The University of Manitoba

THE DISPOSITION OF ETHYLESTRENOL IN THE RAT

by

LESLIE JULES BOUX

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

Faculty of Pharmacy

Winnipeg, Manitoba, Canada

February, 1979

THE DISPOSITION OF ETHYLESTRENOL IN THE RAT

BY

LESLIE JULES BOUX

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ABSTRACT

The disposition of ethylestrenol in the rat and the *in vitro* metabolism by rat liver homogenate was studied utilizing ^{3}H-ethylestrenol as a radioactive tracer.

It was determined that 33% of an oral dose was absorbed from the alimentary canal and that a total of 17% of an oral dose was excreted in the urine and 83% in the faeces within ten days. Distribution experiments showed that oral doses of ^{3}H-ethylestrenol were circulated throughout the rat, but kidney tissue was found to contain 2 1/2 - 3 times and liver tissue 5 - 7 times the specific radioactivity of all other tissues. Samples of urine and faeces from orally dosed rats were examined for metabolites. Unchanged ethylestrenol was the only unconjugated drug-related compound detected in the urine and faeces, but two dihydroxylated dihydro-ethylestrenol metabolites and a trihydroxylated dihydro-ethylestrenol metabolite were detected from the glucuronide conjugates in the faeces.

Measurement of the excretion after intravenous doses showed that the drug excretory system could handle high concentrations of the drug. It was also found that 39.7% of an intravenous dose was excreted in the urine and 50.6% in the faeces in five days. Excretion after an intramuscular dose was found to be very slow. The average $t_{1/2}$ for the excretion of the radioactivity after an intramuscular dose of ^{3}H-ethylestrenol was 5.5 days. The major metabolite

produced by the *in vitro* metabolism of ethylestrenol by rat liver homogenate was identified as 17α-ethyl-17β-hydroxy-4-estren-3-one and 17α-ethyl-5ξ-estrane-3ξ,17β-diol was tentatively identified as a minor metabolite.

ACKNOWLEDGEMENT

I wish to express my gratitude for the considerable guidance given by Dr. J. W. Steele on performing this research and preparing this thesis, the technical criticisms and enlightenment given by Diane Smith and Ricky Araneda, and the extensive encouragement given by my wife, Heather.

Financial assistance from the Medical Research Council and the Faculty of Pharmacy is also gratefully acknowledged.

CONTENTS

page

ABSTRACT. i

ACKNOWLEDGEMENT iii

CONTENTS. iv

INTRODUCTION. 1

A. Anabolic Steroids. 1

1. Evaluation and Classification 1

2. Uses and Abuses 4

3. Mode of Action. 9

4. Undesirable Effects 12

B. Ethylestrenol. 13

C. Aims of this Study 16

METHODS 18

A. Chemicals. 18

B. Instruments and Equipment. 19

C. Animals. 19

D. Dosing 19

1. Oral. 19

2. Intramuscular 20

3. Intravenous 20

E. Excreta Collection 21

F. Liquid Scintillation Counting. 21

1. Urine 22

2. Faeces. 22

3. Tissues 22

CONTENTS

page

4. Solutions and Extracts. 22

G. Chromatography 23

1. Gas-liquid Chromatography 23

2. Thin-layer Chromatography 24

3. Adsorption Chromatography 24

H. Extraction of Metabolites. 25

1. Urine 25

2. Faeces. 27

I. Liver Homogenate Incubations 29

1. NADPH-Regenerating Medium 29

2. NADPH-Non-regenerating Medium 31

3. Incubation and Extraction 31

RESULTS 32

A. Absorption 32

B. Distribution 33

1. Short Term. 33

2. Long Term 35

C. Excretion. 35

1. Oral Dose 35

2. Intramuscular Dose. 37

3. Intravenous Dose. 37

D. Metabolism 44

1. Urine 44

2. Faeces. 46

3. In Vitro. 49

CONTENTS page

DISCUSSION. 57

A. Absorption 57

B. Distribution 59

C. Excretion. 61

D. Metabolism 64

1. Urine 65

2. Faeces. 66

3. In Vitro. 69

SUMMARY 74

REFERENCES. 77

LIST OF TABLES. 80

LIST OF FIGURES 81

APPENDIX. 83

A. Abbreviations and Symbols. 83

INTRODUCTION

A. Anabolic Steroids

Since a report in 1935 (Kolchakian and Murlin, 1935), stating that an extract of male human urine, when injected into dogs, could cause weight gain and a marked drop in urinary nitrogen, the belief that certain male hormones possess an "anabolic" action has led to the development of a class of drugs known as anabolic steroids. They have been developed to "promote the synthesis and storage of cytoplasmic protein and stimulate the growth of tissues," i.e. the anabolic effect (Camerino and Sciaky, 1975). It has also been the aim to separate this effect from the action inherent in male sex hormones of promoting development of the secondary sex characteristics of the male, i.e. the androgenic effect. This class of drugs has been the topic of some controversy in the last few years, due to the lack of knowledge concerning their mode of action, coupled with the fact that they are subject to substantial abuse by the athletic community.

1. Evaluation and Classification

The main problem in anabolic steroid development has been to synthesize analogues of sex hormones, such that these compounds have enhanced anabolic activity and the least possible androgenic effect. This has necessitated the development of a variety of tests to differentiate between the two drug actions.

The assay for androgenic activity was the simplest to design, as any measurement of the growth of an accessory sex organ or sex characteristic would suffice. The most commonly used indicator is the weight of the ventral prostate and/or the seminal vesicles of the rat (Kolchakian, 1975).

The methods to measure anabolic activity were much more difficult to design and in recent years have come under some criticism. Two immediate possibilities, total body weight and nitrogen balance, have both had limited use due to the many technical problems inherent in these methods. Because the objective of anabolic drugs is to increase the amount of the body's lean tissue, other tests measuring the growth or mass of specific muscles have been developed. Most of the work done today is based on a method of this type. The method, developed in 1950 (Eisenberg and Gordon, 1950) and since modified (Hershberger et al., 1953; Desaulles and Krahenbuhl, 1962), utilizes the weight gain of the levator ani muscle of castrated rats, after seven days of drug treatment. For the sake of comparison, the weights of the ventral prostate and of the seminal vesicles are also taken at this time as an indicator of androgenic activity. This enables researchers to compare the two effects, and the resulting ratio of the gain in weight of the muscle to the gain in weight of the other organs is called the anabolic/androgenic ratio.

This method, however, has been severely criticized by several researchers. It was shown (Hayes, 1965) that the muscle used is the "dorsal bulbocavernosa" and is not the levator ani muscle, the former being definitely sex linked. Thus the method, although relatively simple and inexpensive, may yield somewhat misleading results and should now only be used for initial laboratory screening (Potts et al., 1977). Some researchers feel that all these drugs may not have the anabolic activity that is attributed to them, and that a few are on the market today only by virtue of the simplified test (Hervey et al., 1976). Thus, the need is obvious for more specific tests to accurately determine the anabolic activity of drugs.

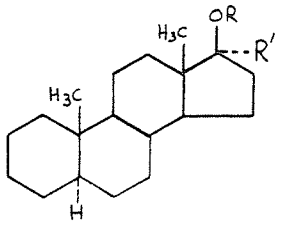
Anabolically active steroids can usually be classified into two large groups depending on their basic structure:
(a) Androstanes: These are C-19 steroids having a methyl group at position 10;
(b) Estranes: These are C-18 steroids having a hydrogen at position 10. These are sometimes called 19-nor-androstanes.

In each of these two main groups are two subgroups (Figure 1):
(i) 17β-hydroxysteroids: Members of this group often have the 17-hydroxyl group esterified, with the length of the ester chain determining the duration of anabolic action. Short chains of approximately two to three carbon atoms give rise to shorter-acting compounds, while those with chains of

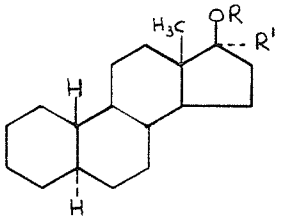
length seven to ten carbon atoms give longer-acting compounds. The products in this subgroup are not orally active and therefore see limited clinical use. More often the compound is converted to its analogue in the second subgroup, conferring oral activity.

(ii) 17α-alkyl-17β-hydroxysteroids: The alkyl group is usually a methyl or ethyl substituent. Members of this class are orally active and can be used for both oral and parenteral administration.

Figure 1. A classification system for anabolic steroids



androstane type



estrane type

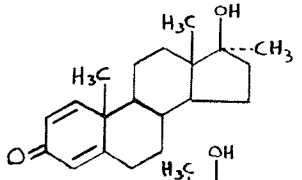
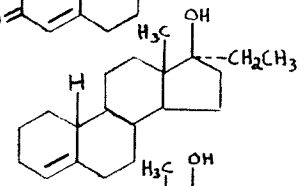
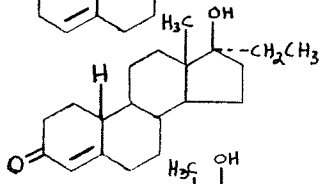
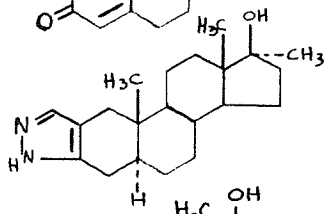
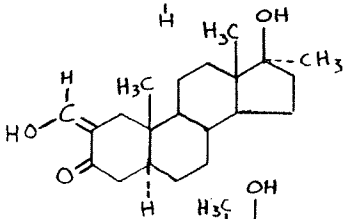
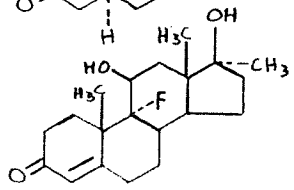
substituent	subgroup (i)	subgroup (ii)
R	-H or ester	-H
R'	-H	$-CH_3$ or $-CH_2CH_3$

Of the six steroids available in Canada for use as anabolic agents (Rotenburg, 1978), all have 17α-alkyl groups and are thus orally active (see Table 1). Four of these are androstane derivatives and the remaining two are estrane derivatives.

2. Uses and Abuses

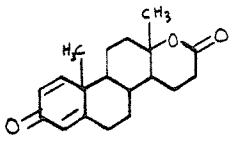
Although anabolic steroids have a wide influence on

Table 1. The anabolic steroids available in Canada

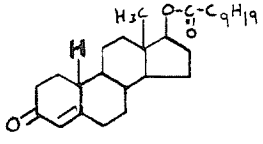
Common Name	Chemical Structure	I.U.P.A.C. Name	Brand Name/ Manufacturer
Methandrostenolone		17β-hydroxy-17α-methyl-1,4-androstadien-3-one	Danabol/ Ciba-Geigy
Ethylestrenol		17α-ethyl-4-estren-17β-ol	Maxibolin/ Organon
Norethandrolone		17α-ethyl-17β-hydroxy-4-estren-3-one	Nilevar/ Searle
Stanozolol		17α-methyl-2'H-5α-androst-2-eno[3,2-c] pyrazol-17β-ol	Winstrol/ Winthrop
Oxymetholone		17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one	Adroyd/ Parke Davis and Company
Fluoxymesterone		11β,17β-dihydroxy-9α-fluoro-17α-methyl-4-androsten-3-one	Halotestin/ Upjohn

metabolic processes and have been suggested for treatment of a vast range of conditions, their clinical importance remains limited to the few areas in which there has been some evidence of success. These areas include the treatment of advanced metastatic breast cancer, aplastic anemia, and wasting diseases in the elderly. In addition, there is the controversial use by athletes to increase their muscle size and strength (Gribbin and Flavell Matts, 1976).

The use of anabolic steroids in the treatment of breast cancer developed from the use of more powerful androgens. These had originally been used to obtain regression of tumour growth, but had the side effect of serious masculinization in many patients (Segaloff et al., 1953). The shift to anabolic agents lowered the incidence of these side effects and still maintained a reasonable level of tumour regression. The use of the drugs, however, remains relatively empirical because the mode of action in producing regression is unclear and the methods for predicting which patient will respond to a given treatment not well developed (Gordan, 1977). The drugs which are now being used to treat this disease, such as Δ^1-testololactone and nandrolone decanoate are probably better classed as weak androgens than as anabolic steroids



Δ'-testololactone



nandrolone decanoate

(Gribbin and Flavell Matts, 1976). It still remains uncer-

tain whether a purely anabolic steroid would produce tumour regression if free of androgenic activity, as it is possible that it is this latter effect which accounts for the anti-cancer activity.

The treatment of aplastic anemia is another use of anabolic steroids in clinical practice (Young et al., 1977). Here, again, the influences of androgenic and anabolic steroids are extremely variable. Some researchers report remission rates as high as 50% (Sanches-Medal et al., 1969) while others have reported that steroid therapy had little influence in the outcome of the disease (Li et al., 1972). It is now generally thought that the treatment of aplastic anemia by anabolic therapy is likely only to influence the milder cases (Lancet, 1975), but that until more definite treatment is available for severe cases, anabolic treatment might as well continue.

The use of anabolics in geriatric practice resulted from a special problem in the treatment of the elderly. For a variety of reasons, many of them neglect their diet and allow themselves to fall into a state of apathy in which catabolism exceeds anabolism. It is these cases in which physicians see a benefit from anabolic steroids. It is suggested that anabolics be given along with other measures, such as a high protein diet and vitamin supplements, to effect improved muscle tone and bulk. In studies where this has been done, under conditions of weight loss, depression,

and muscular wasting, there has been some degree of success, producing general weight gain, increased activity, and overall clinical improvement (Lye and Ritch, 1977; Kopera, 1977).

Although anabolic steroids obviously have limited use in general clinical practice, much research is still being done due to the widespread and often unsupervised use by athletes. The belief that the use of steroids during training will increase both muscle mass and strength, coupled with the fact that the detection of these drugs can be (made) difficult, has led to increased misuse of the drugs. It has been estimated that as many as 75% of professional football players and 80-90% of all weightlifters in the world are taking steroids (Wade, 1972). Other "power" events such as the rowing and throwing events also have a high percentage of competitors who have repeatedly taken anabolic steroids (Freed et al., 1975). This extensive misuse of the drugs has concerned researchers, because of the reports of toxicity related to long-term use of anabolic steroids. The risk that athletes might do themselves serious injury by taking anabolics has led researchers to attempt to determine the actual toxic limits and mode of action of the drugs. It is hoped that this will shed some light on the causes and extent of any hazardous side-effects.

There has been much controversy recently in the scientific community as to whether or not there is an actual anabolic action of these drugs in healthy individuals, such as

athletes. A number of workers have tried to test the effect in athletes, some using "clinical" doses and others using doses more typical of actual use (sometimes as high as twenty to one hundred times the recommended therapeutic dose). Some have concluded that the drugs help to increase strength and body weight, while others report no effect (Tahmindis, 1976; Freed et al., 1975; Ryan, 1977). Even two recent papers, measuring hormone levels and endocrine functions in order to measure physiological effects, reported conflicting results for several indicators (Hervey et al., 1976; Holma and Aldercreutz, 1976). This situation in the literature, along with the consideration of ethics in sports (which led to sports administration bodies banning the use of these drugs by athletes), has resulted in a dilemma for the individual athlete. Although the general trend today is still to take anabolic steroids, it has been suggested that more athletes should approach researchers to supervise their drug-taking and monitor physiological parameters in order to avoid any permanent damage resulting from drug use (editorial, Med. S. Aust., 1976).

3. Mode of Action

The mode of action of the anabolic steroids which might produce increased lean weight is, for the most part, unknown. Although a fair body of knowledge concerning metabolic effects has been collected over the years, very little is yet clearly explained by events at the cellular level.

After the initial work by Kolchakian, it was found that paralleling the retention of nitrogen was a decrease in the excretion of calcium, potassium, phosphate, creatinine, and water (Camarino and Sciaky, 1975). More recent studies showed that during the administration of anabolic steroids plasma testosterone, aldosterone, and some pituitary hormone levels were lowered (Kilshaw et al., 1975; Holma and Aldercreutz, 1976). The retention of water could be explained by the increased binding capacities of newly formed proteins, although water retention has also been observed after high doses of other steroids. An explanation of the observed reduced hormone levels could be inhibition of synthesis related to high levels of pseudo-androgen in the system, which may be the cause of such side-effects as lowered sex libido. The retention of calcium, potassium, and phosphate ions may result from either increased protein production or mere water retention in the tissues. It has been suggested that an anabolic effect would involve one or more of the following factors:

(a) a higher rate of protein synthesis;

(b) a lower rate of protein catabolism;

(c) a lower rate of amino acid catabolism.

Bartlett (1953) showed that during increased nitrogen retention, both an elevated rate of protein synthesis and a lowered rate of amino acid breakdown to urea could be observed.

In a paper by Mayer and Rosen (1975), a theory was postulated that incorporates all three aspects, two of them being secondary to the initial effect. They proposed that the anabolic effects of all androgens is mediated through the interaction of these hormones with the glucocorticoid receptors in the muscle. Their proposal was based on three observations:

(a) that treatment with glucocorticoids produces a severe catabolic response in muscles, and androgens can reverse this;

(b) that specific binding proteins have been shown to exist in rat skeletal muscle for glucocorticoids, but have not been found for androgens; and

(c) that androgens can compete with the glucocorticoids for binding to the specific glucocorticoid receptors in the muscle tissue.

Thus the androgens (and anabolics) might interfere with and block the catabolic response of tissue to circulating glucocorticoids.

Another, similar hypothesis proposes that anabolic steroids interfere with glucocorticoids by inducing enzymes in the liver to increase the metabolism of glucocorticoids to inactive metabolites (Colby and Kitay, 1972). This proposal is supported by other work which indicates that there may be a mechanism by which anabolic steroids enhance the activity of drug metabolizing enzymes in the liver (Gillette, 1963;

Selye, 1970).

Although these theories begin to elucidate a mode of action for the effects of anabolic steroids, some researchers still regard the existence of a purely anabolic action to be an open question. This is based on publications which report finding no "permanent" weight gain in animals after drug treatment (Hervey and Huchinson, 1973).

4. Undesirable Effects

Most of the undesirable effects of taking anabolic steroids result either from the residual androgenic activity of the drugs or some type of liver toxicity. Whereas the effects of the first variety are relatively minor and usually reversible by termination of the drug administration, the effects of anabolics on the liver are viewed as potentially more serious.

Since it has been impossible to completely separate anabolic from androgenic activity, a number of side-effects resulting from residual androgenicity have been documented. Some of the effects that have been associated with anabolic steroids are acne, flushing, decreased libido, testicular atrophy, salt and water retention, and hypertension (Percy, 1977). Other effects such as lowered sperm count and temporary sterility have also been reported (Cooper and Craig, 1975). In women, side-effects resulting from an increase in androgenic activity in the body have also been seen. Such effects as virilization (skin coarsening, facial hair growth,

voice deepening), clitoral enlargement, and interference with the menstrual cycle are all effects to be expected when taking anabolic steroids. All of the effects caused by the androgenicity of the anabolic steroids in women also seem to be reversible on termination of the administration of the drugs.

With increasing frequency, anabolic steroids have been implicated in various forms of liver toxicity. Although it is only the 17α-alkylated steroids that show this characteristic, it is these drugs which are being most widely used, as they are orally active. Hospital surveys (Westaby et al., 1977), review articles (Johnson, 1975; Scheuer and Lehmann, 1977), and case studies (Young et al., 1977) all mention anabolic steroids as causes of cholestatic jaundice, peliosis hepatitis, leukemia, and hepatocellular carcinoma. Complications with anticoagulant therapy have also been reported (Howard et al., 1977). Liver toxicity is usually seen after prolonged use of anabolic steroids, so it is rarely reported in conjunction with athletes, since athletes usually stop taking steroids after noticing the milder, more reversible symptoms.

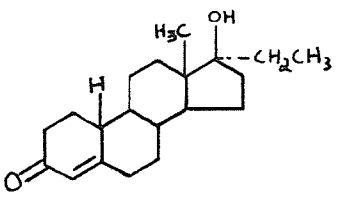
B. Ethylestrenol

Ethylestrenol (17α-ethyl-4-estren-17β-ol) was first synthesized in 1959 (DeWinter et al., 1959). A preliminary study (Overbeek et al., 1962), using the levator ani assay, stated that ethylestrenol possessed high myotropic (anabolic) and low androgenic properties that would make it useful

as a potent anabolic steroid. Another study (Ruchelman and Ford, 1963) demonstrated that there was a trend towards a positive nitrogen balance when ethylestrenol was given, and that ethylestrenol could reverse a negative nitrogen balance induced by dexamethasone (a glucocorticoid). Thus ethylestrenol came to be used medically for conditions characterized by wastage of protein and abused non-medically to improve muscle development and athletic performance.

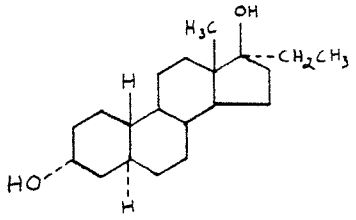
During the last ten years, very little research has been done with ethylestrenol. Only about forty-five research articles have been published in which ethylestrenol was studied. One-sixth of these deal with the use of ethylestrenol in the treatment of blood disorders, while three-quarters deal with the ability of ethylestrenol to induce hepatic microsomal enzymes. Most of this latter work was done at the University of Montreal, by a group of researchers headed by Selye and Tache. A recent review (Kourounakis *et al.*, 1977) summarized this work.

Only a few papers have been published that deal with the detection of ethylestrenol or its metabolites. Okada *et al.* (1969) reported the *in vitro* metabolism of ethylestrenol by rabbit liver slices to norethandrolone (17α-ethyl-17β-hydroxy-4-estren-3-one) and a more polar Δ^4-3 keto

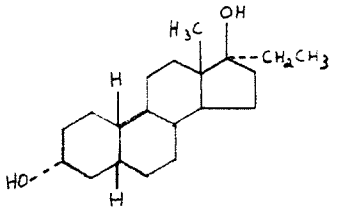


norethandrolone

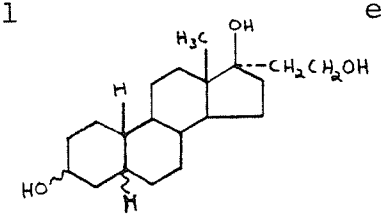
metabolite. Brooks and Middleditch (1971) reported on the suitability of gas-liquid chromatography/mass spectrometry for the detection of estrenols and their chloromethyl(dimethyl)silyl ethers. Hara and Mibe (1975) reported on the suitability of high pressure liquid chromatography in the detection of natural and synthetic steroid pharmaceuticals, including ethylestrenol. In vivo urinary metabolites from man and the marmoset monkey were reported by Ward et al. (1975, 1977) who found that ethylestrenol was metabolized to norethandrolone and its metabolites. These secondary metabolites were two tetrahydro metabolites (5α and 5β-17α-ethyl-estrane-3α, 17β-diol), a hydroxy-norethandrolone, a hydroxy-tetrahydro metabolite, and 17α-ethyl-5ξ-estrane-3ξ,17β,21-triol.



17α-ethyl-5α-estrane-3α,17β-diol



17α-ethyl-5β-estrane-3α,17β-diol



17α-ethyl-5ξ-estrane-3ξ,17β,21-triol

There is little available information concerning the disposition of ethylestrenol in human or any other animals. Aside from the small amount of metabolism work mentioned

above, there has been no research on the fate of ethylestrenol after it enters the body. It is not known what percentage is absorbed into the blood system, whether it is circulated throughout the body, and by which routes and how quickly it is excreted. Indeed, very little of this information is available for any of the anabolic steroids.

C. Aims of this Study

This study forms a part of a much larger research project, outlining the absorption, distribution, metabolism, and excretion of the anabolic steroids in the rat. Because the anabolic steroids are widely used and hepatotoxic, it is hoped that research into the disposition of the drugs will give some indications as to the causes of this toxicity. The aim of this study is to provide disposition data for ethylestrenol in the rat, utilizing tritium-labelled ethylestrenol as a radioactive tracer. This study is intended to provide information about:

(a) the percentage of drug absorbed after an oral dose;

(b) the distribution of the drug after an oral dose and any tissue localization;

(c) the time course and route of excretion of the drug and its metabolite(s) after oral, intramuscular, and intravenous dosing;

(d) the quantity of unchanged drug excreted and the quantities and identities of any metabolites;

(e) the identity of *in vitro* metabolites, and the differences

and/or similarities between *in vivo* and *in vitro* metabolism; (f) comparison of the above with other anabolic steroids.

METHODS

A. Chemicals

^{3}H-ethylestrenol [20,21-^{3}H-17α-ethyl-4-estren-17β-ol] and non-radioactive ethylestrenol were gifts of the Drug Metabolism and Development Department of Organon, N.V., Oss, Holland. Norethandrolone was a gift from G. D. Searle and Co., Chicago, Ill., U.S.A. All other chemicals were of reagent grade or better and most were purchased from Canadian Laboratory Supplies (Canlab), Fisher Scientific, or Sigma Chemical Co.

^{3}H-ethylestrenol (6.3 mCi/mg*) was stored as received in benzene solution. A working stock solution was prepared by evaporating approximately one-quarter of the solution to dryness and redissolving in 25 ml of 95% ethanol. This gave a solution of approximately 5.5$_{\mu}$Ci/100$_{\mu}$l) (12.2 x 10^{6} dpm/100$_{\mu}$l). Radiochemical purity of the working stock solution was determined by thin-layer chromatography followed by extraction of all spots and liquid scintillation counting of the extracts. Only one spot other than ^{3}H-ethylestrenol was found (at the origin) and accounted for 3% of the radioactivity. A gas-liquid chromatogram of this stock solution was also obtained and showed only one peak, corresponding to ^{3}H-ethylestrenol.

The chemical purity of the non-radioactive ethylestrenol was also determined by thin-layer and gas-liquid chromato-

---

*for a list of the symbols and abbreviations used, see Appendix.

graphy and showed only one spot on the thin-layer plate and one peak on the gas-liquid chromatogram.

B. Instruments and Equipment

The following is a list of the brand names and models of the instruments and equipment used:

liquid scintillation counter - Beckman, LS-9000

freeze-dryer - Virtis, Model 10-100

tissue blender - Brinkmann, Willems Polytron

carbon-hydrogen analyzer - Coleman, Model 33

gas-liquid chromatograph - Varian, 1840

rotary evaporator - Buchi, Rotavap-R

mass spectrometer - Finnigan Quadrupole, Model 1015

refrigerated centrifuge - International Equipment Co. (IEC), Model B-20.

C. Animals

The rats used were male, Sprague-Dawley strain, obtained from Bio Laboratories, St. Paul, Minn., U.S.A. The rats were 50-53 days old and had been fasted 18-24 hours at the time of dosing. The average weight of the rats used was 170±30 g.

D. Dosing

1. Oral

Oral doses were prepared by dissolving 30-60 mg of ethylestrenol in an aliquot of the ^{3}H-ethylestrenol stock solution (100 or 200 μl) in a 4 ml test tube. Another aliquot of the stock solution was taken at the same time

and diluted to 25 ml in order to determine the gross dose. The dose was administered by gavage using a Plastipak^{R}, 1 ml, disposable syringe and a curved, stainless steel feeding tube (8.0 cm, 16 gauge). Immediately after administration, the syringe, feeding tube, and the test tube were washed with several millilitres of 95% ethanol and the combined washings diluted with toluene to 5 ml in order to determine the amount of the prepared dose which was not administered. This enabled a calculation of the net dose from the gross dose.

2. Intramuscular

Intramuscular doses were prepared by dissolving 8 mg of ethylestrenol in a 100 µl aliquot of the ^{3}H-ethylestrenol stock solution in a 4 ml test tube. The rats were anaesthetized with ether and the muscles surrounding the femoral vein exposed. The total dose was injected in one lot into the muscle surrounding the vein using a Hamilton #710 syringe (100 µl). This procedure caused some bleeding and no estimate of the exact net dose was made at the time of dosing. The wound was closed using 11x2mm wound clips.

3. Intravenous

One rat was dosed with a 100 µl aliquot of a solution consisting of 32 mg of ethylestrenol dissolved in 400 µl of the ^{3}H-ethylestrenol stock solution and the others were dosed with 100 µl of the ^{3}H-ethylestrenol stock solution (0.87 µg). The rats were anaesthetized with ether and the femoral vein exposed. The desired volume of dosing solu-

tion was injected as accurately as possible from a Plasti-pak^{R}, 1 ml, disposable tuberculin syringe with a 3/8 inch, 26 gauge needle. The reproducibility of this injection procedure was uncertain and no estimate of the exact net dose was made at the time of dosing. The wound was closed using wound clips.

E. Excreta Collection

After dosing, the rats were placed in stainless-steel metabolism cages and allowed food (Purina Laboratory Chow) and water ad libitum. The metabolism cages effected separation of the urine from faeces. The separated excreta were collected daily and the faeces immediately placed in a freezer (-20°C). The urine was assayed for radioactivity and stored frozen. The cages were rinsed at the time of collection with approximately 25 ml of distilled water and the rinse also assayed for radioactivity. The values obtained for the rinse were added to the urine values.

F. Liquid Scintillation Counting

Two liquid scintillation cocktails were used. The first was Scintiverse^{R} (Fisher), a ready-made cocktail, and the second was prepared by dissolving 14.4 g of butyl-PBD (Sigma) in one litre of redistilled toluene. Both counting efficiencies and cpm data were determined and converted to dpm data automatically by the liquid scintillation counter. All sample were counted to a 1% (2S) statistical error (40,000 counts) or 20 minutes, whichever came first. Stan-

dard volume polyethylene vials (Fisher) were used and background levels were 20±2 cpm.

1. Urine

Urine samples were prepared for counting by diluting the daily output to a known volume and a 1 ml aliquot was then counted in 10 ml of Scintiverse. Efficiencies ranged between 20% and 35%. Metabolism cage washings were treated in the same manner, efficiencies being about 5% higher.

2. Faeces

Total daily samples of faeces were freeze-dried (72 h), weighed, and then pulverized. Approximately 100 mg of the powder were then accurately weighed and combusted in a modified carbon-hydrogen analyzer. Tritiated water resulting from the combustion was trapped in a U-tube which had been placed in an acetone-dry ice bath. The water was then rinsed into a scintillation vial with 3-4 ml of distilled methanol and counted in 100 ml of the butyl-PBD cocktail. Counting efficiencies for this mixture were about 32±1%.

3. Tissues

Organs and tissue samples were weighed wet, homogenized (Polytron homogenizer) in 10-30 ml of distilled water, and freeze-dried (48 h). The dried samples were then weighed and up to 100 mg taken for combustion as described earlier (page 22) for faeces samples.

4. Solutions and Extracts

Radioactivity in other aqueous solutions and extracts

was assayed by diluting to a known volume and counting a small aliquot (100-1000 µl) in 10 ml of Scintiverse. Organic solutions were counted similarly in the butyl-PBD cocktail.

G. Chromatography

1. Gas-liquid Chromatography (G-LC)

G-LC was performed on a Varian 1840 gas-liquid chromatograph equipped with a flame ionization detector and an effluent splitter (1:10) for sample collection. The column used was a glass, 1.8 m x 2 mm internal diameter, 3% OV-17 on Chromosorb G-HP (100/120 mesh). Gas flow rates were 30 ml/min for hydrogen and nitrogen (carrier gas) and 300 ml/min for air. The injector temperature was 330°C and the detector and collector temperatures were 350°C. Two temperature programmes were used for the column oven temperature:

(a) 200°C for 6 min, rising at 6°C/min to 320°C, and remaining at 300°C for 4 min,

(b) 220°C for 6 min, rising at 6°C/min to 320°C, and remaining at 320°C for 7 min.

The second temperature programme was used for the *in vitro* metabolism study only.

Samples for G-LC were prepared by first evaporating extracts, etc. to dryness and redissolving in 100-500µl of anhydrous pyridine or anhydrous dimethylformamide. Some of this mixture (2-5 µl) was then injected and a 100 µl portion reserved for derivatisation. These portions were derivatized by adding 100 µl of Tri-Sil Concentrate (Pierce) and

heating at approximately 50°C for 30 minutes. Part of the derivatized portion (2-5 μl) was then injected into the gas-liquid chromatograph.

Peaks of interest were collected via the effluent splitter in 3 cm pieces of glass capilliary tubing (2 mm internal diameter) placed over the collector outlet. The collected material was rinsed out of the tubing using dichloromethane and either assayed for radioactivity or transferred into a glass, mass spectrometry sample container and the solvent evaporated. Mass spectra (70 eV) were obtained from a Finnigan Quadrupole mass spectrometer with the dried sample placed in the solid sample probe.

2. Thin-layer Chromatography (T-LC)

T-LC was performed in 10% v/v or 20% v/v ethyl acetate in benzene on silica gel GF-254 plates, 250 μm thick for analytical work and 1000 μm for preparatory work. Two methods were used for visualization of the spots:

(a) the plates were sprayed with a 3% v/v concentrated sulphuric acid in methanol solution and heated for 3 minutes at 100°C. The plates were viewed under both white and ultraviolet lights.

(b) the plates were sprayed with a saturated solution of antimony trichloride in chloroform and viewed under both white and ultraviolet lights.

3. Adsorption Chromatography (Amberlite XAD-2)

Amberlite XAD-2 resin (Mallinckrodt) is a non-ionic

polymeric adsorbent for use with aqueous and organic solvents. It was used to separate steroids from aqueous samples (urine, etc.). A 16x5 cm column was prepared by placing a slurry of the resin in water into a 250 ml cylindrical separatory flask. The aqueous sample was siphoned through the column, followed by distilled water until the eluate was colorless (approx. 250 ml). Methanol was then passed through the column until the eluate was again colorless. All eluates were collected in 25 ml fractions and 1 ml of each fraction was assayed for radioactivity. All fractions that contained more than 1% of the amount of radioactivity found in the most active fraction were bulked and evaporated to dryness *in vacuo* (rotary evaporator). The aqueous fractions were discarded as they contained no radioactivity. The column was cleaned by washing successively with 1% v/v hydrochloric acid (200 ml), methanol (500 ml), and distilled water (one litre).

H. Extraction of Metabolites

1. Urine

The urine from orally dosed or control rats was passed through an Amberlite XAD-2 column (page 24) and the steroid fraction obtained. This residue was resuspended in 50 ml of 0.1 M phosphate buffer (pH 7.4) and extracted with dichloromethane (2x30 ml). The organic layers were bulked, diluted to 100 ml, assayed for radioactivity, dried over anhydrous sodium sulphate, filtered, and the solvent evaporated *in*

vacuo. This constituted the unconjugated (free) fraction from urine.

Approximately 100,000 units of β-glucuronidase (Type 1, bacterial, Sigma) and 3 drops of chloroform were then added to the aqueous phase and the mixture incubated for 48 hours at 37°C. After incubation the mixture was extracted with dichloromethane (3x30 ml). The organic layers were bulked, diluted to 100 ml, assayed for radioactivity, dried over anhydrous sodium sulphate, filtered, and the solvent evaporated in vacuo. This constituted the glucuronide aglycone fraction from urine.

The aqueous fraction from the above step was saturated with sodium chloride and centrifuged to remove precipitated proteins (20 min at 10,000 rpm, 20°C). The supernatant was adjusted to pH 0.5 by dropwise addition of concentrated hydrochloric acid and incubated for 24 hours at 50°C. After cooling to room temperature, the solution was adjusted to pH 7.0 with 20% w/v sodium hydroxide solution and extracted with dichloromethane (3x30 ml). The extracts were bulked and treated as above. This constituted the mild hydrolysis fraction from urine.

Concentrated hydrochloric acid (10 ml) was added to the aqueous phase, which was left at room temperature for two weeks. The solution was filtered and extracted with dichloromethane (2x25 ml) and ethyl acetate (2x25 ml). The two dichloromethane extracts were bulked and the two ethylacetate

extracts were bulked. These fractions were assayed, dried, and the solvent evaporated to constitute the strong hydrolysis fractions A and B from urine.

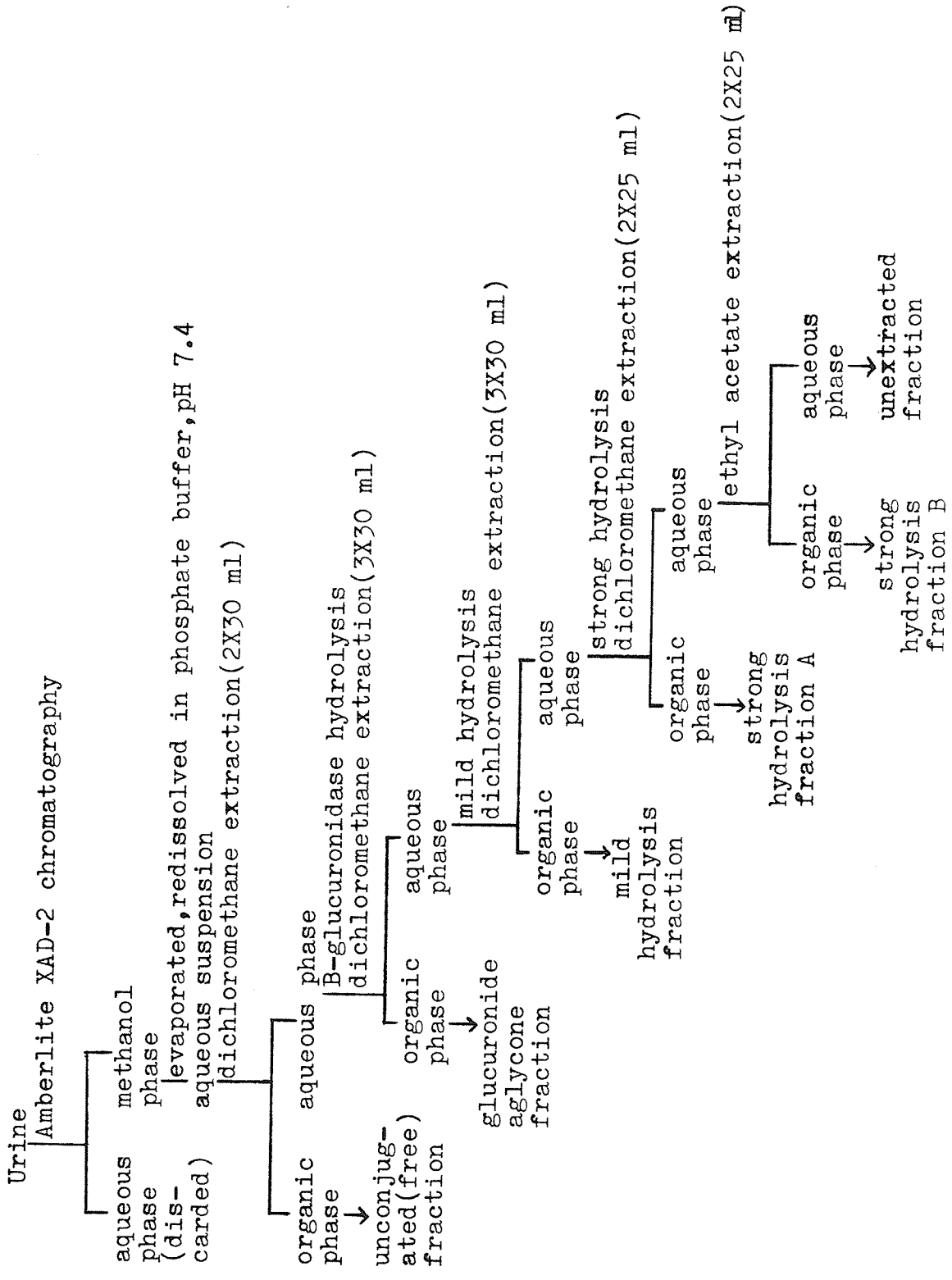
The residual aqueous solution from above was assayed for radioactivity to determine the amount of unextracted radioactivity remaining after the above procedures. A flow diagram (see Figure 2) summarizes the urinary metabolites extraction procedures.

2. Faeces

Faeces from orally dosed or control rats were freeze-dried (72h), pulverized, and assayed for radioactivity (see page 22). The powder was extracted with chloroform in a soxhlet extractor for 24 hours. The chloroform extract was assayed for radioactivity and the solvent was driven off by heating under a stream of nitrogen. This constituted the unconjugated (free) fraction from faeces. This fraction was partitioned between hexane and methanol (200 ml each) to give two fractions, which were assayed and evaporated in vacuo. These fractions constituted the hexane (free) and methanol (free) fractions from faeces.

The faeces residue after the chloroform extraction was allowed to dry (air, room temperature) followed by extraction with methanol in a soxhlet extractor (24h). The methanol extract was evaporated in vacuo and the residue resuspended in 100 ml of distilled water. The mixture was centrifuged to remove undissolved material which was redissolved in me-

Figure 2. A flow diagram of the extraction procedures for urinary metabolites of ethylestrenol



thanol and assayed for radioactivity. The supernatant was also assayed, then passed through an Amberlite XAD-2 column to obtain the conjugated steroid fraction.

This fraction was treated with β-glucuronidase and subjected to the mild hydrolysis procedure as described for urine (see page 26). This produced the glucuronide aglycone and mild hydrolysis fractions from faeces. A flow diagram (Figure 3) summarizes the faecal metabolites extraction procedures.

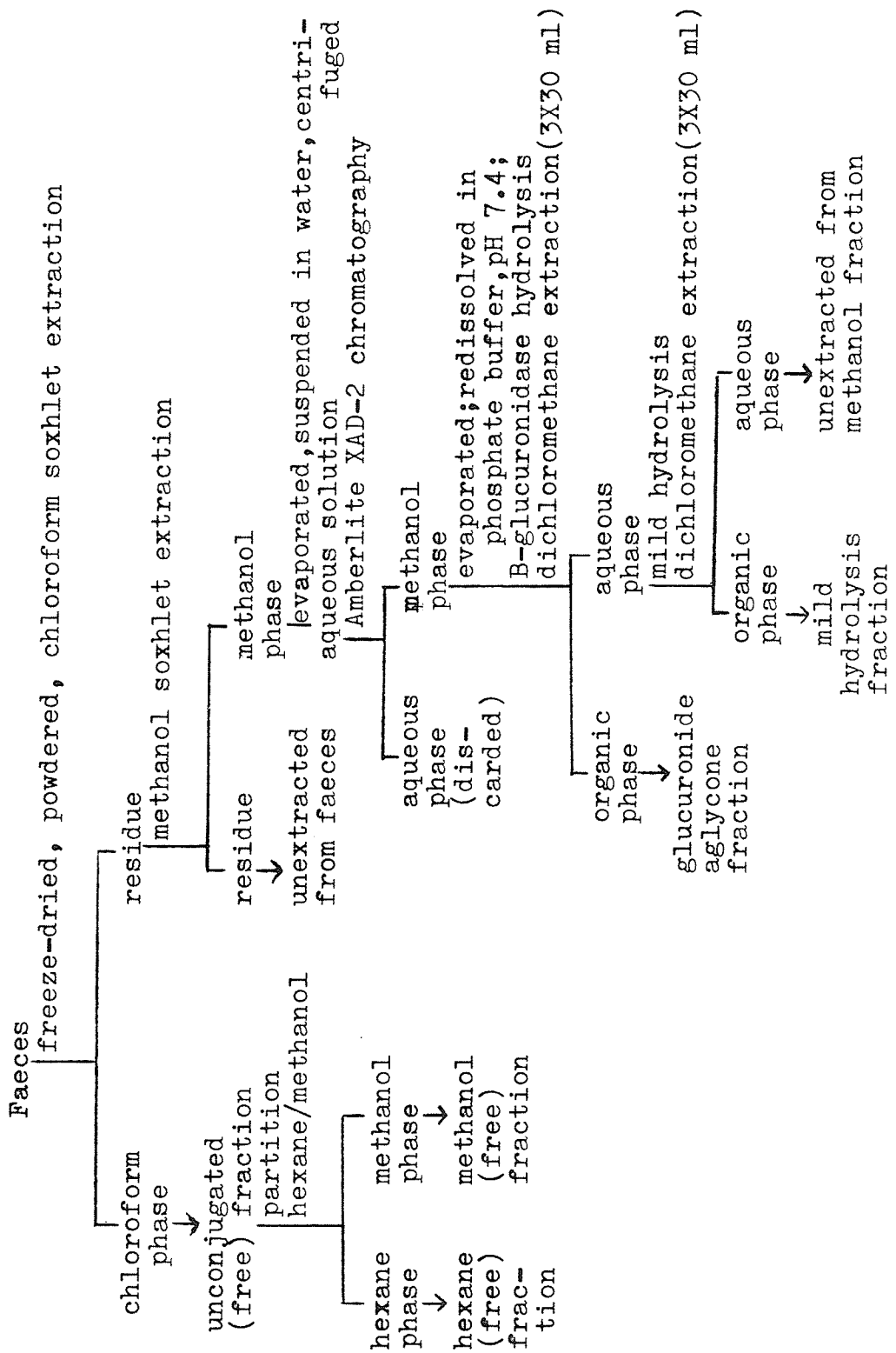
I. Liver Homogenate Incubations

Livers from four freshly killed rats (cranial crush method) were excised and weighed (33 g total). The livers were homogenized in three volumes (100 ml) of 0.25 M sucrose solution, then centrifuged for 30 minutes at 14,000 rpm (25,000 x G) in a refrigerated centrifuge maintained at 4°C. The supernatant was decanted and used in the incubation mixtures. Two media were used. For each medium, four incubations were prepared containing ^{3}H-ethylestrenol and one was prepared containing no ethylestrenol to serve as a control (blank).

1. NADPH-Regenerating Medium

Liver homogenate supernatant (10 ml) was added to a 50 ml Erlenmeyer flask containing 20 mg of magnesium chloride and 6 mg of ^{3}H-ethylestrenol (6×10^{5} dpm) in 0.3 ml of 95% ethanol. To this mixture was added a solution of 6 mg NADP (Sigma) 11.2 mg glucose-6-phosphate (Sigma), and 36.4

Figure 3. A flow diagram of the extraction procedures for faecal metabolites of ethylestrenol.



mg nicotinamide in 6 ml of 0.1 M phosphate buffer (pH 7.4). The addition of 80 units of glucose-6-phosphate dehydrogenase (Sigma) in 0.1 ml distilled water completed the incubation medium.

2. NADPH-Non-regenerating Medium

Liver homogenate supernatant (10 ml) was added to a 50 ml Erlenmeyer flask containing 4 mg of ^{3}H-ethylestrenol ($4x10^{5}$ dpm) in 0.2 ml of 95% ethanol, 0.4 ml of a 100 mM magnesium chloride solution, and 0.4 ml of a 1 M nicotinamide solution. The addition of 100 mg of NADPH in 0.4 ml of Trizma Saline buffer (Sigma, 0.56% NcCl in 0.05 M Tris, pH 7.4 at 25°C) completed the incubation medium.

3. Incubation and Extraction

The ten incubation mixtures were placed in a water-bath shaker and incubated for 2 hours at 37°C under an oxygen atmosphere. After incubation the four regenerating mixtures with steroid were bulked together and the four non-regenerating mixtures with steroid were bulked together. These large mixtures were then extracted with dichloromethane (2x40 ml) and ethyl acetate (40 ml). The two bulked extracts were washed with 40 ml of distilled water, then 40 ml of saturated sodium chloride solution. The extracts were then dried over anhydrous sodium sulphate, filtered, and the solvents evaporated *in vacuo*. The blank incubations were treated similarly except that 10 ml lots of each solvent were used instead of 40 ml lots, as the blanks consisted of only one incubation mixture each.

RESULTS

A. Absorption

In order to determine the amount of ethylestrenol absorbed into general circulation after an oral dose, a bile ligation experiment was conducted. The common bile ducts on two rats were ligated under ether anaesthesia. After regaining consciousness, the rats were allowed to recover for approximately fifteen minutes, then dosed orally with approximately 40 mg of ^{3}H-ethylestrenol.

BL1 (bile ligated): weight - 185 g
net dose - 39.7 mg
net activity - 10.02×10^{6} dpm

BL2: weight - 184 g
net dose - 40.4 mg
net activity - 10.43×10^{6} dpm

The dosed rats were placed in metabolism cages and urine and faeces were collected. BL2 died after 3.5 days and BL1 was sacrificed after 7 days. The results of this experiment are shown in Table 2.

Table 2. Recovery of radioactivity from bile duct ligated rats after an oral dose of ^{3}H-ethylestrenol.

	BL1	BL2
% of dose recovered in urine	16.43	23.36
% of dose recovered in faeces	66.83	46.40
total % of dose recovered	83.26	69.76
% of recovered activity in urine	19.73	33.49
% of recovered activity in faeces	80.27	66.51

B. Distribution

Two distribution experiments were performed in order to follow the distribution of ^{3}H-ethylestrenol in the rat after an oral dose. The first was a short-term study (4-18h) and the second was a long-term study (1-5 days).

1. Short Term

Three rats were orally dosed with 45-50 mg of ^{3}H-ethylestrenol and sacrificed, one after 4h, one after 8h, and one after 18h. Organs and tissue samples were removed and assayed for radioactivity.

SD1 (short distribution): weight - 150 g
net dose - 45.8 mg
net activity - 21.92×10^{6} dpm
dosed - 10 a.m.
sacrificed - 2 p.m.

SD2: weight - 166 g
net dose - 48.4 mg
net activity - 22.59×10^{6} dpm
dosed - 10 a.m.
sacrificed - 6 p.m.

SD3: weight - 136 g
net dose - 45.4 mg
net activity - 20.98×10^{6} dpm
dosed - 4 p.m.
sacrificed - 10 a.m. next day

The results of this experiment are shown in Table 3.

Table 3. Short term distribution of radioactivity in the rat after an oral dose of ^{3}H-ethylestrenol.

Tissue or Organ	Wet Weight (g)	% of dose found			specific activity of dried tissue (dpm/mg)		
		SD1 (4h)	SD2 (8h)	SD3 (18h)	SD1 (4h)	SD2 (8h)	SD3 (18h)
stomach	1.5-3	77.46	40.97	7.70	-	-	-
small intestine	4.8-6	13.50	25.44	32.51	-	-	-
caecum	1.7-3.1	0.13	12.08	13.34	-	-	-
large intestine	1-1.1	0.06	0.44	2.10	-	-	-
liver	7-9	0.81	1.86	1.79	82.6	164.0	169.7
kidneys	1.3-1.8	0.31	0.42	0.18	174.0	211.7	111.7
spleen	0.3-0.5	0.03	0.04	0.05	66.5	82.8	99.0
heart	0.7-1	a.	0.06	0.06	a.	82.1	82.8
lungs	1-1.5	0.04	0.10	0.10	42.8	85.1	96.7
testes	2-2.5	0.09	0.07	0.09	64.4	47.5	57.8
seminal vesicles	0.2-0.3	0.01	0.01	0.01	100.7	61.6	38.2
brain	1.4-1.6	0.03	0.04	0.06	20.6	29.3	36.7
eyes	0.2-0.3	0.01	0.01	0.01	40.5	38.6	56.5
hind leg muscles	4-5.2	0.07	0.11	0.16	14.5	19.1	29.5
peritoneum	3.2-5.5	0.07	0.14	0.09	14.4	25.1	24.9
urine	-	1.02	1.44	5.36	-	-	-
faeces	-	b.	0.01	13.89	-	-	-

a. sample lost
b. no sample

2. Long Term

Three rats were orally dosed with approximately 8 µg of ^{3}H-ethylestrenol and sacrificed, one after one day, one after 2 days, and one after 5 days. Organs, tissue samples, and excreta were removed and assayed for radioactivity.

LD1 (long distribution): weight - 134 g
net dose - 7.68 µg
net activity - 106×10^{6} dpm
sacrificed after one day

LD2: weight - 146 g
net dose - 8.05 µg
net activity - 112×10^{6} dpm
sacrificed after two days

LD3: weight - 128 g
net dose - 8.16 µg
net activity - 113×10^{6} dpm
sacrificed after five days

The results of this experiment are shown in Table 4.

C. Excretion

Three experiments were performed in order to follow the time course of excretion of ^{3}H-ethylestrenol and to determine the major route of excretion.

1. Oral Dose

Two rats were dosed orally with 56-57 mg of ^{3}H-ethylestrenol and placed in metabolism cages. Urine and faeces were collected for 10 days and assayed for radioactivity.

Table 4. Long term distribution of radioactivity in the rat after an oral dose of ^{3}H-ethylestrenol.

Tissue or Organ	Wet Weight (g)	% of dose found			specific activities of dried tissues (dpm/mg)		
		LD1 (1d)	LD2 (2d)	LD3 (5d)	LD1 (1d)	LD2 (2d)	LD3 (5d)
stomach	5-8	11.10	0.01	a.	-	-	-
small intestine	8-10	13.38	3.45	0.06	-	-	-
caecum	4.5-5.5	11.37	1.76	0.05	-	-	-
large intestine	2.5-4	2.85	1.13	0.03	-	-	-
liver	8-9	1.43	0.41	0.10	580.4	179.1	47.2
kidneys	1.7-1.9	0.12	0.03	0.01	283.1	76.3	23.6
spleen	0.5-0.7	0.01	a.	a.	82.2	21.8	12.1
heart	0.6-1	0.01	a.	a.	76.8	20.8	9.0
lungs	1.6-2	0.05	0.01	a.	121.7	30.6	11.6
testes	1.8-2.2	0.03	0.01	a.	95.1	22.5	8.3
seminal vesicles	0.2-0.3	a.	a.	a.	121.4	31.0	15.1
brain	1.5-2.2	0.02	a.	a.	66.5	9.4	4.9
eyes	0.5	a.	a.	a.	42.0	27.7	5.7
hind leg muscles	3.5-5	0.07	0.01	a.	66.9	13.1	5.9
peritoneum	2-2.5	0.06	0.01	a.	118.8	23.0	5.6
skin	2.5-4	0.12	0.05	0.01	117.7	38.6	10.2
urine	-	13.47	19.48	20.62	-	-	-
faeces	-	35.52	57.67	64.73	-	-	-
averages ± S.D.[b]	-	-	-	-	90.9±26.9	23.8±8.2	8.9±3.2
total recovered		89.61	84.03	85.61	-	-	-

a. <0.01%
b. not including liver or kidneys

OE1 (oral excretion): net dose - 56.2 mg

net activity - 12.06×10^6 dpm

OE2: net dose - 56.5 mg

net activity - 12.14×10^6 dpm

The results of this experiment are shown in Tables 5 and 6 and Figures 4 and 5.

2. Intramuscular Dose

Two rats were dosed intramuscularly with 8.3 mg of ^{3}H-ethylestrenol and placed in metabolism cages. Urine and faeces, collected daily for 21 days, then every two days for 14 more days, were assayed for radioactivity.

ME1 (intramuscular excretion): weight - 325 g

dose - 8.3 mg

activity - 11.03×10^6 dpm

ME2: weight - 320 g

dose - 8.3 mg

activity - 11.03×10^6 dpm

The results of this experiment are shown in Tables 7 and 8 and Figures 6 and 7.

3. Intravenous Dose

One rat was dosed with 8 mg of ^{3}H-ethylestrenol and three rats were dosed with 0.87 µg of ^{3}H-ethylestrenol intravenously. Urine and faeces were collected for five days and assayed for radioactivity.

VE1 (intravenous excretion): weight - 215 g

dose - 8.0 mg

activity - 11.80×10^6 dpm

Table 5. Excretion of radioactivity from rats after an oral dose of 3H-ethylestrenol

	% of dose recovered									
	OE1 Urine		OE1 Faeces		OE1 Total	OE2 Urine		OE2 Faeces		OE2 Total
Day	Daily	Cumulative	Daily	Cumulative	Cumulative	Daily	Cumulative	Daily	Cumulative	Cumulative
1	12.39	-	50.11	-	62.50	9.83	-	43.55	-	53.38
2	3.76	16.15	24.21	74.32	90.47	2.87	12.70	29.23	72.78	85.48
3	1.32	17.47	4.89	79.21	96.68	1.02	13.72	6.68	79.46	93.18
4	0.46	17.93	2.45	81.66	99.59	0.61	14.33	1.84	81.30	95.63
5	0.47	18.40	0.76	82.42	100.82	0.32	14.65	0.83	82.13	96.78
6	0.42	18.82	0.51	82.93	101.75	0.28	14.93	0.35	82.48	97.41
7	0.28	19.10	0.17	83.10	102.20	0.20	15.13	0.13	82.61	97.74
8	0.22	19.32	0.10	83.20	102.52	0.13	15.26	0.13	82.74	98.00
9	0.15	19.47	0.13	83.33	102.80	0.14	15.40	0.12	82.86	98.26
10	0.12	19.59	0.10	83.43	103.02	0.11	15.51	0.04	82.90	98.41

Table 6. Excretion of radioactivity from rats after an oral dose of ^{3}H-ethylestrenol (OE1, OE2 averaged).

Day	% of dose			
	Urine Daily	Faeces Daily	Total Cumulative	Remaining[a]
1	11.11	46.83	57.94	42.88
2	3.32	26.72	87.98	12.74
3	1.17	5.79	94.89	5.83
4	0.54	2.15	97.61	3.11
5	0.40	0.80	98.80	1.92
6	0.35	0.43	99.58	1.14
7	0.24	0.15	99.97	0.75
8	0.18	0.12	100.26	0.46
9	0.15	0.13	100.53	0.19
10	0.12	0.07	100.72	-

[a]Remaining = Total Cumulative (∞)-Total Cumulative
Total Cumulative (∞) = 100.72% in this experiment

Figure 4. Cumulative excretion of radioactivity from rats after an oral dose of ^{3}H-ethylestrenol.

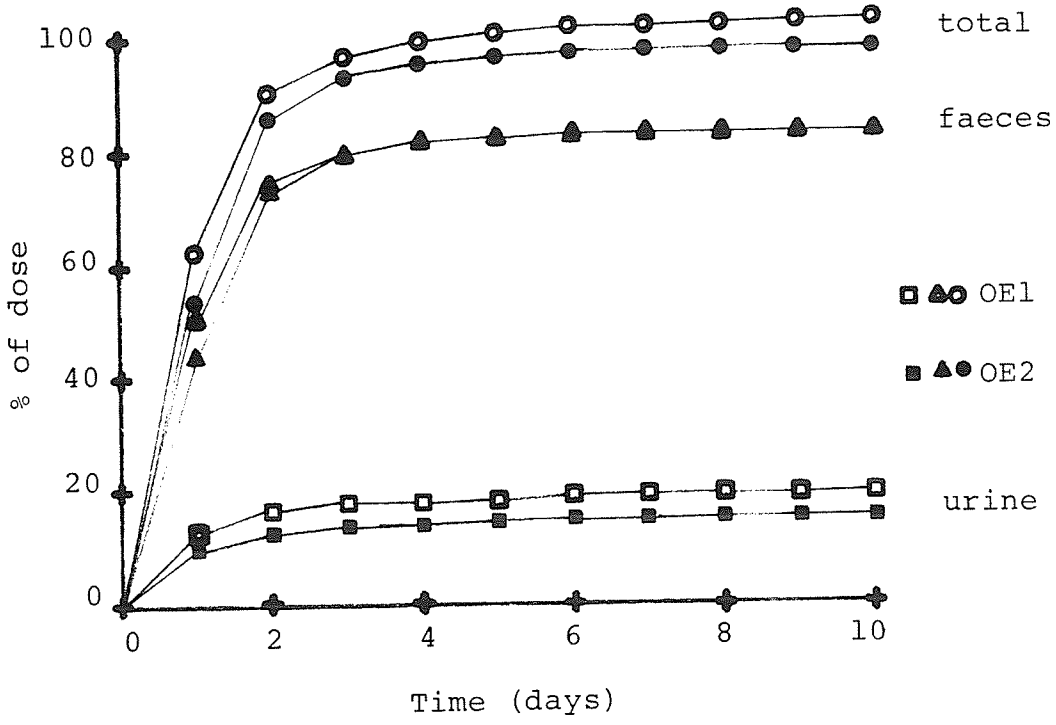


Figure 5. Percentage of radioactivity remaining in rats after an oral dose of 3H-ethylestrenol.

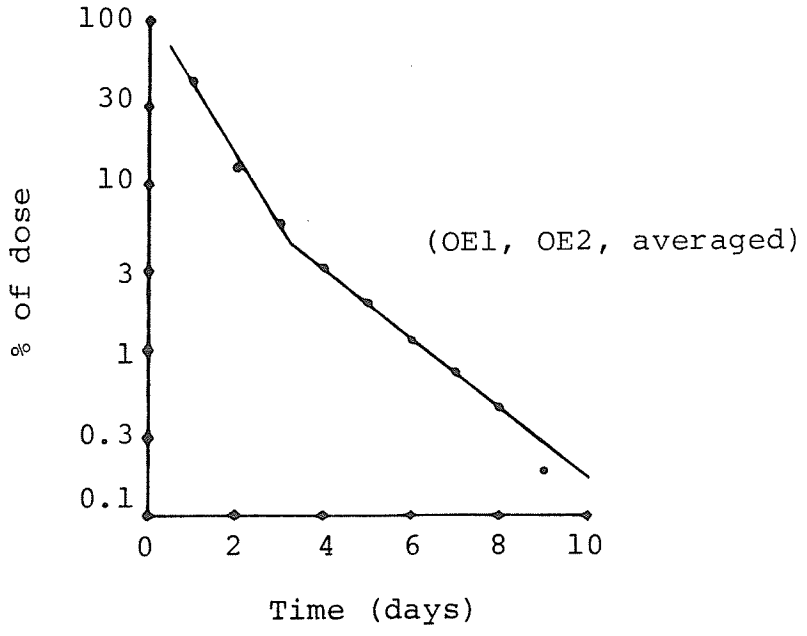


Table 7. Excretion of radioactivity from rats after an oral dose of ^{3}H-ethylestrenol

	% of dose recovered									
	ME1 Urine		ME1 Faeces		ME1 Total	ME2 Urine		ME2 Faeces		ME2 Total
Day	Daily	Cumulative	Daily	Cumulative	Cumulative	Daily	Cumulative	Daily	Cumulative	Cumulative
1	3.53		4.50		8.03	2.42		5.30		7.72
2-3	3.61	6.96	5.59	10.09	17.05	1.44	3.86	7.04	12.34	16.20
4	1.37	8.33	1.79	11.88	20.21	0.88	4.74	2.36	14.70	19.44
5	2.15	10.48	1.86	13.74	24.22	1.29	6.03	3.30	18.00	24.03
6	3.22	13.70	3.04	16.78	30.48	2.37	8.40	4.87	22.87	31.27
7	2.15	15.85	2.29	19.07	34.92	2.62	11.02	4.23	27.10	38.12
8	2.17	18.02	3.58	22.65	40.67	2.20	13.22	6.02	33.12	46.34
9-10	3.59	21.61	8.00	30.65	52.26	2.61	15.83	3.49	36.61	52.44
11	1.16	22.77	2.61	33.26	56.03	1.07	16.90	4.76	41.37	58.27
12	1.02	23.79	1.26	34.52	58.31	1.10	18.00	2.37	43.74	61.74
13	1.03	24.82	0.99	35.51	60.33	0.93	18.93	2.63	46.37	65.30
14	1.08	25.90	1.08	36.59	62.49	0.86	19.79	1.36	47.73	67.52
15	0.84	26.74	1.30	37.89	64.63	0.82	20.61	2.34	50.07	70.68
16	0.81	27.55	1.05	38.94	66.49	0.76	21.37	1.93	52.00	73.37
17	0.73	28.28	0.63	39.57	67.85	0.55	21.92	0.66	52.66	74.58
18	0.49	28.77	1.08	40.65	69.42	0.50	22.42	1.61	54.27	76.69
19	0.54	29.31	0.52	41.17	70.48	0.36	22.78	0.68	54.95	77.73
20	0.36	29.67	0.81	41.98	71.65	0.25	23.03	0.64	55.59	78.62
21	0.69	30.36	0.53	42.51	72.87	0.25	23.28	0.53	56.12	79.40
22-23	1.01	31.37	1.45	43.96	75.33	0.33	23.61	0.78	56.90	80.51
24-25	1.13	32.50	1.40	45.36	77.86	0.24	23.85	0.47	57.37	21.22
26-27	1.04	33.54	1.17	46.53	80.07	0.25	24.10	0.34	57.71	81.81
28-29	0.80	34.34	0.92	47.45	81.79	0.11	24.21	0.22	57.93	82.14
30-31	0.32	34.66	0.80	48.25	82.91	0.02	24.23	0.20	58.13	82.36
32-33	0.48	35.14	0.70	48.95	84.09	0.06	24.29	0.10	58.23	82.52
34-35	0.27	35.41	0.29	49.24	84.65	0.02	24.31	0.12	58.35	82.66

Table 8. Excretion of radioactivity from rats after an intramuscular dose of ^{3}H-ethylestrenol (ME1, ME2 averaged)

Day	% of dose			
	Urine Daily	Faeces Daily	Total Cumulative	Remaining[a] (TC∞-TC)
1	2.98	4.90	7.88	77.12
2-3	2.53	6.32	16.63	68.37
4	1.13	2.08	19.83	65.17
5	1.72	2.58	24.13	60.87
6	2.80	3.96	30.88	54.12
7	2.39	3.26	36.52	48.48
8	2.19	4.80	43.51	41.49
9-10	3.10	5.75	52.35	32.65
11	1.12	3.69	57.15	27.85
12	1.06	1.82	60.03	24.97
13	0.98	1.81	62.82	22.18
14	0.97	1.22	65.01	19.99
15	0.83	1.82	67.66	17.34
16	0.79	1.49	69.93	15.07
17	0.64	0.65	71.22	13.78
18	0.50	1.35	73.06	11.94
19	0.45	0.60	74.11	10.89
20	0.31	0.73	75.14	9.86
21	0.47	0.53	76.14	8.86
22-23	0.67	1.12	77.92	7.08
24-25	0.69	0.94	79.54	5.46
26-27	0.65	0.76	80.94	4.06
28-29	0.46	0.57	81.97	3.03
39-31	0.17	0.50	82.64	2.36
32-33	0.27	0.40	83.31	1.69
34-35	0.15	0.21	83.66	1.34

[a]Remaining = Total cumulative (∞)-Total cumulative
Total cumulative (∞) ≃ 85% in this experiment

Figure 6. Excretion of radioactivity from rats after an intramuscular dose of 3H-ethylestrenol.

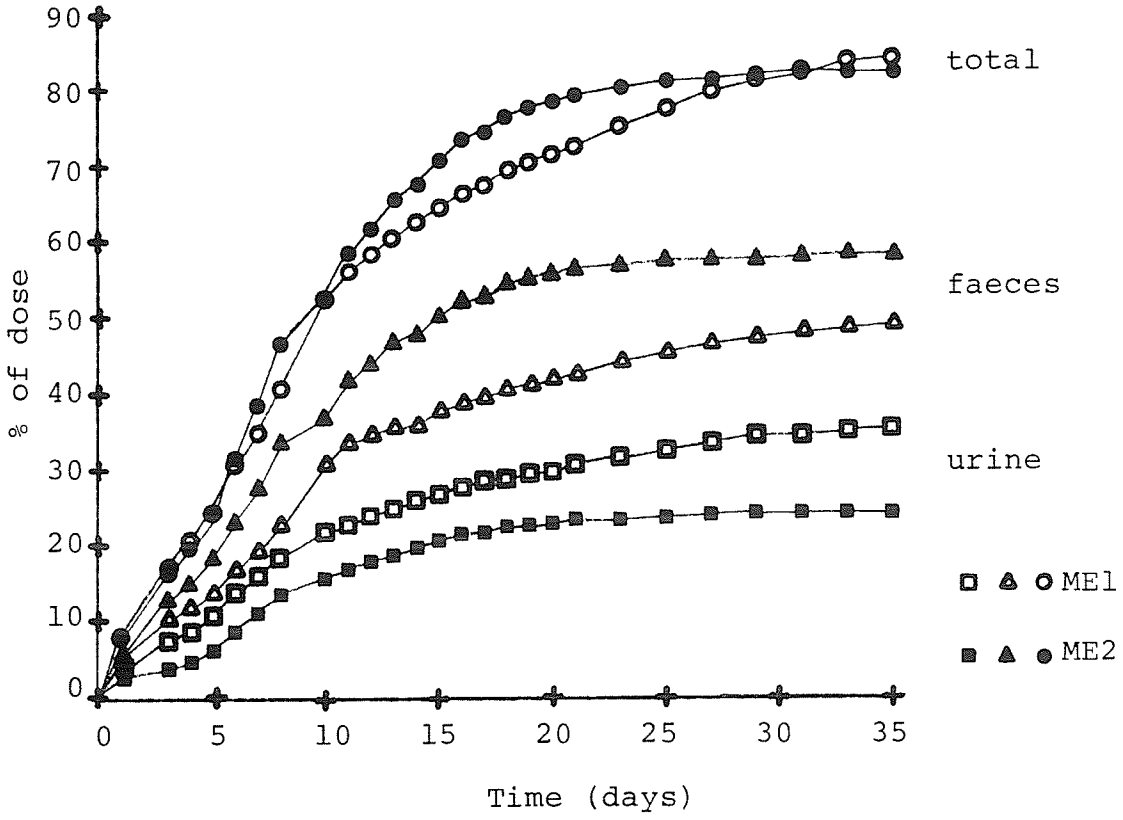
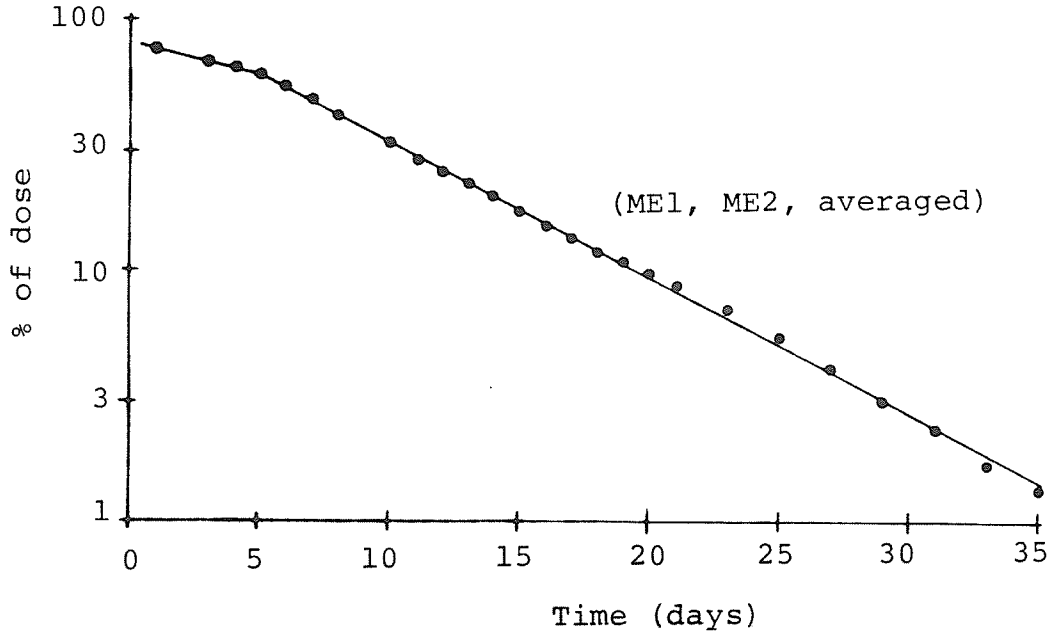


Figure 7. Percentage of radioactivity remaining in rats after an intramuscular dose of 3H-ethylestrenol.



VE2: weight - 193 g

dose - 0.87 µg

activity - 11.80×10^6 dpm

VE3: weight - 214 g

dose - 0.87 µg

activity - 11.80×10^6 dpm

VE4: weight - 144 g

dose - 0.87 µg

activity - 11.80×10^6 dpm

The results of this experiment are shown in Table 9 and Figure 8. The results presented are an average for all four rats as there was little difference between the percentages of radioactivity excreted after the 8 µg dose and the 0.87 µg dose.

D. Metabolism

1. Urine

Three rats were orally dosed with a total of 100 mg of ^{3}H-ethylestrenol. Urine and faeces were collected for 3 days, bulked, and assayed for radioactivity. The urine was examined for metabolites according to the procedures outlined in the Methods (p. 25).

UM1-3 (urine metabolites): total net dose - 99.92 mg

total net activity - 48.92×10^6 dpm

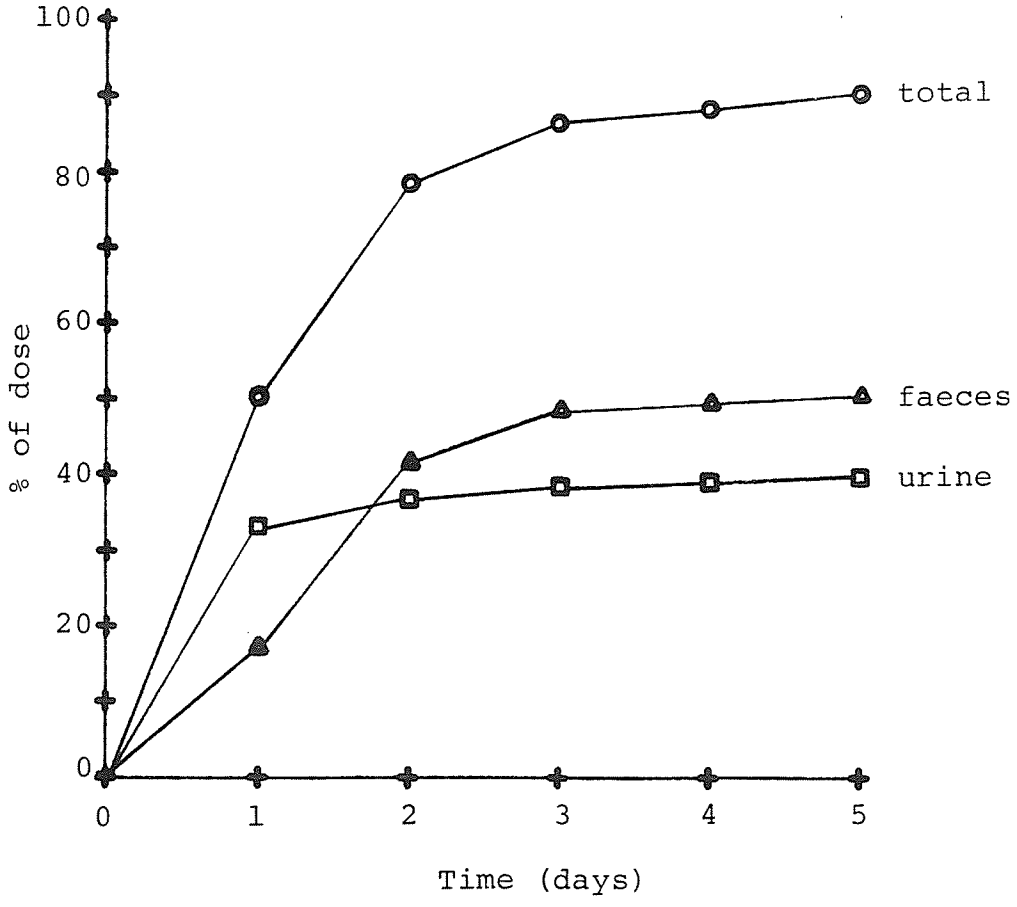
The results of the extractions are shown in Table 10.

Gas-liquid chromatography of the unconjugated fraction from urine showed only one drug related peak by comparison

Table 9. Excretion of radioactivity from four rats after intravenous doses of 3H-ethylestrenol.

	% of dose (averaged)				
	Urine		Faeces		Total
Day	Daily	Cumulative	Daily	Cumulative	Excretion
1	33.10	-	17.09	-	50.19
2	3.59	36.69	24.40	41.49	78.18
3	1.56	38.25	6.89	48.38	86.63
4	0.70	38.95	1.18	49.56	88.51
5	0.79	39.74	0.99	50.55	90.29

Figure 8. Excretion of radioactivity from rats after an intravenous dose of 3H-ethylestrenol (VE1-4 averaged).



with similar extracts from the faeces of rats fed only vehicle (ethanol). The peak had the same retention time as an authentic sample of ethylestrenol (see Figure 9) and co-chromatography produced only one G-LC peak. The G-LC scan of a derivatized portion of the unconjugated fraction also showed only one drug related peak, having the same retention time as derivatized ethylestrenol.

Table 10. Extraction of radioactivity from the urine of rats orally dosed with 3H-ethylestrenol.

Fraction	% of dose
total excretion (urine + faeces)	64.77
total urine	11.82
unconjugated (free)	1.80
glucuronide aglycone	0.65
mild hydrolysis	2.36
strong hydrolysis (A)	1.20
strong hydrolysis (B)	1.56
unextracted	4.24

2. Faeces

Four rats were orally dosed with approximately 40 mg of ^{3}H-ethylestrenol and urine and faeces collected for three days and assayed for radioactivity. The bulked faeces were then examined for metabolites according to the procedures outlined in the Methods (p. 27).

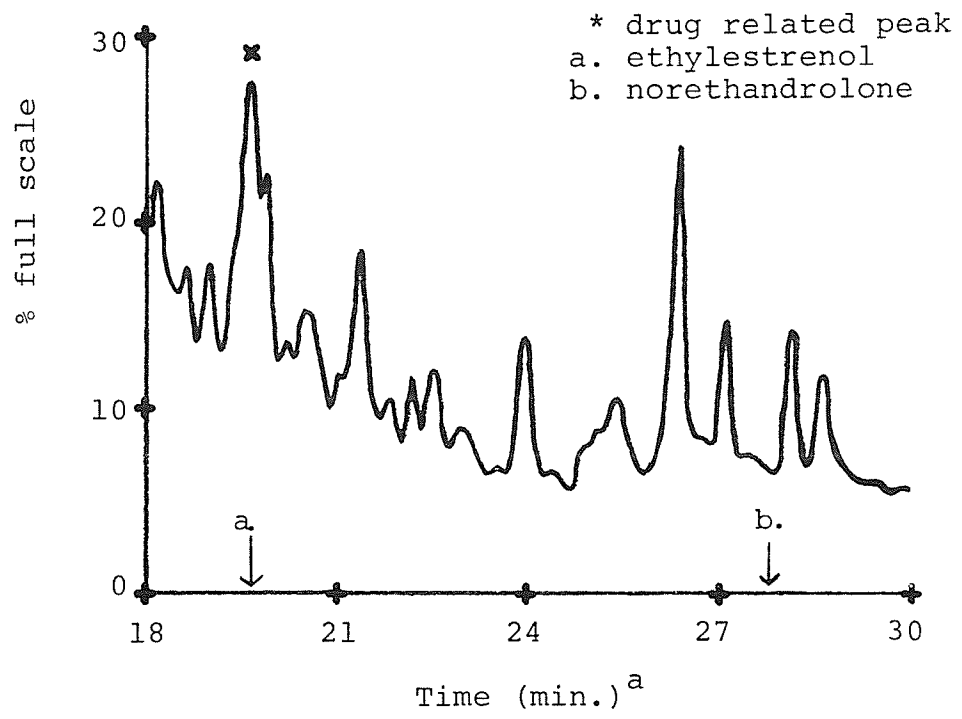
FM1 (faeces metabolites): weight - 144 g

net dose - 36.3 mg

net activity - 8.42×10^{6} dpm

FM2: weight - 152 g

Figure 9. Gas-liquid chromatogram of the unconjugated fraction from the urine of orally dosed rats.



[a]In order to obtain better separation of the steroid portion, the temperature programme for this chromatogram was 120°C initial temperature, rising at 6°/min to 320°C.

net dose - 43.1 mg

net activity - 9.96×10^6 dpm

FM3: weight - 148 g

net dose - 42.0 mg

net activity - 9.58×10^6 dpm

FM4: weight - 166 g

net dose - 40.5 mg

net activity - 9.59×10^6 dpm

The results of the extractions are shown in Table 11.

Table 11. Extraction of radioactivity from the faeces of rats orally dosed with 3H-ethylestrenol.

Fraction	% of dose
total excretion (urine + faeces)	78.23
total faeces	66.93
unconjugated (free)	31.82
hexane (free)	8.34
methanol (free)	23.48
glucuronide aglycone	3.16
mild hydrolysis	5.52
unextracted from faeces	12.18
unextracted from methanol	14.25

Gas-liquid chromatography of the hexane and methanol (free) fractions from faeces showed only one drug related peak in each fraction by comparison with similar extracts from the faeces of rats fed only vehicle. The peaks had the same retention times as an authentic sample of ethylestrenol and co-chromatography produced only one G-LC peak. The G-LC scan of derivatized portions of the hexane (free) and methanol (free) fractions from faeces also showed one

drug related peak in each fraction, which had the same retention times as a derivatized sample of authentic ethylestrenol.

A G-LC scan of the glucuronide aglycone fraction from faeces showed one drug related peak, but a G-LC scan of a derivatized portion of the same fraction showed three drug related peaks. A portion of the G-LC scan containing the three peaks is shown in Figure 10. The three peaks were collected via the streamsplitter and mass spectra obtained (see Figures 11, 12, and 13).

3. In vitro (liver)

G-LC scans of the extracts after incubation (see Methods, p. 31) showed one major metabolite peak. The peak height ratio of this peak to the ethylestrenol peak was 0.3:1 for the NADPH regenerating system and 2:1 for the non-regenerating system. The extract from the NADPH non-regenerating system was used for further identifications.

The extract was streaked onto a 20x20 cm thick-layer chromatography plate and run in 20% ethyl acetate in benzene. A 3 cm portion of the plate was sprayed with the antimony trichloride spray and the plate was observed under white, shortwave ultraviolet, and longwave ultraviolet lights. On this basis the plate was divided into fourteen bands. The results of the TLC are shown in Table 12.

The bands were scraped from the plate and eluted succesively with dichloromethane, methanol, and acetone. The three eluates were bulked and checked for radioactivity.

Figure 10. Gas-liquid chromatogram of a silylated portion of the glucuronide aglycone fraction of faeces from orally dosed rats.

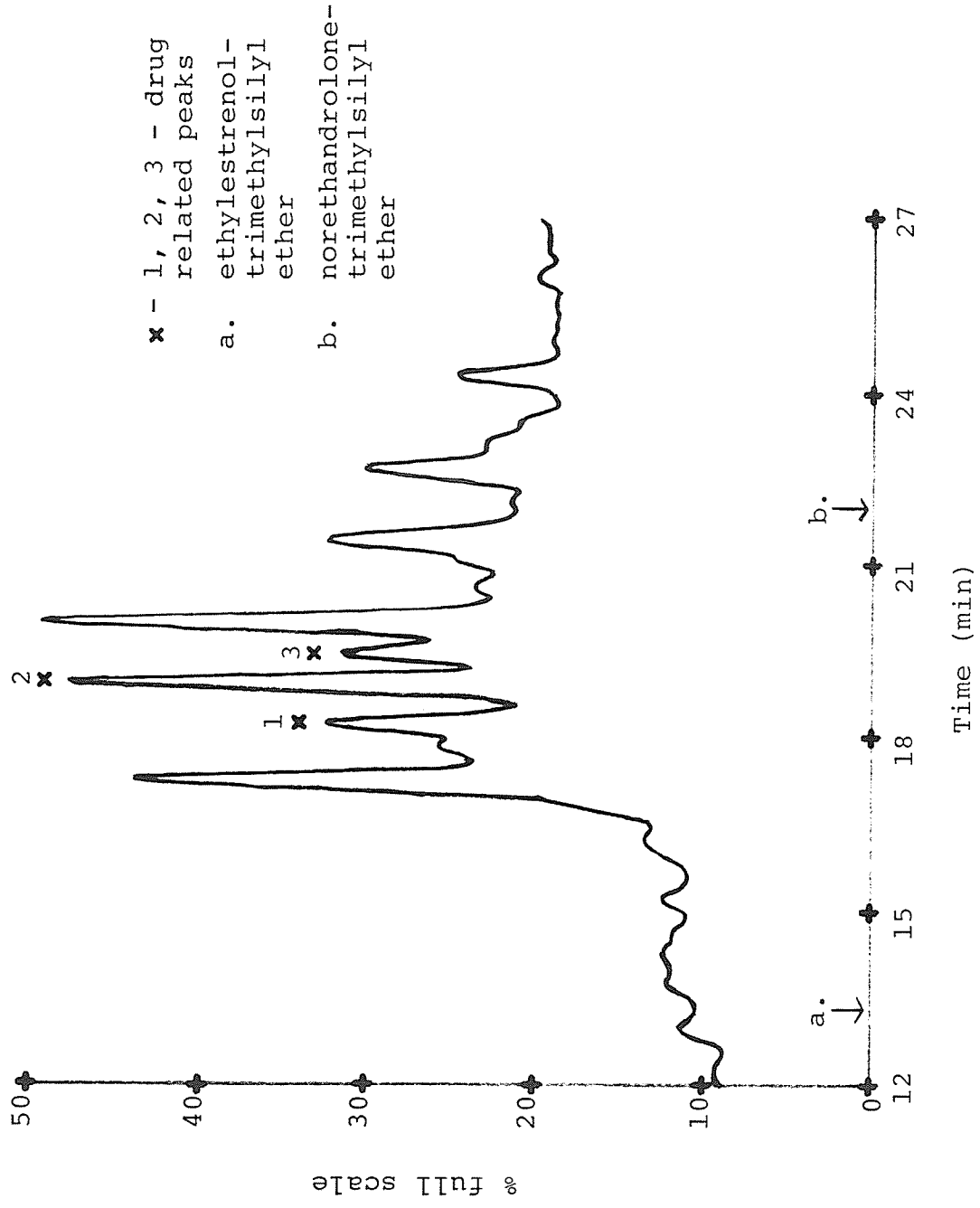


Figure 11. Normalized 70eV mass spectrum of a faecal metabolite from the glucuronide aglycone fraction of faeces from rats orally dosed with ethylestrenol (Peak 1).

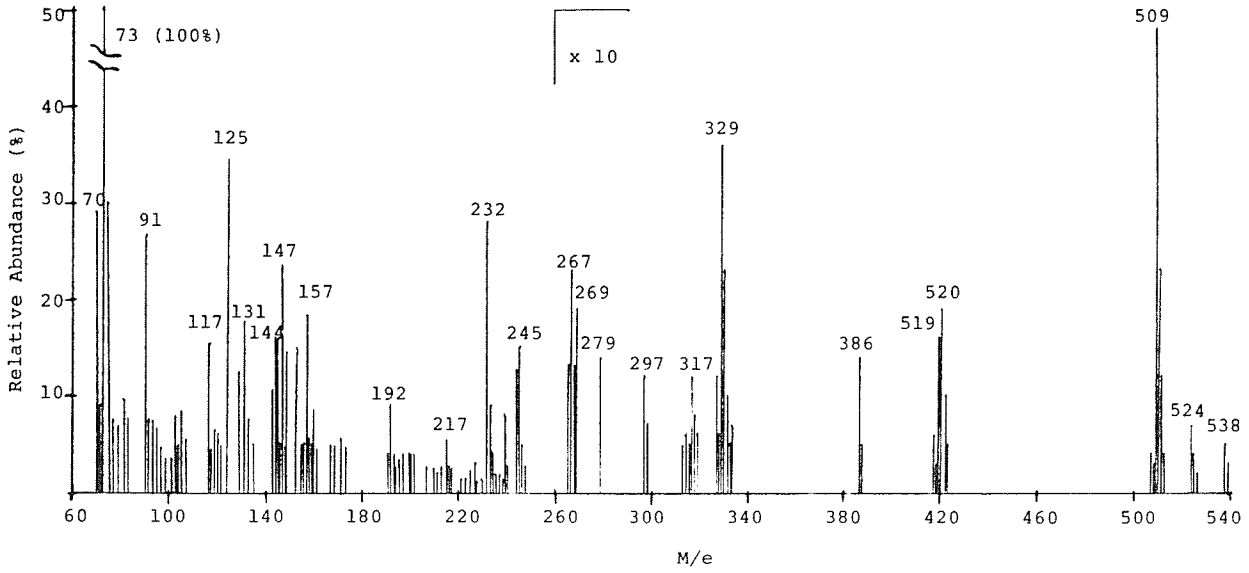


Figure 12. Normalized 70eV mass spectrum of a faecal metabolite from the glucuronide aglycone fraction of faeces from rats orally dosed with ethylestrenol (Peak 2).

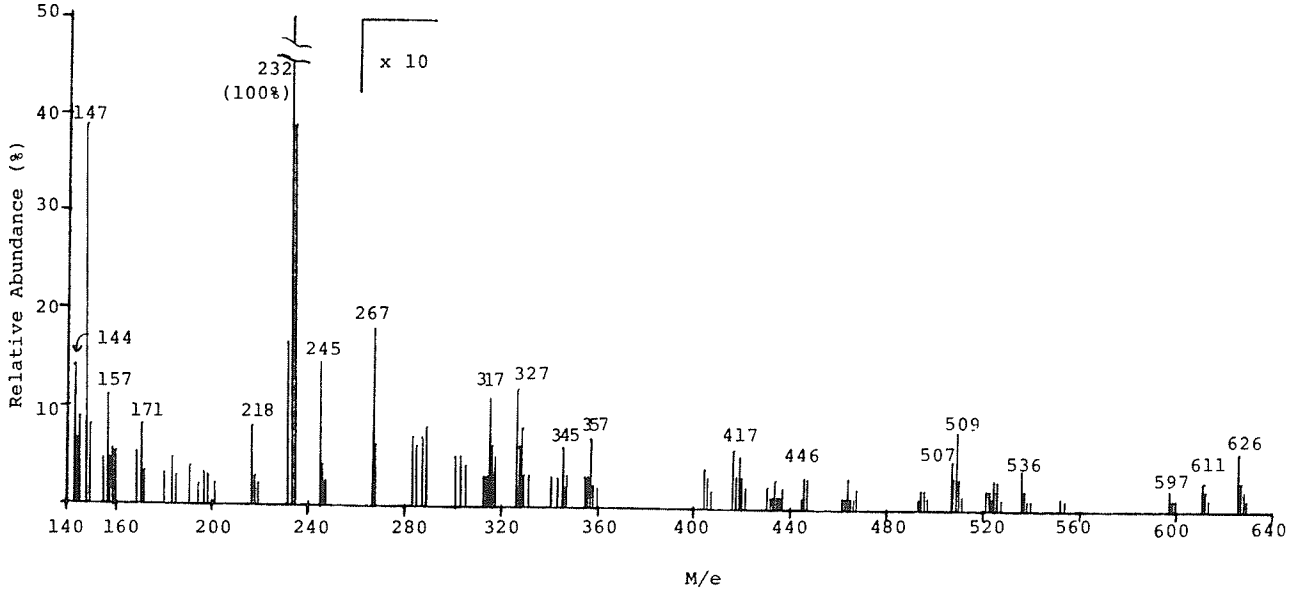
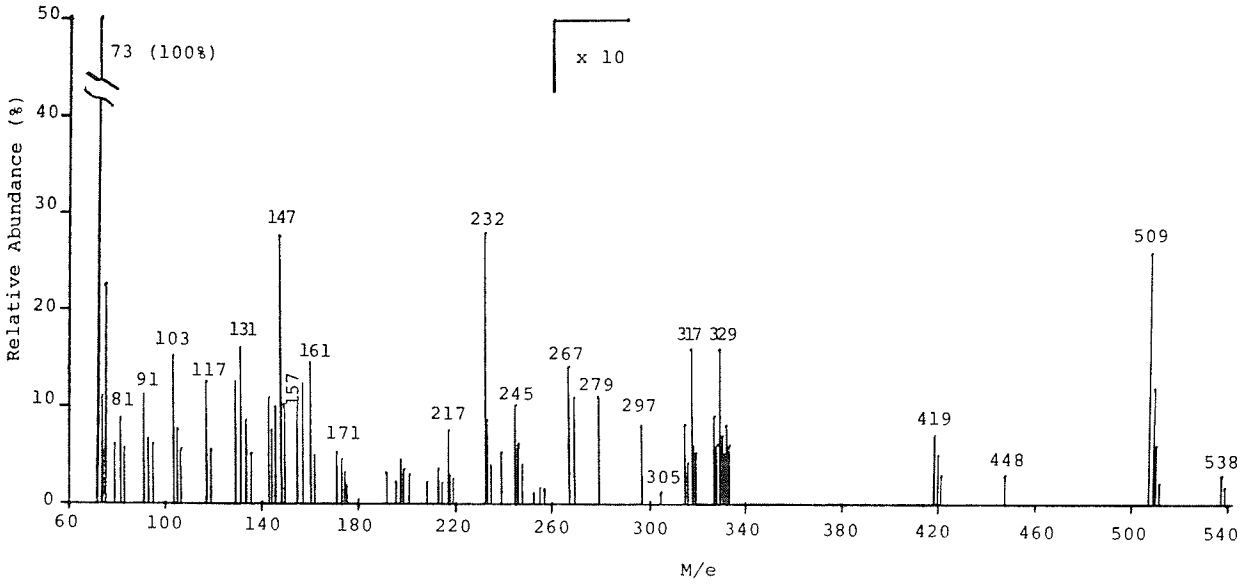


Figure 13. Normalized 70eV mass spectrum of a faecal metabolite from the glucuronide aglycone fraction of faeces from rats orally dosed with ethylestrenol (Peak 3).



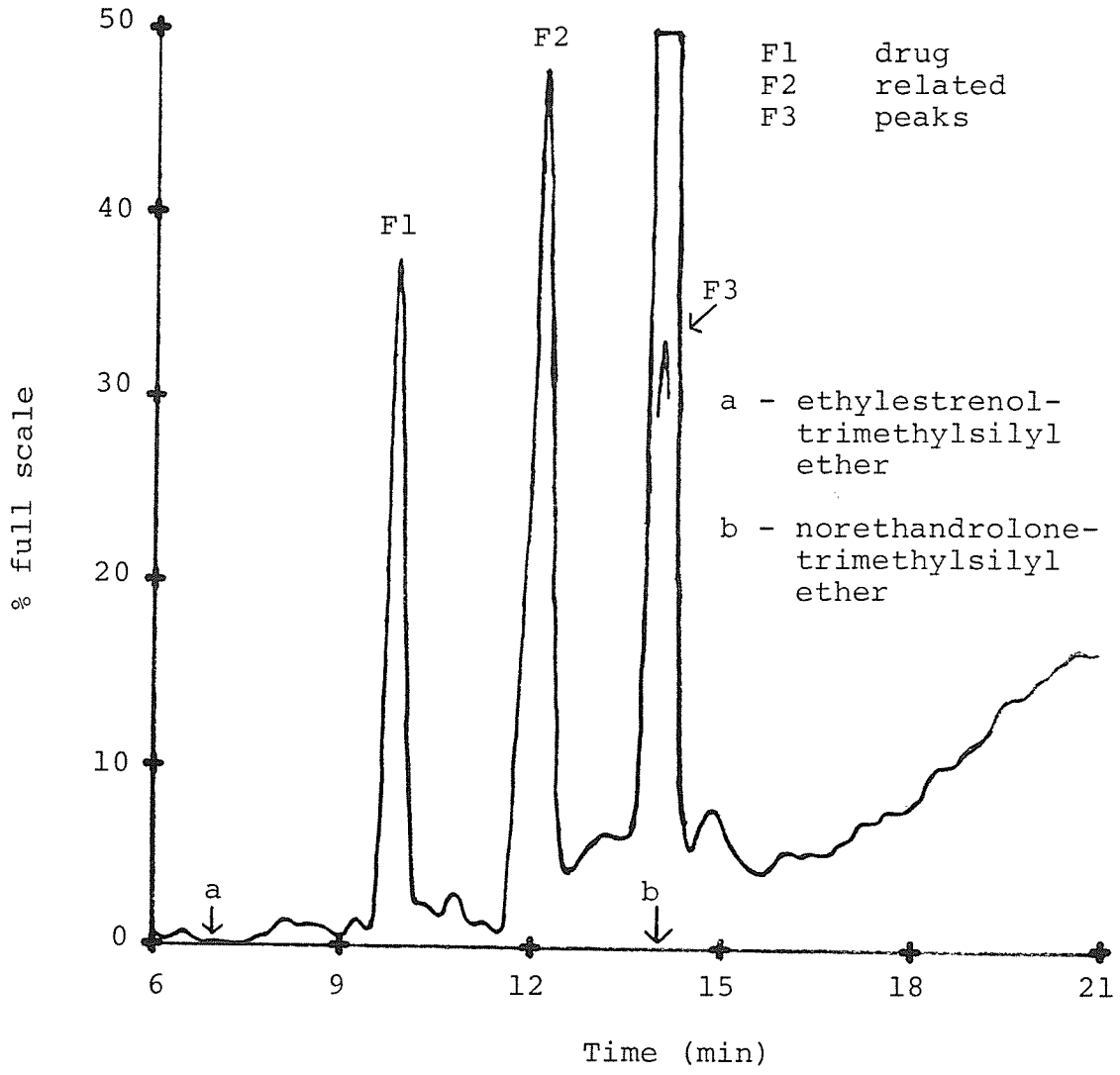
Radioactivity was found in five of the fractions (see Table 12) and the fractions corresponding to fractions I and J were bulked, as it appeared they probably contained the same compound(s).

Gas-liquid chromatography of the resulting four fractions (from bands B, D, F, and IJ) showed that fraction IJ contained only unchanged ethylestrenol (co-chromatography, mass spectroscopy) and that fraction F contained the major metabolite peak. Fractions B and D showed only traces of material by GLC and no more was done with them. A portion of fraction F was derivatized (in pyridine) and a gas-liquid chromatogram of this silylated fraction showed three major peaks (F1, F2, and F3) in a peak height ratio of 0.8:1:3. Figure 14 shows the

Table 12. Results from the thick layer chromatography of the extract from in vitro liver homogenate after incubation with 3H-ethylestrenol.

Band	Distance from origin (cm)	Color				Radioactivity present
		Untreated		Antimony trichloride treated		
		Short U.V. light	Long U.V. light	White light	Long U.V. light	
A	0-0.4	-	-	-	-	no
B	0.4-1.6	absorption	green-yellow	orange-brown	-	yes
C	1.6-2.5	-	-	-	-	no
D	2.5-3.2	absorption	-	yellow-orange	green	yes
E	3.2-4.8	-	-	-	-	no
F	4.8-5.9	absorption	-	orange	orange-yellow	yes
G	5.9-8.4	-	-	light blue	-	no
H	8.4-9.4	-	light yellow	pink-orange	pink	no
I	9.4-11.7	-	-	light yellow	light yellow-green	yes
J	11.7-12.7	-	-	yellow	yellow green	yes
K	12.7-15.7	-	light blue	-	blue	no
L	15.7-16.3	-	-	-	-	no
M	16.3-17.2	absorption	yellow-orange	black	-	no
N	17.2-19.1	-	-	-	-	no

Figure 14. Gas-liquid chromatogram of the major metabolites from the in vitro liver metabolism of 3H-ethylestrenol (Fraction F-derivatized).



portion of the chromatogram containing these peaks.

Peak F3 had the same retention time as norethandrolone-trimethylsilyl ether and co-chromatographed with a derivatized authentic sample of norethandrolone. The mass spectrum of derivatized peak F3 had the highest mass ion at m/e 374, base peak at m/e 73, and major peaks at m/e 359 (m^+-15), 345 (m^+-29), 303 (m^+-42-29), 290, 284 (m^+-90), 255, 213, 157, and 144. The normalized mass spectrum of Peak F3 is shown in Figure 15.

The mass spectrum of derivatized peak F1 had the highest mass ion at m/e 450, the base peak at m/e 157, and major peaks at m/e 435 (m^+-15), 421 (m^+-29), 360 (m^+-90), 331 (m^+-90-29), 305 (m^+-145), 270 (m^+-90-90), 241 (m^+-90-90-29), 219, 144, 75, and 73. The normalized mass spectrum of peak F1 is shown in Figure 16.

The mass spectrum of peak F2 had the highest mass ion at m/e 446 with ions at m/e 431 (m^+-15) and 417 (m^+-29). Another sample of fraction F was derivatized in dimethylformamide and showed no G-LC peak corresponding to peak F2. An authentic sample of norethandrolone was derivatized in pyridine and contained a small G-LC peak with a retention time the same as peak F2.

Figure 15. Normalized 70eV mass spectrum of the major metabolite from the in vitro liver metabolism of 3H-ethylestrenol (Peak F3).

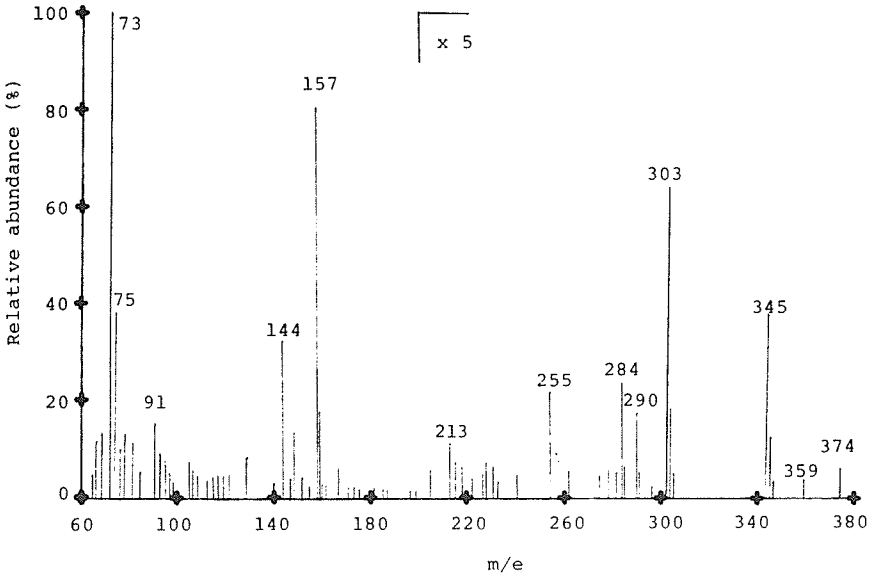
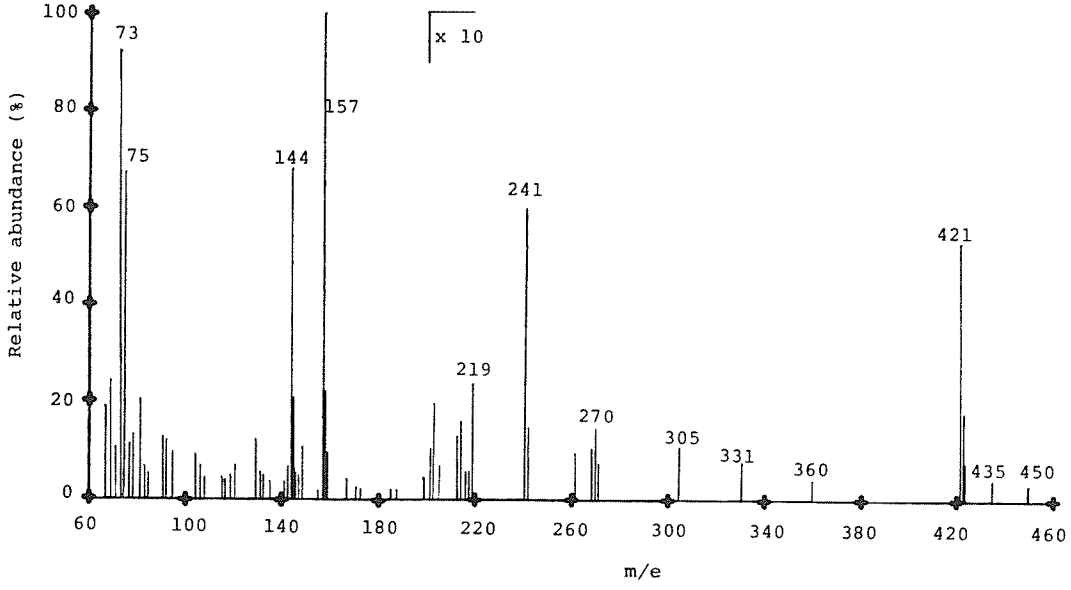


Figure 16. Normalized 70eV mass spectrum of a metabolite from the in vitro liver metabolism of 3H-ethylestrenol (Peak F1).



DISCUSSION

Before experimentation could begin using the tritium-labelled ethylestrenol, some consideration of the possibility of loss of label resulting from exchange had to be made, as any exchange would cause low recoveries. It was not expected that ^{3}H-exchange would be very significant as the label was not attached to relatively active positions, such as oxygen or nitrogen atoms, or on carbon atoms adjacent to or bonded by double bonds. It is generally accepted that attached to inactive carbon atoms, tritium labels are relatively permanent (Chase and Rabinowitz, 1967). The ^{3}H-label was tested for exchange with ethanol in the working stock solution by evaporating an aliquot of the solution to dryness, redissolving it in toluene, and assaying the toluene solution for radioactivity. No loss of activity of the stock solution because of exchange was found during the entire study.

A. Absorption

There are several experimental methods to obtain estimates of the absorption of a labelled drug into general circulation after an oral dose by measurement of the total radioactivity in the excreta. Most of the methods involve measuring the differences in biliary or urinary excretion after oral and intravenous administrations of the drug. These methods assume that the distribution ratio between biliary and urinary excretion will remain the same after oral and intravenous administrations, but this assumption may frequently

prove to be invalid (Nimmerfall and Rosenthaler, 1976). The circumstance that a compound may be eliminated differently after oral and after intravenous administration has been discussed by various authors (Gibaldi and Feldman, 1969; Rowland, 1972).

Nimmerfall and Rosenthaler (1976) have described a method for estimation of absorption that bypasses this difficulty. Their method involves the quantitation of cumulative biliary and urinary excretion from cannulated animals as an estimate of the absorption after oral doses. The total amount of faecal excretion is also determined and is an estimate of the unabsorbed portion of the drug.

Initial experiments to measure the biliary excretion of radioactivity after an oral dose of ^{3}H-ethylestrenol showed that the excretion through the biliary route was too slow to accurately estimate total biliary excretion (2-3.5% of the dose was excreted in 12-24 hours). Therefore, the method was altered to avoid this difficulty. The common bile ducts were ligated on orally dosed rats, blocking excretion of any absorbed drug through the bile system into the faeces. Any radioactivity then appearing in the faeces was the result of unabsorbed drug and an estimate of the percentage of the dose absorbed could be made.

As reported in Table 2, the recoveries of radioactivity in the faeces were 67% (BL1) and 46% (BL2), but because BL2 died before faecal excretion of the radioactivity was complete,

the figure from that rat is not an accurate representation of the unabsorbed dose. Using the figure from rat BL1, the estimate of the absorption can be calculated as 33% (100% minus 67%).

Research with another anabolic steroid, methandrostenolone, has shown that essentially all of that drug was absorbed after oral dosing (Steele, et al.). Therefore, it seems that lack of an oxygen function on the A ring of the steroid and a slightly longer 17-alkyl chain has retarded absorption of ethylestrenol. This is reasonable, as lack of an oxygen function on the A ring would severely affect the partition coefficient, a parameter known to have a major effect on the transmembrane passage of chemicals (Goldstein, et al., 1974).

B. Distribution

Two experiments were performed in order to follow the distribution of ^{3}H-ethylestrenol in the rat after oral dosing. The first was a short term study to follow the initial movement of the drug within the first twenty-four hours. The second experiment was a long term study to look for any tissue localization of the drug. In the latter case, a small dose of high specific radioactivity was used in order to be able to detect very small amounts of tissue binding.

The results from the short term study were similar to what had been expected. The highest levels were found in the alimentary canal, in part due to the unabsorbed portion of

the dose merely passing through the rats, and partly due to the small amount of biliary excretion of some of the absorbed portion. It can be noted from Table 3 that after four hours the dose was mainly in the stomach with a small amount in the small intestine, and as time passed there was less drug in the stomach and increasing amounts in the lower parts of the alimentary tract. It can also be seen from these results that increasing radioactivity gradually appears in the urine and faeces and that there is a general trend for radioactivity to build up in the tissues after eight and eighteen hours. The same time sequence of the passing of the drug through the alimentary canal was found in the long term study (Table 4).

The information to be obtained from the specific activities of the individual tissues during the short term study is somewhat limited. The most obvious observation is that the levels of radioactivity in the liver and kidneys were higher than in the other tissues. This may not be very significant as there was a general trend for the highest levels to be found in the tissues with the greatest blood supply (relatively high levels were also found in the spleen, heart, and lungs).

The results from the long term study are very interesting. It is notable that, while the radioactivity dropped to quite low levels in most of the tissues, there remained a substantially larger amount situated in the liver and kidneys, even after five days. The radioactivity that remained

in the kidneys was 2.5-3 times higher and in the liver 5-7 times higher than the average of the rest of the tissues for the duration of the study. These higher levels, especially for the liver, may be important as it has been suggested that ethylestrenol causes stimulation of the hepatic mono-oxidase system (Gillette, 1963; Selye, 1970). It is therefore a possibility that some ethylestrenol may be bound in the hepatic cells to stimulate the production of hepatic enzymes.

C. Excretion

The excretion experiments were performed in order to follow the time course of excretion of ethylestrenol and to determine the major routes of excretion. It might be assumed that the major route of excretion of circulating drug can be determined by following the excretion of an oral dose, however, all of the drug may not be absorbed and the results could be misleading. For this reason, the excretion after intramuscular and intravenous doses was also followed. These latter experiments were performed to give a representation of the division of excretion between liver and kidneys, since it was expected that in these experiments, all of the drug would eventually enter the circulatory system.

The oral dose excretion experiment demonstrated recovery of 100% of the dose fed. It is obvious from the resulting data (Tables 5 and 6) that the majority of the dose was excreted in the faeces. This is important as previous work (Ward, et al., 1975; 1977) has focused mainly on the urine,

even though this accounted for only a small proportion of the dose (17%). Although a large proportion of the dose was excreted in the faeces (83%), it should be remembered that a large part of this was due to unabsorbed drug.

The data also showed that the majority of the drug dose was excreted within the first three days. This is shown in Figures 4 and 5. The data (discarding day 9) can be fitted by a first-order two-compartment model, the equation for the line (method of residuals) being $D_R = 5.236e^{-0.5213T} + 20.66e^{-0.4762T}$ ($r = 0.9957$), where D_R is the average percent of drug remaining in the rats at any time T (Gibaldi and Perrier, 1975). The increased excretion rate over the first few days was due in part to the unabsorbed portion of the dose passing relatively quickly into the faeces. The $t_{1/2}$ for the excretion of radioactivity during the second phase was 1.46 days ($\ln 2/0.4762$).

The intramuscular dose experiment showed clearly that once the drug was outside the general circulation it was very slowly excreted. Indeed, it appears that excretion from rat ME1 was not complete even after five weeks (Figure 6). By extrapolating the lines in Figure 6, the average cumulative excretion at time ∞ can be estimated at 85%. This may, in fact, represent close to 100% of the administered dose as a calculation of the net dose was impossible at the time of dosing due to bleeding. Thus, the cumulative excretion of the dose was 35.12% of the dose in the urine and 63.28% of

the dose in the faeces after 35 days. The linear relationship in Figure 7 indicates that the first-order excretion rate was reasonably constant over most of the study period. The data for days five to thirty-five are fitted by the straight line $D_R = 115.2e^{-0.1253T}$ ($r = 0.9992$). The $t_{1/2}$ for the excretion of the radioactivity during this period was 5.53 days. This straight line is no doubt due to a combination of several parameters (kinetics of the release from depot, metabolism, excretion, etc.), all having reached a steady state. One of these factors must also be the rate limiting step and because the intravenous experiment suggests that high concentrations of the drug can be handled by the excretory system, the limiting factor would therefore be the release of the drug.

The intravenous dose experiment was performed in order to determine whether the excretory systems could handle a large dose if it was present in the blood stream all at once. It was found that 39.74% of the dose was excreted in the urine and 50.55% of the dose in the faeces after five days. The observation that there was little difference between the higher and lower doses suggests that the pathways by which the rats excreted the drug were probably the same for the two doses. It also implies that the drug handling system was able to manage a large amount of drug (the 8 mg dose was 37.2 mg/kg or 521 times the human therapeutic dose) and was not easily saturated. This indicates that the drug was not

handled or bound in a compartment which consisted of a limited number of binding sites.

In excretion experiments using methandrostenolone, it was found that about 5-7% of an oral dose was recovered in the urine and the remainder in the faeces. This is interesting when compared with the approximately 60:40 (faeces:urine) split found in the intramuscular and intravenous administrations of ethylestrenol and the 83:17 (67% unabsorbed) split found after the oral dose of ethylestrenol. It has been suggested (Plaa, 1971) that the deciding influence on the division between urinary and faecal (via biliary) excretion is a drug's molecular weight. The dividing weight has been placed at 300 and it is reasoned that drugs having lesser molecular weights are excreted only in negligible amounts in the bile, because they are reabsorbed from the primary bile as it passes through the smallest canaliculi (Goldstein, et al., 1974). Most steroids are extensively conjugated, increasing their molecular weights to well over 300 and it would thus be expected that they would almost exclusively be excreted in the bile. Since this was not the case found with ethylestrenol, other factors must play an important role in affecting the amount of biliary excretion (i.e., the lack of an A-ring oxygen function would affect the partition coefficient).

D. Metabolism

A great many drugs, steroids included, are conjugated as a step in their metabolism. This usually involves the attach-

ment of molecules of glucuronic acid or sulphuric acid to the hydroxyl groups on the steroid resulting in increased water-solubility and improved urinary excretion (Hadd and Blickenstaff, 1969). In order to identify the metabolic changes that the steroid moiety has undergone before being excreted, it is necessary that any conjugating moieties be removed. This often necessitates the separation of the metabolites into unconjugated (free) and conjugated portions and the conjugated portion into sulphate and glucuronide portions. The conjugating moieties can then be removed by various chemical and enzymatic methods.

1. Urine

Although the proportion of metabolites found in each form (unconjugated, glucuronide, or sulphate) is in itself not of considerable importance, a few observations can be made by comparing the results found for ethylestrenol with those found for methandrostenolone (Steele, et al.).

The unconjugated fraction of the urinary ethylestrenol metabolites represented approximately 2% of the dose or 15% of the amount of radioactivity excreted into the urine. The percentage of the dose found in the unconjugated form after methandrostenolone administration was also about 2%, but because only 5-7% of the dose was excreted into the urine this represents 30-40% of the total urinary metabolites. It is possible that the higher percentage of conjugated drug with ethylestrenol is related to a lower biliary excretion rate.

In that case, a conjugate which had been formed in the liver would have a greater chance of being circulated and excreted in the urine.

Also in contrast with methandrostenolone results are the data showing that very little of the ethylestrenol urinary metabolites were present as glucuronides (0.65% of the dose, 5.5% of total urinary metabolites). After methandrostenolone administration almost all of the conjugated fraction was shown to be glucuronides by hydrolysis with β-glucuronidase.

The mild acid hydrolysis fraction from urine (Table 10) represented the drug present as sulphate conjugates (Layne, et al., 1963). There is no evidence as to what conjugated form(s) the strong hydrolysis procedures hydrolyzed.

The large non-extractable portion of the radioactivity remaining in the aqueous phase after the hydrolytic procedures is similar to that found by others studying the metabolism of contraceptive steroids (Hanasono and Fisher, 1974; Layne, et al., 1963). They have suggested that these polar nonhydrolyzable metabolites could be products of other conjugation pathways or non-conjugated polyhydroxy steroids.

2. Faeces

It was expected that the faeces would contain a large proportion of unchanged drug especially during the first few days after dosing, due to the portion of the drug that would pass through the rats without being absorbed.

In the experiment reported (Table 11), about 67% of the

dose was recovered in the faeces and 11% recovered in the urine during the first three days after dosing. Because it has already been shown that approximately one-third of an oral dose was absorbed and one-half of that was found in the urine, approximately 11% of the dose that appeared in the faeces in this experiment was the result of biliary excretion of absorbed drug. This would imply that the other 56% of the dose found in the faeces was the result of unabsorbed drug.

The extraction with chloroform, to obtain the unconjugated fraction, however, only extracted about 32% of the dose, although it appeared by G-LC (page 48) that most, if not all, of the radioactivity was due to unchanged ethylestrenol. This lower proportion can be explained by intestinal bacterial metabolism. Results with methandrostenolone (Steele, et al.) showed that a great deal of bacterial metabolism took place in the intestines with that drug. It is therefore reasonable to postulate that part of the unabsorbed portion had been metabolized by intestinal bacteria to form part of the other fractions (conjugates, unextractable from methanol, etc.). Some of the unabsorbed portion may even have formed a part of the unextractable from faeces fraction, having been tightly bound to other faecal material (Hanasono and Fischer, 1974).

The small glucuronide fraction (3.16% of the dose) found as ethylestrenol faecal metabolites is again in contrast with the results found with methandrostenolone. With that drug, all of the conjugates found were glucuronides (Steele, et al.).

The faeces, like the urine, contained a large polar fraction which could not be hydrolyzed and extracted into medium polarity solvents. This is also similar to results found for some contraceptive steroids (Hanasono and Fischer, 1974).

The detection of drug related compounds, other than unchanged ethylestrenol, in the derivatized glucuronide aglycone fraction from faeces is the first report of faecal ethylestrenol metabolites. The mass spectra of the derivatives of these compounds after fraction collection off a G-LC column (Figures 11, 12, and 13) indicate that the compounds are hydroxy-metabolites. The highest mass ion for peaks 1 and 3 (m/e 538) is consistent with the molecular ion as expected for the trimethylsilyl derivative of a dihydroxylated dihydro-ethylestrenol metabolite and the highest mass ion for peak 2 (m/e 626) is consistent with the molecular ion as expected for the trimethylsilyl derivative of a trihydroxylated dihydro-ethylestrenol metabolite. The fragmentation pattern seen in all three mass spectra (m^+-29, m^+-90-29, m^+-180-29) would indicate that the 17α-ethyl group was intact and unsubstituted, but intense ions of m/e 232 and m/e 245 would indicate hydroxylation at the 16, 20, or 21 position (Ward et al., 1977). From the gas-liquid chromatogram of these compounds (Figure 10), it can be seen that the derivatized compounds have longer retention times and are thus larger or more polar than ethylestrenol trimethylsilyl ether and have shorter retention times (smaller or less polar) than norethandrolone trimethylsilyl ether. These data, along with the mass spectra obtained for the derivatives of these

the derivatives of these compounds, are consistent with those expected for hydroxy-metabolites.

The detection of hydroxy-metabolites was not unexpected. Ward et al. (1975, 1977) reported finding several hydroxy- and dihydroxy- metabolites of ethylestrenol in the urine of the marmoset monkey and man (see Introduction, p.15). They also reported norethandrolone as a urinary metabolite of ethylestrenol, but no evidence of norethandrolone was found in any of the faecal or urinary fractions examined from the rat, in this study.

3. In Vitro

The study of ethylestrenol metabolism in rat liver homogenate was initiated in order to obtain large amounts of metabolites for identification purposes. It would then be known that the liver could be capable of producing these metabolites and they could be compared with metabolites found in the urine and faeces of intact animals.

Several in vitro metabolites were detected and two were partially characterized in this experiment. The major metabolite was undoubtedly norethandrolone. This identification is based on the fact that the metabolite co-chromatographed with an authentic sample of norethandrolone, the metabolite's trimethylsilyl ether derivative co-chromatographed with the trimethylsilyl derivative of an authentic sample of norethandrolone, and the derivative of the metabolite had a mass spectrum identical with that of the derivative of an authentic

sample of norethandrolone and also with that reported by Ward et al. (1977) for the trimethylsilyl derivative of norethandrolone. As mentioned earlier, norethandrolone was identified as a urinary metabolite of ethylestrenol in marmoset monkeys and man by Ward et al. (1977). The in vitro metabolism of ethylestrenol has also been studied by Okada et al. (1969) and they reported the metabolism of ethylestrenol by rabbit liver slices to norethandrolone.

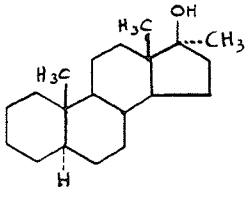
The minor metabolite has been tentatively identified as a 17α-ethyl-5ξ-estrane-3ξ,17β-diol. The mass spectrum of the trimethylsilyl derivative of the minor metabolite fits very well with that which would be expected for this compound (molecular ion at m/e 450 fits the chemical formula, loss of m/e 29=ethyl, loss of m/e 90=TMS-OH, etc.), although the mass spectrum does not match identically with that reported by Ward et al. for 17α-ethyl-5α-estrane-3α,17β-diol. Ward et al. reported both the 5α and 5β compounds as in vivo metabolites of ethylestrenol and it is possible that the difference in mass spectra is due to the metabolite found in this study having been the 5β compound or a mixture of isomers (5β,3α; 5α, 3β; etc.).

A third compound from the extract of in vitro metabolites was partially characterized (peak F2, Figure 14). This compound proved to be an artifact of the silylation procedure due to pyridine having been used as the solvent (Pierce Chem. Co., 1972). The peak disappeared when dimethylformamide was used

as the solvent and appeared when an authentic sample of norethandrolone was derivatized in pyridine. The compound was a di-TMS derivative of norethandrolone. This would also fit the mass spectrum, which was the same as that obtained by silylating in pyridine an authentic sample of norethandrolone.

Although other, more polar metabolites of ethylestrenol were detected (Fractions B and D), not enough material was present to make identifications.

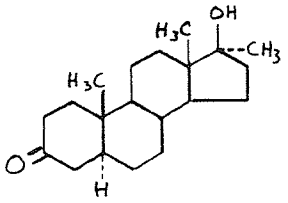
The mass spectrum of the minor metabolite's trimethylsilyl derivative shows that the metabolite was a diol with the double bond of ethylestrenol reduced, but it is not possible at this stage to be certain that hydroxylation was at position 3. The tentative assignment was based on the assumption that this was the most likely position, as norethandrolone (a 3-one derivative) was identified as the major *in vitro* metabolite. Ward *et al*. reasoned that ethylestrenol was first hydroxylated in the 3 position to give a 3-hydroxy-4-ene steroid, then oxidized to the ketone. Supporting this theory is work done with 17α-methyl-5α-androstan-17β-ol (Wolff and



17α-methyl-5α-androstan-17β-ol

Kasuya, 1972). Incubation of this compound with rabbit liver homogenate produced initially 3α,17β and 3β,17β diols with subsequent conversion to a 3-ketone compound, 17β-hydroxy-17α-

methyl-5α-androstan-3-one. Indeed, Templeton and Kim (1975)



17β-hydroxy-17α-methyl-5α-androstan-3-one

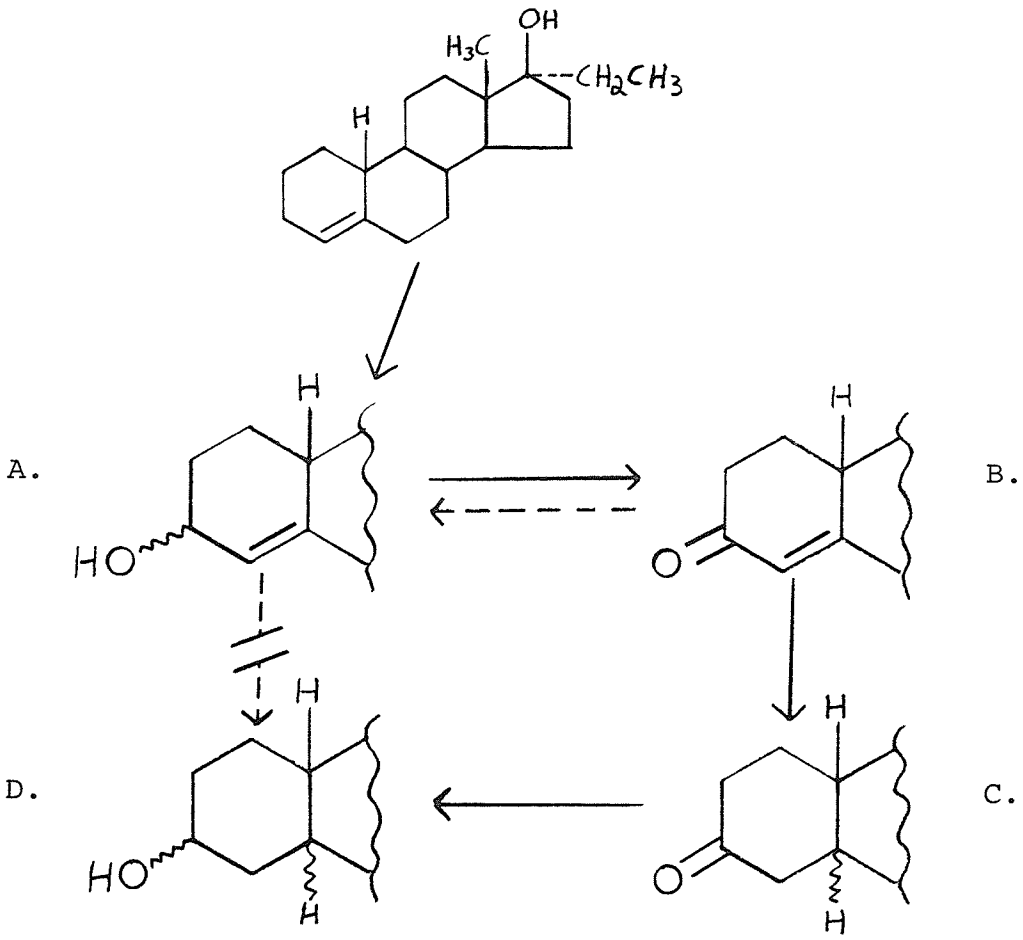
have suggested that for any mono-oxygenated steroid, metabolic hydroxylation will take place at the position farthest from the initial oxygen function. The appropriate position for ethylestrenol is C_3.

If it is accepted that the initial step in the metabolism of ethylestrenol was hydroxylation at the 3 position, it is still unclear whether the minor metabolite characterized arose by direct reduction of the proposed 3-hydroxy-4-ene intermediate or by reduction of the norethandrolone. It would appear at first that the simpler reduction via the initial intermediate would be the more likely pathway, but it has been demonstrated that the incubation of 4-androsten-3α(or 3β),17β-diol under similar conditions gives no reduced metabolites (Breuer, et al., 1963). This suggests the possibility that the minor metabolite was formed as a secondary product from norethandrolone. The detection of 3-hydroxy-4,5-dihydro metabolites of norethandrolone in men and the marmoset monkey (Ward et al., 1977) supports this theory. The suggested pathways of the metabolism of ethylestrenol are illustrated in Figure 17.

In comparing the in vitro metabolites with those found in urine and faeces, it is surprising that norethandrolone

was not detected as an *in vivo* metabolite in the rat, since it forms the major product *in vitro*. The most reasonable explanation for this is that the conversion of norethandrolone to reduced metabolites must be rapid in the rat.

Figure 17. Possible pathways of ethylestrenol metabolism.*



*Structures B. and D. were identified by Ward *et al.* as *in vivo* metabolite in man and marmoset monkey. Structure B. was identified in this study as *in vitro* metabolite in rats. Structure D. was tentatively identified in this study as *in vitro* metabolite in rats.

SUMMARY

Anabolic steroids are used very sparingly in general clinical practice, but are reported to be widely abused by athletes who believe that the use of these drugs during training will increase muscle mass and strength. Reports of liver toxicity related to the long-term use of anabolic steroids indicate that these athletes run the risk of doing themselves serious injury. Before researchers can attempt to determine the extent and causes of the toxic effects, it is important to know the disposition of the drug in the body. With regards to anabolic steroids, however, this information is, for the most part, unavailable. For these reasons, this study of the disposition in rats of the anabolic steroid, ethylestrenol, was initiated.

Experiments were performed to determine the absorption, distribution, metabolism, and excretion of an oral dose of ethylestrenol in the rat, utilizing 20, 21-^{3}H-ethylestrenol as a radioactive tracer. The excretion after intramuscular and intravenous doses and the *in vitro* metabolism of ^{3}H-ethylestrenol by rat liver homogenate were also studied.

The absorption of ethylestrenol from the alimentary tract was determined to be approximately one-third of the dose by measuring the percentage of radioactivity excreted in the faeces of bile duct ligated rats dosed orally with ^{3}H-ethylestrenol.

A lower specific activity level distribution experiment

was performed to follow the initial movements of the drug within the first twenty-four hours and a higher specific activity level experiment was performed to detect any long term tissue binding. Highest levels of radioactivity were found in the alimentary tract as a result of non-absorption of the greater proportion of the dose, but measurable levels were found in all tissues indicating general distribution of the drug. In the higher specific activity level experiment, kidney tissue was found to contain 2 1/2 - 3 times and liver tissue 5-7 times the specific radioactivity of all other tissues, excluding the alimentary tract.

It was found that all of an oral dose of ethylestrenol was excreted within 10 days, 83% in the faeces and 17% in the urine. Excretion of the dose was fastest during the first three days as a result of the unabsorbed portion of the dose passing relatively quickly out of the body in the faeces. The $t_{1/2}$ for the excretion of the radioactivity during the last seven days was 1.46 days.

Examination for metabolites of a sample of urine containing 11.8% of an oral dose found that 1.8% of the dose was unconjugated, 0.7% of the dose was present as glucuronide conjugates, 2.4% of the dose was present as sulphate conjugates, 2.8% of the dose was present as unidentified conjugates which could be hydrolyzed by strong acid treatment, and 4.2% of the dose was unextractable from aqueous media. Unchanged ethylestrenol was the only drug-related

compound found in the unconjugated fraction.

In a sample of faeces containing 66.9% of an oral dose, it was found that 31.8% of the dose was unconjugated, 3.2% of the dose was present as glucuronide conjugates, 5.5% as sulphate conjugates, 14.3% was soluble in methanol and water but could not be extracted into less polar solvents, and 12.2% of the dose could not be extracted from the faecal material. The only drug-related compound detected in the unconjugated fraction was unchanged ethylestrenol. Examination of a derivatized portion of the glucuronide aglycone fraction indicated the presence of three drug-related compounds. Mass spectra, obtained for these three metabolite compounds, indicated that two were dihydroxylated dihydro-ethylestrenol derivatives and one was a trihydroxylated dihydro-ethylestrenol derivative.

The excretion of ^{3}H-ethylestrenol after intravenous doses was studied and indicated that the pathways which handled the excretion of ethylestrenol were capable of handling the drug when present in high concentrations in the blood. An experiment to follow the excretion of an intramuscular dose showed that the excretion was very slow; the average $t_{1/2}$ for the excretion of the radioactivity was 5.5 days.

Examination of the metabolites produced by the <u>in vitro</u> metabolism of ethylestrenol by rat liver homogenate identified 17α-ethyl-17β-hydroxy-4-estren-3-one as the major metabolite and tentatively identified 17α-ethyl-5ξ-estrane-3ξ,17β diol as a minor metabolite.

REFERENCES

Bartlett, P. D. Endocr. 55, 77-84 (1953).

Breuer, H., Dehm, K., Norymberski, J. K. J. Endocr. 27, 357-8 (1963).

Brooks, C. J. W. and Middleditch, B. S. Clin. Chim. Acta 34, 145-57 (1971).

Camerino, B. and Sciaky, R. Pharm. Therap. B 1, 233-75 (1975).

Colby, H. D. and Kitay, J. I. Endocr. 90, 473-8 (1972).

Cooper, D. L. and Craig, T. T. J. Am. Med. Ass. 233, 463 (1975).

Desaulles, P. A. and Krahenbuhl, C. Acta Endocr. 40, 217-31 (1962).

DeWinter, M. S., Siegman, C. M. and Szpilfogel, S. A. Chem. Ind., 905 (1959).

Eisenburg, E. and Gordon, G. S. J. Pharmac. exp. Therap. 99, 38-44 (1950).

Freed, D. L. J., Banks, A. J., Longson, D. and Burley, D. M. B. Med. J. 2, 471-3 (1975).

Gibaldi, M. and Feldman, S. J. Pharm. Sci. 58, 1477-80 (1969).

Gibaldi, M. and Perrier, D. Pharmacokinetics Marcel Dekker Inc. (1975).

Gillette, J. R. Adv. Enz. Regulation 1, 215-23 (1963).

Goldstein, A., Aronow, L. and Kalman, S. M. Principles of Drug Action 2nd ed. Harper and Row (1974).

Gordan, G. S. Handb. Exp. Pharm. 43, 499-513 (1977).

Gribbin, H. R. and Flavell Matts, S. G. B. J. Clin. Prac. 30, 3-9 (1976).

Hadd, H. E. and Blickenstaff, R. T. Conjugates of Steroid Hormones Academic Press (1969).

Hanasono, G. K. and Fischer, L. J. Drug Metab. Dispos. 2, 159-68 (1974).

Hara, S. and Mibe, K. Chem. Pharm. Bull 23, 2850-9 (1975).

Hayes, K. J. Acta Endocr. 48, 337-47 (1965).

Hershberger, L. G., Shipley, E. G. and Meyer, R. K. Proc. Soc. exp. Biol. Med. 83, 175-80 (1953).

Hervey, G. R. and Huchinson, I. J. Endocr. 57, xxiv-v (1973).

Hervey, G. R., Huchinson, I., Knibbs, A. V., Burkinshaw, L., Jones, P. R. M., Norgan, N. G. and Levell, M. J. Lancet, 699-702 (1976).

Holma, P. and Aldercreutz, H. Acta Endocr. 83, 856-64 (1976).

Howard, C. W., Hanson, S. G. and Wahed, M. A. B. Med. J., 1659-60 (1977).

Johnson, F. L. Med. and Sci. in Sports 7, 284-6 (1975).

Kilshaw, B. H., Harkness, R. A., Hobson, B. M. and Smith, A. W. M. Clin. Endocr. 4, 537-41 (1975).

Kolchakian, C. D. Pharm. Therap. B 1, 149-77 (1975).

Kolchakian, C. D. and Murlin, J. R. J. Nutr. 10, 437-59 (1935).

Kopera, H. Handb. Exp. Pharm. 43, 535-625 (1977).

Kourounakis, P., Selye, H. and Tache, Y. Adv. Steroid Biochem. and Pharmacol. 6, 35-57 (1977).

Lancet 1, 22-3 (1975).

Li, F. P., Alter, B. P. and Nathan, D. G. Blood 40, 153-62 (1972).

Layne, D. S., Golab, T., Arai, K. and Pincus, G. Biochem. Pharmacol. 12, 905-11 (1963).

Lye, M. D. W. and Ritch, A. E. S. Rheum. Rehab. 16, 62-9 (1977).

Mayer, M. and Rosen, F. Am. J. Physio. 229, 1381-6 (1975).

Medical Journal of Australia 1, 984-5 (1976).

Nimmerfall, F. and Rosenthaler, J. J. Pharmacokin. Biopharm. 4, 57-66 (1976).

Okada, H., Sumi, M., Ahara, M. and Ishihara, M. Nippon Naibumpi Gakki Zasshi 44, 1274-6 (1969) from Chem. Abs. 71: 109429.

Overbeck, G. A., Delver, A. and deVisser, J. Acta Endocr. 40, 133-9 (1962).

Percy, E. C. Can. Med. Ass. J. 117, 601-5 (1977).

Pierce Chemical Co. Handbook of Silylation (Handbook GPE-3a) (1972).

Plaa, G. L. Fundamentals of Drug Metabolism and Drug Disposition LaDu, B. N. ed., 131-45 (1971)

Potts, G. O., Arnold, A. and Beyler, A. L. Handb. Exp. Pharm. 43, 361-406 (1977).

Rotenburg, G. N. ed. Compendium of Pharmaceuticals and Specialities (Canada), 13th ed. Southam-Murray (1978).

Rowland, M. J. Pharm. Sci. 61, 70-4 (1972).

Ruchelman, H. and Ford, R. V. Metabolism 12, 846-9 (1963).

Ryan, A. J. Handb. Exp. Pharm. 43, 515-34 (1977).

Sanchez-Medal, L., Gomez-Leal, A., Duarte, L. and Rico. M. G. Blood 34, 283-300 (1969).

Scheuer, A. and Lehmann, F.,-G. Internist 18, 208-14 (1977).

Segaloff, A., Horwitt, B. N., Carabasi, R. A., Murison, P. J. and Schlosser, J. V. Cancer 6, 483-7 (1953).

Selye, H. Can. Rev. Biol. 29, 49-102 (1970).

Steele, J. W., Audette, R. C. S., Smith, D., Araneda, F. and Boux, L. J. unpublished results.

Tahmindjis, A. J. Med. J. Aust. 1, 991-3 (1976).

Templeton, J. F. and Kim, R.-S. S. Steroids 25, 403-11 (1975).

Wade, N. Science 176, 1399-1403 (1972).

Ward, R. J., Lawson, A. M. and Shackleton, C. H. L. J. Steroid Biochem. 8, 1057-63 (1977).

Ward, R. J., Shackleton, C. H. L. and Lawson, A. M. Br. J. Sports Med. 9, 93-7 (1975).

Westaby, D., Ogle, S. J., Paradinas, F. J., Randell, B. J., Murray-Lyon, I. M. Lancet, 261-3 (1977).

Wolff, M. E. and Kasuya, Y. J. Med. Chem. 15, 87-8 (1972).

Young, G. P., Bathel, P. S., Sullivan, J. R., Wall, A. J., Fone, D. J. and Hurley, T. H. Aust. N. Z. J. Med. 7, 47-51 (1977).

LIST OF TABLES

page

1. The anabolic steroids available in Canada. 5

2. Recovery of radioactivity from bile duct ligated rats after an oral dose of 3H-ethylestrenol. 32

3. Short term distribution of radioactivity in the rat after an oral dose of 3H-ethyl-estrenol. 34

4. Long term distribution of radioactivity in the rat after an oral dose of 3H-ethyl-estrenol. 36

5. Excretion of radioactivity from rats after an oral dose of 3H-ethylestrenol. 38

6. Excretion of radioactivity from rats after an oral dose of 3H-ethylestrenol (OE1, OE2 averaged) 39

7. Excretion of radioactivity from rats after an intramuscular dose of 3H-ethylestrenol . . . 41

8. Excretion of radioactivity from rats after an intramuscular dose of 3H-ethylestrenol (ME1, ME2 averaged) 42

9. Excretion of radioactivity from four rats after intravenous doses of 3H-ethyles-trenol. 45

10. Extraction of radioactivity from the urine of rats orally dosed with 3H-ethylestrenol 46

11. Extraction of radioactivity from faeces of rats orally dosed with 3H-ethylestrenol 48

12. Results from the thick layer chromatography of the extract from *in vitro* liver homo-genate after incubation with 3H-ethyles-trenol. 53

LIST OF FIGURES

page

1. A classification system for anabolic steroids. 4

2. A flow diagram of the extraction procedures for urinary metabolites of ethylestrenol. . . . 28

3. A flow diagram of the extraction procedures for faecal metabolites of ethylestrenol 30

4. Cumulative excretion of radioactivity from rats after an oral dose of 3H-ethylestrenol . . 40

5. Percentage of radioactivity remaining in rats after an oral dose of 3H-ethylestrenol. 40

6. Excretion of radioactivity from rats after an intramuscular dose of 3H-estrenol 43

7. Percentage of radioactivity remaining in rats after an intramuscular dose of 3H-ethylestrenol. 43

8. Excretion of radioactivity from rats after an intravenous dose of 3H-ethylestrenol (VE1-4 averaged) 45

9. Gas-liquid chromatogram of the unconjugated fraction from the urine of orally dosed rats. 47

10. Gas-liquid chromatogram of a silylated portion of the glucuronide aglycone fraction of faeces from orally dosed rats 50

11. Normalized 70eV mass spectrum of a faecal metabolite from the glucuronide aglycone fraction of faeces from rats orally dosed with ethylestrenol (Peak 1) 51

12. Normalized 70eV mass spectrum of a faecal metabolite from the glucuronide aglycone fraction of faeces from rats orally dosed with ethylestrenol (Peak 2) 51

13. Normalized 70eV mass spectrum of a faecal metabolite from the glucuronide aglycone fraction of faeces from rats orally dosed with ethylestrenol (Peak 3) 52

LIST OF FIGURES page

14. Gas-liquid chromatogram of the major metabolites from the *in vitro* liver metabolism of 3H-ethylestrenol (Fraction F-derivatized). 54

15. Normalized 70eV mass spectrum of the major metabolite from the *in vitro* liver metabolism of 3H-ethylestrenol (Peak F3). 56

16. Normalized 70eV mass spectrum of a metabolite from the *in vitro* liver metabolism of 3H-ethylestrenol (Peak F1) 56

17. Possible pathways of ethylestrenol metabolism. 73

APPENDIX

A. Abbreviations and Symbols

butyl-PBD	2-(4- butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole
cm	centimetre(s)
cpm	counts per minute
°C	degree(s) Celsius
dpm	disintegrations per minute
g	gram(s)
G	gravitational constant (980 cm/sec^2)
h	hour(s)
l	litre(s)
m	metre(s)
µCi	microcurie(s)
µg	microgram(s)
µl	microlitre(s)
µm	micrometre(s)
mCi	millicurie(s)
mg	milligram(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
min	minute(s)
M	molar
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced form)
%	per cent
rpm	revolutions per minute
Trizma or tris	tris(hydroxymethyl)amino methane
eV	electron volt(s)
% V/V	ml of active substance in 100 ml of product
% W/V	g of active substance in 100 ml of product