95.6	0									
		92.2	0									
		60.2	0									
mean$\pm$SD	92.0$\pm$5.5	90.6$\pm$10.4	0	92.5$\pm$7.3	0	94.7$\pm$2.5	0	94.1$\pm$1.8	0	93.6$\pm$2.9	0	0
	17.7	65.4	28.2	61.0	32.0	54.8	34.4	92.0	0.6	59.2	16.4	13.6
	73.4	66.8	24.2	63.2	28.4	60.2	30.8	90.0	1.8	62.4	18.6	15.1
10	40.0	59.1	28.0	61.8	24.4	58.8	36.0	94.2	0.8	67.5	19.0	14.0
		59.3	29.8	58.8	29.6	78.2	16.0	94.4	0.9			
		74.0	16.2	56.3	30.8							
		82.8	10.9	82.8	12.8							
				60.2	24.8							
				58.8	28.8							
	43.7$\pm$28.0	67.9$\pm$9.1	22.9$\pm$7.6	62.8$\pm$8.3	26.4$\pm$6.1	63.0$\pm$10.3	29.3$\pm$9.1	92.6$\pm$2.0*	1.0$\pm$0.5	63.0$\pm$4.1	18.0$\pm$1.4	14.2$\pm$0.7
	37.6	69.5	19.2	65.8	28.4	77.6	16.2	92.0	0.4	58.4	20.0	14.1
	43.8	67.2	18.1	39.8	32.8	60.7	28.2	92.0	0	56.2	22.0	15.2
15	66.0	34.2	20.0	59.7	36.2	64.8	26.4	94.9	0.8	52.8	28.0	17.1
	53.0			60.6	24.8	69.1	22.8	93.7	0.7	56.4	26.0	20.0
				57.2	25.9							
				80.1	16.8							
mean$\pm$SD	50.1$\pm$12.3	56.9$\pm$19.7	19.1$\pm$1.0	60.5$\pm$13.0	27.5$\pm$6.7	68.0$\pm$7.2*	23.4$\pm$5.2	93.1$\pm$1.4*	0.48$\pm$0.4	55.9$\pm$2.3	24.0$\pm$3.6	16.6$\pm$2.5

APPENDIX TABLE 2 continued. from 1.

STEROID INCUBATED	19-NORTESTOSTERONE	NORETHISTERONE		dl-NORGESTREL		d-NORGESTREL		l-NORGESTREL		LYNESTRENOL		
STEROID RECOVERED	19-NORT	NET	THNET	dl-Ng	d-Ng	d-Ng	THNg	l-Ng	THNg	Lyn	NET	THNET
20	48.3 53.7 58.9	61.2 61.8 63.7	18.8 17.1 16.1	53.7 31.9 56.9 48.2 55.4	40.2 42.0 36.4 30.8 40.4	45.5 43.4 43.0 51.1	56.2 48.2 46.4 40.2	90.9 87.7 93.4 93.7	1.8 2.4 1.4 1.8	52.0 52.0 54.9	25.4 26.4 27.2	21.0 17.1 16.5
mean±SD	53.6±5.3	62.2±1.3	17.3±1.3	49.2±10.2	37.9±4.4	45.5±3.9	47.7±6.6	91.4±2.7*	1.9±0.4	52.0±1.6	26.3±0.9	18.2±2.4
30	37.8 26.6 65.1 42.4 47.2 56.5 41.3 40.4 39.2	14.5 46.5 34.7 33.0 43.1 43.1 72.2 65.4	76.2 42.4 46.4 54.4 46.2 50.4 32.8 28.4	65.1 38.4 37.7 83.6 74.2 79.5 36.0 53.7	28.4 32.2 56.4 4.2 18.2 17.8 46.8 37.0	68.9 30.1 55.7 44.9	20.2 60.1 24.2 49.7	90.8 90.2 95.4 92.8	1.9 1.6 0 1.9	40.4 41.3 42.1 37.9 38.4 39.1	32.1 31.2 32.7 34.2 36.2 32.4	20.1 24.2 22.1 26.4 21.2 23.4
mean±SD	45.1±11.3	42.2±15.5	47.1±14.5	58.5±19.7	30.1±16.8	52.4±18.3	38.5±9.3	92.3±2.3*	1.3±0.9	40.2±1.6	33.0±1.8	22.9±2.2
60	26.6 35.8 0 36.3	11.2 54 1 12.3 40.0	82.4 40.0 74.2 46.4	56.5 35.4 38.2 62.2 54.6 23.5 58.5 37.2	37.8 33.0 28.4 36.0 32.8 62.0 30.8 46.8	18.6 20.4 30.9 30.2	62.8 68.8 50.8 53.6	88.2 83.2 91.3 91.8	4.0 5.8 3.8 2.0	36.0 40.0 42.0 34.1 26.2	27.1 29.4 30.1 27.2 25.5	12.0 14.2 16.9 17.6 14.8
mean±SD	24.7±17.0	29.4±21.1	60.7±20.7	45.9±13.8*	38.4±11.0	27.0±9.0	60.5±6.6	88.6±3.9	3.9±1.5	35.6±6.1	27.8±1.8	15.1±2.2

APPENDIX TABLE 3 continued from 1.

STEROID INCUBATED	19-NORTESTOSTERONE	NORETHISTERONE		dl-NORGESTREL		d-NORGESTREL		l-NORGESTREL		LYNESTRENOL		
STEROID RECOVERED	19-NORT	NET	THNET	dl-Ng	THNg	d-Ng	THNg	l-Ng	THNg	Lyn	NET	THNET
180	13.0	0	96.4	54.3	36.2	13.1	72.8	87.0	3.6	28.3	32.1	20.1
	12.0	9.9	92.2	54.3	34.8	18.7	68.4	85.2	5.1	26.0	38.5	22.4
	6.9	9.9	76.4	82.1	12.4	25.9	64.2	91.4	3.8	24.6	35.4	28.1
		9.9	78.4	9.3	80.0	31.7	60.2	92.0	2.0	31.3	37.2	29.5
				35.4	54.4							
				51.9	44.2							
				48.9	36.2							
				36.6	44.4							
mean$\pm$SD	13.0$\pm$12.0	9.9$\pm$0	85.8$\pm$9.9	44.2$\pm$18.1*	42.8$\pm$19.2	22.3$\pm$8.1*	66.4$\pm$5.9	88.9$\pm$3.3*	3.6$\pm$1.3	27.5$\pm$2.9*	35.8$\pm$2.7	25.0$\pm$4.4
300	0	0	87.1	65.2	28.2	8.6	82.0	88.0	3.9	16.0	35.1	39.1
	0	0	85.2	38.1	32.8	6.8	80.5	85.1	6.0	13.4	38.2	37.4
	0	0	84.2	45.8	48.2	12.7	78.4	90.5	3.8	17.5	42.1	39.1
				36.4	46.8	18.3	72.4	91.8	3.0	33.4	43.4	36.4
				47.0	44.2							
				53.0	42.0							
				40.8	62.8							
				20.7	64.0							
mean$\pm$SD	0	0	85.5$\pm$1.4	43.4$\pm$13.0*	46.1$\pm$12.6	11.6$\pm$5.0*	78.3$\pm$9.9	88.8$\pm$3.0*	4.2$\pm$1.2	20.0$\pm$9.0*	39.7$\pm$3.8	38.0$\pm$1.3

APPENDIX TABLE 4.

COMPARISON OF THE RATE OF METABOLISM OF 19-NORTESTOSTERONE, LYNESTRENOL ACETATE, NORETHISTERONE ACETATE, dl-NORGESTREL ACETATE AND NORETHISTERONE OENANTHATE EXPRESSED AS PERCENT STEROID RECOVERED AT VARIOUS TIME INTERVALS (*denotes value significantly different from 19-nortestosterone $p < 0.05$)

STEROID INCUBATED	LYNESTRENOL ACETATE				NORETHISTERONE ACETATE			dl-NORGESTRAL ACETATE			NORETHISTERONE OENANTHATE		
STEROID RECOVERED	Lyn-AC	Lyn	NET	THNET	NET-AC	NET	THNET	dl-Ng-AC	Ng	THNg	NET-EN	NET	THNET
TIME (min)	91.4	–	–	–	90.4	–	–	96.6	–	–	88.0	–	–
	95.6	–	–	–	96.2	–	–	93.1	–	–	89.5	–	–
0	92.0	–	–	–	86.8	–	–	95.3	–	–	90.4	–	–
											94.8	–	–
											86.2	–	–
											80.4	–	–
mean$\pm$SD	93.0$\pm$2.1	–	–	–	91.1$\pm$4.7	–	–	95.0$\pm$1.7	–	–	86.5$\pm$3.6	–	–
	83.4	2.3	5.1	0	79.5	9.0	2.2	83.9	3.1	2.9	65.1	16.8	7.2
10	86.6	3.4	4.2	0	88.1	6.2	2.4	81.2	3.6	3.4	66.0	14.6	7.0
	88.1	3.3	3.0	0	76.5	5.8	4.0	86.9	5.3	3.1	70.2	12.2	5.2
											60.2	14.8	7.2
											62.0	16.6	5.8
											56.0	16.8	6.0
mean$\pm$SD	86.0$\pm$2.4	3.0$\pm$0.6	4.0$\pm$1.0	0	81.3$\pm$5.9	7.0$\pm$1.7	3.0$\pm$1.2	84.0$\pm$2.8	4.0$\pm$1.1	3.1$\pm$0.2	63.3$\pm$4.9	15.3$\pm$1.8	6.4$\pm$0.8
	80.1	3.5	5.1	2.1	73.2	6.4	4.4	76.6	5.1	5.1	–	–	–
15	84.6	4.1	6.2	1.9	74.4	8.2	4.2	78.7	6.4	4.9	–	–	–
	82.8	5.9	4.0	2.0				82.1	7.1	5.3	–	–	–
mean$\pm$SD	82.5$\pm$2.2*	4.5$\pm$1.2	5.1$\pm$1.1	2.0$\pm$0.1	73.8$\pm$0.8	7.3$\pm$1.2	4.2$\pm$0.3	79.1$\pm$2.7*	6.2$\pm$1.0	5.1$\pm$0.2	–	–	–
	81.1	5.1	9.1	3.9	75.9	10.2	4.2	75.2	6.6	8.1	50.0	20.4	20.4
20	79.8	5.9	11.2	4.5	79.5	11.8	2.9	71.4	8.9	6.4	46.2	25.0	25.0
	77.6	7.0	10.3	4.0	71.3	9.8	4.3	72.4	8.5	7.1	40.2	17.0	17.0
											44.2	18.0	18.0
											42.4	18.4	18.4
											40.4	19.2	19.2
mean$\pm$SD	79.5$\pm$1.7*	6.0$\pm$0.9	10.2$\pm$1.0	4.1$\pm$0.3	75.6$\pm$4.1*	10.6$\pm$1.0	3.8$\pm$0.7	73.0$\pm$1.9*	8.0$\pm$1.2	7.2$\pm$0.8	43.9$\pm$3.7*	19.6$\pm$2.8	17.1$\pm$1.4

APPENDIX TABLE 5 continued from 4.

COMPARISON OF THE RATE OF METABOLISM OF 19-NORTESTOSTERONE, LYNESTRENOL ACETATE, NORETHISTERONE ACETATE, dl-NORGESTREL ACETATE AND NORETHISTERONE OENANTHATE EXPRESSED AS PERCENT STEROID RECOVERED AT VARIOUS TIME INTERVALS (*denotes value significantly different from 19-nortestosterone $p < 0.05$)

STEROID INCUBATED	LYNESTRENOL ACETATE				NORETHISTERONE ACETATE			dl-NORGESTREL ACETATE			NORETHISTERONE OENANTHATE		
STEROID RECOVERED	Lyn-AC	Lyn	NET	THNET	NET-AC	NET	THNET	Ng-AC	Ng	THNg	NET-EN	NET	THNET
TIME	72.6	7.6	12.6	8.1	63.8	18.4	6.2	75.2	12.1	10.3	42.0	25.2	18.4
	71.4	9.1	11.9	9.1	69.8	17.0	8.4	71.4	13.4	12.0	32.0	32.6	24.4
30	75.4	8.6	14.5	5.3	59.7	18.2	9.8	72.4	14.1	11.0	36.8	22.8	17.0
											34.4	22.2	24.2
											36.4	23.0	22.4
											32.8	22.8	21.6
mean$\pm$SD	$73.1 \pm 2.0^{*}$	8.4 ± 0.7	13.0 ± 1.3	7.5 ± 1.9	$64.4 \pm 5.0^{*}$	17.9 ± 0.7	8.1 ± 1.8	$73.0 \pm 1.9^{*}$	13.2 ± 1.0	11.1 ± 0.8	35.7 ± 3.6	24.7 ± 3.9	21.3 ± 3.0
	63.2	8.1	15.1	9.1	66.2	14.8	14.2	66.2	18.4	13.1	54.0	7.2	25.4
	64.5	10.5	16.2	7.4	62.1	16.2	17.8	71.2	16.1	14.5	33.0	4.6	27.2
60	66.3	11.4	17.9	10.6	54.6	12.8	19.4	72.6	17.5	11.9	40.4	10.8	30.2
											38.2	8.8	27.2
											40.4	8.6	26.0
											32.8	9.6	25.6
mean$\pm$SD	$64.6 \pm 1.5^{*}$	10.0 ± 1.7	16.4 ± 1.4	9.0 ± 1.6	$58.9 \pm 3.8^{*}$	14.6 ± 1.7	17.1 ± 2.6	$70.0 \pm 3,2^{*}$	17.3 ± 1.1	13.1 ± 1.3	$39.8 \pm 7.7^{*}$	8.2 ± 2.1	26.9 ± 1.7
120	-	-	-	-	-	-	-	-	-	-	52.9	2.0	31.2
											50.9	2.2	36.2
											46.4	4.2	34.0
											44.2	3.0	32.8
											46.4	2.6	31.0
											42.4	2.4	30.8
mean$\pm$SD											$47.2 \pm 3.9^{*}$	2.7 ± 0.7	32.6 ± 2.1
180	58.4	11.2	18.5	12.1	53.6	12.4	24.2	51.2	16.8	14.2	-	-	-
	59.6	10.1	19.4	11.4	58.0	16.2	20.2	56.4	17.9	17.2			
	62.0	14.7	17.1	10.6	46.7	17.8	21.8	58.1	19.6	13.9			
mean$\pm$SD	$60.0 \pm 1.8^{*}$	12.0 ± 2.4	18.3 ± 1.1	11.3 ± 0.7	$52.7 \pm 5.6^{*}$	15.5 ± 2.7	22.0 ± 1.9	$55.2 \pm 3.5^{*}$	18.1 ± 1.4	15.1 ± 1.8			
300	54.6	13.0	22.0	13.0	46.2	8.0	28.2	48.1	19.3	16.9	-	-	-
	55.4	16.4	17.1	15.1	49.8	14.0	27.0	51.2	21.8	17.4			
	58.1	14.1	21.0	12.2	39.6	15.0	24.0	50.9	18.9	19.7			
mean$\pm$SD	$56.0 \pm 1.8^{*}$	14.5 ± 1.7	20.0 ± 2.5	13.4 ± 1.0	$45.2 \pm 5.1^{*}$	12.3 ± 3.7	24.4 ± 2.1	$50.0 \pm 1.7^{*}$	20.0 ± 1.5	18.0 ± 1.4			

APPENOIX TABLE 6.

COMPARISON OF THE RATE OF METABOLISM OF 19-NORTESTOSTERONE, NORETHISTERONE, dl-, d-, l-NORGESTREL IN FEMALE RABBIT TISSUES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT 120 min. (* denotes value significantly different from 19-nortestosterone $p < 0.05$)

STEROID INCUBATED	19-NORTESTOSTERONE	NORETHISTERONE		dl-NORGESTREL		d-NORGESTREL		l-NORGESTREL		LYNESTRENOL		
STEROID RECOVERED	19-NORT	NET	THNET	Ng	THNg	Ng	THNg	Ng	THNg	Lyn	NET	THNET
TISSUE												
	20.1	32.1	66.4	42.1	58.2	47.1	56.1	89.1	4.1	40.1	20.1	36.1
LIVER	22.4	44.2	52.4	48.1	44.4	36.4	50.2	88.4	6.1	42.1	22.4	34.2
	18.1	40.1	64.2	43.4	45.1	42.0	48.4	90.1	2.0	32.1	26.2	31.4
mean$\pm$SD	20.2 ± 2.1	$38.8\pm6.1^{*}$	61.0 ± 7.5	$44.5\pm3.1^{*}$	49.2 ± 7.7	$41.8\pm5.3^{*}$	51.5 ± 4.0	$89.2\pm0.8^{*}$	4.0 ± 2.0	$38.1\pm5.2^{*}$	22.9 ± 3.0	33.9 ± 2.3
	84.2	89.4	1.1	78.4	0	74.2	0	88.2	0	80.2	0	0
KIDNEY	81.5	94.1	0.9	86.4	0	76.0	0	86.1	0	86.4	0	0
	80.2	92.1	1.8	90.1	0	84.2	0	89.2	0	83.1	0	0
mean$\pm$SD	81.9 ± 2.0	$91.8\pm2.3^{*}$	1.2 ± 0.4	84.9 ± 5.9	0	78.1 ± 5.3	0	$87.8\pm1.5^{*}$	0	83.2 ± 3.1	0	0
	62.1	68.4	30.4	84.2	9.0	70.4	20.1	88.4	0	91.0	0	0
SKELETAL	64.2	66.1	33.8	86.1	10.2	73.1	18.0	89.2	0	87.2	0	0
MUSCLE	58.4	73.0	25.8	94.1	5.0	78.0	14.0	87.2	0	86.1	0	0
mean$\pm$SO	61.5 ± 2.9	$69.1\pm3.5^{*}$	30.0 ± 4.0	$88.1\pm5.2^{*}$	8.0 ± 2.7	$75.8\pm2,4^{*}$	17.3 ± 3.0	$88.2\pm1.0^{*}$	0	$88.1\pm2.5^{*}$	0	0
	42.1	42.1	48.2	89.4	7.3	74.2	16.2	86.4	0	94.1	0	0
LUNG	39.2	39.1	54.0	89.1	9 1	83.4	13.9	88.2	0	93.4	0	0
	37.4	50.1	45.0	90.1	10.9	88.1	12.9	89.4	0	92.1	0	0
mean$\pm$SO	39.5 ± 2.3	43.7 ± 5.6	49.0 ± 4.5	$89.5\pm0.5^{*}$	9.1 ± 1.8	$81.9\pm7.0^{*}$	14.3 ± 1.6	$88.0\pm1.5^{*}$	0	$93.2\pm1.0^{*}$	0	0

APPENDIX TABLE 7 continued from 6.
COMPARISON OF THE RATE OF METABOLISM OF 19-NORTESTOSTERONE, NORETHISTERONE, dl-, d-, l-NORGESTREL AND LYNESTRENOL IN FEMALE RABBIT TISSUES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT 120 min. (* denotes value significantly different from 19-nortestosterone $p < 0.05$).

STEROID INCUBATED	19-NORTESTOSTERONE	NORETHISTERONE		dl-NORGESTREL		d-NORGESTREL		l-NORGESTREL		LYNESTRENOL		
STEROID RECOVERED	19-NORT	NET	THNET	Ng	THNg	Ng	THNg	Ng	THNg	Lyn	NET	THNET
TISSUE	89.2	86.2	0	90.1	0	88.4	0	86.4	0	83.2	0	0
	88.4	90.1	0	86.4	0	90.1	0	81.2	0	86.4	0	0
SPLEEN	89.1	87.2	0	89.2	0	79.4	0	87.4	0	70.1	0	0
mean$\pm$SD	88.9$\pm$0.4	87.8$\pm$2.0	0	88.5$\pm$1.9	0	85.9$\pm$5.7	0	85.0$\pm$3.3	0	79.9$\pm$8.6	0	0
SMALL	44.2	60.1	32.5	90.1	10.1	80.4	15.1	86.2	0	93.4	0	0
INTESTINE	46.1	50.4	34.8	86.4	8.6	81.2	14.2	88.4	0	96.1	0	0
	45.2	52.1	38.2	89.2	6.5	84.1	12.1	89.2	0	97.2	0	0
mean$\pm$SD	45.2$\pm$0.9	56.2$\pm$4.0	35.1$\pm$2.8	88.5$\pm$1.9*	8.4$\pm$1.8	81.9$\pm$1.9*	13.8$\pm$1.5	87.9$\pm$1.5*	0	95.5$\pm$1.5*	0	0
ADIPOSE	83.1	81.2	13.0	88.1	0	84.2	0	88.2	0	89.2	0	0
	90.2	84.2	9.1	86.4	0	86.1	0	86.1	0	86.4	0	0
	91.4	85.1	11.1	74.2	0	90.1	0	89.2	0	70.9	0	0
mean$\pm$SD	88.2$\pm$4.4	83.5$\pm$2.0	11.0$\pm$1.9	82.9$\pm$7.5	0	86.8$\pm$3.0	0	87.8$\pm$1.5	0	82.1$\pm$9.8	0	0
HEART	82.5	83.2	0	89.1	0	84.2	0	87.4	0	86.4	0	0
	85.2	84.1	0	86.4	0	60.4	0	88.2	0	83.1	0	0
	87.1	86.5	0	82.1	0	79.4	0	92.1	0	89.1	0	0
mean$\pm$SD	84.9$\pm$2.3	84.6$\pm$1.7	0	85.8$\pm$3.5	0	74.6$\pm$12.5	0	89.2$\pm$2.5	0	86.2$\pm$3.0	0	0

APPENDIX TABLE 8.

COMPARISON OF THE RATE OF METABOLISM OF LYNESTRENOL ACETATE, NORETHISTERONE ACETATE, dl-NORGESTREL ACETATE AND NORETHISTERONE OENANTHATE IN FEMALE RABBIT TISSUES. (* denotes value significantly different from 19-nortestosterone $p < 0.05$)

STEROID INCUBATED	LYNESTRENOL ACETATE				NORETHISTERONE ACETATE			dl-NORGESTREL ACETATE			NORETHISTERONE OENANTHATE		
STEROID RECOVERED	Lyn-AC	Lyn	NET	THNET	NET-AC	NET	THNET	Ng-AC	Ng	THNg	NET-EN	NET	THNET
TISSUE													
	64.2	18.2	12.1	5.6	42.8	25.4	19.4	63.1	14.2	16.2	52.4	9.8	24.1
	65.1	20.5	14.2	8.1	56.0	19.1	16.1	60.1	15.1	18.4	48.2	8.4	24.9
LIVER	70.2	21.2	13.2	7.2	40.1	18.2	34.0	53.2	17.2	15.1	54.4	12.4	29.1
											48.2	9.2	30.5
											50.1	6.2	29.1
											48.2	4.6	30.5
mean$\pm$SD	$66.5 \pm 3.2^{*}$	19.9 ± 1.5	13.2 ± 1.0	6.9 ± 1.2	$46.3 \pm 8.5^{*}$	20.9 ± 3.9	23.1 ± 9.5	$58.8 \pm 5.0^{*}$	15.5 ± 1.5	16.5 ± 1.6	$50.2 \pm 2.6^{*}$	8.4 ± 2.7	27.8 ± 3.0
	84.1	14.1	0	0	96.4	0	0	88.2	7.2	0	80.2	7.9	0
	85.2	12.2	0	0	94.1	0	0	85.2	9.1	0	74.4	8.1	0
KIDNEY	87.1	10.1	0	0	80.2	0	0	88.1	6.2	0	74.2	6.6	0
											68.4	8.2	0
											77.1	6.6	0
											75.4	8.2	0
mean$\pm$SD	85.4 ± 1.5	12.1 ± 2.0	0	0	90.2 ± 8.7	0	0	87.1 ± 1.7	7.5 ± 1.4	0	74.9 ± 3.9	7.5 ± 0.7	0
	70.2	21.2	0	0	76.4	18.0	4.1	63.2	16.2	5.2	70.4	4.2	0
	72.2	23.4	0	0	76.8	22.0	2.1	61.2	18.1	6.1	76.2	5.1	0
SKELETAL	73.2	25.2	0	0	80.0	20.0	1.2	65.4	17.1	9.5	70.4	2.5	0
MUSCLE											66.4	3.5	0
											83.1	2.5	0
											80.0	3.5	0
mean$\pm$SD	$71.8 \pm 1.5^{*}$	23.2 ± 2.0	0	0	$77.7 \pm 1.9^{*}$	20.0 ± 2.0	2.4 ± 1.4	63.2 ± 2.1	17.1 ± 0.9	6.9 ± 2.2	$74.4 \pm 6.4^{*}$	3.6 ± 0.9	0
	80.2	12.1	2.1	0	88.0	10.0	2.1	81.2	8.2	4.2	60.2	21.0	2.4
	86.1	13.2	4.2	0	76.0	4.1	7.4	82.4	9.1	4.1	62.2	20.2	2.6
LUNG	88.2	10.6	5.1	0	89.0	2.0	1.8	83.1	10.2	2.2	56.2	31.2	1.8
											54.4	30.0	2.0
											54.2	31.2	1.8
											50.1	30.0	2.2
	$84.8 \pm 4.1^{*}$	11.9 ± 1.3	3.8 ± 1.5	0	$84.3 \pm 7.2^{*}$	5.3 ± 4.1	3.7 ± 3.1	$82.2 \pm 0.9^{*}$	9.1 ± 1.0	3.5 ± 1.1	$56.2 \pm 4.3^{*}$	$27.1 \pm 5,1$	2.0 ± 0.5

APPENDIX TABLE 9 continued from 8.

COMPARISON OF THE RATE OF METABOLISM OF LYNESTRENOL ACETATE, NORETHISTERONE ACETATE, dl-NORGESTREL ACETATE AND NORETHISTERONE OENANTHATE

THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED at 120 min. (* denotes values significantly different from 19-nortestosterone $p < 0.05$)

STEROID INCUBATED	LYNESTRANOL ACETATE				NORETHISTERONE ACETATE			dl-NORGESTRAL ACETATE			NORETHISTERONE OENANTHATE		
STEROID RECOVERED	Lyn-AC	Lyn	NET	THNET	NET-AC	NET	THNET	Ng-AC	Ng	THNg	NET-EN	NET	THNET
TISSUE	20.1	61.5	9.2	4.1	9.4	82.0	2.1	16.2	69.2	3.2	44.4	20.2	18.2
SMALL	21.2	65.2	10.1	2.4	8.1	78.4	3.9	17.1	68.1	4.1	39.6	21.1	18.1
INTESTINE	22.1	74.1	11.2	3.2	8.9	86.0	4.1	20.2	70.2	5.2	40.2	22.3	18.4
											42.0	25.3	19.2
											40.1	22.3	18.4
											41.9	26.3	19.2
mean$\pm$SD	$21.1 \pm 1.0^{*}$	66.9 ± 6.4	10.1 ± 1.0	3.2 ± 1.0	$8.8 \pm 0.6^{*}$	82.1 ± 3.8	3.3 ± 1.1	$17.8 \pm 2.0^{*}$	69.1 ± 0.1	4.1 ± 1.0	41.3 ± 1.7	22.9 ± 2.3	18.5 ± 0.4
SPLEEN	-	-	-	-	96.2	0	0	-	-	-	-	-	-
					98.4	0	0						
					90.1	0	0						
mean$\pm$SD					94.9 ± 4.3								
ADIPOSE	91.2	4.5	0	0	100.0	0	0	97.1	0	0	72.4	2.0	0
	94.4	4.9	0	0	99.8	0	0	96.4	0	0	68.4	2.1	0
	95.9	5.1	0	0	100.0	0	0	99.1	0	0	66.2	0	0
											76.4	0	0
											81.2	0	0
											80.1	0	0
mean$\pm$SD	93.8 ± 2.4	4.8 ± 0.3	0	0	$99.9 \pm 0.1^{*}$	0	0	$97.5 \pm 1.4^{*}$	0	0	74.4 ± 6.1	2.0 ± 0.01	0
	76.1	19.4	3.2	0	92.0	2.4	0	85.2	16.1	0	70.2	9.0	0
	80.2	18.2	4.1	0	86.4	6.9	0	89.1	12.2	0	69.8	8.9	0
HEART	86.1	16.1	5.2	0	88.0	8.4	0	86.4	13.5	0	62.2	17.3	0
											50.4	18.2	0
											69.2	17.3	0
											63.4	18.3	0
	80.8 ± 5.0	17.9 ± 1.6	4.1 ± 1.0	0	88.8 ± 2.8	5.9 ± 3.1	0	86.9 ± 1.9	13.9 ± 1.9	0	65.2 ± 5.5	12.6 ± 4.1	0

APPENDIX TABLE 10. COMPARISON OF THE RATE OF METABOLISM OF 19-NORTESTOSTERONE, NORETHISTERONE, dl-, d-, l-NORGESTREL AND LYNESTRENOL IN MALE RABBIT TISSUES. RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED IN 120 mins. (* denotes values significantly different from 19-nortestosterone, $p < 0.05$)

STEROID INCUBATED	19-NORTESTOSTERONE	NORETHISTERONE		dl-NORGESTREL		d-NORGESTREL		l-NORGESTREL		LYNESTRENOL		
STEROID RECOVERED	19-NORT	NET	THNET	Ng	THNg	Ng	THNg	Ng	THNg	Lyn	NET	THNET
TISSUE												
LIVER	21.2	22.1	72.4	50.1	52.0	40.4	50.9	89.1	0.9	38.2	17.2	42.0
	18.4	32.4	66.1	48.2	47.0	43.0	52.1	88.4	1.0	42.1	14.4	32.0
	17.2	34.1	68.1	51.2	46.1	43.1	57.8	92.1	1.2	24.0	30.2	34.2
mean$\pm$SD	18.9$\pm$2.0	29.5$\pm$6.4	68.8$\pm$3.2	49.8$\pm$1.5*	48.3$\pm$3.1	42.1$\pm$1.5*	53.6$\pm$3.6	89.9$\pm$1.9*	1.0$\pm$0.2	34.7$\pm$9.5*	20.6$\pm$8.4	36.0$\pm$5.2
KIDNEY	81.2	81.4	3.1	88.1	0	88.7	0	90.2	0	83.4	0	0
	78.1	89.2	4.5	81.2	0	84.2	0	92.1	0	87.6	0	0
	69.2	88.1	2.1	71.7	0	83.9	0	98.4	0	89.4	0	0
mean$\pm$SD	76.1$\pm$6.2	86.2$\pm$4.2	3.2$\pm$1.2	80.3$\pm$8.2	0	85.6$\pm$2.6	0	93.5$\pm$4.2*	0	86.8$\pm$3.0	0	0
SKELETAL MUSCLE	61.2	70.1	25.8	90.1	10.0	78.4	18.4	98.2	0	90.1	0	0
	59.4	64.2	27.4	90.2	9.0	82.1	13.1	92.1	0	98.2	0	0
	56.2	58.1	30.9	86.4	12.1	83.2	14.2	95.2	0	79.4	0	0
mean$\pm$SD	58.9$\pm$2.5	64.1$\pm$5.9	28.0$\pm$2.6	88.9$\pm$2.1*	10.3$\pm$1.5	81.2$\pm$2.5*	12.4$\pm$2.7	95.7$\pm$3.0*	0	89.2$\pm$9.4*	0	0
LUNG	40.2	40.1	47.1	87.1	8.2	88.2	10.0	91.2	0	93.4	0	0
	38.2	42.1	40.1	89.2	6.2	81.2	15.4	94.1	0	96.1	0	0
	36.9	42.5	35.2	78.1	11.0	83.4	12.0	96.4	0	98.0	0	0
mean$\pm$SD	38.4$\pm$1.6	41.5$\pm$1.2	40.8$\pm$5.9	84.8$\pm$5.8*	8.4$\pm$2.4	84.2$\pm$3.5*	12.2$\pm$2.7	93.9$\pm$2.6*	0	95.8$\pm$2.3*	0	0

APPENDIX TABLE 11 COMPARISON OF THE RATE OF METABOLISM OF 19-NORTESTOSTERONE, dl-, d-, l-NORGESTREL AND LYNESTRENOL IN MALE RABBIT TISSUES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT 120 mins. (*denotes value significantly different from 19-nortestosterone $p < 0.05$)

continued from 10.

STEROID INCUBATED	19-NORTES TOSTERONE	NORETHISTERONE		dl-NORGESTREL		d-NORGESTREL		l-NORGESTREL		LYNESTRENOL		
STEROID RECOVERED	19-NORT	NET	THNET	Ng	THNg	Ng	THNg	Ng	THNg	Lyn	NET	THNET
TISSUE												
SPLEEN	88.2	90.1	0	86.1	0	90.1	0	93.2	0	86.2	0	0
	92.1	92.1	0	80.2	0	91.0	0	94.4	0	84.1	0	0
	94.5	83.4	0	90.1	0	83.2	0	96.2	0	89.2	0	0
mean$\pm$SD	91.6$\pm$3.1	88.5$\pm$4.5	0	85.4$\pm$4.9	0	88.1$\pm$4.2	0	94.6$\pm$1.5	0	86.5$\pm$2.5	0	0
SMALL INTESTINE	40.2	75.1	28.2	87.1	9.0	88.4	10.2	98.2	0	93.1	0	4.0
	44.1	64.2	32.1	86.4	8.0	83.2	12.1	99.1	0	93.0	0	5.8
	43.2	62.1	30.1	88.9	4.0	80.0	14.1	92.2	0	90.1	0	5.6
mean$\pm$SD	42.5$\pm$2.0	67.1$\pm$6.9*	30.1$\pm$1.9	87.4$\pm$1.2*	7.0$\pm$2.6	83.8$\pm$4.2*	12.1$\pm$1.9	96.5$\pm$3.7*	0	92.1$\pm$1.7*	0	5.1$\pm$0.9
ADIPOSE	82.1	80.2	9.2	79.1	0	88.2	0	93.8	0	89.2	0	0
	72.1	86.4	10.4	81.2	0	83.1	0	92.1	0	88.1	0	0
	70.2	87.2	11.4	81.4	0	70.2	0	88.1	0	94.2	0	0
mean$\pm$SD	74.8$\pm$6.3	84.6$\pm$3.8	10.3$\pm$1.1	80.5$\pm$1.2	0	80.5$\pm$9.2	0	91.3$\pm$2.9*	0	90.5$\pm$3.2*	0	0
HEART	80.2	70.2	0	91.2	0	87.4	0	89.2	0	87.8	0	0
	79.2	84.1	0	90.1	0	81.2	0	92.1	0	86.4	0	0
	75.1	85.4	0	64.2	0	89.1	0	93.2	0	83.1	0	0
mean$\pm$SD	78.1$\pm$2.7	79.7$\pm$8.4	0	81.8$\pm$15.2	0	85.9$\pm$4.1	0	91.5$\pm$2.0*	0	85.7$\pm$2.4*	0	0

APPENDIX TABLE 12 COMPARISON OF THE RATE OF METABOLISM OF LYNESTRENOL ACETATE, NORETHISTERONE ACETATE AND dl-NORGESTREL ACETATE IN MALE RABBIT TISSUES. RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED IN 120 min (* denotes value significantly different from 19-nortestosterone $p < 0.05$).

STEROID INCUBATED	LYNESTRENOL ACETATE				NORETHISTERONE ACETATE			dl-NORGESTREL ACETATE		
STEROID RECOVERED	Lyn-Ac	Lyn	NET	THNET	NET-Ac	NET	THNET	Ng-Ac	Ng	THNg
TISSUE										
LIVER	62.1	20.1	14.1	14.1	53.0	22.1	20.4	62.1	16.1	18.1
	65.2	22.0	15.1	5.4	48.6	19.1	26.1	58.1	15.2	16.2
	67.1	18.2	17.2	7.1	43.0	17.4	19.4	59.2	14.0	13.1
mean $\pm$ SD	$64.8 \pm 2.5^*$	20.1 ± 1.9	15.5 ± 1.5	5.5 ± 1.5	$48.2 \pm 5.9^*$	19.5 ± 2.3	21.9 ± 3.6	$59.8 \pm 2.0^*$	15.1 ± 1.1	15.8 ± 2.5
KIDNEY	85.2	12.1	0	0	98.4	0	0	82.2	8.0	0
	86.4	10.2	0	0	68.4	10.0	0	83.1	6.2	0
	88.1	9.1	0	0	78.2	9.2	0	89.1	6.1	0
mean $\pm$ SD	86.5 ± 1.4	10.4 ± 1.5	0	0	81.6 ± 15.3	9.6 ± 0.6	0	84.8 ± 3.7	6.7 ± 1.0	0
SKELETAL MUSCLE	71.2	22.1	0	0	66.2	24.4	8.2	60.1	21.2	6.1
	70.1	26.4	0	0	58.2	28.8	9.1	57.2	26.1	7.2
	64.2	80.2	0	0	61.0	30.8	6.4	61.1	20.2	9.1
mean $\pm$ SD	$68.5 \pm 3.7^*$	26.2 ± 4.0	0	0	61.8 ± 4.1	28.0 ± 3.2	7.9 ± 1.3	59.4 ± 2.0	22.5 ± 3.1	7.4 ± 1.5
LUNG	85.1	10.2	3.1	0	76.0	12.0	4.0	80.1	7.1	2.0
	86.4	12.1	2.4	0	82.4	6.0	3.0	82.1	8.0	3.4
	80.2	15.2	5.2	0	84.2	5.4	2.9	84.2	8.2	3.0
mean $\pm$ SD	$83.9-3.2^*$	12.5-2.5	3.5-1.4	0	$80.8-4.3^*$	7.8-3.6	3.3-0.6	$82.1-2,0^*$	7.7-0.5	2.8-0.7

APPENDIX TABLE 13 COMPARISON OF THE RATE OF METABOLISM OF LYNESTRENOL ACETATE, NORETHISTERONE ACETATE AND dl-NORGESTREL ACETATE
continued from 12 IN MALE RABBIT TISSUE. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT 120 min (*denotes value significantly different from 19-nortestosterone $p < 0.05$).

STEROID INCUBATED	LYNESTRENOL ACETATE				NORETHISTERONE ACETATE			dl-NORGESTREL ACETATE		
STEROID RECOVERED	Lyn-Ac	Lyn	NET	THNET	NET-Ac	NET	THNET	Ng-Ac	Ng	THNg
TISSUE										
SMALL INTESTINE	18.2	60.2	8.2	2.1	12.0	74.0	4.4	15.4	70.1	2.1
	19.4	64.1	9.1	1.2	10.0	84.4	6.9	18.2	72.2	3.2
	20.2	70.2	8.9	3.4	9.4	84.2	2.1	19.1	76.4	2.0
mean $\pm$ SD	$19.2 \pm 1.0^*$	64.8 ± 5.0	8.7 ± 0.4	2.2 ± 1.1	$10.5 \pm 1.3^*$	80.8 ± 5.9	4.4 ± 2.4	$17.5 \pm 1.9^*$	72.9 ± 3.2	2.4 ± 0.6
ADIPOSE	94.0	4.1	0	0	98.0	1.0	0	98.1	0	0
	96.8	3.1	0	0	98.0	0.9	0	96.4	0	0
	92.1	6.1	0	0	97.0	1.4	0	94.2	0	0
mean $\pm$ SD	$94.3 \pm 2.3^*$	4.4 ± 1.5	0	0	$97.6 \pm 0.5^*$	1.1 ± 0.2	0	$96.2 \pm 1.9^*$	0	0
HEART	75.4	18.2	2.1	0	70.0	21.0	0	84.2	12.1	0
	79.1	16.2	1.4	0	86.2	14.2	0	86.1	14.3	0
	76.4	15.1	2.1	0	84.2	12.1	0	80.2	11.1	0
mean $\pm$ SD	76.9 ± 1.9	16.5 ± 1.5	1.8 ± 0.4	0	80.1 ± 8.8	15.7 ± 4.6	0	83.8 ± 3.0	12.5 ± 1.6	0

APPENDIX TABLE 14.

INCUBATION OF (4-^{14}C) TESTOSTERONE WITH FEMALE RABBIT LIVER MICROSOMES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT VARIOUS TIME INTERVALS; ALL VALUES ARE MEANS OF DUPLICATE DETERMINATIONS.

STEROID INCUBATED	(^{14}C) TESTOSTERONE.			
% STEROID RECOVERED	TESTOS-TERONE	DIHYDROTES TOSTERONE	TETRAHYDRO TESTOSTERONE	'POLAR METABOLITE'
TIME (min)				
10	19.4	8.1	12.1	56.1
	18.1	9.2	11.2	53.4
	22.0	9.4	12.4	49.2
	18.4	9.1	11.1	54.6
	19.8	8.8	10.2	55.4
	25.1	7.5	12.2	56.1
	18.1	9.8	14.6	48.2
	19.2	10.1	12.2	51.1
(mean$\pm$SD)	20.0$\pm$2.4	9.0$\pm$0.8	12.0$\pm$1.2	53.0$\pm$3.1
20	10.1	8.1	14.1	61.2
	9.4	7.9	12.2	57.1
	11.2	7.2	14.4	60.2
	8.3	8.4	12.1	58.1
	12.1	8.1	11.0	61.0
	12.0	8.0	10.2	59.0
	9.5	8.1	16.6	68.3
	8.9	8.2	13.4	71.2
(mean$\pm$SD)	10.2$\pm$1.4	8.0$\pm$0.3	13.0$\pm$2.0	62.0$\pm$5.0
	4.9	3.1	14.2	70.2
	6.1	3.0	14.1	72.1
	7.2	2.9	15.1	73.4
	5.4	2.4	14.1	69.1
	5.8	3.8	12.0	74.2
	6.7	3.1	13.2	68.1
	7.7	3.1	15.0	71.4
	8.2	2.6	15.3	70.2
(mean$\pm$SD)	6.5$\pm$1.1	3.0$\pm$0.4	14.1$\pm$1.1	70.9$\pm$2.0

APPENDIX TABLE 15 : INCUBATION OF (^{14}C) d-NORGESTREL WITH FEMALE RABBIT LIVER MICROSOMES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT VARIOUS TIME INTERVALS; ALL VALUES ARE MEANS OF DULPICATE DETERMINATIONS. (* Indicates value significantly different from dl- and l-Ng, $p < 0.05$)

STEROID INCUBATED	(^{14}C) d-NORGESTREL.					
% STEROID RECOVERED	d-Ng	3β,5β-hydroxy-Ng.	3α,5β-hydroxy-Ng.	16α-hydroxy-Ng.	16β-hydroxy-Ng.	16ε-hydroxy-Ng.
TIME (min)	44.2	9.1	12.1	2.1	13.1	6.1
	41.2	8.2	13.2	4.1	14.2	6.2
	46.1	6.5	13.1	2.9	13.2	7.1
10	48.1	8.1	12.2	2.5	10.1	6.9
	43.0	9.2	11.0	3.5	13.9	5.9
	47.1	8.5	10.5	2.1	14.2	6.7
	45.2	6.2	11.9	2.9	13.1	8.2
	45.1	8.2	12.6	3.9	12.2	8.9
(mean±SD)	45.0±2.2	8.0±1.1*	12.0±1.0*	3.0±0.8*	13.0±1.4*	7.0±1.1
	30.1	9.0	18.1	5.1	16.1	7.1
	29.8	10.5	14.2	6.2	17.2	8.1
20	33.2	12.4	15.1	7.4	18.2	7.5
	32.1	10.1	13.1	7.6	18.1	7.9
	30.9	10.9	16.2	6.3	17.1	8.9
	30.8	11.1	14.1	4.1	16.2	18.2
	29.9	9.2	18.3	5.2	15.8	8.4
	35.2	8.4	18.9	6.1	17.3	7.9
(mean±SD)	31.5±1.9	10.2±1.3*	16.0±2.2*	6.0±1.1*	17.0±1.0*	8.0±0.5
	18.1	11.0	18.1	6.1	25.1	8.1
	16.2	12.9	22.1	7.2	23.1	9.2
30	18.1	12.1	20.1	8.4	22.4	10.1
	16.4	13.4	24.2	7.8	24.8	9.0
	19.4	10.8	25.1	9.1	19.2	12.2
	18.6	12.8	17.2	9.2	18.2	12.1
	17.1	10.8	16.1	8.8	25.0	10.1
	20.1	12.4	17.1	7.4	26.2	9.2
(mean±SD)	18.0±1.4	12.0±1.1*	20.0±3.4*	8.0±1.1*	23.0±2.9*	10.0±1.5

APPENDIX TABLE 16.

INCUBATION OF (^{14}C) dl-NORGESTREL WITH FEMALE RABBIT LIVER MICROSOMES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT VARIOUS TIME INTERVALS; ALL VALUES ARE MEANS OF DULPICATE DETERMINATIONS.

STEROID INCUBATED	:(^{14}C) dl-NORGESTREL.					
% STEROID RECOVERED	dl-Ng	3β,5β-hydroxy-Ng	3α,5β-hydroxy Ng	16α-hydroxy-Ng	16β-hydroxy-Ng	6ε-hydroxy-Ng.
TIME (min)	64.0	2.0	4.8	10.2	8.4	6.1
	59.6	1.5	3.5	13.1	8.9	7.5
10	68.2	1.3	5.1	10.2	9.7	7.8
	63.1	1.9	7.2	9.5	10.1	5.9
	61.2	3.2	4.2	12.2	9.3	6.9
	58.2	1.8	6.1	13.0	8.1	8.4
	60.1	1.9	4.0	10.8	8.9	8.0
	62.1	2.4	5.5	9.1	8.6	5.4
(mean±SD)	62.0±3.1	2.0±0.6	5.0±1.2	11.0±1.6	9.0±0.7	7.0±1.1
	45.0	4.0	5.1	16.1	10.1	9.1
	50.8	3.1	6.1	13.1	12.1	7.9
20	48.8	4.5	7.2	15.2	9.2	9.4
	47.1	6.1	4.2	16.1	13.1	9.0
	48.2	3.2	3.9	10.2	10.1	8.5
	50.0	3.1	8.4	14.9	11.2	10.1
	51.0	3.8	6.0	15.0	12.8	8.8
	44.0	4.1	7.1	12.0	9.4	9.2
(mean±SD)	48.1±2.6	4.0±1.1	6.0±1.6	14.0±2.1	11.0±1.5	9.0±0.6
	37.0	5.4	6.1	14.1	11.0	12.1
	42.1	5.9	7.2	15.1	15.2	8.1
30	41.3	6.1	8.1	18.3	13.1	12.1
	42.1	6.8	6.4	13.4	12.1	11.2
	39.2	4.1	8.1	14.2	10.3	12.4
	37.1	7.1	5.9	15.1	9.4	10.1
	41.1	8.0	7.4	14.1	10.9	11.2
	40.0	4.6	6.8	15.7	14.0	10.8
(mean±SD)	40.0±2.0	6.0±1.3	7.0±0.8	15.0±1.5	12.0±2.0	11.0±1.4

APPENDIX TABLE 17.

INCUBATION OF (^{14}C) 1-NORGESTREL WITH FEMALE RABBIT LIVER MICROSOMES. THE RESULTS ARE EXPRESSED AS PERCENT OF STEROID RECOVERED AT VARIOUS TIME INTERVALS; ALL VALUES ARE MEANS OF DUPLICATE DETERMINATIONS.

STEROID INCUBATED	(^{14}C) 1-NORGESTREL.					
% STEROID RECOVERED	1-Ng.	3β,5β-hydroxy-Ng.	3α,5β-hydroxy-Ng.	16α-hydroxy-Ng	16β-hydroxy-Ng.	6ε-hydroxy-Ng.
TIME (min)	68.1	0.9	2.2	9.1	3.5	6.5
	71.2	0.8	3.1	9.4	2.1	7.1
10	68.1	1.1	2.1	9.7	2.9	7.2
	69.8	1.4	1.9	10.1	3.1	6.4
	71.1	0.7	1.7	8.1	3.5	7.3
	73.1	0.9	1.2	11.2	2.0	8.0
	68.5	2.0	2.2	11.9	2.9	6.5
	70.1	0.6	1.8	10.5	4.0	7.0
(mean±SD)	70.0±1.7	1.0±0.4	2.0±0.5	10.0±1.2	3.0±0.7	7.0±0.5
	59.1	1.1	3.6	13.2	5.1	9.1
	60.1	2.1	2.9	15.1	5.4	10.2
20	62.1	2.4	2.4	17.2	6.1	9.9
	58.2	2.1	2.7	12.1	5.8	10.2
	57.4	1.5	3.4	13.2	7.0	12.1
	63.2	2.1	3.1	14.2	6.5	8.6
	60.8	1.6	3.2	14.0	5.9	9.4
	59.2	3.1	3.0	13.0	6.2	10.5
(mean±SD)	60.0±2.0	2.0±0.6	3.0±0.4	14.0±1.5	6.0±0.6	10.0±1.1
	48.2	4.5	6.1	16.0	7.1	9.4
	52.1	3.0	6.7	16.2	7.8	10.0
30	51.2	4.4	7.2	18.1	9.1	12.3
	49.1	4.1	8.1	19.1	9.3	13.2
	47.2	3.8	7.4	20.2	8.4	8.9
	50.1	3.7	8.1	17.2	8.5	11.9
	51.2	5.4	6.3	18.1	6.6	12.1
	52.1	3.1	6.1	19.1	7.2	10.2
(mean±SD)	50.0±1.8	4.0±0.8	7.0±0.9	18.0±1.5	8.0±1.0	11.0±1.5

APPENDIX TABLE 18

COMPARISON OF THE RATE OF SULPHOCONJUGATION OF DEHYDROEPIANDROSTERONE AND VARIOUS SYNTHETIC STEROIDS IN THE HEPATIC TISSUE OF THE FEMALE RABBIT. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AS SULPHATE. (* denotes value significantly different from $DHASO_4$ $p < 0.05$).

TIME OF INCUBATION	PERCENT STEROID RECOVERED AS SULPHATE						
	$DHASO_4$	$EESO_4$	$NETSO_4$	d-Ng-SO_4	dl-Ng-SO_4	l-Ng-SO_4	$lynSO_4$
30 min	10.0	11.9	8.0	3.4	2.0	2.2	1.5
	8.2	12.1	7.0	2.9	3.2	1.8	1.4
	11.1	13.2	6.1	5.1	1.9	1.5	1.0
	11.7	13.8	6.4	4.2	4.8	3.0	1.2
	9.0	14.2	7.8	2.1	2.2	1.6	0.9
mean$\pm$SD	10.0$\pm$1.4	13.0$\pm$1.0*	7.0$\pm$0.8*	3.5$\pm$1.1*	2.8$\pm$1.2*	2.0$\pm$0.6*	1.2$\pm$0.2*
60 min	12.0	13.9	9.0	7.2	4.1	3.0	3.1
	10.2	14.1	8.6	5.4	5.2	4.5	1.9
	12.1	14.9	7.2	9.2	4.1	2.9	2.3
	12.2	15.0	7.4	7.1	4.1	6.1	1.8
	10.0	16.1	8.5	6.2	6.8	3.4	1.9
mean$\pm$SD	11.3$\pm$1.1	14.8$\pm$0.8*	8.5$\pm$0.7*	7.0$\pm$1.4*	5.0$\pm$1.1*	3.9$\pm$1.3*	2.2$\pm$0.5*
120 min	17.8	18.2	14.1	8.0	6.2	5.8	5.1
	15.7	19.1	12.7	9.3	8.9	4.7	3.0
	16.2	17.4	10.9	8.0	7.0	7.5	6.0
	14.4	22.1	7.4	11.5	7.4	6.5	4.1
	11.0	16.2	11.0	10.5		7.2	5.2
	12.4	16.0	10.8	12.0		6.7	4.5
	11.5	20.0	10.2	10.0		5.7	2.0
	16.0	15.0	12.1	9.2		2.9	3.0
		14.2	11.4	8.2		3.0	4.3
		17.1	15.2	8.0		3.0	5.0
		16.2	14.1	9.4		4.2	
		18.1	10.1	9.0			
		16.4	12.9				
		19.0	11.4				
		15.1	10.3				
		22.2	13.0				
		13.4	12.8				
		20.2	11.1				
		15.9	12.2				
		15.8	13.4				
		16.7	12.0				
		16.6	13.0				
mean$\pm$SD	14.3$\pm$2.4	17.3$\pm$2.3*	11.8$\pm$1,7*	9.4$\pm$1.3*	6.8$\pm$0.4*	5.2$\pm$1.7*	4.2$\pm$1.2*

APPENDIX TABLE 19

SYNTHESIS OF STEROID SULPHATES BY VARIOUS TISSUES OF THE FEMALE RABBIT. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AS SULPHATE IN 120 MIN. (* denotes value significantly different from DHA SO_4 $p < 0.05$)

STEROID RECOVERED AS SULPHATE	TISSUES EXAMINED									
	LIVER	STOMACH	ILLEUM	CAECUM	LUNG	HEART	SPLEEN	ADIPOSE	SKELETAL MUSCLE	KIDNEY
DEHYDROEPIANDROSTERONE SULPHATE		11.2 13.1 12.0	4.1 5.2 6.0	2.6 3.0 4.0	13.2 11.3 12.3	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
mean$\pm$SD	(8) 14.3$\pm$2.4	12.1$\pm$0.9	5.1$\pm$0.9	3.2$\pm$0.7	12.2$\pm$1.0	0	0	0	0	0
ETHYNYLOESTRADIOL SULPHATE		7.0 6.2 6.0	2.1 1.2 3.0	2.4 3.3 3.0	1.6 2.8 2.2	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
mean$\pm$SD	(*22)17.3$\pm$2.3	6.4$\pm$0.5*	2.1$\pm$0.9*	2.9$\pm$0.4	2.2$\pm$0.6*	0	0	0	0	0
NORETHISTERONE SULPHATE		4.1 6.2 4.7	0 0 0	0 0 0	1.1 1.9 2.1	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
mean$\pm$SD	(*12)11.8$\pm$1.7	5.0$\pm$1.0*	0	0	1.7$\pm$0.5*	0	0	0	0	0
d-NORGESTREL SULPHATE		9.4 12.0 11.0	0 0 0	0 0 0	0.9 1.1 1.0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
mean$\pm$SD	(*12)9.4$\pm$1.3	10.8$\pm$1.3	0	0	1.0$\pm$0.1	0	0	0	0	0
dl-NORGESTREL SULPHATE		3.1 4.2 5.0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
mean$\pm$SD	(*4)6.8$\pm$0.4	4.1$\pm$0.9*	0	0	0	0	0	0	0	0
l-NORGESTREL SULPHATE		2.5 3.5 4.0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
mean$\pm$SD	(*11)5.2$\pm$1.7	3.3$\pm$0.7*	0	0	0	0	0	0	0	0
LYNESTRENOL SULPHATE		0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
mean$\pm$SD	(*10)4.2$\pm$1.2	0	0	0	0	0	0	0	0	0

APPENDIX TABLE 20

COMPARISON OF THE RATE OF METABOLISM OF NORETHISTERONE OXIME RACEMATE, 'ANTI'- AND 'SYN'-ISOMER IN FEMALE RABBIT LIVER. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT VARIOUS TIME INTERVALS (**denotes value significantly different from norgestrel oxime $p < 0.05$)

STEROID INCUBATED	NET - OX RACEMATE			NET-OX 'ANTI'-ISOMER			NET - OX 'SYN'-ISOMER.		
STEROID RECOVERED	NET-OX	NET	THNET	NET-OX	NET	THNET	NET-OX	NET	THNET
TIME (min)	85.1	0	0	89.1	0	0	92.0	0	0
	89.3	0	0	88.2	0	0	90.6	0	0
0	87.0	0	0	88.0	0	0	95.4	0	0
	89.1	0	0	90.2	0	0	94.0	0	0
mean$\pm$SD	87.6-1.9	0	0	88.8-1.0	0	0	92.6-2.4	0	0
	78.0	1.2	1.0	80.1	3.2	2.1	84.0	1.1	1.1
	82.8	3.0	1.9	81.0	4.0	1.1	86.2	2.2	2.3
10	79.0	2.9	1.3	80.1	2.4	2.2	84.0	3.3	1.2
	81.2	3.5	2.9	82.2	3.6	3.0	86.2	3.0	1.4
mean$\pm$SD	80.2$\pm$2.1**	2.6$\pm$1.0	1.7$\pm$0.8	80.8$\pm$0.9**	3.3$\pm$0.6	2.1$\pm$0.7	85.1$\pm$2.6**	2.4$\pm$0.9	1.5$\pm$0.5
	74.0	3.8	2.9	78.1	5.4	5.2	80.2	4.9	3.1
	80.0	5.9	3.1	80.0	6.0	4.1	80.0	5.1	2.2
20	78.3	6.0	3.1	79.2	5.2	3.2	80.2	6.0	4.1
	74.1	6.8	5.3	78.0	7.0	3.1	76.0	4.0	4.1
mean$\pm$SD	76.6$\pm$3.0**	5.6$\pm$1.2	3.6$\pm$1.1	78.8$\pm$0.9**	5.9$\pm$0.8	3.9$\pm$0.9	80.1$\pm$4.3**	5.0$\pm$0.8	3.4$\pm$0.9
	75.3	4 1	2.8	76.1	6.0	6.1	79.2	5.1	4.2
	77.1	7.1	3.0	78.0	7.4	4.0	77.1	6.1	3.3
30	75.3	5.9	6.2	75.1	5.6	3.0	81.0	5.6	3.2
	73.0	7.9	4.6	76.2	7.0	4.0	74.1	4.4	4.1
mean$\pm$SD	75.1$\pm$1.6**	6.2$\pm$1.6	4.1$\pm$1.5	76.3$\pm$1.2**	6.5$\pm$0.8	4.3$\pm$1.2	78.0$\pm$5.1**	5.3$\pm$0.7	3.7$\pm$0.5
	72.0	6.9	2.4	75.1	9.5	7.1	75.0	6.1	5.2
	76.1	8.3	3.2	76.1	7.3	4.3	74.1	7.2	4.4
60	69.3	7.9	7.2	74.1	6.2	5.2	80.0	5.3	4.1
	73.1	5.9	5.0	74.0	7.0	3.0	71.0	5.0	3.1
mean$\pm$SD	72.6$\pm$2.8**	7.2$\pm$1.0	4.4$\pm$2.1	74.8$\pm$0.9**	7.5$\pm$1.4	4.9$\pm$1.7	75.0$\pm$3.7**	5.9$\pm$0.4	4.2$\pm$0.8
	71.3	8.3	3.0	71.1	10.0	7.2	71.1	6.2	4.2
	75.1	9.7	1.2	72.2	8.2	5.1	72.0	7.4	5.0
120	75.0	8.3	7.5	72.1	9.4	4.3	79.0	6.3	3.4
	65.1	8.9	5.5	73.2	7.2	5.0	70.0	6.1	4.6
mean$\pm$SD	71.6$\pm$4.6**	8.8$\pm$0.6	4.3$\pm$2.7	72.1$\pm$0.8**	8.7$\pm$1.2	5.4$\pm$1.2	73.2$\pm$6.9**	6.5$\pm$0.6	4.3$\pm$0.6

APPENDIX TABLE 21

COMPARISON OF THE RATE OF METABOLISM OF NORGESTREL OXIME RACEMATE, 'ANTI'- AND 'SYN'-ISOMER IN FEMALE RABBIT TISSUE AT VARIOUS TIME INTERVALS. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED (** denotes value significantly different from the previous value $p < 0.05$)

STEROID INCUBATED	Ng-OX RACEMATE		Ng-OX 'ANTI'-ISOMER		Ng-OX 'SYN'-ISOMER	
STEROID RECOVERED	Ng-OX	Ng	Ng-OX	Ng	Ng-OX	Ng
TIME (min)						
0	92 2	0	96.6	0	93.2	0
	93.2	0	95.2	0	90.1	0
	89.9	0	91.1	0	94.2	0
	90.6	0	92.1	0	93.2	0
mean$\pm$SD	91.4$\pm$1.5	, 0	93.2$\pm$1.9	0	92.6$\pm$1.7	0
	88.8	2.2	86.4	2.0	88.2	2.0
	86.9	3.2	86.7	3.1	86.1	3.8
10	84.2	1.1	83.4	4.0	90.2	3.1
	89.2	2.1	88.1	2.9	88.1	3.6
mean$\pm$SD	87.2$\pm$2.1	2.1$\pm$0.8	86.1$\pm$1.9	3.0$\pm$0.8	88.1$\pm$1.6	3.0$\pm$0.8
	85.2	4.1	84.0	4.8	86.0	4.1
	86.1	5.0	82.9	6.0	84.0	4.2
20	82.2	3.4	82.2	8.1	88.0	5.2
	87.1	4.3	85.3	5.9	86.0	6.1
mean$\pm$SD	85.1$\pm$2.1	4.2$\pm$0.6	83.8$\pm$2.1	6.2$\pm$1.3	86.0$\pm$2.1	4.9$\pm$0.9
	83.9	6.4	83.1	5.4	84.9	5.3
	84.9	7.2	81.1	7.4	83.0	6.0
30	81.9	4.4	80.2	9.6	87.1	6.9
	85.8	6.4	84.7	7.2	85.2	7.9
mean$\pm$SD	84.1$\pm$1.6	6.1$\pm$1.1	82.2$\pm$2.0	7.4$\pm$1.4	85.0$\pm$1.6	6.5$\pm$1.1
	82.2	8.2	80.8	6.6	81.6	6.9
	81.2	10.1	80.1	8.2	80.8	7.1
60	79.8	7.1	78.2	10.9	84.7	7.5
	83.5	9.8	81.2	7.1	83.1	8.2
mean$\pm$SD	81.6$\pm$1.5	8.8$\pm$1.4	80.0$\pm$1.3	8.2$\pm$1.9	82.5$\pm$1.7	7.4$\pm$0.5
	80.3	9.4	78.2	9.4	80.8	7.2
	80.9	12.4	76.1	8.2	78.2	8.0
120	78.0	8.0	75.2	10.2	81.2	9.0
	81.2	10.2	77.2	12.9	80.0	10.2
mean$\pm$SD	80.1$\pm$1.4 **	10.0$\pm$1.8	76.6$\pm$1.3 **	9.9$\pm$1.6	80.0$\pm$1.3 **	8.6$\pm$1.2

APPENDIX TABLE 22 continued from 20.

COMPARISON OF THE RATE OF METABOLISM OF NORETHISTERONE AND NORGESTREL EXPRESSED AS PERCENT STEROID RECOVERED AT VARIOUS TIME INTERVALS (* denotes value significantly different from the oxime derivatives $p < 0.05$)

STEROID INCUBATED	NORETHISTERONE		d-NORGESTREL			NORETHISTERONE		d-NORGESTRAL	
STEROID RECOVERED	NET	THNET	Ng	THNg		NET	THNET	Ng	THNg
TIME (min)	96.0	-	94.7	-	TIME (min)	48.6	50.0	58.0	39.2
	94.8	-	90.2	-		45.6	54.0	54.1	37.1
0	90.1	-	96.4	-	30	44.1	52.0	56.2	38.2
	92.1	-	91.2	-		46.2	56.0	58.2	36.1
	93.0	-	89.4	-		42.1	57.1	57.1	34.2
	90.2	-	88.2	-		45.2	54.2	56.2	36.1
	89.2	-	-	-		47.2	50.1	-	-
mean $\pm$ SD	92.2 ± 2.5	-	91.6 ± 3.2	-	mean $\pm$SD	45.5 ± 2.1*	53.3 ± 2.7	56.6 ± 1.5*	36.8 ± 1.7
	60.2	40.4	74.0	22.1		24.4	72.8	52.0	46.0
	66.8	32.2	72.0	20.9		20.8	76.0	50.0	45.2
10	61.2	35.1	76.0	21.4	60	22.4	74.0	51.2	42.1
	63.4	36.7	74.2	26.1		26.1	73.2	53.4	42.8
	68.1	34.2	72.1	21.2		28.2	70.1	58.1	46.2
	59.1	31.9	70.2	20.0		24.3	69.2	50.0	47.8
	57.1	36.2	-	-		23.0	73.1	-	-
mean $\pm$SD	62.2 ± 3.9*	35.2 ± 2.9	73.0 ± 2.1*	$21.9 \pm$	mean $\pm$ SD	$24.1 \pm 2,4$*	72.6 ± 2.3	52.4 ± 3.0*	44.8 ± 1.9
	56.8	42.4	63.0	34.0		10.0	86.0	45.0	52.0
	52.2	46.6	61.2	30.0		6.0	92.4	42.0	51.0
20	54.2	45.1	62.4	32.0	120	8.0	88.2	45.8	55.2
	53.1	42.2	64.2	31.0		8.9	86.1	46.1	54.1
	50.2	46.1	61.9	29.1		6.2	89.1	40.0	50.2
	54.2	45.2	62.4	33.2		7.1	82.1	48.2	54.1
	51.2	41.0	-	-		8.4	80.3	-	-
mean $\pm$ SD	53.1 ± 2.1*	44.0 ± 2.2	62.5 ± 0.9*	$31.5 \pm$	mean $\pm$ SD	7.8 ± 1.4*	86.3 ± 4.1	44.5 ± 2.9*	53.7 ± 1.9

APPENDIX TABLE 23

COMPARISON OF THE RATE OF METABOLISM OF NORETHISTERONE, NORETHISTERONE OXIME RACEMATE, 'ANTI'- AND 'SYN'-ISOMERS IN FEMALE RABBIT TISSUES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT 120 MIN. (*denotes value significantly different from norgestrel and ** denotes value significantly different from d-norgestrel oxime racemate $p < 0.05$)

STEROID INCUBATED	NORETHISTERONE		NET-OX RACEMATE			NET-OX 'ANTI'-ISOMER			NET-OX 'SYN'-ISOMER		
STEROID RECOVERED	NET	THNET	NET-OX	NET	THNET	NET-OX	NET	THNET	NET-OX	NET	THNET
TISSUE	45.1	54.1	70.1	9.0	3.1	68.2	10.2	2.1	64.2	12.1	4.1
	57.1	47.0	76.2	7.2	4.4	67.1	12.1	2.5	67.1	10.2	5.2
LIVER	43.0	49.2	70.1	7.8	2.1	70.2	13.2	3.4	66.4	11.2	3.1
mean±SD	48.4±7.6*	50.1±3.6	72.1±3.5**	8.0±0.9	3.2±1.1	68.5±1.5	11.8±1.5	2.6±0.6	65.9±1.5	11.1±0.9	4.1±1.0
	81.2	8.1	71.2	2.3	2.1	72.2	3.0	4.1	73.1	4.2	6.2
	88.2	6.1	75.1	3.1	1.2	78.1	3.9	5.1	72.5	4.1	6.1
STOMACH	83.2	7.4	67.2	4.2	3.0	70.2	5.2	5.9	76.4	5.1	7.2
mean±SD	84.2±3.6*	7.2±1.0	71.1±3.9**	3.2±0.9	2.1±0.9	73.5±4.1	4.0±1.1	5.0±0.9	74.0±2.1	4.4±0.4	6.5±0.6
	68.1	10.0	79.1	4.1	3.1	76.4	5.9	5.2	75.4	6.1	4.1
DUODENUM	60.2	8.0	72.2	3.0	4.0	78.2	6.1	3.1	73.2	7.2	5.2
	61.0	9.6	89.2	3.1	2.2	77.1	7.4	2.2	77.1	8.1	5.9
mean±SD	63.1±4.3*	9.2±1.0	80.1±8.5**	3.4±0.6	3.1±0.9	77.2±0.9	6.4±0.8	3.1±1.5	75.2±1.9	7.1±1.9	5.0±0.9
	80.2	10.0	81.0	4.1	1.5	76.2	7.1	1.1	72.1	4.1	4.1
ILEUM	78.3	9.1	79.1	5.0	2.0	80.1	6.4	1.9	76.4	5.2	5.1
	80.0	12.1	66.2	3.2	3.1	88.2	7.9	2.1	77.1	3.1	3.2
mean±SD	79.5±1.0	10.4±1.5	75.4±8.0**	4.1±0.9	2.2±0.9	81.5±6.1	7.1±0.7	1.7±0.5	75.2±2.7	4.1±1.0	4.1±0.9
	86.6	8.2	78.4	1.2	1.4	80.1	2.1	0	82.1	2.8	0
CAECUM	85.4	7.4	80.1	2.2	1.2	82.1	2.4	0	81.2	3.1	0
	81.5	6.0	82.1	3.0	1.3	86.1	2.9	0	79.1	1.8	0
mean±SD	84.5±2.6	7.2±1.1	80.2±1.8**	2.1±0.9	1.3±0.1	82.7±3.0	2.4±0.4	0	80.8±1.5	2.5±0.6	0

APPENDIX TABLE 24 continued from 23.

COMPARISON OF THE RATE OF METABOLISM OF NORETHISTERONE AND NORETHISTERONE OXIME RACEMATE, 'ANTI'- AND 'SYN'-ISOMERS IN VARIOUS FEMALE RABBIT TISSUES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT 120 MIN. (* denotes value significantly different from norgestrel and ** value significantly different from d-norgestrel oxime racemate $p < 0.05$).

STEROID INCUBATED	NORETHISTERONE		NET-OX RACEMATE			NET-OX 'ANTI'-ISOMER			NET-OX 'SYN'-ISOMER		
STEROID RECOVERED	NET	THNET	NET-OX	NET	THNET	NET-OX	NET	THNET	NET-OX	NET	THNET
TISSUE	82.4	2.1	80.1	1.4	1.2	88.1	2.1	0	89.2	4.1	0
	86.9	3.2	79.0	1.8	1.4	86.4	3.2	0	86.4	5.1	0
KIDNEY	95.3	4.0	90.2	2.8	1.3	81.2	3.5	0	88.1	2.1	0
mean±SD	88.2±6.5	3.1±0.9	83.1±6.1	2.0±0.7	1.3±0.1	85.2±3.5	2.9±0.7	0	87.9±1.4	3.7±1.5	0
	90.1	0	81.4	2.5	0	82.4	4.1	0	84.2	4.5	0
HEART	88.2	0	84.2	2.8	0	86.1	2.1	0	86.1	3.4	0
	89.0	0	77.0	3.7	0	88.2	3.3	0	87.2	3.4	0
mean±SD	89.1±0.9*	0	80.8±3.6**	3.0±0.6	0	85.5±2.9	3.1±0.1	0	85.8±1.5	3.3±1.2	0
	68.1	4.1	81.0	2.2	1.2	88.4	4.2	2.2	81.2	2.2	1.1
SKELETAL	69.0	3.8	87.6	1.1	1.1	83.2	3.1	3.2	80.1	2.3	1.5
MUSCLE	52.2	2.3	89.4	2.7	1.9	81.9	2.7	4.1	83.4	3.9	1.9
mean±SD	63.1±9.4*	3.4±0.9	86.0±4.4**	2.0±0.8	1.4±0.4	84.5±3.4	3.3±0.7	3.1±0.9	81.5±1.6	2.8±0.9	1.5±0.4
	47.1	8.0	83.1	9.2	1.2	81.2	8.1	2.1	82.2	9.2	1.2
LUNG	45.0	7.6	78.1	3.8	2.5	80.1	9.2	3.4	81.2	9.1	1.9
	37.2	6.0	80.0	4.0	3.5	82.2	10.1	6.1	86.1	10.8	3.4
mean±SD	43.1±5,2*	7.2±1.0	80.4±2.5**	5.6±3.0	2.4±1.1	81.1±1.0	3.8±2.0	3.8±2.0	83.1±2.5	9.7±0.9	2.1±1.1
	86.2	0	80.1	0	0	89.2	0	0	91.2	0	0
ADIPOSE	88.0	0	70.1	0	0	91.4	0	0	93.4	0	0
	74.8	0	80.2	0	0	89.9	0	0	94.5	0	0
mean±SD	83.0±7.1	0	76.8±5.8	0	0	90.1±1.1	0	0	93.0±1.6	0	0

APPENDIX TABLE 25

COMPARISON OF THE RATE OF METABOLISM OF d-NORGESTREL AND d-NORGESTREL OXIME RACEMATE IN FEMALE RABBIT TISSUES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT 30 min. (* denotes value significantly different from d-norgestrel oxime racemate $p < 0.05$).

STEROID INCUBATED	d-NORGESTREL		d-NORGESTREL OXIME RACEMATE		
STEROID RECOVERED	Ng	THNg	Ng-OX	Ng	THNg
TISSUE					
	56.4	42.0	86.0	7.1	0
LIVER	53.6	47.6	89.0	6.0	0
	46.0	45.4	90.1	5.2	0
mean$\pm$SD	52.0$\pm$5.3*	45.0$\pm$2.8	88.3$\pm$2.0	6.1$\pm$0.9	0
	87.6	6.0	89.6	2.1	1.5
STOMACH	91.4	4.3	88.8	2.2	1.7
	88.7	5.0	92.0	2.0	2.8
mean$\pm$SD	89.2$\pm$1.9	5.1$\pm$0.8	90.1$\pm$1.6	2.1$\pm$0.1	2.0$\pm$0.7
	87.1	4.3	94.4	0	0
DUODENUM	91.1	4.5	96.6	0	0
	88.0	3.5	97.0	0	0
mean$\pm$SD	88.7$\pm$2.0	4.1$\pm$0.5	96.0$\pm$1.4	0	0
	82.0	7.1	92.3	0	0
ILEUM	85.4	6.2	92.6	0	0
	86.6	5.0	91.1	0	0
mean$\pm$SD	84.6$\pm$2.3*	6.1$\pm$1.1	92.0$\pm$0.8	0	0
	92.4	0	93.3	0	0
CAECUM	94.6	0	94.6	0	0
	89.0	0	91.1	0	0
mean$\pm$SD	92.0$\pm$2.8	0	93.0$\pm$1.7	0	0

APPENDIX TABLE 26 continued from 25
COMPARISON OF THE RATE OF METABOLISM OF d-NORGESTREL AND d-NORGESTREL OXIME RACEMATE IN FEMALE RABBIT TISSUES. THE RESULTS ARE EXPRESSED AS PERCENT STEROID RECOVERED AT 30 min. (" denotes value significantly different from d-norgestrel oxime racemate $p < 0.05$).

STEROID INCUBATED	d-NORGESTREL		d-NORGESTREL OXIME RACEMATE		
STEROID RECOVERED	Ng	THNg	Ng-OX	Ng	THNg
TISSUE					
	91.3	0	92.0	0	0
KIDNEY	93.6	0	91.5	0	0
	89.1	0	90.5	0	0
mean$\pm$SD	91.3$\pm$2.2	0	91.3$\pm$0.7	0	0
	94.0	0	92.0	0	0
HEART	93.4	0	94.6	0	0
	90.0	0	93.1	0	0
mean$\pm$SD	92.4$\pm$2.1	0	93.2$\pm$1.3	0	0
	81.0	8.0	93.4	0	0
SKELETAL	79.6	9.2	94.6	0	0
MUSCLE	85.4	7.1	96.0	0	0
mean$\pm$SD	82.0$\pm$3.0*	8.1$\pm$1.0	95.0$\pm$1.4	0	0
	80.1	8.1	86.4	2.1	3.0
LUNG	84.6	7.1	85.6	3.2	1.2
	83.0	6.8	89.0	4.0	2.1
mean$\pm$SD	82.5$\pm$2.3	7.3$\pm$0.7	87.0$\pm$1.7	3.1$\pm$0.9	2.1$\pm$0.9
	89.2	0	90.6	0	0
ADIPOSE	88.8	0	89.4	0	0
	92.0	0	91.0	0	0
mean$\pm$SD	90.0-1.7	0	90.3-0.8	0	0

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