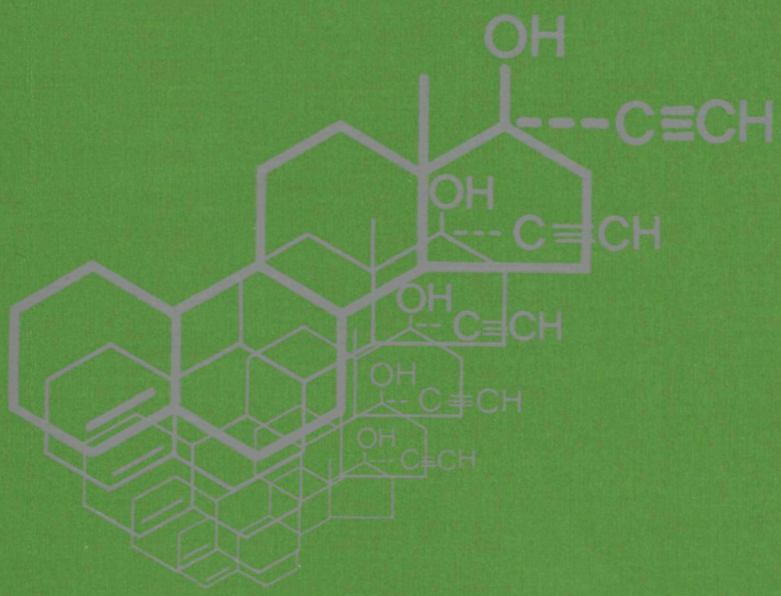
ESTROGEN RECEPTOR INTERACTION AND PHARMACOKINETICS OF CONTRACEPTIVE STEROIDS

in vivo and in vitro studies on lynestrenol and related compounds



J.M.G. van Kordelaar

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ESTROGEN RECEPTOR INTERACTION AND PHARMACOKINETICS OF CONTRACEPTIVE STEROIDS

IN VIVO AND IN VITRO STUDIES ON LYNESTRENOL AND RELATED COMPOUNDS

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ESTROGEN RECEPTOR INTERACTION AND PHARMACOKINETICS OF CONTRACEPTIVE STEROIDS

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR A. J. H. VENDRIK
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IN HET OPENBAAR TE VERDEDIGEN
OP VRIJDAG 9 JUNI 1978,
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GEBOREN TE BRUNSSUM

1978
DRUKKERIJ BRAKKENSTEIN, NIJMEGEN
THE NETHERLANDS

ter nagedachtenis van mijn vader
aan mijn moeder
voor José en Suzanne

Bij de voltooiing van dit proefschrift wil ik uitdrukking geven aan mijn gevoelens van erkentelijkheid jegens eenieder, die op direkte dan wel indirekte wijze een bijdrage aan het onderzoek heeft geleverd. Hierbij denk ik op de eerste plaats aan de voortdurende belangstelling, adviezen en waar nodig daadwerkelijke hulp van velerlei aard van de zijde van de medewerkers en medewerksters van het Farmacologisch Laboratorium.

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STEROID NOMENCLATURE*)

The basic steroid nucleus consists of three fused cyclohexane rings and one cyclopentane ring as shown in Fig. 1a for pregnane, which illustrates the atomic numbering and ring nomenclature. The name of the steroid skeletone having no carbon side chain at C_{17} is androstane (Fig. 1b). When additionally also the carbon atoms numbered 19 and 18 are lacking, the skeletone is named estrane and gonane respectively.

In Figs. 1c and 1d pictures are shown indicating the three-dimensional structure of androstane. Of the two possible substituents on any given carbon atom in the steroid nucleus the one emanating from the nucleus on the same side as the C_{18} and C_{19} angular methyl groups is called β-oriented, whereas the one on the opposite side is called α-oriented. A β-oriented bond is pictorially represented by a solid line and an α-oriented bond by a broken line. A bond whose orientation is unknown is represented by a wavy line (~~~) and denoted by the letter ξ. Substituents lying in the plane of the steroid nucleus are equatorial substituents, whereas those extending away from the central plane are axial substituents. The configuration of hydrogen at position 5 should always be designated by adding α, β, or ξ after the numeral 5, with the numeral and letter being placed immediately before the stem name. The configuration of a substituent attached at any other center of asymmetry in the nucleus is stated by adding α, β or ξ after the numeral denoting the position. Most of the naturally occurring steroids including the sex hormones are derivatives of 5α-androstane.

Derivatives formed by modification of, or introduction of substituents into, a parent compound (e.g. androstane, estrane, gonane, pregnane) are named by the usual methods of organic chemistry. Unsaturation is indicated by changing terminal "-ane" to "-ene", "-adiene", "-yne", etc. or "-an" to "-en", "-adien", "-yn", etc. and a positional numeral placed immediately before the stem name. Most substituents can be designated either as prefixes or suffixes, a few only as prefixes, e.g. halogens and alkyl groups. When possible one type of substituent must be designated as suffix. When more types are present, the other type(s) must be designated as prefix(es). The choice for suffix is made according to an order of preference, that is laid down in the Rules referred to below.

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*) Nomenclature according to IUPAC conventions as far as pertinent to this thesis. For the complete guidelines see IUPAC-IUB 1967 Revised Tentative Rules for Steroid Nomenclature, *Biochim. Biophys. Acta 164*, 453 (1968).

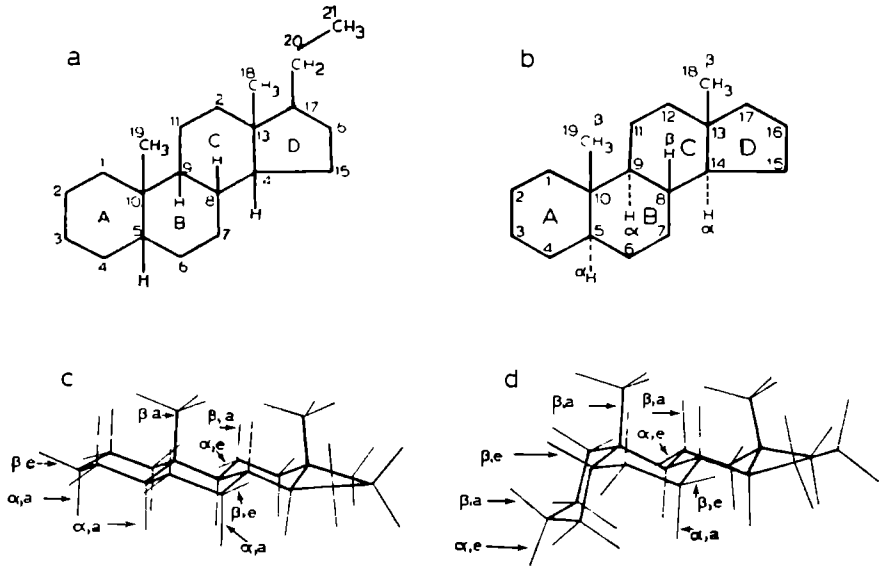


Fig. 1 — a. Pregnane skeletone, showing atomic numbering and ring nomenclature.

b. Formula of 5α-androstane according to the conventional projection on the plane of the paper, showing the α and β orientation of hydrogen atoms and C_{18} and C_{19} carbon atoms.

c, d. Three-dimensional structure of 5α-androstane (c) and 5β-androstane (d), indicating equatorial (e) and axial (a) bonds.

SURVEY OF THE TRIVIAL NAMES USED IN THE TEXT AND THE CHEMICAL NAMES OF THE STEROIDS

Chlormadinone	6-chloro-17α-hydroxy-4,6-pregnadiene-3,20-dione
Chlormadinone acetate	6-chloro-3,20-dioxo-4,6-pregnadien-17α-yl acetate
Corticosterone	11β,21-dihydroxy-4-pregnene-3,20-dione
Estradiol	1,3,5(10)-estratriene-3,17β-diol
Estradiol-17α	1,3,5(10)-estratriene-3,17α-diol
Estrenol	4-estren-17β-ol
Estrenone	4-estren-17-one
Estriol	1,3,5(10)-estratriene-3,16α,17β-triol
Estrone	3-hydroxy-1,3,5(10)-estratrien-17-one
Ethinylestradiol	17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol
Ethisterone	17α-ethynyl-17β-hydroxy-4-androsten-3-one
Ethynodiol	17α-ethynyl-4-estrene-3β,17β-diol
Ethynodiol diacetate	17α-ethynyl-4-estrene-3β,17β-diol diacetate
Hydroxyprogesterone	17α-hydroxy-4-pregnene-3,20-dione
Lynestrenol	17α-ethynyl-4-estren-17β-ol
Lynestrenol acetate	17α-ethynyl-4-estren-17β-yl acetate
Medroxyprogesterone	6α-methyl-17α-hydroxy-4-pregnene-3,20-dione
Medroxyprogesterone acetate	6α-methyl-3,20-dioxo-4-pregnen-17α-yl acetate
Megestrol	6-methyl-17α-hydroxy-4,6-pregnadiene-3,20-dione
Megestrol acetate	6-methyl-3,20-dioxo-4,6-pregnadien-17α-yl acetate
Methyllynestrenol	6α-methyl-17α-ethynyl-4-estren-17β-ol
Norethindrone	17α-ethynyl-17β-hydroxy-4-estren-3-one
Norethindrone acetate	17α-ethynyl-3-oxo-4-estren-17β-yl acetate
Norethynodrel	17α-ethynyl-17β-hydroxy-5(10)-estren-3-one
Norgestrel	13β-ethyl-17α-ethynyl-17β-hydroxy-4-gonen-3-one
Nortestosterone	17β-hydroxy-4-estren-3-one
Progesterone	4-pregnene-3,20-dione
Testosterone	17β-hydroxy-4-androsten-3-one

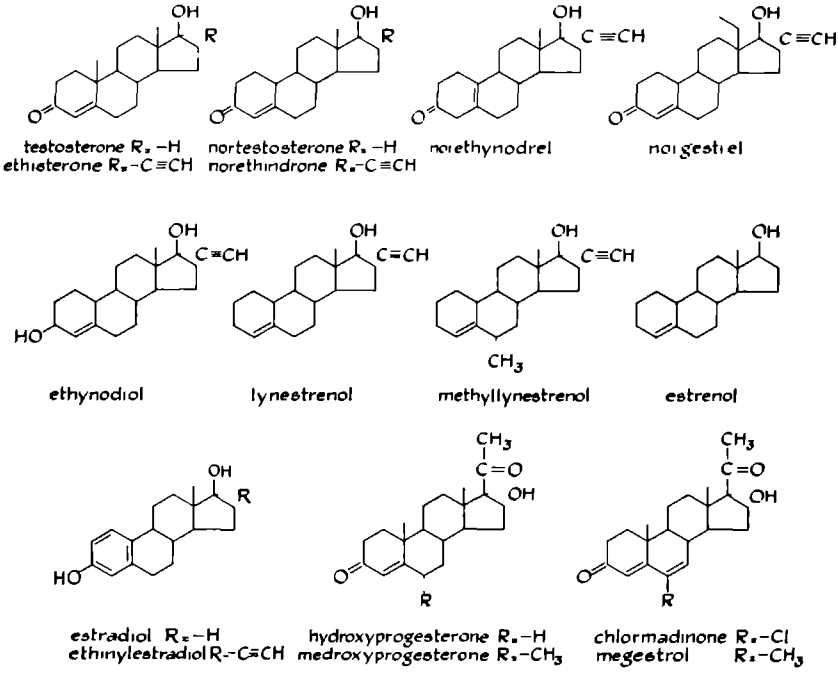


Fig. 2 — Formulae of steroids used in the present investigations.

SECTION I

SCOPE AND INTENT

CHAPTER 1

GENERAL INTRODUCTION

PRINCIPLES OF DRUG ACTION

The effect of most drugs in biological systems must be regarded as the ultimate consequence of a physicochemical interaction between that drug and certain functionally important molecules in the living organism (Ariëns et al., 1964). These sites of action are localized in what is called the target tissue of the drug. The tissue elements with which the drug combines in a more or less specific way and which convert chemical information into a biological response are called receptors.

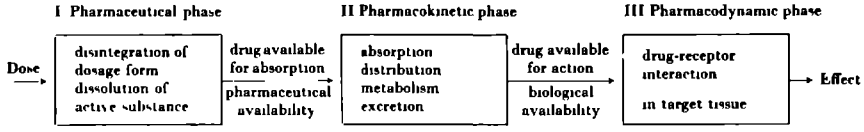
The strenght of the biological response elicited by a bioactive agent is for most drugs dose dependent and may, therefore, be thought to be related with the drug concentration in the direct environment of the receptors, viz. the target tissue or receptor compartment. The concentration of the drug in the target tissue is governed by the time course of the free drug concentration in the blood, i.e. the fraction of the drug not bound to plasma proteins.

The drug may enter the blood circulation system directly by intravascular administration or by absorption from a depot like occurs in case of oral, rectal and intramuscular administration. Immediately after drug administration and during the absorption process the drug is distributed with the blood over the organism according to a pattern, that initially will be highly dependent on the relative blood supply to the various organs and tissues. This first stage distribution pattern may considerably differ from the pattern under steady-state conditions, when a constant blood level of the drug is maintained, e.g. by means of zero-order infusion. Under these circumstances the distribution pattern will depend on the mutual affinity of tissue and drug for each other. Generally, however, there will be no equilibrium between drug supply and elimination of the drug from the circulation. Then progressive elimination of the drug from the circulation and from the biosystem will occur as a result of continuing biotransformation and/or excretion processes. The rate of each of these processes of distribution, biotransformation and excretion, that thus will contribute

References p. 20

simultaneously to the termination of drug action, is determined by the physicochemical properties of the drug and its interaction with the tissues responsible for the elimination (Goldstein et al., 1974).

So, between the administration of a certain drug product and drug action three main phases can be distinguished as depicted in the following scheme (Ariëns & Simonis, 1974):



It follows that the effective concentration of the drug at the receptor sites and therefore the time course of drug action depends in a complex way upon the relative rates of the processes comprising the pharmaceutical and pharmacokinetic phase.

The pharmaceutical phase

The pharmaceutical phase covers the release of the drug from its dosage form as a result of the disintegration of the drug product and/or dissolution of the active ingredient. The extent and the rate of this process may be influenced by various factors such as the particle size and crystal structure of the drug as well as the specific properties of the dosage form itself, and determine the pharmaceutical availability of that particular drug product.

The pharmacokinetic phase

Pharmacokinetics is the quantitative study of the processes of drug absorption, distribution, biotransformation and excretion. Mathematical models are used to describe these processes with the ultimate aim to provide a tool for a proper understanding of the relationship between on the one hand the dose, dosage form and route of administration and on the other hand the drug concentration in the receptor compartment as a function of time (see e.g. Dost, 1968; Notari, 1975; Van Rossum, 1971; Wagner, 1971, 1975).

Various experimental techniques may be used dependent on the kind of in-

formation looked for. Obviously, animal studies offer more possibilities than studies in humans, particularly with respect to the investigation of the time course of drug distribution and metabolism (this thesis, Chapters 14, 15). Because repetitive sampling directly in the receptor compartment is generally not possible without loss of the integrity of the biosystem, drug concentrations *in vivo* are usually monitored by the analysis of blood and other body fluids and excreta. Although this approach is less straightforward, the information thus obtained is a quite useful substitute. For most drugs the kinetics of the drug-receptor interaction is very fast in comparison with the kinetics of drug absorption, distribution and elimination (Van Ginneken, 1977) and in these cases the time course of drug action will be reflected in the time course of the drug in the blood. This relationship has enabled in a number of selected cases even the use of drug levels as therapeutic guides in the individualization of drug dosage (Koch-Weser, 1975).

Complications, however, arise when drugs are retained in the target tissue for a relatively long period of time and when the evoked response results from a time-consuming multi-step biochemical process. Then the response may appear and persist after the elimination of the drug. For example, a long-term retention in the target cell nuclei has been shown to be a prerequisite for obtaining a full biological response in case of the estrogenic hormones as will be outlined in the second part of this chapter. For these compounds the relationship between dose and effect is largely obscured owing to pharmacokinetic as well as pharmacodynamic aspects of drug action.

The pharmacodynamic phase

Pharmacodynamics deals with the interaction of the drug with the molecular sites of action in the target tissue and the consequences thereof. This interaction is generally a reversible process. As a result of the interaction conformational changes occur in the receptor molecule, which constitute the stimulus finally leading via a sequence of biochemical events to the pharmacological response within the target tissue or elsewhere in the organism (Ariëns et al., 1964; Ariëns & Simonis, 1974; Ariëns & Beld, 1977). The relationship between the stimulus and the response is believed to be independent of the properties of the drug molecule. It is not quite clear, whether this concept is valid for the mechanism of action of steroid hormones. It was demonstrated by Anderson et al. (1975) that in contrast with the early estrogenic response the initiation of late estrogenic responses require prolonged nuclear retention of the estradiol-receptor complex (see second part of this chapter). So, in this model of action later events induced

by the estrogen-receptor complex are depending not only upon earlier events, but upon the continued binding and action of the hormone itself.

On the other hand it is the goodness of the fit of the drug molecule to the receptor site and, therefore, the chemical structure of the drug molecule, that will determine the affinity of the binding and that is essential for drug action. Depending on the particular structure of a drug molecule and the specificity of the binding sites of the numerous receptors in the organism one and the same drug may bind to more than one kind of receptors and may so display a mixture of activities. Dissociation of activities may be obtained by modification of the chemical structure using the results of receptor-binding studies as a guide-line (Ariëns, 1971; Baulieu, 1975; Raynaud, 1977; Van Kordelaar, 1973; this thesis, Chapter 11).

Besides the affinity to the receptor a drug needs what is called intrinsic activity to induce a stimulus. This characteristic indicates the efficacy of the drug-receptor interaction. This concept was introduced as it appeared necessary to discriminate between on the one hand agonists and on the other hand competitive antagonists. The latter compounds share with the agonists a certain affinity to the receptor site, but they lack, once bound, the capacity to induce in the receptor those conformational changes indispensable for the induction of the stimulus.

MECHANISM OF ACTION OF SEX STEROID HORMONES

The regulation of protein synthesis in the target tissues is undoubtedly the principal action of steroid hormones. After entering the target cell the hormone is bound to a specific hormone receptor. This hormone-receptor complex is then transferred to the nucleus in an "activated" form, where it is bound to the target cell genome. Subsequently, by a yet undefined process, the target cell responds by increased RNA synthesis followed by increased protein synthesis. Several excellent and extensive reviews have appeared recently, covering the properties of the steroid hormone receptors as well as the effects of the steroid-receptor interaction on target cell metabolism (Baulieu et al., 1975; Buller & O'Malley, 1976; Gorski & Gannon, 1976; Jensen & DeSombre, 1973; King & Mainwaring, 1974). In this chapter we will confine us to the estrogen receptor. Attention is mainly focused on the localization and properties of this receptor and general pharmacological implications thereof as far as pertinent to the investigations described in Section III of this thesis. Most of the studies reported thus far have made use of rats. Results obtained with other species including man are essentially similar.

Estradiol binding to target tissues

Estrogen responsive tissues have been shown to contain proteins with a high affinity and a limited capacity for the binding of estrogenic compounds. Their presence was first indicated by the striking affinity of these tissues for the endogenous hormone, estradiol, *in vivo*. Following the administration of physiological amounts of tritiated estradiol to immature rats (Jensen & Jacobson, 1960) or tritiated hexestrol, a synthetic non-steroidal estrogen, to goats and sheep (Glascock & Hoekstra, 1959) a selective accumulation and retention of radioactivity from the blood against a marked concentration gradient was found to occur in the uterus, vagina and anterior pituitary, but not in non-target tissues like e.g. skeletal muscle and diaphragm. It was subsequently demonstrated that in spite of its rapid biotransformation only unchanged estradiol was retained (Jensen & Jacobson, 1962; Stone, 1964). This selective process was found to be estrogen specific and stereospecific, since simultaneous administration of non-labelled estradiol or other estrogenic compounds like diethylstilbestrol markedly decreased estradiol binding, whereas the biologically inactive stereoisomer estradiol-17α as well as other non-estrogenic steroid hormones like testosterone and corticosterone were inactive in this respect (Noteboom & Gorski, 1965). Estrogen-specific accumulation and retention was afterwards also demonstrated in the anterior pituitary and various brain regions involved in estrogen action such as the preoptic area and hypothalamus (Eisenfeld & Axelrod, 1965, 1966; Kato & Villee, 1967; King et al., 1965; McEwen & Pfaff, 1970; Stumpf, 1970). A variety of steroids with estrogenic activity (for review see Jensen & DeSombre, 1972) as well as non-steroidal estrogenic and anti-estrogenic compounds like ethamoxytriphetol (Jensen, 1962), clomiphene (Eisenfeld & Axelrod, 1967; Kato et al., 1968; Roy et al., 1964), Parke Davis CI-628 (Callantine et al., 1968) and nafoxidine (Eisenfeld & Axelrod, 1967; Jensen et al., 1966; Terenius, 1970) prevented the characteristic preferential retention of estradiol in the uterus and vagina as well as in the anterior pituitary and hypothalamus.

The intracellular binding sites for estradiol

1. The cytosol receptor

The radioactive estradiol taken up by the rat uterus appeared to be associated with a different form of the receptor substance in the high speed (105,000xg) supernatant (cytosol) of the tissue homogenate than in the heavy, nuclear, sediment (Noteboom & Gorski, 1965; Talwar et al., 1964). The cytosol was

found to contain a macromolecule with a high affinity (dissociation constant 10^{-9}-10^{-10} M for estradiol) and specificity for the binding of estrogens (Gorski et al., 1968; Toft & Gorski, 1966). The remarkably tight binding of estradiol appeared to result from an extremely low rate of dissociation of the complex (Korach & Muldoon, 1974a; Sanborn et al., 1971; Truong & Baulieu, 1971). The destructive effect of proteinases (Noteboom & Gorski, 1965; Toft & Gorski, 1966) and the inhibition of estradiol binding by SH group blocking agents (Jensen et al., 1967; Kahwanago et al., 1970; Shyamala & Gorski, 1969) as well as the protective effect of the reducing agent mercaptoethanol (Mester et al., 1970) suggested that the receptor molecule contains an essential protein portion and that SH groups are important for its binding activity. The binding itself is of non-covalent character as the hormone could be easily extracted by organic solvents (Jensen et al., 1966; Maurer & Chalkley, 1967).

On ultracentrifugation in a sucrose density gradient the cytoplasmic estradiol-receptor complex could be localized in the 8-9S region (Erdos, 1968; Gorski et al., 1968; Rochefort & Baulieu, 1968). In the presence of 0.2-0.4 M KCl or NaCl the 8S complex is reversibly transformed into a more slowly sedimenting 4S entity (Erdos, 1968; Jensen et al., 1969b; Korenman & Rao, 1968; Rochefort & Baulieu, 1968). Forms with intermediate sedimentation characteristics can be obtained depending on the experimental conditions (Chamness & McGuire, 1972; Sica et al., 1976; Stancel et al., 1973). The native form of the free as well as of the estradiol-bound receptor in the cytoplasm is still unknown.

In agreement with previous *in vivo* findings estrogen binding macromolecules with properties similar to the estrogen binding sites of the uterus appeared also to be present in the cytosol of the anterior pituitary (Korach & Muldoon, 1974a; Mowles et al., 1971; Notides, 1970), hypothalamus (Eisenfeld, 1970; Korach & Muldoon, 1974b; Mowles et al., 1971), the lactating mammary gland (Gardner & Wittliff, 1973; Hsueh et al., 1973), liver (Chamness et al., 1975; Eisenfeld et al., 1977), adrenal (Müller & Wotiz, 1978) and some other tissues of the rat as well as of other species (for review see Baulieu et al., 1975).

2. *The nuclear receptor*

The major part of the uterine estradiol, which is found in the nuclear fraction of the rat uterus up to 6 hours after estradiol administration, appeared to be associated with chromatin (King et al., 1966; Maurer & Chalkley, 1967; Teng & Hamilton, 1968). Solubilization and extraction of the hormone, unaccompanied by DNA, can be accomplished with 0.4 M KCl at pH 8.5 (Puca & Bresciani, 1968), yielding an estradiol-receptor complex sedimenting at about

5S on sucrose density gradient centrifugation (Giannopoulus & Gorski, 1971b; Jensen et al., 1968; Steggles & King, 1970).

3. *Relationship between the nuclear and cytosol receptor*

Incubation of rat uteri with tritiated estradiol at 37°C revealed an intracellular distribution of radioactivity as well as the formation of 8S and 5S complexes in agreement with the observations after hormone administration *in vivo*. When, however, the incubation was carried out at 2°C, the major portion of the radioactivity appeared to be present in the extranuclear region and appears as 8-9S complex in the cytosol on gradient centrifugation (Gorski et al., 1968; Jensen et al., 1968, 1969a, 1969b; Rochefort & Baulieu, 1969; Shyamala & Gorski, 1969). Following brief warming to 37°C redistribution of the steroid within the cell takes place yielding predominantly nuclear bound estradiol extractable as the 5S complex. Similarly, when estradiol was administered to immature rats *in vivo*, there was a progressive loss of uterine cytoplasmic receptor accompanied by a gradual increase in the amount of estrogen-receptor complex found in the nucleus (Anderson et al., 1973; Gorski et al., 1971; Sarff & Gorski, 1971). Exposure to high levels of estradiol caused almost complete disappearance of the cytosol receptor *in vivo* as well as *in vitro* (Giannopoulos & Gorski, 1971a).

Further support for the direct transport of cytosol receptor to the nucleus can be derived from the demonstration that formation of the nuclear receptor was dependent on the presence of both estradiol and uterine cytosol (Jensen et al., 1968, 1972a; Musliner et al., 1970). Transformation of the 4S cytosol receptor to the 5S form without loss of its nuclear binding affinity could be accomplished by incubation of the cytosol with estradiol in the absence of nuclei at 25-37° but not at 0°C (Jensen et al., 1972a). No evidence for the presence of the 5S binding protein in the uterine nuclei before estradiol administration could be obtained.

At present, it is not yet clear whether the change of sedimentation coefficient of 4S to 5S is related to a purely conformational change or to the addition of a subunit (Notides & Nielsen, 1974). Also the relevance of the temperature dependence of the receptor "activation" as observed *in vitro* for hormone action *in vivo* is still questionable.

Significance of estradiol receptor binding for estradiol action

Receptor binding and transformation and subsequent translocation of the estradiol-receptor complex to the nucleus of the target cell appears to be an

indispensable step for estradiol action (Buller & O'Malley, 1976). It has been shown to occur *in vivo* in the rat during physiological fluctuations of estradiol levels during the estrous cycle (Clark et al., 1972). Estrogen receptor binding to nuclear acceptor sites was also found to be positively related to early and late uterotrophic responses (Anderson et al., 1972a, 1973, 1974). Growth responses were proportional to the quantity of estrogen-receptor complex, which remained bound to the nucleus for 6 hours (Anderson et al., 1975). Recently, the different response of the uterus to the "impeded" estrogen estriol (Huggins & Jensen, 1955) as compared with estradiol could be correlated with the short time of half-life of the estriol-nuclear receptor complex (Clark et al., 1977). Thus nuclear retention rather than just nuclear binding may determine the cellular response.

The precise nature of the nuclear binding is still unclear and the presence and nature of specific acceptor sites for the steroid hormone-receptor complex is still controversial (Chamness et al., 1974; Müller et al., 1977) and will not be discussed.

Some pharmacological considerations

Assuming the general importance of estrogen and other steroid hormone receptors, the processes described above, although not quite understood, provide a rational and useful basis to explain the actions of estrogenic drugs and anti-estrogens on a molecular level. Conversely, exploring the nature of the drug-receptor interaction and of the corresponding target cell response will contribute to a better understanding of the relationship between the chemical structure and drug action, badly needed for the design of highly active and, perhaps equally important in the development of drugs with steroid hormone action, "pure" drugs with a small spectrum of activities (Raynaud, 1977).

The binding and the binding parameters of a variety of estrogenic and anti-estrogenic compounds to the estrogen receptor have been studied *in vivo* and *in vitro* (Black & Kraay, 1973; Chernayaev et al., 1975; Geynet et al., 1972; Hähnel et al., 1973a,b; Jensen & DeSombre, 1972; Jensen et al., 1972b; Makler & Eisenfeld, 1974; Raynaud et al., 1973; Rochefort et al., 1972; Shutt & Cox, 1972; Skidmore et al., 1972; Van Kordelaar et al., 1975a,b). Estrogenic compounds or their metabolites all display binding to a varying extent depending on their chemical structure. In view of the model of estrogen action introduced by Jensen et al. (1968, 1972a) and Gorski et al. (1968) as outlined above, the nature, the magnitude, and the duration of the response evoked will be dependent on various factors, including, besides physiological factors like

the presence and the intracellular concentration of receptor and the endogenous hormone concentration, pharmacological factors like the drug concentration in the target tissue, the binding affinity of the drug to the receptor and the efficacy thereof.

1. Receptor availability

After the entrance of the drug molecule into the target cell the responsiveness of the cell to an estrogenic or anti-estrogenic drug is determined in the first place by the availability of the cytosol receptor for binding. If the intracellular concentration of receptor molecules is depleted one way or another, the cell will not show a response normal to the hormone or to the drug.

The concentrations of unoccupied specific binding sites in the uterus and anterior pituitary are of similar order of magnitude in the castrated and cycling rat (Ginsburg et al., 1975; Korach & Muldoon, 1974a; Morris, 1976; Notides, 1970). The concentration in the hypothalamus is at least ten times lower (Ginsburg et al., 1975; Korach & Muldoon, 1974a; Morris, 1976). Uterine concentrations decrease with age, highest levels being found in the prepuberal rat at the age of 20-30 days (Feherty et al., 1970; Lee & Jacobson, 1971), although there seems to be not a great difference in the binding capacity of the uterus between the groups of varying hormonal state. The total number of specific binding sites for estradiol is in the uterus about 10^{12}, in the anterior pituitary about 10^{11} and about 10^{10} in the hypothalamus. Literature data in this respect, however, are not unequivocal and do not always allow a valid comparison to be made, because the binding capacities are generally expressed on basis of DNA, tissue or cytosol protein contents instead of a quantity per whole organ.

Physiological fluctuations in the available or free cytoplasmic binding sites of the uterus, pituitary and hypothalamus occur during the estrous cycle (Feherty et al., 1970; Sen & Menon, 1978; Ginsburg et al., 1975; Kielhorn & Hughes, 1977; Lee & Jacobson, 1971). The fall in cytosol receptor levels closely approximates the phase of the cycle when the highest plasma levels of estradiol are found, namely at pro-estrus (Brown-Grant et al., 1970; Butcher et al., 1974), and coincides with the period of maximal accumulation of estradiol receptor in the uterine and hypophyseal nuclei (Clark et al., 1972; Jacobelli, 1973; Sen & Menon, 1978).

Apart from a reduction of available binding sites as a consequence of estradiol binding, the re-synthesis or replenishment of the cytoplasmic receptor is an important factor in receptor availability. Replenishment has been shown to be stimulated by estradiol (Clark et al., 1973; Morris, 1976; Sarff & Gorski,

1971) and is most markedly at estrus (Sen & Menon, 1978), whereas evidence is accumulating that progesterone can antagonize or suppress this process (Coulson & Pavlik, 1977; Hsueh et al., 1975, 1976).

In the light of this physiological variability of the available specific binding sites it seems probable that in addition to the dose, the timing of drug administration, particularly for those drugs, which are rapidly eliminated, may be of critical significance for the intensity of the effect.

2. *Receptor binding*

Binding of estrogens to the cytosol receptor proteins is considered to be a prerequisite for action. According to the occupation theory for drug action there is a direct relationship between the number of drug-receptor complexes formed and the pharmacological response. In the absence of estradiol and under conditions of equilibrium the relative number of occupied binding sites is dependent on the affinity of the drug to the receptor. An estrogenic response may then be observed when the drug under consideration is an agonist or a partial agonist. The anti-estrogenic activity of an antagonist without intrinsic activity will become apparent only in the presence of the agonist.

In the presence of estradiol, binding of a drug to the receptor will interfere to some extent with estradiol receptor binding. As a consequence of this interaction and depending on the total number of bindings sites occupied, the ratio between the sites occupied by estradiol on the one hand and the drug on the other hand and the nature of the stimulus-effect relationship an estrogenic response may be observed, when the drug involved is an agonist, an estrogenic or an anti-estrogenic response when the drug is a partial agonist and an anti-estrogenic response when the drug is an antagonist without intrinsic activity.

Recently, it has been shown by Bouton & Raynaud (1978) that besides the affinity of an estrogen to the estradiol receptor also the single rate constants of association and dissociation should be taken into account. The dissociation and association constant of the drug-receptor interaction reflect the binding affinity of the drug under equilibrium conditions, which may be quite different from the actual situation within the cell *in vivo*. Depending, therefore, on the kinetics of the receptor transformation and the nuclear translocation of the drug-receptor complex and on the other hand on the rate of elimination of the unbound drug from the target tissue, the kinetic rate constants of the drug-receptor complex may become relevant parameters for the initiation of drug action and the evaluation of drug activity.

3. *Intrinsic activity*

The intrinsic activity of a drug or the efficacy of the drug-receptor interaction is generally thought to be materialized in the conformational change and concurrent "activation" of the receptor molecule induced by the binding of the drug. Although a direct analysis and quantification of this efficacy at the level of the drug-receptor complex is not yet possible, the efficacy may be assumed to be reflected somehow in one of the consecutive steps subsequent to the formation of the drug-receptor complex. Investigations with respect to the mechanism of action of estrogenic and anti-estrogenic compounds have provided some interesting examples, which are recorded below.

a. Nuclear translocation

Depending on the nature of the drug-receptor complex nuclear translocation may be inhibited. This kind of inefficacy of the drug-receptor interaction has been demonstrated to be the mechanism of action for the anti-mineralocorticoid spirolactone SC-26304 (Marver et al., 1974). This spirolactone and aldosterone, antagonist and agonist respectively, appeared to compete for the same binding sites in renal cells. In contrast, however, with the rapid translocation of the aldosterone-receptor complex to the nucleus under appropriate conditions, no such nuclear translocation was observed for the spirolactone-receptor complex.

b. Time of half-life of the chromatin-bound complex

It has been demonstrated by Anderson et al. (1975) that estrogenicity as reflected by stimulation of uterine growth, is directly related to the length of time, which the estradiol-receptor complex is retained in the nucleus of uterine cells and that there is a requirement of at least 4-6 hours for the induction of true growth. Certain estrogens such als estriol are incapable to induce long-term uterine growth responses in the bioassay and are therefore called "impeded" estrogens (Huggins & Jensen, 1955). Recently, Clark et al. (1977) could show that the limited uterotrophic response when estriol is administered acutely by injection, was due to the short half-life of the estriol-nuclear receptor complex. When estriol was administered continuously the substance produced an uterotrophic response quantitatively and qualitatively indistinghuishable from that induced by estradiol itself.

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c. Replenishment of the estrogen receptor

The synthesis of the estrogen receptor, which is regulated by estradiol among others, has been shown to be also sensitive to drug action. After an injection of estrogen in the rat, the uterine cytoplasmic receptor is depleted owing to the translocation of the hormone-receptor complex to the nucleus. Maximal depletion of cytoplasmic receptor has been found to occur at one to four hours (Cidlowski & Muldoon, 1974; Gorski et al., 1971). During the period when cytoplasmic receptor is reduced the uterus is insensitive to additional exogenous estrogen (Anderson et al., 1974). This state is followed by replenishment and eventually overshoot in the number of binding sites 24 h later (Sarff & Gorski, 1971). It is obvious, that any agent interfering with this replenishment process is a potential anti-estrogen. Some of the non-steroidal anti-estrogens, e.g. nafoxidine, clomiphene and CI-623, act probably via this mechanism (Clark et al., 1973, 1974) and also progesterone (Hsueh et al., 1975, 1976). A point of major interest is, that the latter compound is devoid of estrogenic activity in contrast with the former ones. The simultaneous administration of progesterone and estradiol results in inhibition or modification of estrogen-induced growth and differentiation of target organs. Progesterone, however, does not compete for the estrogen receptor binding sites (Toft & Gorski, 1966) nor does it interfere with the translocation of the estrogen-receptor complex to the nucleus (Anderson et al., 1972b). So it may be inferred that interference with estradiol binding to cytoplasmic receptor sites is not essential for the inhibition of an estrogenic response.

On the other hand nafoxidine, given alone or together with estradiol, appeared to be at least as effective as estradiol alone in stimulating uterine growth. The antagonistic properties of nafoxidine were observed only when a second injection of estradiol was administered 24 hours after nafoxidine treatment. Clark et al. (1974) concluded that nafoxidine was an estrogen-antagonist not because it competed with estradiol for the receptor binding sites, as it actually does (Jensen et al., 1966; Rochefort et al., 1972; Terenius, 1970), to produce a complex that is a poor stimulator of growth in the target cell nucleus, but because it failed to stimulate the replenishment of the receptor.

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CHAPTER 2

THE CONTRACEPTIVE STEROIDS

HISTORY AND MEDICAL USE

The synthetic progestins which are the subject of the present investigations, have been the product of extensive research efforts in the pursue of orally active steroid hormones. Although these compounds are also used in the treatment of various gynaecological disorders with notable therapeutic success (Murad & Gilman, 1975) and although, actually, the treatment of infertility by the temporarily inhibition of ovulation under the influence of progestational agents was an important aspect of the initial studies of Rock et al. (1957), their principal application is in the field of oral contraception.

The physiological parallel of their activity is encountered in the anti-ovulatory action of progesterone, which becomes manifest in pregnancy, when huge quantities of this hormone are produced in the corpus luteum and later on in the placenta and as a result ovulation is abolished. The same effect can be obtained in normally menstruating women by the oral administration of progesterone (Pincus, 1956, 1959; Tyler, 1961). Very high doses, however, of 300 mg and more were needed to achieve this purpose, since progesterone undergoes rapid biotransformation in the organism. Large-scale oral contraception became thus a reality first, when highly potent orally active steroids were synthetized.

A very potent orally active estrogen became available as early as 1938, when ethinylestradiol was synthetized by Inhoffen & Hohlweg (1938). During the same year in the search for an orally active androgen they succeeded in the synthesis of 17α-ethynyltestosterone or ethisterone, the first orally active progestin (Inhoffen et al., 1938). Derivatives of this compound, lacking the angular methyl group at C_{10}, the so-called 19-nortestosterone derivatives, appeared to be much more active. The synthesis of 17α-ethynyl-19-nortestosterone, norethisterone or norethindrone, was achieved by Djerassi et al. (1954) in 1952. Almost at the same time Colton (1953, 1954) reported the synthesis of norethynodrel. Both compounds proved to be very potent progestins and retained their activity when

given by mouth. Inhibition of ovulation by these compounds was demonstrated in rabbits by Pincus et al. (1956). Extension of these studies (Pincus et al., 1958; Rock et al., 1956, 1957) revealed their effectiveness also in humans. Oral administration in doses of 10 mg a day from the 5th to 25th day of the cycle suppressed ovulation. Fertility returned on cessation of treatment.

In addition to preventing ovulation any practical therapy should also provide for withdrawal bleeding with properties approaching the promptness, amount and duration of normal menstruation. Continued research revealed (Pincus, 1959, 1965) that regular menstrual-like bleedings were obtained most frequently when progestins were administered together with a synthetic orally active estrogen, ethinylestradiol or mestranol (*combination-type* contraceptive). It is to accomplish this goal that both estrogen and progestin are still included in most contraceptive regimens. In 1957 Enovid®, containing 10 mg norethynodrel and 0.15 mg mestranol, was released by the US government first for the treatment of menstrual disorders, and subsequently in 1960 as an oral contraceptive.

The other compounds belonging to the same class of 19-nortestosterone derived progestins and currently still in use represent later developments. Ethynodiol diacetate was also synthetized by Colton (1958), being followed by lynestrenol in 1959 (De Winter et al., 1959). In contrast with the former steroids norgestrel, which appeared to be the most potent progestin of this group (Edgren et al., 1966, 1967), was obtained by total synthesis (Smith et al., 1963) according to a novel procedure developed by Hughes & Smith (1960). Comparison of the biological activity of the so obtained racemic *dl*-norgestrel with the resolved enantiomers showed, that the activity resides in the "natural" *d*-form and that the *l*-form is devoid of activity (Edgren et al., 1963). These observations are consistent with the high binding affinity of *d*-norgestrel to the progesterone receptor and the absence of binding in the *l*-form (Terenius, 1974).

The second class of synthetic progestins in use for contraception is the group derived from 17α-hydroxyprogesterone, comprising medroxyprogesterone acetate, megestrol acetate and chlormadinone acetate (Babcock et al., 1958; Ellis et al., 1960; Ringold et al., 1959). The latter compounds have been recently withdrawn from medical use after the reported appearance of mammary nodules in beagles during chronic treatment with these drugs. The relevance, however, of these findings for the human situation has been questioned (Briggs, 1977). The former compound, medroxyprogesterone acetate, is principally in use for parenteral administration as a long acting depot contraceptive.

When through the years experience was gained it appeared possible to reduce the dose of the progestin and later on also the estrogen content considerably without loss of the contraceptive effectiveness (Bye & Elstein, 1973; Elstein et al., 1974, 1976; Preston, 1972; Schneider et al., 1974; Virtavuo et al., 1977).

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A still further decrease of the progestin content may be feasible by choosing a route of administration which avoids the first passage through the liver (this thesis, Chapter 16).

A successful modification of the combined preparations with fixed doses of the active constituents over the entire cycle are the *step-up* pill and the *sequentials*. With the former type the progestin content is reduced during the first 11 days of the contraceptive regimen and in the sequentials the progestin is even completely omitted during the first 7 days of treatment. This method has the appeal of a more close mimicry of the normal cycle. Both treatments, the combination method and the sequential method act by suppressing the secretion of the gonadotrophin releasing hormone, which in turn decreases the circulating FSH and LH levels. As a result follicular growth in the ovary is largely suppressed and the mid-cycle surge of LH, assumed to be of paramount significance for the occurrence of ovulation, is prevented (Becker et al., 1973; Dufau et al., 1970; Elstein et al., 1974; Schally et al., 1970; Thomas & Ferin, 1972; Thomas et al., 1972).

The rationale behind the introduction of another variant, the *minipill*, which contains only a progestin in a low dose, was the elimination of the estrogen. The estrogenic component is thought to be responsible for a large deal of the side effects of the former preparations. Although, taking the minipill, slight to profound changes in the pattern of gonadotrophin secretion have been observed (Collins et al., 1974; Friedrich et al., 1975; Moghissi & Marks, 1971; Taymor & Levesque, 1971) and inhibition of ovulation also at this low dose level, e.g. 0.5 mg lynestrenol daily, may occur, its principal mode of action is supposed to be the induction of a change in the consistency of the cervical mucus, rendering it hostile to sperm penetration. This and other actions may contribute also to the anti-fertility activity of the combined-type contraceptives, as the incidence of escape ovulations with these products is too high to explain their more than 99.9% effectiveness.

PHARMACOLOGY

Progesterone may evoke a variety of biological effects, each of which may be used to evaluate and characterize the synthetic progestins, too. Although progestational agents share by definition with progesterone the potency to induce the secretory transformation of the estrogen-primed endometrium, which is the yardstick for the evaluation of activity in the Clauberg test (Clauberg, 1930), other progesterone-like activity such as the maintenance of pregnancy, deciduomagenic activity, inhibition of ovulation, may be absent or less pronounced,

whereas some actions of progesterone may be antagonized. In addition to progestational properties some of the synthetic compounds display also markedly estrogenic, androgenic and anabolic activity. For this reason a range of bioassays is necessary to characterize adequately the biological profile of each newly synthetized steroidal compound. It is beyond the scope of this thesis to present here a survey of the *scala* of biological actions exhibited by the contraceptive progestins in the various bioassays and observed in clinical trials. Comprehensive reviews covering this subject have been edited by Junkmann (1968/1969) and Tausk (1972). The interpretation and the extrapolation of the outcome of the animal tests to the human have appeared to be complicated very much by inter-species differences, probably in part due to species differences in the pharmacokinetics of the compounds, and are hampered by a still incomplete understanding of the physiology of the endocrine systems involved (Neumann et al., 1977).

Confining us in this chapter to the progestational and estrogenic activity of the contraceptive steroids in animals and humans, then two classes can be discerned, viz. the hydroxyprogesterone derivatives and *d*-norgestrel on the one hand and the other 19-nortestosterone derivatives on the other hand. The compounds belonging to the former class have shown to be more potent in the Clauberg test (Edgren et al., 1967; Kontula et al., 1975; Neumann et al., 1968) and to exhibit a more progesterone-like character, effective as they are also in maintaining pregnancy. Furthermore, the estrogenic component present in the spectrum of activities of the nortestosterone derivatives is absent in *d*-norgestrel and chlormadinone acetate, medroxyprogesterone acetate and megestrol acetate (Edgren et al., 1967; Overbeek et al., 1962).

Estrogenic activity

The estrogenic activity of the 19-nortestosterone derivatives has been generally attributed to their *in vivo* conversion into phenolic metabolites, among others ethinylestradiol, which is as active as estradiol itself (Fotherby & James, 1972; Paulsen, 1965; Petrow, 1966). Ethinylestradiol and other phenolic metabolites were reported to be present in the urine of subjects treated with norethindrone or norethindrone acetate (Braselton et al., 1977; Breuer et al., 1960; Brown & Blair, 1960; Fotherby, 1970; Kamyab et al., 1968a; Langecker, 1961; Okada et al., 1964). Similarly, partial conversion of *dl*-norgestrel (Littleton et al., 1968) and lynestrenol (Kamyab et al., 1968b) to phenolic compounds has been reported. The *in vivo* aromatization of the contraceptive progestins to potentially estrogenic compounds has been a matter of concern because of the possible

correlation between estrogen content and side effects of the contraceptive pill, particularly the increased risk of thrombo-embolic disease (Inman et al., 1970).

The formation, however, of estrogenic metabolites has been questioned by Breuer (1970), who considered the formation of ethinylestradiol from norethindrone as an artefact arising during the fractionation of the urinary metabolites. His assumption was based on the observation by Townsley & Brodie (1966) that treatment of 1β-hydroxy-19-norandrostenedione with either acid or base results in the formation of estrone. Recently, Sisenwine et al. (1973, 1974) have isolated 1β-hydroxynorgestrel from the urine of women treated with *dl*-norgestrel and could confirm the aromatization of this steroid under acidic and mildly alkaline conditions. After reduction of the 1β-hydroxy-4-en-3-one grouping in the A ring of this metabolite with $NaBH_4$ the fraction of the phenolic excretion products appeared to be decreased to only 0.2-0.3% of the dose administered. Less than 0.01% of the dose could be identified as 18-homo-ethynylestradiol and 16β-hydroxy-18-homo-ethynylestradiol. Although the formation of phenolic compounds cannot be totally ruled out and has still to be studied for the other contraceptive progestins, a significant contribution to the estrogenic activity of the nortestosterone derivatives can hardly be expected on account of these findings.

Investigation of the interaction of the contraceptive progestins with the estrogen receptor is a useful and direct approach from the opposite direction, which may shed light on the question, whether the estrogenic activity observed *in vivo* is the result of bioactivation of these compounds or may be an intrinsic property inherent to their chemical structure (this thesis, Section III).

That bioactivation, on the other hand, may be an important condition for progestational activity is indicated for lynestrenol by the experiments of Kontula et al. (1975). The relative binding affinity of lynestrenol to the progesterone receptor of the rabbit uterus was found to be ten times lower than the affinity of norethindrone, whereas both compounds appeared to be equipotent in the Clauberg assay. The high disappearance rate of lynestrenol from the blood, its extensive biotransformation and poor bioavailability after oral administration as observed in the present investigations (this thesis, Section IV) is consistent with this assumption.

THE PRESENT INVESTIGATIONS AND THE STRUCTURE OF THIS THESIS

As outlined in Chapter 1 the relationship between chemical structure and drug action and on the other hand dose and effect is dependent upon the affinity of a drug to the receptor binding site and its pharmacokinetic behaviour. In the

present investigations attention has been paid to both of these aspects with the aim to gather more information about the properties of the contraceptive steroids. The interaction of these and other, structurally related, compounds with the estrogen receptor of the rat and calf has been studied *in vivo* and *in vitro* and the binding parameters of the binding steroids to this receptor have been determined (Section III). On the other hand the pharmacokinetics of one of these widely-used drugs, lynestrenol, has been thoroughly explored in the rat and mouse (Section IV). For this latter part of the investigations the synthesis of tritiated lynestrenol had to be accomplished, because no analytical procedure was available, which permitted the determination of the low blood concentrations of this compound in the experiments envisaged. The synthesis of lynestrenol-^{3}H and the determination of the lipophilicity and protein binding of this compound are described in Section II. This section comprises furthermore a description and account of those materials and methods, which have been employed in more than one chapter of Sections III and IV. In order to preserve the integrity of these chapters as much as possible a description of technical and analytical procedures pertinent to only one of these chapters has been incorporated in the chapter concerned.

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SECTION II

GENERAL EXPERIMENTAL PART

SPECIFICATION OF CHEMICALS, SOLVENTS AND STEROIDS

CHEMICALS

The chemicals and solvents were of analytical reagent grade (Merck, Darmstadt, GFR or Baker, Deventer, The Netherlands) and used without further purification, unless otherwise indicated.

STEROIDS

Radioactive steroids

Estradiol-6,7-^3H —. Tritiated estradiol with a specific activity of 40 Ci/mmol, prepared by the reduction of estrone-6,7-^{3}H with sodium borohydride, was obtained from the Radiochemical Centre, Amersham, U.K. The compound was stored as a solution in benzene/ethanol 90/10 in tightly closed silanized counting vials at a temperature of 1-4°C. The identity and radiochemical purity of the hormone were authenticated by thin-layer chromatography over silicagel pre-coated glass plates (Kieselgel F_{254}, Merck) after the addition of 25 μg estradiol to serve as carrier for the labelled compound in order to avoid adsorption phenomena. The solvent systems used were chloroform/acetone 70/30 (R_f value estradiol 0.41) and chloroform/ethylacetate 80/20 (R_f value estradiol 0.25). An estradiol marker was run on the same plate. Radioactive spots were localized by scanning (Berthold Zählratenmesser LB 2722), scraped off the plate and assayed for radioactivity by liquid scintillation counting. The radiochemical purity was always more than 98%. In experiments, where a lower specific activity sufficed, the specific activity was reduced by the addition of an aliquot of the non-labelled hormone.

Lynestrenol-^3H —. Tritiated lynestrenol with a specific activity of 800 mCi/mmol, was prepared from estrenone-16-^{3}H by reaction with acetylene and

potassium t-butoxide in tetrahydrofurane. The tritiation of estrenone and the synthesis of lynestrenol-^{3}H are described in Chapter 4. The compound was stored as a solution in benzene/ethanol 90/10 in tightly closed silanized counting vials at a temperature of 1-4°C. The radiochemical purity was checked regularly by means of thin-layer chromatography over silicagel as described above. A co-chromatographed lynestrenol marker was localized by exposing the plate to iodine vapour. The solvent systems used were toluene/ethanol 90/10 (R_f value lynestrenol 0.7) and cyclohexane/ethylacetate 90/10 (R_f value lynestrenol 0.3). Whenever the purity decreased below 97% the material was purified by thin-layer chromatography in the solvent system n-heptane/acetone 80/20 (R_f value lynestrenol 0.5) and subsequent extraction of the lynestrenol from the scraped silicagel with dichloromethane/methanol 70/30. Following this procedure more than 95% of the tritiated lynestrenol could be recovered. During the investigations the specific activity of the compound was corrected for the radioactive decay of the tritium. In experiments, where a lower specific activity of the compound sufficed, the specific activity was reduced by the addition of an aliquot of the non-labelled steroid.

Lynestrenol-4-^{14}C —. Lynestrenol-4-^{14}C with a specific activity of 32.5 mCi/mmol was generously supplied by Organon International, Oss, The Netherlands. It was stored as a solution in benzene/ethanol 90/10 at 1-4°C. The authentication of the identity and radiochemical purity and the, rarely necessary, purification of the compound were accomplished as described above for the tritiated steroid. The radiochemical purity of the material used in the experiments was always more than 98%. In experiments, where a lower specific activity sufficed, the specific activity was reduced as described above.

Non-labelled steroids

The non-labelled steroids have been obtained from the following sources. We gratefully acknowledge a gift of lynestrenol, lynestrenol acetate, estrenol, estrenone, methyllynestrenol and 17α-ethynyl-5α-estran-17β-ol from Organon International (The Netherlands), chlormadinone and chlormadinone acetate from Syntex Corp. (USA), megestrol and megestrol acetate from Novo Industri (Denmark), medroxyprogesterone and medroxyprogesterone acetate from Upjohn Comp. (USA), ethynodiol and ethynodiol diacetate from G. D. Searle & Co. (USA) and *d*-norgestrel and *dl*-norgestrel from Schering (The Netherlands). The other steroids were purchased from Sigma Chem. Corp. (USA) with the exception of hydroxyprogesterone (Merck, GFR).

The purity of these compounds was checked by thin-layer chromatography over silicagel (pre-coated plates Kieselgel F_{254}, Merck) and/or gas-liquid chromatography (Hewlett & Packard, Model 402). Additional purification, if necessary, was carried out by preparative thin-layer chromatography over silicagel (pre-coated plates Kieselgel F_{254}, layer thickness 2 mm, Merck).

CHAPTER 4

SYNTHESIS OF LYNESTRENOL-^{3}H*)

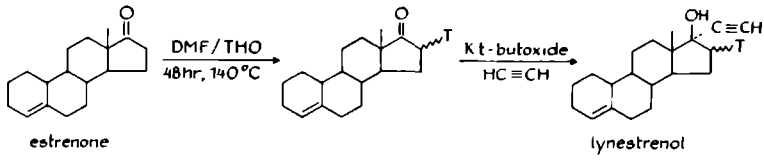
INTRODUCTION

In view of unsolved problems encountered in the development of an analysis of sufficient sensitivity and specificity for the assay of lynestrenol in biological samples the availability of tritiated lynestrenol with high specific activity was considered to be an indispensable tool.

Although lynestrenol is chemically closely related to norethindrone, norethynodrel and ethynodiol, tritium labelling as described for these compounds by Kepler & Taylor (1971) or Chaudhuri & Gut (1969) is not possible because of the lack of the oxygen atom at C_3. This also holds for tritium labelling of norethindrone as described by Rao (1971). For the same reason various other methods, based on partial reduction of unsaturated precursors, are not feasible. The only other way to obtain a high specific labelled product, apart from a multistep synthesis, would be a halogen-tritium replacement (Chaudhuri & Gut, 1969; Evans, 1966), but this again would result in complete saturation of the $C_{4,5}$ double bond.

The pharmacological studies envisaged required a compound with high specific activity, even at the expense of non-specificity of labelling. We considered that this may be best achieved by tritium labelling of estrenone (estr-4-en-17-one) via exchange with tritiated water (THO) in dimethylformamide followed by an ethynylation reaction at C_{17} to yield lynestrenol. The C_{16} hydrogen atoms of estrenone are susceptible to exchange for tritium under conditions causing enolization of the C_{17} oxo group. After subsequent incorporation of the 17α-ethynyl group these labile tritium atoms will be stabilized, provided they are not lost in the procedure. The introduction of the ethynyl group can be achieved in essentially the same way as described by

*) In co-operation with Mr. J. S. Favier (R & D Laboratories, Organon Scientific Development Group, Oss, The Netherlands) and Mr. J. P. Kitcher (Organic Department, The Radiochemical Centre, Amersham, U.K.).



Djerassi et al. (1954), but with some adaptations for radio-isotope work on a micro-scale. The sequence of the reactions that was followed is depicted above.

The specific activity attained depends very much on the temperature and duration of the exchange reaction (Evans, 1966). For estrenone this appeared to be no problem: heating the non-labelled compound for a period of 16 hours at 160°C in an evacuated ampoule with dimethylformamide and water did not result in the formation of other products.

The contamination of estrenone-^{3}H with estranone-^{3}H after the exchange reaction, although not very probable, required special attention. Even small amounts of estranone-^{3}H, present in estrenone-^{3}H, would, if not removed, cause a serious radiochemical contamination of lynestrenol-^{3}H. The two C_{17} oxo compounds can be separated by thin-layer chromatography (TLC) on silicagel silver nitrate plates (Lisboa & Palmer, 1967), but this method cannot be used after the introduction of the 17α-ethynyl group.

The radioactive work described below was carried out under contract at the Radiochemical Centre (Amersham, U.K.) by Mr. J. P. Kitcher.

MATERIALS

All reagents were of analytical reagent grade.

Estrenone was obtained from Steraloids Ltd., Croydon, U.K. Lynestrenol, used as a carrier during the identification of lynestrenol-^{3}H, was obtained from Organon, Oss, The Netherlands. Identity and purity of these compounds was authenticated by IR spectroscopy, melting point, mass spectrometry, and gas-liquid chromatography.

The following solvents were specially purified. Dimethylformamide (DMF) was stored over molecular sieve 4A (BDH Ltd., Poole, Dorset, U.K.) for 2 days, decanted and then distilled. Tetrahydrofuran (THF) was distilled under nitrogen from lithium aluminium hydride, after standing over this compound for 24 hours. The distillate was stored under nitrogen, in the dark, in a phosphorous pentoxide desiccator and used within five days. Potassium t-butoxide was prepared by reacting cleaned potassium (Pearson, 1967) with t-butanol under nitrogen. The solution was pumped to dryness on a manifold

and the white residue purified by sublimation under reduced pressure. Benzene and ethanol used for storage of tritiated materials in solution were redistilled prior to use.

Acetylene was purified by passing it through a cold trap (-80°C), a mercury safety valve, an empty bottle, concentrated sulphuric acid, and a soda-lime tower.

RADIOACTIVITY MEASUREMENTS

Radioactivity measurements were made with a Nuclear-Chicago Mark I counter using the toluene: triton X100 scintillator of Patterson & Greene (1965).

THIN-LAYER CHROMATOGRAPHY

Analytical chromatography was performed on Merck 0.25 mm silicagel precoated plates, or with plates spread with 10% silver nitrate impregnated silicagel (thickness 0.25 mm). Active regions were located by scanning the developed plate with a windowless gas flow proportional counter. Steroid markers were visualized by spraying with a 2.5% w/v solution of ceric sulphate in 1 M sulphuric acid and heating at 110°C for about 10 minutes.

Preparative chromatography was performed on 1 mm silicagel PF_{254} coated plates. Active regions were located on developed plates by autoradiography. Labelled steroids were recovered by scraping off the silicagel and extracting with benzene/ethanol 90/10.

Table 4.1 shows the solvent systems, which were used, together with typical R_f values of steroids likely to occur in the crude reaction product.

RESULTS

Estrenone-16-^3H

Estrenone (144 mg) was dissolved in a mixture of purified dimethylformamide (1 ml) and tritiated water (0.3 ml, 200 Ci). This mixture was sealed in an ampoule under vacuum and heated at 140°C for 48 hours. After cooling the solvent was removed under vacuum, followed by removal of labile tritium from the product by repeated distillation under vacuum with benzene/ethanol 80/20. The residue was taken up in chloroform (1 ml) and purified by TLC on two 1 mm silicagel plates, developed in system C.

Table 4.1 — TLC data steroids

System	Solvents	Adsorbent	R_f values steroids[1])				
			I	II	III	IV	V
A	benzene/ethanol 99/1	silicagel	0.38	0.23	0.60	—	—
B	cyclohexane/ethyl acetate 90/10	silicagel	0.32	0.20	0.54	—	0.45[2])
C	cyclohexane/acetone 95/5	silicagel	0.29	0.23	0.60	—	—
D	cyclohexane/acetone 95/5	silicagel/$AgNO_3$	0.00	0.32	0.66	0.85	—

[1]) I lynestrenol, II estrenol, III estrenone, IV estranone, V 17β-ethynyl-estr-4-en-17α-ol.

[2]) Broess et al., 1975.

The purified estrenone-16-^{3}H was extracted from the silicagel with benzene/ethanol 90/10 and weighed after lyophilisation of the solution. The sample was redissolved in benzene/ethanol 90/10, the radioactivity measured and its specific activity calculated. The solution of estrenone-16-^{3}H was analysed by TLC in systems A, B, C and D.

Yield:	estrenone-^{3}H	1.8 Ci 40.5 mg
	specific activity	12 Ci/mmol
	radiochemical purity	98% in systems A, B, C and D

Lynestrenol-^{3}H

The reagents required for the conversion of estrenone-^{3}H to lynestrenol-^{3}H were manipulated in an enclosure filled with dry nitrogen. A solution of potassium t-butoxide in purified tetrahydrofuran (THF) (20 ml) was prepared. Aliquots (2 ml) of this were removed from the enclosure, diluted with water (1 ml) and then evaporated under a nitrogen stream to remove excess THF. These samples were then titrated against 0.1 M hydrochloric acid to determine the base concentration.

The solution of estrenone-^{3}H in benzene/ethanol was evaporated to dryness and 0.12 mmol (31.5 mg) of the residue was dissolved in purified THF (3 ml). This together with a rinsing of THF (2 ml) was transferred to a small separating funnel. The acetylene flask was evacuated and filled with acetylene (volume about 20 ml = 1 mmol). Potassium t-butoxide (0.2 mmol of base) in THF (5 ml) was placed in the reaction flask (Fig. 4.1) which was temporarily frozen with liquid nitrogen. The space above the solution in the separating funnel was partially evacuated and the main system was evacuated to a final pressure of about 10 Pa. The acetylene was condensed into the reaction flask and the stopcocks were closed. The system was warmed to 0°C and with vigorous stirring the estrenone-^{3}H solution was added from the separating funnel. This mixture was stirred for 3 hours. Nitrogen was admitted to the system and THF/water 20/1 (0.1 ml) was added to the reaction flask, cooled to 0°C. About 1 minute later Amberlite AG50 H^+ resin (0.5 g) was added and the mixture was stirred for a further 20 minutes, whereupon it was filtered and the filter washed with THF and benzene. The combined filtrate was rotary evaporated under reduced pressure to dryness. The residue was taken up in chloroform, a sample was taken for analysis, and the bulk of this solution applied to two 1 mm silicagel plates. These and also the analytical plate were developed with system B.

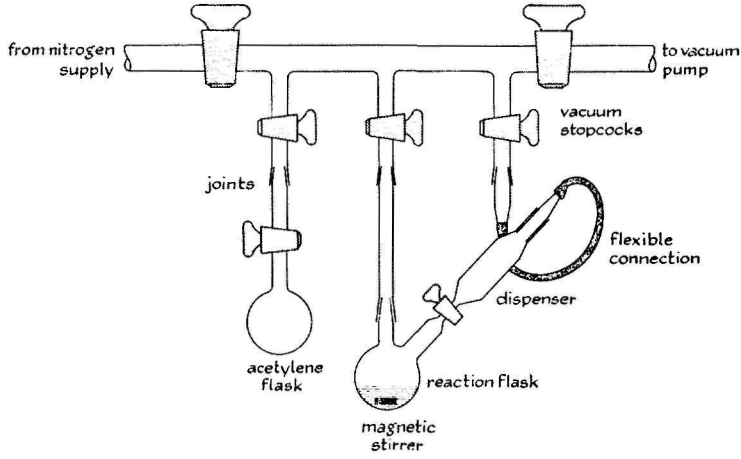


Fig. 4.1 — Schematic drawing of the apparatus used in the ethynylation of estrenone-16-^{3}H.

Lynestrenol-^{3}H and estrenone-^{3}H were removed separately from the plate and eluted from the silica with benzene/ethanol 90/10. The lynestrenol-^{3}H solution obtained was rotary evaporated to small bulk and transferred to a tared flask. The residual solvent was removed *in vacuo* at room temperature and pumped to constant weight. The crystalline residue was weighed, redissolved in benzene and the radioactivity was measured and the specific activity of the product calculated. Subsequently, the solution was analysed in systems A, B and C.

Yield:	lynestrenol-^{3}H	29 mCi	10.4 mg
	specific activity	800 mCi/mmol	
	recovered estrenone-^{3}H	52 mCi	

Radiochemical purity

After determination of the specific activity the lynestrenol-^{3}H, cochromatographed with carrier, was 98% pure in systems A and B, 97% pure in system C. The impurities were minor decomposition products which had arisen during the manipulation of the sample.

The recovered estrenone-^{3}H was 95% pure in system C. The principle impurity was lynestrenol-^{3}H.

The dimethylformamide/tritiated water exchange reaction was carried out three times in all, the quality (age and previous use) of the solvent mixture being varied. The best result was obtained with a freshly prepared solution. These results together with the yields in the subsequent ethynylation reactions are shown in Table 4.2. The conditions of tritiation in all cases resulted in loss of the starting material estrenone, but this was acceptable for the resultant decomposition products exhibited low R_f values in system C and were easily separated from estrenone-^{3}H.

In all cases the ethynylation reaction was accompanied by considerable loss of tritium from the C_{16} position. This is demonstrated by the activity yields of both lynestrenol-^{3}H and estrenone-^{3}H shown in Table 4.2. In the case where the specific activity of the recovered estrenone-^{3}H was measured, it was found to be similar to the product, lynestrenol-^{3}H. This result indicates that the activity found in the products after the ethynylation reaction could be in positions other than C_{16}. This non-specific introduction of tritium is perhaps to be expected, considering the vigorous conditions (140°C, 2 days) of the initial tritiation.

Loss of tritium from the C_{16} position during the ethynylation of estrenone is not quite unexpected. It is known, that in the ethynylation of carbonyl compounds which have a proton in the α-position, enolization competes with the desired reaction (Ziegenbein, 1969) and thus in the present synthesis of lynestrenol may lead to loss of label from C_{16}.

The present results were confirmed by Broess et al. (1975) in model experiments using deuterium instead of tritium. No significant change in deuterium content appeared to occur, when the ethynylation was carried out with an excess of ethynyl magnesium bromide in THF. Following this procedure also in the synthesis of tritiated lynestrenol from estrenone-16-^{3}H as described in the present study, Broess et al. (1975) obtained lynestrenol-16-^{3}H with a specific activity of 13.3 Ci/mmol in comparison with 14.0 Ci/mmol for the estrenone-16-^{3}H.

SUMMARY

Estrenone-16-^{3}H with a specific activity of 12.0 Ci/mmol was prepared from estrenone by an exchange procedure with tritiated water in dimethylformamide. Lynestrenol-^{3}H (specific activity 800 mCi/mmol) was obtained from estrenone-^{3}H by reaction with acetylene and potassium t-butoxide in tetrahydrofuran.

Table 4.2 — Yields of estrenone-^{3}H and lynestrenol-^{3}H

Experiment	Quantity estrenone	Yield of estrenone-^{3}H			Yield of lynestrenol-^{3}H			Recovered estrenone-^{3}H	
		by weight	activity	specific activity	by weight	activity	specific activity	activity	specific activity
	mg	%	mCi	mCi/mmol	%	mCi	mCi/mmol	mCi	mCi/mmol
1	220	61	440	880	23	23.5	230	34	240
2	230	60	1020	2000	60	77	270	28	—
3	144	30	1700	12000	32	28	800	52	—

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ASSAY OF RADIOACTIVITY

INTRODUCTION

Radioactivity was measured throughout the investigations by means of liquid scintillation counting (Horrocks, 1974). This technique is based upon the conversion of part of the kinetic energy of an ionizing particle (usually originating from the decay of a radionuclide) into photons as a result of its interaction with scintillator molecules present in the scintillation solution. These photons are collected and measured by multiplier phototubes and subsequently the pulses from these tubes are summed up, sorted and counted. Although the energies of the radiation of a certain radioisotope are always the same, the response, which they produce in a particular liquid scintillation sample and, therefore, the counting rate measured will differ to a variable extent from the actually occurring number of nuclear desintegrations per unit of time. The term commonly used to denote the various processes causing a decrease in the photon yield of a liquid scintillator solution is "quenching". As a consequence of this ever present quenching phenomenon the number of counts per minute (cpm), that is measured with the liquid scintillation spectrometer, has to be corrected with the help of a calibration curve or otherwise in order to obtain the actual number of nuclear desintegrations per minute (dpm).

MATERIALS AND METHODS

Apparatus

The liquid scintillation spectrometer used was a Packard Tri-Carb Model 3380 (Packard Instruments S.A., Bruxelles, Belgium) equipped with an automatic external standardization system.

Sample preparation

The samples were prepared for liquid scintillation counting after digestion, if necessary, with Soluene® (Packard) and using Instagel® (Packard) as scintillation liquid. Glass counting vials were used (Packard).

Aliquots of aqueous protein-free solutions and dried aliquots of organic solvent extracts were counted after the addition of 10 ml Instagel. In case of volumes larger than 0.2 ml the volume was made up to 5 ml with water and then thoroughly mixed with 10 ml Instagel.

The silicagel samples obtained from thin-layer chromatograms were prepared for liquid scintillation counting by addition of 5 ml water and 10 ml Instagel followed by vigorous shaking.

Blood plasma, perfusate plasma and protein-containing dialysis samples were treated with 1 ml Soluene and warmed for 30 minutes in a water bath at 40°C. Tissue samples were digested similarly with Soluene at a temperature of 55°C until a clear solution was obtained. After dissolution 15 ml of a mixture of Instagel/1.0 M HCl 90/10 was added.

For the assay of radioactivity in blood and perfusate an aliquot of not more than 0.2 ml was mixed with 1.5 ml Soluene/isopropanol 50/50. After digestion at room temperature for 10 minutes with occasional swirling 0.5 ml hydrogen peroxide 35% (OPG, Utrecht, The Netherlands) was added. After another 10 minutes at room temperature and gently swirling the loosely capped vial was placed in a water bath at 40°C for about 15 minutes. Finally, 15 ml Instagel/1.0 M HCl 90/10 was added and mixed with the sample under vigorous shaking.

The radioactivity of faecal samples was determined after the preparation of a suspension in water. An aliquot of the suspension (0.05-0.15 ml) was digested in triplicate with 1.0 ml Soluene in a water bath at 50°C for 2 hours. Subsequently, 0.5 ml isopropanol was added and the solution was decolorized by the addition of 0.2 ml hydrogen peroxide 35%. After standing for 15 minutes at room temperature and 2 hours in the water bath at 50°C, 15 ml Instagel/1.0 M HCl 90/10 was added and radioactivity was determined.

Liquid scintillation counting

Radioactivity was measured at preselected optimum spectrometer settings for the determination of ^{3}H and ^{14}C. Quenching of the samples was corrected according to the external standard channels-ratio method using a calibration curve. This curve was obtained and checked at least once a week by the

assay of blank samples containing hexadecane-^{3}H or hexadecane-^{14}C (The Radiochemical Centre, Amersham, U.K.) as an internal standard. Generally, a similar curve was obtained with standards quenched with increasing amounts of nitromethane.

Blank samples were used to correct for background activity.

RESULTS AND DISCUSSION

Following the procedures outlined above no particular problems were encountered in the course of the investigations. An example of a calibration curve for quench correction of tritium samples is shown in Fig. 5.1. The points in the curve represent the mean of 5 successive measurements of the counting efficiency and the external standard channels-ratio of a series of hexadecane-^{3}H standards. The relative error did not exceed ± 0.5% and demonstrates the

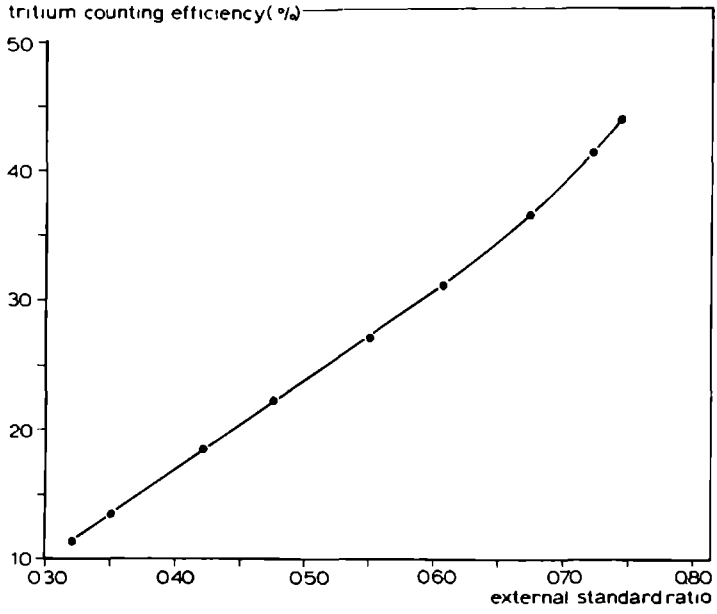

Fig. 5.1 — Quench correction curve for tritium counting efficiency versus external standard channels-ratio. Points in the curve represent the mean efficiency and channels-ratio of 5 successive measurements of a series of nitromethane quenched standards. The relative error for each point is smaller than ± 0.5%.

reliability of this technique. The counting efficiency of tritium samples varied between 15 and 47%, for carbon-14 samples between 80 and 92%. Incidental counting efficiencies for tritium samples below 15% were corrected by means of the internal standard method.

Apart from the applied quench correction the precision of the radioactivity value measured for any sample is determined by the total number of counts (gross counts) measured during the counting period of the sample and is expressed in the relative standard deviation of counting. This relative standard deviation is represented by the quotient of 100 and the square root of the gross count value. Generally, this figure was less than $\pm$ 1.0%. Counting of some samples, however, with a very low level of radioactivity, like blank samples, silicagel samples scraped outside the zones of high radioactivity and certain tissue samples, resulted in standard deviations of 2.5% and more, because the maximal counting period applied was 20 minutes.

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CHAPTER 6

THE RADIOLIGAND BINDING ASSAY USED IN THE RECEPTOR BINDING STUDIES

INTRODUCTION

The availability of radiolabelled estradiol of high specific activity enables a direct measurement of its binding to the estrogen receptor. After preliminary experiments it appeared also possible to study the interaction of the other compounds under investigation with the receptor using the principle of competition between a radioligand (tritiated estradiol) and a radioinert substance for the receptor binding sites (Korenman, 1968). The bound fraction of the radioligand is measured by means of liquid scintillation counting after adsorption and precipitation of the unbound ligand with dextran-coated charcoal (Herbert et al., 1965; Korenman, 1968). This technique is widely used in studies dealing with the binding of steroid hormones to their receptors as well as to antibodies. Several points pertinent to the applicability and a correct use of the competitive binding assay deserve further consideration. In this chapter special attention is given to the incubation time needed for the radioligand to reach binding equilibrium under the experimental conditions applied, the reversibility of binding and the equilibration time for the binding of the competing steroids. The binding capacity of the dextran-coated charcoal suspension, the effectiveness of this separation procedure and the stability of the radioligand binding in the presence of the adsorbant have also been studied.

EXPERIMENTAL PROCEDURE

Steroids, dissolved in ethanol, were transferred to silanized conical glass tubes. The solvent was evaporated with a weak stream of air. When the initial volume did exceed 0.2 ml, the wall of the tube was consecutively rinsed with 0.2, 0.2 and 0.1 ml ethanol to concentrate the steroids at the bottom. The indicated amount of estradiol-^{3}H alone or together with a certain excess of

another steroid was incubated with 0.2 ml cytosol (see Chapters 10, 11) under continuous and gentle shaking at a temperature of 1-4°C. After a period of 20 hours, unless otherwise indicated, 0.2 ml charcoal-dextran suspension (0.1% Aktivkohle, Merck, and 0.05% Dextran T 70, Pharmacia, in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.0015 M EDTA) was added and the incubation was continued for another 15 minutes. Subsequently, the charcoal was spun down by centrifugation for 10 minutes at 600xg and 0.2 ml of the clear supernatant was put into a counting vial for the assay of radioactivity.

RESULTS AND DISCUSSION

Unbound ligand adsorption to dextran-coated charcoal

1. Binding capacity of the charcoal suspension

Calf uterine cytosol was incubated overnight with 1.5 nM estradiol-^{3}H and 15 μM estradiol. The effectiveness of the charcoal adsorption of the radioligand was measured by comparing the radioactivity of samples not treated with charcoal and the radioactivity of samples incubated for 15 minutes with either 0.1% or 0.5% dextran-coated charcoal suspension. The same results were obtained irrespective of the charcoal concentration. Only 1-2% of the radioactivity measured in the control samples (5300 cpm), probably estradiol bound to non-specific binding sites, appeared to be not precipitated.

2. Influence of time on radioligand adsorption

Incubation of only the cytosol buffer, containing 1.5 nM estradiol-^{3}H, with an equal volume of the 0.1% dextran-coated charcoal suspension revealed that adsorption of the radioligand was complete within 15 minutes after the addition of the adsorbant. Only 2% of the radioactivity present in the control sample (5400 cpm) could be detected after 5 minutes charcoal treatment. After 15, 30 and 60 minutes the amount left in the solution was less than 0.5% of the control level.

3. Stability of the estradiol-receptor complex

1.5 nM estradiol-^{3}H was incubated overnight with calf uterine cytosol and subsequently for 10, 15 and 30 minutes with an equal volume of 0.1% dextran-

coated charcoal suspension. No decrease of bound radioactivity could be observed over the time period studied, so it can be concluded that the estradiol binding measured in the assay is stabile and resists the charcoal treatment under the experimental conditions.

Equilibration time and reversibility of binding

Calf uterine cytosol was incubated for increasing periods of time with 11 nM estradiol-^{3}H alone or in the presence of 200 nM ethynodiol. After charcoal treatment as described above bound estradiol-^{3}H was measured by liquid scintillation counting. The results presented in Fig. 6.1 (upper curve) demonstrate the high association rate of estradiol to the receptor binding sites. After one hour incubation more than 85% of the binding observed ultimately is attained. The initial steep incline of the estradiol binding curve is followed by a more gradual increase, until a plateau is reached after about 17 hours incubation. That the curve observed actually reflects the interaction of estradiol with its

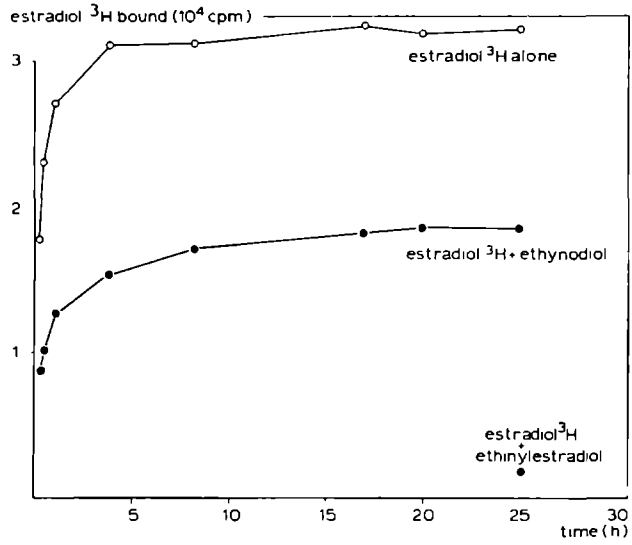

Fig. 6.1 — Time course of estradiol binding to calf uterine cytosol. In the presence of an excess of ethynodiol the binding of estradiol is reduced, but the shape of the binding curve does not change. The points in the curves are the mean of 3 incubations. The residual, non-specific, binding of estradiol-^{3}H in the presence of a 200-fold excess of ethinylestradiol is also shown.

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specific binding sites is evident from the small fraction of the ligand, that is non-specifically bound. This non-specific binding is the estradiol binding measured in the presence of an excess (2 μM) of the strong binding synthetic estrogen ethinylestradiol.

The other, lower, curve in Fig. 6.1 shows on the one hand, that ethynodiol is reversibly bound to the binding sites of the receptor, since inhibition of estradiol binding does not increase with time, and on the other hand, that equilibrium of the binding is reached after an incubation period of about 20 hours.

Under equilibrium conditions the relative error, from which the reproducibility and the precision of the assay may be deduced, appeared to be smaller than ± 5% in binding experiments with only the radioligand. In experiments, however, concerning the study of the influence of other steroids on estradiol-^{3}H receptor binding, the relative error appeared to increase and varied usually between ± 5-10%.

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CHAPTER 7

THE ASSAY OF LYNESTRENOL IN BLOOD AND PERFUSION MEDIUM

INTRODUCTION

In general the use of radiolabelled drugs in pharmacokinetic investigations has to be considered as second choice for several reasons. Apart from economic and practical reasons like the availability of the radiolabelled drugs of interest and the more practical drawback inherent to the daily and safe handling of radioactive materials and samples, which, additionally, will make the overall analysis often more time-consuming than the analysis of radioinert substances, the most serious disadvantage is that pharmacokinetic investigations in humans using radiolabelled drugs are generally not permitted for ethical reasons. Therefore, at the initiation of the present investigations extensive efforts have been undertaken to develop a gas chromatographic assay for the determination of therapeutic blood levels of lynestrenol.

Because the blood concentration of lynestrenol in humans, when a contraceptive dosage regimen is followed, consisting of a daily intake of 2.5 mg of the compound, can be expected to be in the order of nanograms per ml and less, the choice of the analytical technique is limited by the required sensitivity. The sensitivity of gas chromatography with electron-capture detection has been shown previously to meet this requirement in the analysis of compounds with a high electron-absorption coefficient as it is the case for many pesticides (Krejci & Dressler, 1970). Because the electron-absorption coefficient of lynestrenol is too low to permit a sensitive analysis by means of this technique, some derivatives were synthetized with better properties in this respect, viz. lynestrenol heptafluorobutyrate and the chloro-, bromo- and iodomethyldimethylsilylether of lynestrenol. The gas chromatographic properties of the latter compound were superior and the detector response allowed the quantitation of minute amounts of only 25 picogram. The necessary two-step derivatization procedure appeared to result in a quantitative conversion. Problems, however, were encountered with the specificity of the assay and could not be overcome (Van Kordelaar, 1972).

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For this reason the pharmacokinetics of lynestrenol has been studied using the radiolabelled compound. The lynestrenol concentrations may then be determined by measuring the isotope content of the sample with the help of liquid scintillation counting (this thesis, Chapter 5).

EXPERIMENTAL PROCEDURE

Extraction

Immediately after collection the samples were cooled in ice and processed during the same day. A volume of 0.1 ml perfusate, blood or plasma was transferred to a conical glass tube and diluted with an equal volume of water. After the addition of 4 volumes ethanol, containing 100 μg lynestrenol/ml, the mixture was extracted with consecutively 2, 1 and 1 ml n-hexane by vigorous stirring for 1 minute using a whirl-mixer. The combined extracts were evaporated in a counting vial with a weak stream of air and radioactivity was measured by means of liquid scintillation counting.

Thin-layer chromatography

Duplicate hexane extracts obtained from a sample volume of 0.2 ml instead of 0.1 ml were used for the fractionation of radioactivity by means of thin-layer chromatography. Because of the decreasing level of radioactivity during the experiment the sample volume was increased to 1.0 ml and 0.4 ml for the last and the last but one sample respectively. After intraduodenal administration of lynestrenol TLC was performed with only one sample of 0.5 ml volume. The other volumes used in the extraction procedure were accordingly adapted.

Chromatography was carried out over silicagel pre-coated glass plates (Kieselgel F_{254}, Merck) in the solvent system toluene/ethylacetate 80/20 or toluene/ethanol 90/10. Lynestrenol was localized by exposing the developed chromatogram to iodine vapour. The developed area was divided into 10 bands, one corresponding to the marker steroid added initially to the sample, 4 zones of 0.5 cm width adjacent to the lynestrenol region, and another 5 zones of about equal size. The bands were scraped off with a razor blade and the silicagel was transferred to a counting vial for the assay of radioactivity. The fraction of the total plate radioactivity measured in the lynestrenol region and in the narrow zone just above was used for the calculation of the lynestrenol content of the hexane extract.

Recovery

Four blank samples to which a known amount of lynestrenol-^{3}H had been added, were extracted with hexane as described. The mean value of the efficiency of these extractions was used to correct the experimental data.

RESULTS AND DISCUSSION

Specificity

Because the measurement of radioactivity is essentially non-specific, as the result will represent the total isotope content of the sample including lynestrenol itself as well as its conversion or degradation products, the separation of the parent compound from these products prior to the measurement of radioactivity is necessary. In the present analysis a two-step separation was applied, namely extraction of the lynestrenol from the aqueous sample with an organic solvent and thin-layer chromatography of the extract.

1. Extraction procedure

For the extraction procedure the choice of a non-polar solvent was considered to be most promising in view of the lipophilic nature of lynestrenol itself (Chapter 8) and the increased polarity of its conversion products (Chapter 12). On the other hand the use of a rather volatile solvent would facilitate the necessary volume reduction of the extract to a high extent. A very effective extraction of lynestrenol could be attained by using n-hexane, which appeared to yield a recovery of about 90% and more, whereas at the same time the majority of the metabolites remained in the aqueous phase. Lysis of the erythrocytes by dilution of the sample with water and the precipitation of the plasma proteins by the subsequent addition of 4 volumes ethanol prior to the extraction improved the precision of the procedure.

In order to prevent the loss of significant amounts of lynestrenol by adsorption to the glass-ware and to avoid similarly the occurrence of artefacts during chromatography of nanogram and subnanogram amounts, the ethanol added to the sample contained an excess of non-labelled lynestrenol. This latter compound could, moreover, serve as a marker to localize the radiolabelled compound after development of the chromatogram.

The present knowledge of those biotransformation products of lynestrenol, which may be assumed to be extractable to some extent with n-hexane, is limited to the observation of a metabolite excreted in the bile with the tentative structure 17α-ethynyl-5ξ-estrane-3ξ,17β-diol (Coert et al., 1975; Mazaheri et al., 1970; this thesis, Chapter 12) and the conversion of lynestrenol to norethindrone, reported by Mazaheri et al. (1970), Murata (1967) and Yamamoto (1968). So in any case an effective separation of lynestrenol from these and other hydroxylated and oxygenated derivatives was required. Because the conversion of lynestrenol to less polar compounds was considered to be not very likely in view of reports concerning the biotransformation of ethynodiol (Cook et al., 1973; Kishimoto et al., 1972), norethindrone (Braselton et al., 1977; Cook et al., 1974; Gerhards et al., 1971), norethynodrel (Cook et al., 1972; Kishimoto et al., 1972) and norgestrel (Gerhards et al., 1971; Hendeles et al., 1972), the choice of a solvent system for chromatography allowing a great mobility to lynestrenol but restricting the mobility of the compounds mentioned above was supposed to give optimal results. The solvent systems toluene/ethylacetate 80/20 and toluene/ethanol 90/10 meet these requirements as is illustrated by a R_f value of lynestrenol in both systems of about 0.7 and on the other hand R_f values for norethindrone, ethynodiol and the metabolite of about 0.25 in the former solvent system and 0.48, 0.40 and 0.34 respectively in the latter solvent system.

In the course of the investigations three out of four of the narrow silicagel zones adjacent to the lynestrenol marker were always devoid of radioactivity. Sometimes, however, and only in extracts of rat blood but never in extracts of perfusion medium, a variable amount of radioactivity, up to 25% of the radioactivity measured in the lynestrenol region on the chromatographic plate, appeared to be present in the zone just above the lynestrenol marker. No regular pattern could be observed in the appearance of this phenomenon in a qualitative and quantitative sense, so the presence of a conversion product is not very probable. Moreover, a similar phenomenon was sometimes observed in thin-layer chromatography of comparable amounts of lynestrenol-^{3}H added to hexane extracts of blank rat blood and subjected to the same experimental procedure. Because, however, 17α-ethynyl-5α-estran-17β-ol appeared to display an analogous mobility in the chromatographic systems applied, the specificity of the assay was further substantiated by means of gas chromatography. To obtain sufficiently high blood levels of lynestrenol and the hypothetical metabolite a rat was injected 3 times at 30 minutes intervals with 0.5 mg lynestrenol and sacrificed 90 minutes after the first injection by decapitation. The blood was collected in a heparinized tube and processed as usual without,

however, the addition of lynestrenol as a marker for chromatography. After extraction and chromatography the silicagel zone corresponding with the lynestrenol spot and the zone of interest just above were scraped off the plate. The silicagel was extracted with dichloromethane/methanol 70/30. After the evaporation of the solvent with a weak stream of air the residue was dissolved in hexane and injected in a gas chromatograph (Hewlett Packard Model 402) equiped with a flame ionization detector, a solid injection system and a capillary column (SE-30, 25 m, LKB no. 74). The gas chromatogram of the blood extract and a standard solution containing lynestrenol and 17α-ethynyl-5α-estran-17β-ol is shown in Fig. 7.1. Obviously, the latter compound is absent in the blood extract. The minor peaks in the gas chromatogram of the extract eluting before lynestrenol were also present in a blank blood sample of the

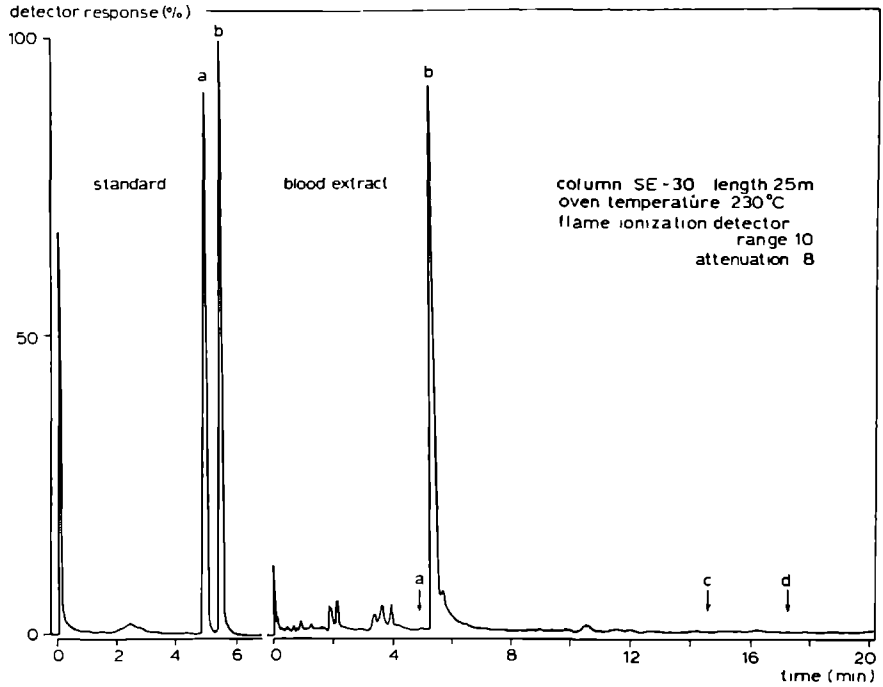

Fig. 7.1 — Gas chromatograms of a standard solution *(left)*, containing 17α-ethynyl-5α-estran-17β-ol (a) and lynestrenol (b), and of an extract of rat blood *(right)* obtained from the animal after lynestrenol injection. For experimental details see text. For comparison, the retention times of norethynodrel (c) and norethindrone (d) are also indicated.

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same rat, which was subjected to an identical procedure. The same results were obtained in a similar experiment with another animal.

In view of these findings we thought it justified to consider also the radioactive material present in the narrow zone just above the lynestrenol marker as lynestrenol.

Sensitivity and sample volume

The sensitivity of the determination of lynestrenol-^{3}H and lynestrenol-^{14}C by means of liquid scintillation counting is limited by the specific activity of the steroid and, like for any assay, by the volume of the sample. Principally, the specific activity of the lynestrenol-^{3}H, about 700 mCi/mmol during the investigations, enables the accurate quantification of amounts of lynestrenol as small as 50 picogram, corresponding with 275 dpm. The comparatively low specific activity of the available lynestrenol-4-^{14}C, 32.5 mCi/mmol, restricted the use of this compound to experiments, where the analysis of excreta was envisaged, and to distribution studies by means of whole-body autoradiography.

Assuming a somewhat higher threshold level of radioactivity of 500 dpm instead of 275 dpm for the experimental samples in the present investigations, then it will be possible to measure lynestrenol concentrations of 1 ng/ml in an extract obtained from a sample of 0.1 ml volume with a standard error of counting of 1.5% at a counting efficiency of 40-45% and using a counting time of 20 min. The chromatographic step, however, in the assay of lynestrenol requires at least double the sample volume as a consequence of the decreased counting efficiency of the silicagel samples (about 25%). Moreover, the fraction of lynestrenol in the hexane extract is calculated as the percentage of the sum of the radioactivity present in 10 silicagel samples after correcting these samples for the background radioactivity (about 200 cpm total). For this reason the sample volume has to be increased above 0.2 ml for blood and perfusate samples with lynestrenol concentrations below 4 ng/ml.

Obviously, therefore, the total volume of blood and perfusate available for sampling will determine ultimately the lowest concentrations which can be measured, as well as the number of samples that can be taken for analysis. The available sample volume is restricted by the total blood volume of the animal or the total volume of the perfusion medium in the experiments with the isolated perfused liver and is determined on the other hand by the number of samples to be taken in order to allow a meaningful pharmacokinetic analysis of the concentration-time course of the drug.

In the experiments with the isolated perfused rat liver the volume of perfusate,

which is available for sampling without disturbance of the physiological state of the liver under the experimental conditions is only determined by the design of the perfusion apparatus and the volume of the perfusion medium. Because, however, the volume of distribution of the drug in these experiments is in the same order of magnitude as the volume of the perfusion medium (Chapter 15), the fraction of the total volume used for analysis should not exceed 5% in order to avoid errors in the estimation of the elimination time constant from the slope of the concentration-time curve (Nagashima et al., 1968). Taking this restriction into account a complete analysis of 8-9 perfusate and perfusate plasma samples could be accomplished in these experiments. This number was sufficient to obtain reliable and significant information about the time course of lynestrenol over a wide concentration range.

With respect, however, to the *in vivo* experiments a complete analysis of each sample, including thin-layer chromatography, appeared to be not feasible for the following reasons. In the first place the number of samples had to be increased as a consequence of the more complex profile of the concentration-time curve of lynestrenol as compared with a mono-exponential decay observed in the isolated liver. Secondly, the number of low level samples was considerably greater, particularly after intraduodenal administration of lynestrenol. Finally, the total blood volume available for analysis was smaller than in the *in vitro* studies: to maintain the physiological state of the animal as much as possible the total blood volume taken for analysis in the course of the *in vivo* experiments did not exceed 2 ml. This volume represents 15-20% of the total blood volume of the rat (about 6 ml/100 g body weight, Altman, 1961). As a consequence of this unfavourable combination of factors a complete analysis including thin-layer chromatography was carried out on 5 out of 13 samples following intra-arterial injection of lynestrenol and on 1 out of 13 samples in case of intraduodenal administration of the compound. The consequences of this procedure for the interpretation of the experimental results are discussed in Chapter 16.

Recovery, reproducibility and precision

The fraction of lynestrenol recovered after the extraction procedure was measured in each experiment using blank samples to which a known amount of lynestrenol-^{3}H was added. The efficiency observed in the course of the investigations varied between 88 and 94% for the extraction of perfusate samples, between 92 and 95% for the extraction of perfusate plasma and between 91 and 96% for the extraction of rat blood. Besides the good reproducibility, which

may be deduced from these figures, the precision of the extraction was equally good as is illustrated by the small relative error, which did not exceed ± 3%. The concentration range of the radiolabelled drug encountered in the experiments was not of significance for the overall performance of the assay as a consequence of the large excess of non-labelled lynestrenol added prior to the extraction.

Pharmacokinetic analysis

Pharmacokinetic analysis of the lynestrenol blood and perfusate levels was accomplished by fitting of the experimental data to the mathematical equations pertinent to the pharmacokinetic model, which was considered to give the best approximation of the concentration-time profile observed. Curve fitting was performed with the aid of the non-linear regression computer program FARMFIT*). Detailed information on this program has been provided previously by Breimer (1974). From the best fit of the data the pharmacokinetic parameters are calculated. Furthermore, the program provides the goodness of fit expressed as the standard error for the ordinate intercept(s) and the elimination time constant(s) of the best-fitting concentration-time curve, assuming a relative error in the experimental data of ± 5%.

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CHAPTER 8

LIPOPHILICITY AND PROTEIN BINDING OF LYNESTRENOL

INTRODUCTION

It is nowadays well established, that the extent of plasma protein binding and the physicochemical properties of a drug under physiological conditions may have an important bearing on its distribution, biotransformation and excretion and, therefore, also on the course of drug activity in a qualitative and quantitative sense (Dayton et al., 1973; Goldstein et al., 1974; Jusko & Gretch, 1976; Raaflaub, 1970; Wilkinson & Shand, 1975).

According to common theories in pharmacology only the unbound or free drug fraction in the blood diffuses through the capillary walls, reaches the site of drug action and is subject to renal elimination from the body. Since generally the binding of drugs to plasma proteins is readily reversible and obeys the law of mass action, particularly the albumin-drug complex, which is quantitatively by far the most important for the great majority of drugs, may serve as a circulating drug reservoir, that will release part of the drug when the free drug concentration in the blood decreases as a consequence of proceeding biotransformation and excretion processes. The possible significance of the carrier and reservoir function of the blood proteins will be greater for drugs displaying a poor solubility in water and a high degree of protein binding. Moreover, protein binding may protect the drug to some extent against biotransformation in the liver, like has been demonstrated for warfarin (Yacobi & Levy, 1975; Yacobi et al., 1976), dicumarol (Lai & Levy, 1977), bilirubin (Øie & Levy, 1975) and phenytoin (Gugler & Azarnoff, 1976), although for a highly protein bound drug like propranolol biotransformation in the liver appeared not to be restricted to the unbound fraction (Evans & Shand, 1975).

The passage of drugs across biological membranes by passive diffusion is generally considered to be limited to the uncharged molecule and to be favoured by a high degree of lipid solubility (Goldstein et al., 1974; Raaflaub, 1970). Drug partitioning between two immiscible liquid phases, one of which water and the other one a non-polar organic solvent like n-heptane, may be used as a

model for the distribution in the organism between the various body fluids and the cell membranes.

In view of the apparent significance of the protein binding and lipophilicity also for the pharmacokinetic investigations envisaged, the partitioning of lynestrenol over n-heptane/water and its binding to serum albumin have been studied and are reported below.

MATERIALS AND METHODS

Assay of protein binding

The binding of lynestrenol to bovine serum albumin (BSA, Povite, Amsterdam, The Netherlands) was determined by means of equilibrium dialysis in the apparatus described by Rodrigues de Miranda (1975). The apparatus consists of six pairs of teflon cells of 3.5 ml volume each, separated by a semi-permeable membrane (cellulose acetate, AKU, Arnhem, The Netherlands) and clamped together. Both cells of a pair contained 3.0 ml 0.1 M phosphate buffer, pH 7.4, and 0.02% NaN_3 as a bacteriostatic. The protein compartment, additionally, contained 2.9% BSA (0.42 mmol/l) and in most experiments also the lynestrenol. In spite of its low solubility in water it appeared possible to obtain lynestrenol concentrations of more than 1 μg/ml by taking advance of the solubilizing properties of the protein. The protein/lynestrenol-^{3}H solution was prepared by incubation of the protein solution with an aliquot of pure lynestrenol-^{3}H for 1 hour at 37°C. Some experiments were carried out starting with lynestrenol-^{3}H in the protein-free compartment. The latter solution was prepared by adding an aliquot of lynestrenol-^{3}H dissolved in ethanol to the buffer solution to a final ethanol concentration of 2%. During the dialysis the cells were placed in a water bath at 37°C and were rotated at a rate of 1 r.p.m. for 24 hours. This equilibration period was chosen from preliminary experiments, where lynestrenol was added alternatively to the protein and to the other compartment. At equilibrium the lynestrenol-^{3}H concentration was measured at both sides of the membrane by liquid scintillation counting of 1.0 ml of the respective solutions. The difference between the total concentrations in the protein and in the protein-free compartment represents the fraction of lynestrenol, that is protein bound. Dividing this fraction by the concentration measured in the protein compartment and multiplying by 100 gives the percentage of binding. The albumin content at equilibrium was measured spectrophotometrically at 279 nm (Zeiss PMQ-II).

Determination of the partition coefficient

Drug partitioning was studied by shaking 3.0 ml 0.1 M phosphate buffer, pH 7.4, saturated with n-heptane, and 1.0 ml n-heptane, saturated with buffer, in stoppered silanized glass tubes for a period of 24 hours at room temperature. Lynestrenol-4-^{14}C, radiochemical purity 98.8%, was dissolved in the organic phase at concentrations of 0.4 and 0.8 μg/ml. The phases were allowed to separate by standing for 1 hour and the equilibrium concentrations were determined by liquid scintillation counting of 0.1 ml n-heptane and 2.5 ml buffer as described in Chapter 5.

RESULTS

Protein binding

The protein binding was determined at equilibrium concentrations varying from 2 to 1700 ng lynestrenol/ml, which were in the range of the blood concentrations encountered in the rat *in vivo* and *in vitro* in the perfusion experiments. The results are shown in Fig. 8.1 and demonstrate that more than 97%

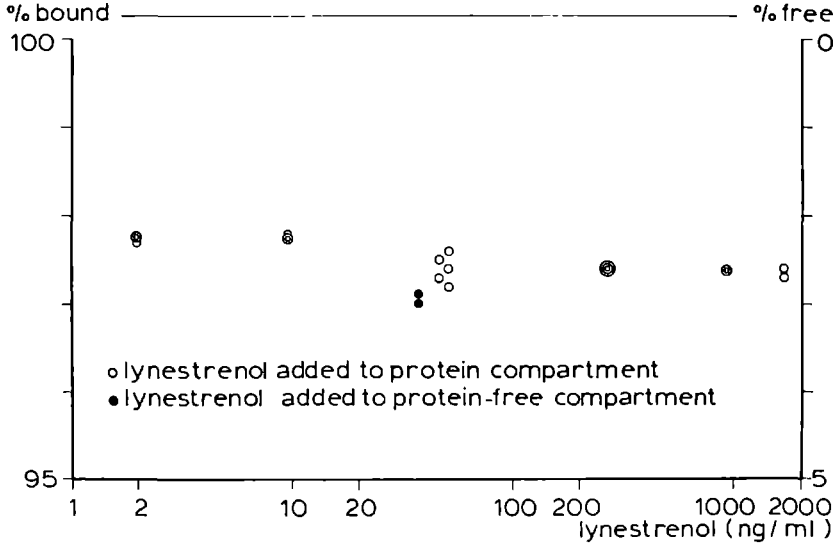

Fig. 8.1 — Diagram illustrating the binding of lynestrenol to bovine serum albumin measured by means of equilibrium dialysis. The lynestrenol concentrations are the concentrations at equilibrium. Each experiment was carried out in duplicate or in triplicate.

of the lynestrenol is bound to the protein at all concentrations. The association constant of the drug-protein complex was determined with the help of the reciprocal plotting method of Klotz (1946), using the equation:

$$1/r = 1/n + 1/n \cdot K_A \cdot 1/[A_F]$$

where r represents the fraction of the macromolecules occupied by drug molecules, n is the number of independent binding places, K_A is the association constant and $[A_F]$ is the unbound drug concentration. When plotting 1/r against $1/[A_F]$ a straight line was obtained in the present study. From its slope a value for K_A could be calculated of 8.9×10^4 M^{-1}, assuming the existence of one binding place for each albumin molecule. The number of binding places per molecule could not be established from the present experiments, because the low solubility of lynestrenol made it impossible to reach high enough drug concentrations.

Lipophilicity

The determination of the lipophilicity of lynestrenol as a partition coefficient between n-heptane and water is complicated by its low solubility in the aqueous phase and a very good solubility in the organic phase. Under these circumstances it is conceivable that at concentrations exceeding the solubility of the compound in the buffer the partition coefficient measured is influenced by the lynestrenol concentration in the organic phase and will correspond ultimately with the ratio of the concentrations of the saturated solutions. In order to avoid this complication the initial concentrations of lynestrenol in the heptane were chosen as low as possible (about 0.4 and 0.8 μg/ml). Using these concentrations the determination of lynestrenol in the buffer appeared to be just feasible.

The results obtained are shown in Table 8.1 and demonstrate, that lynestrenol

Table 8.1 — Partition coefficient of lynestrenol

EQUILIBRIUM CONCENTRATION mean (dpm/ml) ± S.D. (n = 5)		PARTITION COEFFICIENT n-heptane/buffer
n-heptane	buffer	
99,200 ± 700	19.3 ± 1.5	5140 ± 400
204,600 ± 1800	43.5 ± 3.2	4700 ± 350

is a highly lipophilic compound. The values of the partition coefficient measured at both concentrations are an indication, that no saturation of the buffer with lynestrenol did occur.

DISCUSSION

The results presented show unequivocally that protein binding and lipophilicity of lynestrenol are high and may have an important influence on its pharmacokinetic behaviour.

Regarding on the one hand the extremely high lipophilicity of lynestrenol and on the other hand the fairly strong binding of testosterone, progesterone and estradiol to BSA with association constants between 10^4 and 10^5 M^{-1} (Westphal, 1971), the extensive and rather strong binding of the former steroid is not an unexpected finding. An inverse relationship between the binding affinity of steroids to serum albumin and the number of polar groups has been observed previously by Eik-Nes et al. (1954) and Westphal & Ashley (1962). The validity of this *polarity rule* has since been confirmed in many instances (Scholtan, 1968; Westphal, 1971).

The concentration range studied in the dialysis experiments covered the perfusate and blood levels of lynestrenol encountered in the isolated perfused rat liver preparation as well as in the rat *in vivo* (this thesis, Section IV). Because the binding of lynestrenol to albumin within this concentration range appeared to be constant and, moreover, the metabolic clearance of lynestrenol appeared to be of the non-restrictive type (Shand et al., 1976; Wilkinson & Shand, 1975) as far as albumin is concerned (Van Kordelaar et al., 1978; this thesis, Chapter 15), it seems unlikely that this binding could complicate the pharmacokinetic behaviour of the drug. However, following intra-arterial administration of lynestrenol to rats a decrease of the clearance of the compound was observed by us as compared with the isolated rat liver (this thesis, Chapters 15, 16). The liver preparation was perfused with a semi-synthetic medium containing only BSA and no other serum proteins. This raises the question, whether there are additional binding sites for lynestrenol present in rat blood, e.g. globulins, with a binding affinity and capacity high enough to reduce the metabolic clearance rate of the compound to the extent observed. This aspect deserves further investigation and is discussed in Chapter 16.

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SECTION III

INTERACTION STUDIES WITH THE ESTROGEN RECEPTOR

EFFECT OF CONTRACEPTIVE STEROIDS AND RELATED COMPOUNDS ON THE DISTRIBUTION PATTERN OF ESTRADIOL-^{3}H IN THE OVARIECTOMIZED RAT

INTRODUCTION

Since the investigations of Eisenfeld & Axelrod (1965, 1966), Glascock & Hoekstra (1959), Jensen & Jacobson (1962), Kato & Villee (1967), Stone et al. (1963) and others concerning the specific uptake and retention of estrogenic hormones by their target tissues, much progress has been made in the elucidation of the underlying mechanism of their observations. In recent years investigations in several laboratories have established that target tissues for estradiol contain cytoplasmic proteins with a high affinity, a high degree of specificity and a limited capacity for the binding of estrogens. At present, evidence is growing, that these proteins are indispensably implicated in estradiol action, and therefore may be regarded as the estrogen receptor molecules (Baulieu et al., 1975; Buller & O'Malley, 1976; Gorski & Gannon, 1976; Jensen & DeSombre, 1973). Consequently, interference with estradiol binding to these proteins by other compounds could modulate or change the course of processes controlled by estradiol and other estrogens (see Chapter 1).

Endogenous as well as exogenous compounds have been demonstrated to influence the binding of estradiol in its target tissues and to possess either estrogenic or anti-estrogenic properties (see Chapter 1 and references thereof). In this connection, contradictory results have been reported for some contraceptive progestins (Eisenfeld & Axelrod, 1965, 1967; Rosner et al., 1972; Saucier et al., 1970; Watanabe et al., 1968). Because of the possible significance of such an interaction for the mechanism of action and the biological profile of these compounds as well as their pharmacokinetic behaviour, further investigations in this field seemed worth-while. A study has been made of the influence of contraceptive progestins and related compounds on the

binding of estradiol to the estrogen receptor of the rat *in vivo* and *in vitro*. In this chapter the results obtained from *in vivo* experiments are described. Evidence is presented, that the contraceptive progestins of the 19-nortestosterone series (including lynestrenol) are able to interfere with the binding of estradiol to the estrogenic target tissues. Experiments *in vitro* with the 105,000xg supernatant of rat uterus homogenates have confirmed these results (Van Kordelaar et al., 1975) and are the subject of Chapter 10.

MATERIALS AND METHODS

Animal experiments

In all experiments young adult Wistar rats (Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands) were used, 3 weeks after ovariectomy. All compounds were administered via the tail vein under light ether anaesthesia. The unlabelled compounds were given 5 min before estradiol-^{3}H unless otherwise indicated. The control animals received the solvent only. One hour after the injection of 0.1 μg/100 g estradiol-6,7-^{3}H, specific activity 40 Ci/mmol, the animals were killed by decapitation. Blood was collected in a heparinized tube and the heart was removed immediately. Then the skull was opened to remove the brain and the anterior pituitary. The brain was frozen and thereafter dissected according to the scheme given in Fig. 9.1. The cortex sample is not indicated in Fig. 9.1 and was taken from the parietal lobes of the cerebrum. As for the other brain samples, the names mentioned and used throughout the text do not pretend to describe the brain areas in a strictly anatomical sense, as adjacent structures are included. During the dissection of the body the removed organs and tissues were cooled on ice. After rinsing with saline and blotting on filter-paper the whole organ, but not more than 50-100 mg, was put in a pre-weighed counting vial for radioactivity assay by liquid scintillation counting. In each experiment two or three animals served as controls. The control data collected over the whole period of the present study have been grouped together for the statistical evaluation of the results obtained.

Test compounds

Specifications of the steroids and other compounds used have been given in Chapter 3.

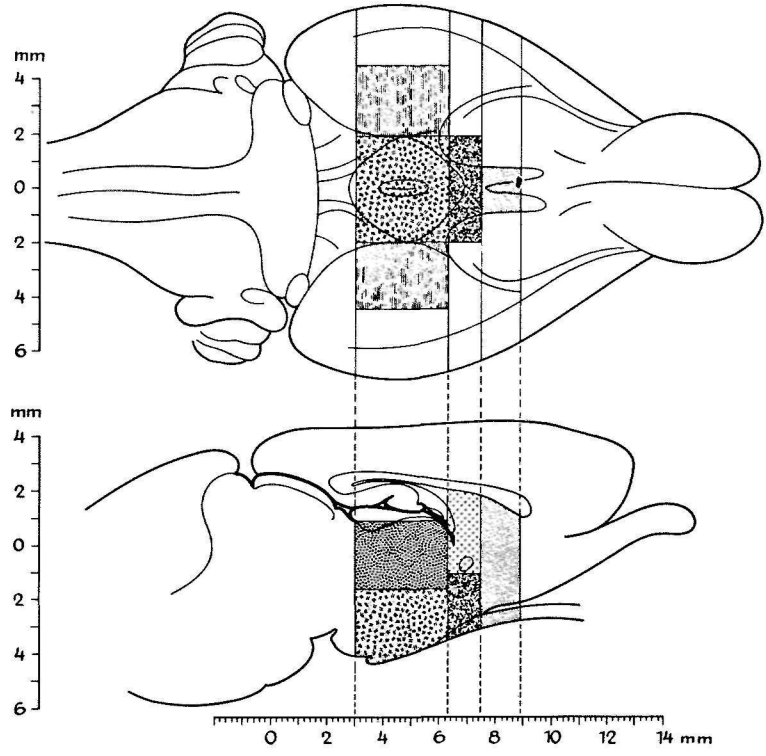

Fig. 9.1 — Dissection scheme of the rat brain illustrated in a mid-sagittal view and an external view from the ventral surface. The shadowed areas indicate the position of the samples taken according to the stereotaxic atlas of the rat brain (König & Klippel, 1963).

amygdala

preoptic area

tractus diagonalis with part of the septum (referred to as septum)

thalamus

septal tissue (referred to as septum)

hypothalamus

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Tissue analysis of estradiol-^3H

The tissues of 5-7 rats were pooled and analysed according to the method used by Jensen & Jacobson (1962) with some modifications. Immediately after sacrifice of the animals the collected tissues were frozen in liquid nitrogen and stored in a freezer at -25°C. After thawing in the cold room the tissues were minced, if necessary, and homogenized in 10 volumes of cold water using a teflon-glass Potter-Elvehjem type homogenizer or an Ultra-Turrax TP 18/2. The homogenate was diluted with 4 volumes of ice-cold ethanol and 25 μg of unlabelled estradiol, estrone and estriol were added to serve as a carrier for the radioactive hormone and the main metabolites. After stirring for 15 min the insoluble material was removed by centrifugation and the supernatant concentrated by evaporation of the ethanol under a stream of nitrogen. The aqueous solution was consecutively extracted twice with light petroleum 80/100 and 3 times with freshly distilled diethylether. The joint ether fractions were subjected to thin-layer chromatography over silicagel (pre-coated plates Kieselgel F_{254}, Merck) in the solvent systems chloroform/ethylacetate 80/20 or chloroform/methanol 97/3. Reference steroids (estradiol, estrone, estriol) were chromatographed on the same plate. The developed area (15 cm) was divided into 30 bands, and the silicagel of each band was scraped off and put into a vial for liquid scintillation counting. The co-chromatographed reference compounds were localized by exposing the plates to iodine vapour. The recovery of estradiol-^3H for the whole procedure was more than 90%.

Injection solutions

Since none of the compounds injected is water soluble, use has been made of a mixture of saline/ethanol 90/10 for the radioactive estradiol and saline/ethanol 50/50 for most of the other steroids. Norgestrel and chlormadinone acetate were administered dissolved in propyleneglycol/ethanol 50/50, because of their poor solubility in the former solvent combination. In all experiments a volume of 0.2-0.3 ml was injected.

In the course of the experiments no differences have been observed between the data collected from the control rats, which received saline/ethanol 90/10, saline/ethanol 50/50 or propyleneglycol/ethanol 50/50 as the solvent mixture.

Assay of radioactivity

Radioactivity was measured by liquid scintillation counting as described in Chapter 5. The standard error of counting was 1.0% for the samples of

Table 9.1 — The distribution pattern of estradiol-^{3}H in the rat one hour after intravenous injection

TISSUE	I[a]	II[b]			III[c]	IV[d]
	dpm/mg	E_2	E_1	E_3	dpm/mg	dpm/mg
Plasma	48.6 ± 1.5 (62)	19	10	2.4	50.9 ± 2.1	48.5 ± 4.6
Heart	46.9 ± 1.1 (62)	25	13	10	43.6 ± 2.4	51.2 ± 2.8
Liver	724 ± 23 (36)	7.4	8.8	5.2	722 ± 26	641 ± 26
Kidney	188 ± 6.2 (36)	47	7.3	4.0	172 ± 14	244 ± 37
Adrenal	219 ± 4.9 (61)	51	21	3.6	165 ± 11**	248 ± 19
Uterus	990 ± 31 (61)	88	1.7	0.8	49.5 ± 2.0***	1194 ± 89
Vagina	336 ± 11 (62)	73	4.5	2.8	34.8 ± 3.9***	421 ± 58*
Anterior pituitary	1458 ± 40 (62)	93	2.1	0.6	86.8 ± 10***	1405 ± 120
Muscle	28.7 ± 0.7 (62)	—	—	—	22.6 ± 1.3*	34.3 ± 2.0
Fat	122 ± 3 5 (58)	57	20	1.0	118 ± 6.6	159 ± 18
Diaphragm	40.8 ± 1.1 (62)	—	—	—	29.9 ± 1.5**	43.2 ± 3.8
Brain cortex	38.4 ± 0.9 (62)	20	10	9.9	42.6 ± 3.1	45.4 ± 3.3*
Thalamus	50.4 ± 2.7 (8)	—	—	—	52.1 ± 1.9	53.3 ± 4.1
Hypothalamus	91.7 ± 1.4 (62)	71	15	1.0	43.0 ± 2.7***	97.5 ± 5.1
Preoptic area	167 ± 2.9 (61)	73	8.1	1.5	53.0 ± 3.1***	160 ± 14
Amygdala	83.6 ± 1.3 (61)	64	14	1.3	43.6 ± 2.5***	76.2 ± 3.8
TD	59.5 ± 1.1 (60)	47	18	6.8	46.6 ± 1.9**	68.1 ± 5.2*
Septum	70.8 ± 1.3 (60)				42.8 ± 2.1***	71.5 ± 3.3

a) Mean ± SE (total radioactivity) in dpm/mg wet tissue of the control data collected over the period covered by the present study. Figures in brackets indicate the number of animals.

b) The tissues of 6 animals were pooled and analysed as described in the experimental part. The results are expressed in percent of the total radioactivity. Abbreviations: E_1 = estrone, E_2 = estradiol, E_3 = estriol.

c) 5 min before estradiol-^{3}H the rats were injected with 20 μg/100 g estradiol (N = 5).

d) 5 min before estradiol-^{3}H the animals were injected with 1 mg/100 g testosterone (N = 5).

Different from control (I) at $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ *** (Student's t-test).

Table 9.2 — Comparison between estradiol

Animals (N)	5	5	5
Survival (min)	5	15	30
TISSUE	dpm/mg wet tissue ± SE		
Plasma	206 ± 13 (47)	98 ± 8.1 (45)	70 ± 2.7 (38)
Heart	301 ± 25 (70)	141 ± 12 (61)	91 ± 6.2 (56)
Cortex	324 ± 28	149 ± 12	78 ± 4.0
Pituitary	1462 ± 139	1493 ± 132	1588 ± 127
Uterus	403 ± 30 (86)	599 ± 68 (83)	812 ± 103 (82)
Preoptic area	546 ± 22	335 ± 17	248 ± 13

The rats received an intravenous injection of 0.1 μg estradiol-^{3}H/100 g body-weight. The figures in

Table 9.3 — Effect of 19-norsteroids

	Control	Ethynodiol	Norethynodrel	Lynestrenol
Number of rats (N)	62	5	5	9
Dose (μg/100 g)		200	200	200
TISSUE	Mean dpm/mg ± SE			
Plasma	48.6 ± 1.5	37.8 ± 2.7	54.4 ± 6.5	59.2 ± 3.9 (8)*
Heart	46.9 ± 1.1	40.6 ± 2.0	43.8 ± 4.0	52.1 ± 2.5
Adrenal	219 ± 4.9 (61)	146 ± 12***	146 ± 11***	194 ± 8.4
Uterus	990 ± 31 (61)	89.2 ± 4.1***	157 ± 14***	464 ± 22***
Vagina	336 ± 11	40.4 ± 2.6***	64.1 ± 3.1***	161 ± 8.0***
Pituitary	1458 ± 40	81.0 ± 3.1***	130 ± 3.1***	300 ± 20***
Brain cortex	38.4 ± 0.9	35.4 ± 2.8	33.1 ± 3.0	38.9 ± 1.9 (8)
Preoptic area	167 ± 2.9 (61)	50.2 ± 2.7***	50.3 ± 3.2***	80.1 ± 3.5***
Hypothalamus	91.7 ± 1.4	43.3 ± 2.6***	42.1 ± 3.3***	58.2 ± 2.8***
Amygdala	83.6 ± 1.3 (61)	44.4 ± 2.8***	44.3 ± 3.6***	56.0 ± 3.1***
TD	59.5 ± 1.1 (60)	46.2 ± 3.1**	44.0 ± 3.8***	52.9 ± 2.4*
Septum	70.8 ± 1.3 (60	43.7 ± 2.5***	41.7 ± 4.0***	53.5 ± 2.8***

The compounds were injected into the tail vein 5 min before the iv administration of 0.1 μg in brackets indicate the number of samples if differing from N. Different from control at $P < 0.05$ *,

distribution with time in target and non-target tissues

62	7	5	5
60	120	240	480
	dpm/mg wet tissue ± SE		
48.6 ± 1.5 (19)	34 ± 2.7 (22)	29 ± 1.2 (13)	25 ± 1.9 (7.3)
46.9 ± 1.1 (25)	25 ± 1.1 (35)	20 ± 0.5	12 ± 0.5
38.4 ± 0.9 (20)	19 ± 1.2	16 ± 0.6	10 ± 0.6
1458 ± 40 (93)	1324 ± 107	1030 ± 80	401 ± 22
990 ± 31 (88)	975 ± 49 (88)	769 ± 44 (87)	268 ± 20 (78)
167 ± 2.9 (73)	102 ± 5.5	69 ± 2.7	22 ± 2.2

brackets give the percentage of the total radioactivity present as estradiol.

on estradiol-^{3}H distribution in the rat

Norethindrone	*d*-Norgestrel	Nortestosterone	Estrenol	Methyllynestrenol
5	8	5	6	6
200	400	400	200	200
		Mean dpm/mg ± SE		
50.2 ± 3.0	48.5 ± 3.3	47.6 ± 5.4	59.6 ± 4.1*	47.3 ± 1.6
52.7 ± 3.7	50.1 ± 2.5	44.6 ± 4.1	63.5 ± 4.1***	47.4 ± 2.5
215 ± 15	207 ± 15	178 ± 17	307 ± 20***	214 ± 11
457 ± 34***	919 ± 50	966 ± 187	900 ± 34	1105 ± 193
170 ± 18***	275 ± 15	296 ± 35	326 ± 32	364 ± 37 (5)
412 ± 25***	1112 ± 59**	996 ± 48**	890 ± 44***	1260 ± 111
43.0 ± 2.9	42.9 ± 2.0	38.3 ± 3.3	57.5 ± 4.2***	39.5 ± 3.2
86.5 ± 6.8***	140 ± 6.8**	110 ± 6.0***	153 ± 8.0	152 ± 4.7
65.0 ± 4.8***	85.6 ± 3.2	64.5 ± 3.2***	92.4 ± 4.9	85.0 ± 3.5
65.7 ± 4.8***	79.4 ± 4.3	57.6 ± 3.8***	87.5 ± 4.5	80.2 ± 2.4
57.0 ± 4.1	62.0 ± 3.9	49.7 ± 3.9*	76.4 ± 5.2***	60.4 ± 4.7 (4)
60.4 ± 4.2*	70.2 ± 4.6	56.9 ± 3.7**	81.2 ± 5.0*	70.8 ± 4.4 (4)

estradiol-^{3}H/100 g body-weight. The rats were sacrificed one hour after the last injection. The figures $P < 0.01$ **, $P < 0.001$ *** (Student's *t*-test).

peripheral tissues and not more than 2.5% for the brain samples and the silicagel samples. The counting efficiency of the tissue samples varied between 15 and 36%. The counting efficiency of the silicagel samples was about 32%.

RESULTS

Distribution pattern of estradiol-^{3}H

With regard to the distribution pattern of estradiol-^{3}H in the rat it can be seen from the results given in Table 9.1, that the anterior pituitary, uterus, vagina, preoptic area, hypothalamus, amygdala and, to a smaller extent, adrenals, tractus diagonalis (TD) and also the septum selectively accumulate and retain unchanged estradiol. Moreover, if 5 min before the tritiated estradiol injection a 200-fold excess of the hormone or another estrogen is administered, the radioactivity in these same tissues is reduced to a level corresponding to the fraction of the metabolites in the first experiment. The radioactivity level in the other tissues, however, remains unchanged. Furthermore, the observed distribution pattern appears to be estrogen specific, because administration of a 10,000-fold excess of testosterone before estradiol-^{3}H has no effect at all. The results mentioned so far are thus in agreement with and complementary to previous studies (Eisenfeld & Axelrod, 1966; Jensen & Jacobson, 1962; Kato & Villee, 1967; McEwen & Pfaff, 1970; Pfaff, 1968; Stumpf & Sar, 1971; Zigmond & McEwen, 1970).

The uptake and retention of unchanged estradiol in the pituitary, uterus and preoptic area against a concentration gradient, in contrast to the heart and brain cortex, is obvious from the data given in Table 9.2. Although the preoptic area does not show an accumulation and retention like that observed for the uterus and pituitary, the difference is, however, evident in comparison with the cortex. In this respect one has to take into consideration, that compared with the capacity of the peripheral organs, the total specific binding capacity for estradiol in the CNS target tissues seems to be rather low as can be derived from the extent to which the estradiol uptake is reduced by a preceding injection of the unlabelled hormone (Table 9.1). Hence the possibility of observing a differential distribution pattern of the hormone within the brain is determined largely by the dissection procedure used: the specific-bound activity is superimposed upon the activity present on account of the non-specific distribution processes. Thus when the former is small relative to the latter, "contamination" of receptor-rich with receptor-poor material will influence the

results to a great extent. For that reason the dissection procedure used has been derived from the radioautographic data reported by Stumpf (1970).

Effect of contraceptive progestins and related compounds

The distribution pattern of estradiol one hour after injection changed very much under the influence of a previous injection of the contraceptive progestins ethynodiol, norethynodrel, norethindrone and lynestrenol. These compounds, administered in 2000-fold excess, reduced the uptake of estradiol significantly in all target tissues (Table 9.3), although with different potency. The other members of the series of the 19-norsteroids tested, however, demonstrated less or no activity in this respect. Methyllynestrenol appeared to be inactive, and nortestosterone was more active than *d*-norgestrel, but in spite of a double dose less active than norethindrone. Estrenol reduced the radioactivity in the pituitary only. The increased levels of radioactivity in the heart, adrenal and CNS tissues in this experiment are probably the result of the high plasma and cortex levels. The same phenomenon was observed after the administration of megestrol, megestrol acetate and the acetate esters of medroxyprogesterone and chlormadinone. These hydroxyprogesterone derivatives did not reduce the radioactivity in the target tissues even after administration in a 4000- to 5000-fold excess over estradiol-^{3}H (Table 9.4), but seemed at this high dose level to have some influence on the clearance of estradiol from the plasma. Similar results have been reported by Eisenfeld & Axelrod (1967) for chlormadinone acetate and medroxyprogesterone acetate.

Because in the case of norethindrone, ethynodiol and lynestrenol the acetate esters are clinically of importance, these compounds have also been studied. From Table 9.5 it is apparent, that the esters have the same properties as the parent alcohols, but in a quantitative way they seem to possess a somewhat lower potency. This activity may be explained either by direct binding to the receptor or it could result from hydrolysis of the esters after the injection. The velocity of the hydrolysis should then be very high, as administration of lynestrenol 5 min after estradiol no longer results in a significant decrease of radioactivity in the hypothalamus and preoptic area (Table 9.6).

Influence of the time of administration

Studying the influence of the time of administration of lynestrenol on the estradiol distribution pattern, it was observed, that the applied sequence of

Table 9.4 — Effect of hydroxyprogesterone derivatives

	Control	Chlormadinone	Medroxy-progesterone
Number of rats (N)	62	5	5
Dose (μg/100 g)	—	435	435
TISSUE		Mean dpm/mg ± SE	
Plasma	48.6 ± 1.5	48.2 ± 2.5	44.9 ± 3.4
Heart	46.9 ± 1.1	51.0 ± 2.4	47.6 ± 3.1
Muscle	28.7 ± 0.7	27.6 ± 1.4	24.6 ± 1.0
Adrenal	219 ± 4.9 (61)	247 ± 15	245 ± 10
Uterus	990 ± 31 (61)	1035 ± 161	885 ± 82
Vagina	336 ± 11	393 ± 24	354 ± 31
Anterior pituitary	1458 ± 40	1407 ± 132	1342 ± 150
Brain cortex	38.4 ± 0.9	42.6 ± 2.8	41.9 ± 4.4
Preoptic area	167 ± 2.9 (61)	175 ± 5.9	163 ± 3.5
Hypothalamus	91.7 ± 1.4	89.4 ± 4.1	90.8 ± 5.3
Amygdala	83.6 ± 1.3 (61)	85.6 ± 3.4	91.1 ± 12
TD	59.5 ± 1.1 (60)	64.5 ± 3.4	61.6 ± 3.1
Septum	70.8 ± 1.3 (60)	74.1 ± 2.6	69.8 ± 2.7

The compounds were injected into the tail vein 5 min before the iv administration of 0.1 μg figures in brackets indicate the number of samples if differing from N. Different from control at

Table 9.5 — Comparison of the effects of steroid alcohols and the corresponding

	N	Plasma	Uterus
		Mean dpm/mg ± SE	
Control	62	48.6 ± 1.5	990 ± 31 (61)
Lynestrenol	9	59.2 ± 3.9 (8)*	464 ± 22***
Lynestrenol acetate	5	48.0 ± 2.5	647 ± 65**
Ethynodiol	5	37.8 ± 2.7	89.2 ± 4.1***
Ethynodiol diacetate	6	60.5 ± 8.3*	179 ± 11***
Norethindrone	5	50.2 ± 3.0	457 ± 34***
Norethindrone acetate	6	65.5 ± 6.2**	564 ± 48***

The progestins were injected into the tail vein 5 min before the iv administration of 0.1 μg progestins were given in a dose of 200 μg/100 g except norethindrone acetate (250 μg/100 g). Different

on estradiol-^{3}H distribution pattern in the rat

Megestrol	Chlormadinone acetate	Medroxy-progesterone acetate	Megestrol acetate
5	7	9	5
500	400	500	500
	Mean dpm/mg ± SE		
54.0 ± 2.9	63.6 ± 3.5**	75.3 ± 4.3***	73.6 ± 6.8***
66.5 ± 2.8***	67.4 ± 2.1***	82.6 ± 2.9***	85.7 ± 4.2***
37.8 ± 2.1***	39.7 ± 1.4 (5)***	46.3 ± 2.4***	46.9 ± 2.9***
262 ± 9.5*	288 ± 15 (5)***	372 ± 16***	283 ± 23***
1014 ± 137	929 ± 97	1191 ± 104	915 ± 66
396 ± 43	371 ± 21	420 ± 30**	366 ± 27
1579 ± 182	1484 ± 150	1763 ± 76**	1256 ± 84
55.3 ± 3.1***	51.9 ± 2.1***	74.3 ± 2.9***	69.0 ± 6.5***
220 ± 11***	207 ± 14***	245 ± 12***	198 ± 12**
119 ± 5.7***	117 ± 4.4***	135 ± 3.9***	129 ± 6.5***
107 ± 6.5**	112 ± 2.5 (5)***	127 ± 2.8***	118 ± 7.7***
77.7 ± 4.6***	83.9 ± 3.1 (5)***	98.0 ± 3.9***	93.1 ± 7.2***
94.6 ± 3.6***	105 ± 7.9 (5)***	116 ± 3.9***	106 ± 19***

estradiol-^{3}H/100 g body-weight. The animals were sacrificed one hour after the last injection. The $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ *** (Student's t-test).

acetates on the uptake of estradiol-^{3}H by the target tissues of the rat

Ant. pituitary	Cortex	Hypothalamus	Preoptic area
	Mean dpm/mg ± SE		
1458 ± 40	38.4 ± 0.9	91.7 ± 1.4	167 ± 2.9 (61)
300 ± 20***	38.9 ± 1.9 (8)	58.2 ± 2.8***	80.1 ± 3.5***
530 ± 67***	44.6 ± 2.3	77.3 ± 2.9**	119 ± 6.8***
130 ± 3.1***	35.4 ± 2.8	43.3 ± 2.6***	50.2 ± 2.7***
93.9 ± 20***	35.5 ± 2.2	43.0 ± 2.8***	51.1 ± 2.6***
412 ± 25***	43.0 ± 2.9	65.0 ± 4.8***	86.5 ± 6.8***
577 ± 79***	49.0 ± 3.8**	79.5 ± 5.1 (4)*	103 ± 5.9***

estradiol-^{3}H/100 g body-weight. The animals were killed one hour after the last injection. The from control at $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ *** (Student's t-test).

administration of these compounds is of crucial significance for the occurrence and the extent of binding inhibition in the target tissues (Table 9.6). Although the maximal effect was obtained, when lynestrenol was administered 5 min before the estrogen, the inhibition of estradiol uptake remained considerable, when lynestrenol was injected 30 and 60 min prior to estradiol. A rapid decrease, however, of the inhibitory effect appeared to occur when lynestrenol was administered after estradiol. The difference is most pronounced in the CNS tissues. As a consequence of the small binding capacity for estradiol in these tissues in comparison with the uterus and the anterior pituitary, which is evident from the data presented in Table 9.1 and has been confirmed later on in investigations using cytosol samples of rat pituitary, hypothalamus and uterus (Korach & Muldoon, 1974; Morris, 1976), it is conceivable, that at similar ligand concentrations the fraction of occupied receptor molecules in the CNS target tissues may exceed the corresponding fraction in the uterus and pituitary. If the occupation of the receptor binding sites is followed by the translocation of the estradiol-receptor complex to the target cell nucleus (see Chapter 1) and, consequently, by a decrease of specific binding sites in the cytosol, this could explain the more rapid abolishment of the lynestrenol effect in the CNS tissues. Moreover, it is obvious from the data in Tables 9.1 and 9.2, that apart from receptor-bound estradiol a relatively large pool of "non-specific" estradiol is present in the CNS target tissues, which may be supposed to be available for an exchange with the receptor-bound hormone. In this respect just an opposite relationship may exist for the ratio between the "non-specific" radioligand and the competing lynestrenol in the pituitary and the uterus, because about 90% of the estradiol in these organs appeared to be specifically bound (Tables 9.1, 9.2). For this reason it may be assumed, that, in case of exchange between bound and unbound hormone, in these tissues an exchange of estradiol for lynestrenol is more likely to occur than in the CNS tissues.

Similarly, the influence of time on the observed effect, when lynestrenol is administered prior to estradiol, may be explained in terms of depletion of cytosol receptor, dissociation of the lynestrenol-receptor complex, and elimination of lynestrenol. Distribution studies with lynestrenol in mice have indicated, that the high lipophilicity of lynestrenol will delay its elimination from the central nervous system (see Chapter 14). This delay may contribute to the comparatively high degree of inhibition in these tissues, that is observed, when the compound is injected 60 min before estradiol. On account of the present findings, however, a differentiation between the relative importance of pharmacokinetic and binding parameters for the results obtained is difficult. Further investigations using other techniques, e.g. chase experiments with radiolabelled and radioinert ligand, may shed more light on this aspect.

Table 9.6 — Effect of time of administration of lynestrenol on estradiol-^{3}H binding in the target tissues

	60 min before	30 min before	5 min before	5 min after	15 min after	30 min after
Number of rats	5	4	9	5	4	4
TISSUE	Mean dpm/mg ± SE					
Plasma	41.8 ± 2.6	58.6 ± 3.9	59.2 ± 3.9*	53.3 ± 4.6	48.0 ± 6.5	53.8 ± 1.2
Heart	40.6 ± 2.5	52.5 ± 2.9	52.1 ± 2.5	50.9 ± 2.9	51.7 ± 6.0	43.2 ± 2.1
Adrenal	209 ± 9.8	233 ± 9.8	194 ± 8.4	214 ± 12	226 ± 24	208 ± 13
Uterus	856 ± 58	586 ± 36**	464 ± 22***	519 ± 28***	582 ± 81**	805 ± 150
Vagina	217 ± 28**	144 ± 29***	161 ± 8.0***	231 ± 19**	263 ± 20	341 ± 45
Anterior pituitary	591 ± 20***	601 ± 166***	300 ± 20***	825 ± 63***	1119 ± 109*	1526 ± 63
Cortex	35.4 ± 2.2	46.5 ± 2.9*	38.9 ± 1.9	41.9 ± 1.1	39.0 ± 3.8	34.6 ± 2.2
Preoptic area	82.0 ± 6.1***	102 ± 9.3***	80.1 ± 3.5***	146 ± 10	148 ± 14	150 ± 3.7
Hypothalamus	54.0 ± 2.3***	69.3 ± 4.6***	58.2 ± 2.8***	85.3 ± 3.9	90.7 ± 6.6	85.9 ± 1.6
Amygdala	52.8 ± 2.6***	69.0 ± 4.9**	56.0 ± 3.1***	83.6 ± 3.7	80.6 ± 9.0	78.7 ± 2.0
TD	46.5 ± 2.4**	62.0 ± 4.0	52.9 ± 2.4*	63.2 ± 2.3	61.8 ± 6.9	55.8 ± 1.8
Septum	51.9 ± 2.6***	65.0 ± 4.5	53.5 ± 2.8***	73.7 ± 4.3	69.1 ± 7.5	71.2 ± 1.9

The compounds (200 µg/100 g lynestrenol and 0.1 µg/100 g estradiol-^{3}H) were administered into the tail vein. Lynestrenol was injected either before or after estradiol as indicated. The animals were killed one hour after the estradiol-^{3}H injection.
The results have been compared for the statistical evaluation with the control data given in Table 9.3.
Different from control at $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ *** (Student's t-test).

The inhibition of estradiol binding in the target tissues increases increase of the lynestrenol dose. In Fig. 9.2 this dose-effect relatio illustrated for the preoptic area, the anterior pituitary and the uterus. latter organs the fraction of the total radioactivity, that is estrogen bound, is 90-95%, for the preoptic area this fraction is about 65% (Ta In order to make a correct comparison of the relationship between t and the inhibition of estradiol binding this difference should be tal account. Therefore, the reduction of specific binding was calculated organ after correcting the total radioactivity measured for the corres non-specific fraction. The residual binding in the tissues observed in the of an excess of non-labelled estradiol (Table 9.1, column III) was co to be a good approximation of this fraction.

Looking at the curves thus obtained (Fig. 9.2) it is evident, that al a dose range of 25-50 μg lynestrenol per 100 g body-weight the inhit estradiol binding in the pituitary and preoptic region is considerable a be of relevance for the activity of lynestrenol and probably also eth norethynodrel and norethindrone. Furthermore, it can be observed degree of inhibition obtained with the same dose of lynestrenol is muc

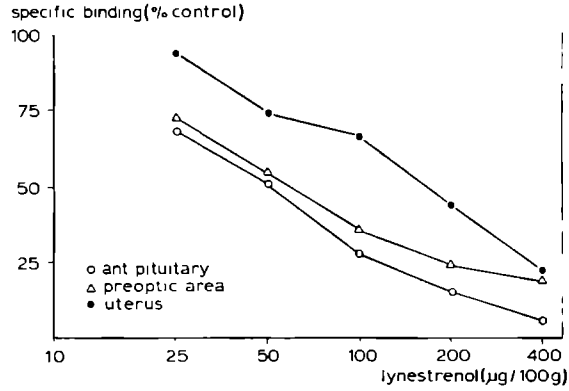

Fig. 9.2 — Relationship between the lynestrenol dose and the reduction o estradiol-^{3}H binding. The experimental procedure and the control animals are th mentioned in Table 9.3. Each point in the curves represents the mean of at least 5 values in per cent of the corresponding control.

for the pituitary and preoptic area than for the uterus. This difference could be caused by a higher ratio of lynestrenol over estradiol in the environment of the receptors or by a difference in the binding affinities of the steroids to the receptors in the respective tissues. This latter possibility does not seem to be very probable in the light of other investigations (Ginsburg et al., 1974; Kahwanago et al., 1970; Korach & Muldoon, 1974; Notides, 1970; Talley et al., 1975). Distribution studies, on the other hand, with tritiated lynestrenol in the mouse revealed, that after intravenous injection the concentration of this compound was much higher in the pituitary than in the uterus (see Chapter 14, Table 14.1). This observation and the different concentration-time course of estradiol uptake in the uterus and pituitary as shown in Table 9.1 are strong indications that distribution factors are involved.

DISCUSSION

Previous studies

The experimental results described above indicate the existence of a competitive interaction between estradiol-^{3}H and the contraceptive progestins of the 19-nortestosterone group with regard to estradiol binding to the estrogen receptor. This interaction may contribute to the spectrum of activity of these steroids. Estrogenic effects of norethindrone, norethynodrel, ethynodiol and lynestrenol are known from bio-assay procedures (Desaulles & Krähenbühl, 1964; Elton & Nutting, 1961; McGinty & Djerassi, 1958; Overbeek et al., 1962; Saunders et al., 1957) as well as from clinical studies (Paulsen et al., 1962; Paulsen, 1965), but have been attributed to their highly questionable (Breuer, 1970; Sisenwine et al., 1974) biotransformation to A-ring aromatized metabolites. Although it has been demonstrated by us (Van Kordelaar et al., 1975) that the active progestins as such can compete with estradiol for the receptor binding sites, *in vivo* estrogenic conversion products may still be involved. The metabolic clearance rate of lynestrenol in the rat seems high enough to account for a contribution of metabolites to the observed effect (this thesis, Chapter 16). A rapid disappearance from the blood of rabbits and humans has also been observed by others after the administration of ethynodiol, norethindrone, norethynodrel and lynestrenol (Cook et al., 1972, 1973; Gerhards et al., 1971; Kamyab et al., 1967, 1968a,b). The elucidation, however, of the chemical structure of a number of these metabolites up to the present does not support this possibility (Breuer, 1970; Coert et al., 1975; Cook et al., 1972, 1973;

Freudenthal et al., 1971; Gerhards et al., 1971; Kishimoto et al., 1972a,b; Mazaheri et al., 1970; Okada, 1972; Palmer et al., 1969; this thesis, Chapter 12). In norethindrone, reduction of the 4-en-3-one group in ring A to the 5α-estran-3β-ol isomer may be expected to give rise to increase of binding affinity. A similar effect may result from the reduction of norethynodrel to 17α-ethynyl-5(10)-estrene-3β,17β-diol (see Chapter 10). At present, however, no information is available with respect to the significance of these metabolic pathways in the overall elimination of the compounds and with respect to the concentration-time course of these metabolites in the blood and target organs. As far as lynestrenol is concerned the data in Table 9.6 also indicate, that the metabolites formed in the first hour after the administration of the compound do not potentiate the ultimate effect.

The results obtained in the present study are in accordance with the observations of Eisenfeld & Axelrod (1965, 1967) for norethynodrel, norethindrone, chlormadinone acetate and medroxyprogesterone acetate, of Saucier et al. (1970) for norethynodrel and of Banerjee et al. (1973) for norethindrone. The last investigators also reported a reduction in estradiol uptake in the uterus, vagina, adrenal, pituitary and hypothalamus by *dl*-norgestrel in a dose of 100 μg/100 g body-weight; in a quantitative sense the effect observed for *dl*-norgestrel was equal to that of the same dose of norethindrone. In the present study 200 μg/100 g norethindrone *in vivo* was far more active than 400 μg/100 g *d*-norgestrel; after *d*-norgestrel administration a small though significant decrease in estradiol uptake was only observed in the pituitary and preoptic area. *In vitro* the reduction of estradiol-^{3}H binding to the rat uterine receptor was marginal for both *d*- and *dl*-norgestrel in contrast to the effect of norethindrone (Van Kordelaar et al., 1975). We have no explanation at the moment for this discrepancy in results. In this connection, however, it should be pointed out, that norgestrel has been claimed to be completely devoid of estrogenic properties (Edgren et al., 1966, 1967).

From the results mentioned in Table 9.3 and in Chapters 10 and 11, it can be derived that the competing progestins have different binding affinities, which are much smaller than the affinity of estradiol to the receptor. These low binding affinities could at least partially explain the negative results reported by Watanabe et al. (1968). In view of the study of Eisenfeld & Axelrod (1965) these investigators did expect a specific uptake and retention of norethynodrel, norethindrone and ethynodiol into the uterus, pituitary and hypothalamus of the rat after the injection of the tritiated progestins in a dose of 0.1 μg/100 g body weight. Such a distribution pattern, however, is only to be expected with the specific activity of 3.5 Ci/mmol used, providing the binding affinity of the progestins is of the same order of magnitude as that of estradiol.

This condition is not required for observing the competitive interaction in the test system used by Eisenfeld & Axelrod (1965, 1967) and in the present study.

In contrast to the data presented here and by Eisenfeld & Axelrod (1967), Rosner et al. (1972) reported a reduction of estradiol uptake in the uterus and pituitary of the rat after the administration of chlormadinone acetate. It was also observed that the decrease in uterine radioactivity 1 h after the estradiol injection was restricted entirely to the nuclear cell fraction. In view of this result these investigators assumed the occurrence of a competition between estradiol and chlormadinone for binding to the estrogen receptor. It is, however, not clear from their assumption why the maximal decrease of radioactivity in the uterus and pituitary (about 50%) already occurred at submaximal doses of chlormadinone administered, whereas the maximal decrease obtained does not correspond with the estradiol fraction that is specifically bound in this organ (cf. Fig. 9.2 and Table 9.1). Since they did not observe a reduction of hypothalamic estradiol either and since no studies with the receptor protein in a cell-free system were performed, the influence of some non-specific process in their experiments cannot be excluded.

Inhibition of ovulation

Most compounds that have been studied, are frequently prescribed for contraceptive purposes because of their anti-ovulatory action. Although the mechanism of action of the contraceptive steroids in current use is still not quite understood, changes in the synthesis, storage and release of the gonadotrophic hormones have been demonstrated in animals and humans and seem to be responsible for the effect (Becker et al., 1973; Dufau et al., 1970; Labhsetwar, 1973; Schally et al., 1968, 1970; Thomas & Ferin, 1972; Thomas et al., 1972). Since estradiol binding to pituitary and CNS receptors may trigger feed-back effects on gonadotrophin release and ethynodiol, norethynodrel, norethindrone and lynestrenol and their acetates interfere with estradiol binding in these same tissues after the administration of anti-ovulatory doses, it seems not improbable that the pattern of attack for the ovulation inhibition is incongruous with that of the non-competing progestins. An additional indication for this assumption may also derive from the observation of a difference in the mechanism of action between lynestrenol and methyllynestrenol as shown by Overbeek & De Visser (1964). Further support for this concept is given by the observations of Baker et al. (1973). After implantation of norethynodrel, norethindrone and medroxyprogesterone in the pars distalis of the rat pituitary, both norethynodrel and norethindrone induced hypertrophy of the prolactin

cells and a reduction in size of the luteinizing hormone synthetizing cells, whereas medroxyprogesterone caused no changes at all. The extrapolation of such a divergent mechanism of action for the competing nortestosterone derivatives and the non-competing steroids from rat to man seems permissible as far as the properties and nature of the estrogen receptors are concerned. Although the receptors have not yet been isolated in a purified form, studies with the human and rat uterine receptor (Evans & Hähnel, 1973; Hähnel, 1971; Hähnel et al., 1973; Korenman, 1970; Martin, 1972; Notides et al., 1972) on the one hand and the receptors of the uterus, hypothalamus and pituitary on the other hand (Ginsburg et al., 1974; Jensen et al., 1969; Kahwanago et al., 1970; Korach & Muldoon, 1974; Kato, 1973; Notides, 1970; Talley et al., 1975) have revealed no substantial differences.

SUMMARY

The distribution pattern of estradiol in ovariectomized rats as a function of time has been studied following intravenous administration of the tritiated hormone. Estrogen specific binding with limited capacity was observed in the uterus, vagina, anterior pituitary, adrenals, preoptic area, hypothalamus, amygdala, septum and tractus diagonalis. Maximal uptake of estradiol in the pituitary occurred within 5 min, in the uterus 60 min after injection and remained almost unchanged at this level for more than two hours. The binding capacity per mg tissue decreased in the order pituitary, uterus, vagina, preoptic area, adrenals, hypothalamus, amygdala, septum and tractus diagonalis.

The hormone concentration in these tissues one hour after estradiol-^{3}H injection was lowered by previous administration of ethynodiol, norethynodrel, lynestrenol and norethindrone, whereas medroxyprogesterone, chlormadinone, megestrol and methyllynestrenol had no effect. The same results were obtained, when instead of the steroid alcohols the corresponding acetate esters were administered. For norgestrel, estrenol and nortestosterone the effect in the dose range studied was limited to the pituitary and preoptic area.

For lynestrenol the inhibition of estradiol binding in the target tissues was almost the same when the progestin was given 60 and 5 min before estradiol, whereas in the case of administration 30 min after estradiol no inhibition was observed. The reduction of estrogen binding appeared to be dose-dependent, but the dose required to obtain a certain effect for the uterus was four times as high as for the pituitary.

Discrepancies between previous studies and the implications of the present findings for the mechanism of action of ovulation inhibition by these progestins are discussed.

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EFFECT OF CONTRACEPTIVE STEROIDS AND RELATED COMPOUNDS ON ESTRADIOL-^{3}H BINDING TO THE RAT UTERINE RECEPTOR IN VITRO

INTRODUCTION

In Chapter 9 of this thesis it has been shown that contraceptive progestins of the 19-nortestosterone series are able to interfere to a varying degree with estradiol binding in the target tissues of the female rat. The extent to which this interaction occurs, is determined by the concentration of the inhibitor in the receptor compartment and its affinity to the receptor. The former parameter depends on the dose, the route of administration and the pharmacokinetics of the compound studied, viz. the rate of absorption, distribution, metabolism and excretion; the latter depends on its molecular structure. Since on the one hand the progestins studied are metabolised extensively and very rapidly (Fotherby, 1974; Fotherby & James, 1972; Thijssen, 1972) and for this reason metabolites could be responsible for the effect, and on the other hand a very short time of half-life could mask an interaction *in vivo*, additional studies have been undertaken using a less complicated test system. Evidence, that the *in vivo* decrease in the binding of estradiol may be caused by a competition of the unchanged progestins with estradiol for the receptor binding sites has been obtained from incubation experiments with the 105,000xg supernatant of rat uterus homogenate. In order to gain an insight into the structural requirements of significance for the occurrence of binding, the behaviour of some related compounds has also been studied.

MATERIALS AND METHODS

Binding studies with rat uterine cytosol

The estrogen receptor preparation was obtained from the uteri of 24-28 days old Wistar rats. The uteri were trimmed of fat and other tissue, minced, washed

with buffer (0.01 M Tris-HCl, pH 7.4, containing 0.0015 M EDTA) and the tissue was then homogenized with carborundum in the same buffer solution. The homogenate was centrifuged for 1 h at 105,000xg in a Spinco L2-75B ultracentrifuge using a Spinco 50Ti rotor. The supernatant was diluted with buffer to a volume of 1.0 ml per uterus in the case of incubation studies and 1.0 ml per 3-5 uteri for incubation experiments followed by density gradient centrifugation, and used on the same day. The protein content was determined according to Lowry et al. (1951). All manipulations with biological material were performed at 1-4°C.

For the incubations silanized glass tubes were used to prevent adsorption phenomena. The indicated amount of estradiol-^{3}H alone or together with a certain excess of a non-labelled steroid was incubated under continuous and gentle agitation with 0.2 ml cytosol until equilibrium was attained; 20 h appeared to be sufficient. Afterwards free and bound hormones were separated by the addition of 0.2 ml of a charcoal-dextran suspension (0.1% Aktivkohle, Merck, and 0.05% Dextran T 70, Pharmacia, in Tris-EDTA buffer) as described by Herbert et al. (1965) and discussed in Chapter 6. After stirring for 15 min the charcoal was spun down (10 min, 600xg) and 0.2 ml of the clear supernatant was put in a counting vial or layered on linear gradients of 5 tot 20% sucrose (SIGMA) in Tris-EDTA buffer, pH 7.4. The gradients, 4.0 ml in polyallomer tubes, were centrifuged for 7 h at 300,000xg in a Spinco L2-75B ultracentrifuge with a Spinco SW 56Ti rotor. After the run 23-25 fractions were tapped from the bottom of the tubes with the aid of a LKB peristaltic pump and collected directly in counting vials (LKB fraction collector Ultrorac). Beef liver catalase (Boehringer) and bovine serum albumin (BSA, Sigma) were used as standards for sedimentation coefficient estimations by the method of Martin & Ames (1961). The proteins were localized with the help of a LKB Uvicord II spectrometer.

Test compounds

Specifications of the steroids and other compounds used have been given in Chapter 3.

Determination of R_M values

The R_M values of the steroids were determined by reversed-phase chromatography on silicagel-coated glass plates (Kieselgel 60 F_{254}, Merck) impregnated

with silicone fluid as a non-polar stationary phase (silicone MS 200, Midland Ltd., U.K.). The impregnation was carried out by developing the plates overnight in a chromatographic chamber containing 200 ml 5 per cent by weight of silicone oil in ether. After the application of the compounds (10 μg/spot) the plate was developed with silicone saturated acetone/water mixture 50/50 at room temperature over a distance of 15 cm. The spots were detected by iodine vapour or UV-absorption.

Assay of radioactivity

The radioactivity of incubation and gradient samples was measured by liquid scintillation counting as described in Chapter 5. The standard error of counting was 2.5% or less and the counting efficiency was about 45%.

RESULTS

Properties of the receptor preparation; effect of drugs on estradiol binding

In Table 10.1 the results are shown of experiments, in which different cytosol batches have been incubated with estradiol-^{3}H and the progestational drugs under discussion. By way of comparison some estrogens have been included. Each compound was incubated with at least two cytosol preparations.

From the results with estradiol-17β, estradiol-17α, ethinylestradiol and diethylstilbestrol on the one hand and progesterone, corticosterone and testosterone on the other hand, it is obvious that 90-95% of the total binding measured is stereospecific for estradiol, estrogen specific and of limited capacity. After incubation of the cytosol with estradiol-^{3}H and centrifugation of the mixture on a density gradient the peak of radioactivity was localized in the 8-9S region (Fig. 10.1a). These observations are in accordance with the characteristics of the estrogen receptor as reported in the literature (Notides, 1970; Toft & Gorski, 1966; Toft et al., 1967).

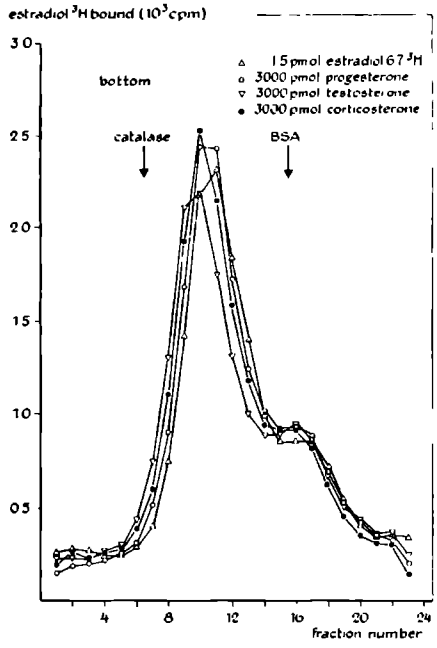
In some experiments a small peak of radioactivity also appeared in the 4S region. As the presence of this peak was also inhibited by estrogenic, but not by other substances, it is presumably the more slowly sedimenting "high salt" receptor species (DeSombre et al., 1969). This phenomenon and also the tendency of the 8S receptor to form more rapidly sedimenting aggregates has been investigated by Chamness & McGuire (1972), Sica et al. (1976) and Stancel et al. (1973), and may be attributed to protein-protein interactions

Table 10.1 — Binding of estradiol-^{3}H to rat uterine cytosol in the presence of estrogenic and non-estrogenic compounds

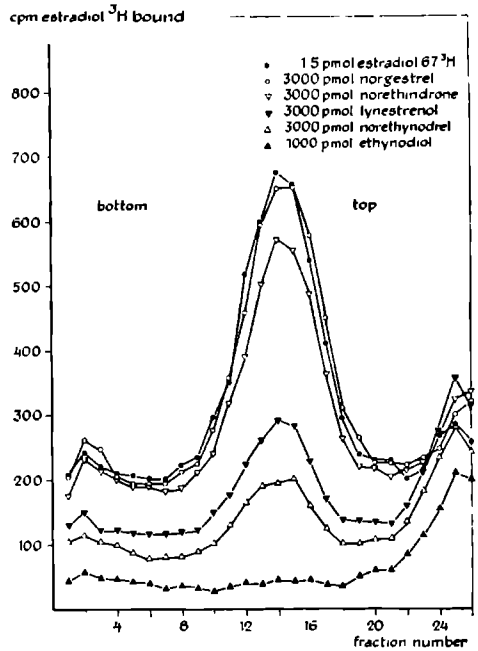
Cytosol preparations		I	II	III	IV	V	VI
Number of incubations (N)		4	5	5	5	5	5
		cpm ± SE					
CONTROL		4280 ± 70	4277 ± 73	3859 ± 41	4569 ± 73	5003 ± 67	4396 ± 141
TEST COMPOUNDS	nmol						
Estradiol-17β	0.10				401 ± 9		
Ethinylestradiol	0.10		332 ± 7		402 ± 16		
Diethylstilbestrol	0.10	369 ±21		377 ± 6			267 ± 8
Estrone	0.10		677 ± 29		587 ± 14		
Estradiol-17α	1.00		576 ± 50		459 ± 37		
Progesterone	1.00	4165 ± 28					4056 ± 74
Testosterone	1.00	4379 ± 34			4543 ± 51		
Corticosterone	1.00				3980 ± 28	4616 ± 35	
Ethynodiol	0.25		553 ± 14			419 ± 14	
Norethynodrel	1.00		913 ± 56			817 ± 18	
Lynestrenol	1.00	885 ± 43		573 ± 27			
Norethindrone	1.00	3304 ± 26		2804 ± 38	3573 ± 43		
d-Norgestrel	1.00	3941 ± 84					4007 ± 227
dl-Norgestrel	1.00						3929 ± 96
Estrenol	1.00				1893 ± 17	2265 ± 92	
Nortestosterone	1.00	3448 ± 15		3188 ± 79		3915 ± 130	

Methyllynestrenol	1.00	3787 ± 44			3802 ± 131			4416 ± 72		
Ethisterone	1.00				2880 ± 105		3810 ± 168	4068 ± 62		
Ethynodiol diacetate	0.25			2745 ± 50				2557 ± 69		
Lynestrenol acetate	1.00				3301 ± 51		3818 ± 86			
Norethindrone acetate	1.00				3577 ± 39		4047 ± 69			
Hydroxyprogesterone	1.00			3983 ± 67			4520 ± 12			
Medroxyprogesterone	1.00			4265 ± 105						4124 ± 85
Chlormadinone	1.00	4373 ±84		4135 ± 132						
Megestrol	1.00			3901 ± 90			4147 ± 41			3863 ± 12
Medroxyprogest. acetate	1.00			4255 ± 23						4221 ± 88
Chlormadinone acetate	1.00			4303 ± 45						4278 ± 68
Megestrol acetate	1.00			4340 ± 36			4563 ± 36	4623 ± 78		

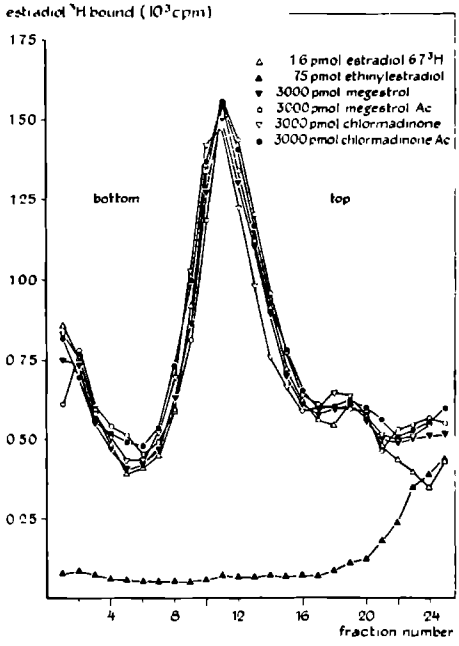
0.2 ml of rat uterine cytosol was incubated with 0.5 pmol of estradiol-^{3}H alone (control) or together with the indicated amount of the test compound. The values are means ± SE of N incubations and represent half of the total bound radioactivity. The protein content of the 6 cytosol preparations varied between 0.6 and 0.7 mg/ml.


estradiol ³H bound (10³ cpm)
30
25
20
15
10
05
bottom
catalase
BSA
1 5 pmol estradiol 6 7 ³H
3000 pmol progesterone
3000 pmol testosterone
3000 pmol corticosterone
4
8
12
16
20
24
fraction number

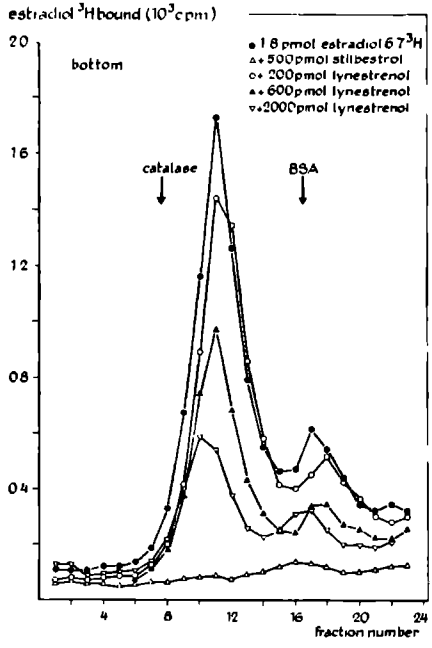
A


cpm estradiol ³H bound
800
700
600
500
400
300
200
100
bottom
top
1 5 pmol estradiol 6 7 ³H
3000 pmol norgestrel
3000 pmol norethindrone
3000 pmol lynestrenol
3000 pmol norethynodrel
1000 pmol ethynodiol
4
8
12
16
20
24
fraction number

B


estradiol ³H bound (10³ cpm)
175
150
125
100
075
050
025
bottom
top
1 6 pmol estradiol 6 7 ³H
75 pmol ethinylestradiol
3000 pmol megestrol
3000 pmol megestrol Ac
3000 pmol chlormadinone
3000 pmol chlormadinone Ac
4
8
12
16
20
24
fraction number

C


estradiol ³H bound (10³ cpm)
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catalase
BSA
1 8 pmol estradiol 6 7 ³H
500 pmol stilbestrol
200 pmol lynestrenol
600 pmol lynestrenol
2000 pmol lynestrenol
4
8
12
16
20
24
fraction number

D

resulting from environmental conditions, including among others the protein concentration, the ionic strenght and aging of the cytosol.

Assuming in the present study that a reduction of estradiol binding of more than 10 per cent in the uterine cytosol, is the result of an occupation of receptor binding sites, ethynodiol, norethynodrel, lynestrenol, estrenol, norethindrone, nortestosterone, ethisterone and the acetate esters of ethynodiol and lynestrenol are to be considered as inhibitors, but not the hydroxyprogesterone derivatives and methyllynestrenol (Table 10.1). As to norgestrel and norethindrone acetate the results obtained indicate almost negligible effects. The density gradient patterns of Figs. 10.1b and 10.1c confirm these results. All the active compounds suppress to some extent the peak of the estradiol-receptor complex. This suppression appeared to be dose dependent as is demonstrated for lynestrenol in Fig. 10.1d. In the presence of a sufficiently high excess of inhibitor, as in the case of ethynodiol in Fig. 10.1b, a total displacement of estradiol from its specific binding sites could be observed.

Competitive interaction

In order to decide whether this inhibitory effect reflects a change in the affinity or in the number of available estradiol binding sites, three lots of cytosol have been incubated with increasing concentrations (0.50-3.75 nM) of the radioactive ligand alone and also in the presence of two concentrations inhibitor. The double reciprocal plots (Lineweaver & Burk, 1934) obtained as the result of experiments with ethynodiol and lynestrenol are shown in Fig. 10.2 and are typical for a competitive interaction. The apparent dissociation constants (K_D) for estradiol as calculated from these plots are 2.5×10^{-10} M for experiment a and 3.8×10^{-10} M for experiment b and indicate strong affinity for the receptor binding sites. Ethynodiol has a markedly higher affinity than lynestrenol as is demonstrated by K_i values of 4.5×10^{-9} M and 1.1×10^{-7} M respectively. The binding capacity of cytosol a for estradiol-^{3}H is 0.75 pmol/mg protein and of cytosol b 0.62 pmol/mg protein.

←

Fig. 10.1a-d — Sucrose density gradient patterns of 105,000xg rat uterine cytosol after 20 h incubation at 1-3°C with estradiol-^{3}H alone or together with one of the unlabelled compounds in the amounts indicated. Before centrifugation (7 h at 300,000xg) the unbound estradiol-^{3}H was removed with dextran-coated charcoal. BSA and beef liver catalase (sedimentation coefficients 4.6 and 11.3 respectively) were used as standards to estimate the sedimentation coefficient.

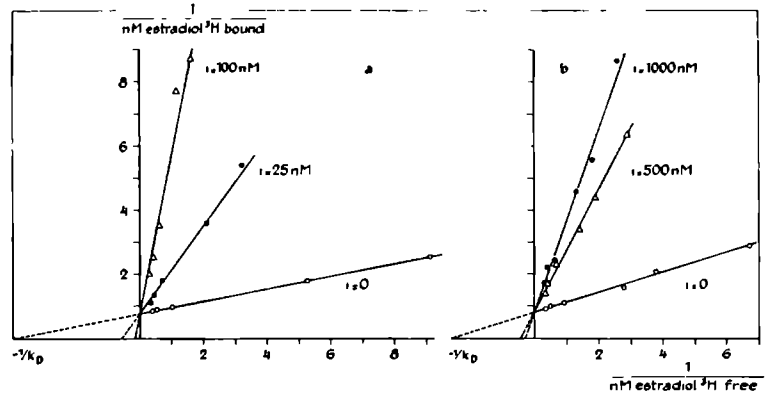

Fig. 10.2 — Competitive inhibition by ethynodiol (a) and lynestrenol (b) of estradiol-^{3}H binding to rat uterine receptor. The cytosol was incubated with increasing concentrations (0.50-3.75 nM) estradiol-^{3}H alone (i = 0) or together with the indicated concentration of inhibitor i. The free estradiol-^{3}H concentration was calculated as the difference between the total and bound estradiol-^{3}H. The double reciprocal plots a and b are from two different cytosols. Each point represents the mean of two (a) or four (b) incubations.

While a report of the present investigations was in the press (Van Kordelaar et al., 1975) Terenius (1974), in a similar study using rabbit uterine cytosol instead of rat uterine cytosol, obtained results in agreement with our observations. The binding affinity of the synthetic progestins to the estrogen receptor decreased in the sequence ethynodiol, norethynodrel, norethindrone. Lynestrenol was not studied. Norgestrel and the hydroxyprogesterone derivatives appeared to be inactive. The K_i value of ethynodiol (2.4×10^{-9} M) compares favourably with our value of 4.5×10^{-9} M.

DISCUSSION

Structural requirements for receptor binding

From the data in Table 10.1 some conclusions may be drawn with regard to the structural requirements of the ligand, which favour receptor binding. Other studies have already stressed the significance of the phenolic hydroxyl group at C_3 and the 17β-hydroxyl function of estradiol for its attachment to the receptor sites (Chernayaev et al., 1975; Hähnel et al., 1973; Katzenellenbogen et al., 1973; Korenman, 1969; Raynaud et al., 1973). Changes in the structure of the molecule at these positions always resulted in a decrease of

binding affinity. Obviously the 17α-ethynyl group has rather unique properties in this respect. The introduction of this substituent not only results in a decrease of metabolism (Fotherby & James, 1972; Thijssen, 1972), but also greatly promotes binding to the estrogen receptor: compare the inhibition of binding by lynestrenol and estrenol, by norethindrone and nortestosterone, and by ethisterone and testosterone. This is consistent with the observations of Korenman (1969), Raynaud et al. (1973) and Shutt & Cox (1972) for ethinylestradiol and estradiol respectively. In the present study estradiol and ethinylestradiol inhibit estradiol-^{3}H binding to the same degree, but this has to be attributed to the fact, that in the presence of a 200-fold excess of these compounds, total displacement of estradiol-^{3}H from the specific binding sites has already occurred.

The high binding affinity of ethynodiol compared with lynestrenol and norethindrone is an indication, that its equatorially oriented 3β-hydroxyl function contributes considerably to a good fit with the receptor binding site. On the other hand, the decrease of binding affinity in comparison with estradiol is probably related with its changed stereochemistry after partial saturation of the A-ring, which has lost the planar conformation present in estradiol. Also the contribution of the alicyclic 3β-hydroxyl may be less effective in non-binding interactions with the receptor site than the phenolic hydroxyl of estradiol.

After removal of the 3β-hydroxyl from ethynodiol binding affinity is decreased (cf. lynestrenol), but still significantly higher than after its replacement with a C_3 oxo group, even in the absence of the 17α-ethynyl group (cf. estrenol). The conformation of this oxo group is apparently an important factor: the conformation present in norethynodrel with the oxo group above the plane of the molecule is opposite to that of the weak binder norethindrone, whereas the interatomic distance between the C_3 oxo and 17β-hydroxyl measured by X-ray crystallography is probably almost equal, cf. 17β-hydroxy-4-androsten-3-one 1.094 nm and 17β-hydroxy-5(10)-estren-3-one 1.087 nm. In estradiol hemihydrate the distance between the C_3 and C_{17} hydroxyl is 1.093 nm (Duax & Norton, 1975). The comparatively small difference of binding affinity between norethynodrel and lynestrenol on the one hand and the large difference between norethynodrel and ethynodiol on the other hand seems to indicate, that the contribution of the C_3 oxo group to the binding is rather small, although steric factors may be involved, too. An explanation for this poor contribution could be, that polarization of the oxo group yields a negative center at the C_3 position in contrast with the charge distribution in ethynodiol and estradiol.

Acetylation of the 3β- and 17β-hydroxyl groups decreases the activity of ethynodiol, lynestrenol and norethindrone. Terenius (1974) reported a K_i value for ethynodiol diacetate of 9.9×10^{-8} M, a 40-fold increase in comparison with

ethynodiol. To what extent the residual activity results from some hydrolysis during the 20 h incubation procedure or has to be considered as an intrinsic property of the acetate ester itself, remains to be studied. Katzenellenbogen et al. (1973) observed a rapid hydrolysis of estradiol-3-acetate when incubated with rat uterine cytosol for 1 h at 0°C.

The lack of activity of chlormadinone, megestrol and medroxyprogesterone is quite understandable in the light of the results obtained with progesterone and methyllynestrenol. The presence of a 6α-methyl substituent seems to be matched with loss of binding ability (compare lynestrenol and methyllynestrenol). A decrease of binding ability is also observed after the introduction of the 19-methyl group as shown by the difference between testosterone and nortestosterone, although in ethisterone the 17α-ethynyl group seems to be able to overcome this to some extent. Previously, a slight inhibition of estradiol binding to the estrogen receptor has been shown by Korenman (1969) and Notides et al. (1972) for 5-androstene-3β,17β-diol, 4-androstene-3β,17β-diol and 5α-androstane-3β,17β-diol. The 3β-hydroxyl in these compounds is equatorially oriented like in ethynodiol. On the other hand, 5β-androstane-3α,17β-diol as well as the 5α-isomer appeared to be inactive (Hähnel et al. 1973). For this reason it may be concluded that steroids of the androstane series may still bind to the estrogen receptor, provided that favourable conditions are preserved at the C_3 or C_{17} position of the molecule. In this connection the 18-methyl group seems to be of less importance than the 19-methyl group. Hähnel et al. (1973) reported equal affinity for estradiol and 18-norestradiol. On the other hand, it is evident from the very weak activity of norgestrel, that the introduction of an ethyl group in this position almost completely eliminates any activity.

Correlation with results obtained in vivo (Chapter 9)

In general the results of the incubation experiments correlate very well with the data of Tables 9.3 and 9.4 in the preceding chapter. The acetate esters, however, of ethynodiol, norethindrone and lynestrenol *in vitro* displayed a much smaller effect than the parent compounds in contrast to an almost equal activity *in vivo* (Chapter 9, Table 9.5). Apparently, the esters *in vivo* are hydrolysed very fast to the respective alcohols.

On the contrary, lynestrenol and estrenol appear to be more active *in vitro* than *in vivo*. An explanation for this difference could be a rather high metabolic clearance rate of lynestrenol and estrenol in comparison with norethynodrel and norethindrone and the generation of inactive or less active biotransformation

products: Mazaheri et al. (1970) and Okada (1972) reported biotransformation of lynestrenol to norethindrone. Analogously, formation of nortestosterone from estrenol could occur via the same mechanism. In the present study, however, we could not get proof for the presence of norethindrone in the bile and urine of the rat after the administration of lynestrenol. Another metabolite could tentatively be identified as 17α-ethynyl-5ξ-estrane-3ξ,17β-diol (see Chapter 12). Binding of the 5α-estran-3β-ol isomer of this structure with the estrogen receptor seems very likely in view of the activity of 5α-androstane-3β,17β-diol mentioned above. So this conversion, if actually of significance, provides no explanation for the question under consideration.

In this connection attention should also be paid to plasma protein binding and physicochemical aspects, particularly lipophilicity, which are also important factors involved in the kinetics of distribution and elimination and therefore in drug action. The determination of the lipophilicity of this group of steroids as a partition coefficient between water and organic solvents is complicated by their extremely low solubility in water and their high solubility in most organic liquids. A good differentiation, however, is also possible with the help of reversed-phase thin-layer chromatography (Boyce & Milborrow, 1965). Between the partition coefficient a and the R_f value obtained from a liquid-liquid partition chromatography system the following relationship exists (Martin, 1949):

$$a = K\,(R_f^{-1} - 1), \text{ where } K = \text{constant for the system.}$$

Bate-Smith & Westall (1950) introduced the term R_M ($= \log (R_f^{-1} - 1)$). The change in the value of R_M with the introduction of a substituent has been shown to be very useful in the characterization of the relative lipophilicity within a series of compounds (Bush, 1961; Stahl, 1969). From the data in Table 10.2 it can be observed that introduction of an oxygen function in the A ring of the steroid molecule results in a very large decrease of the R_M value for all nortestosterone derivatives and so in a marked difference in lipophilicity between lynestrenol and estrenol on the one hand and ethynodiol, norethindrone, norethynodrel, norgestrel and nortestosterone on the other. The lipophilic nature of lynestrenol and estrenol may give rise to a more rapid accumulation in the fatty tissues of the rats immediately after injection and consequently in lower effective concentrations in the receptor compartments as compared with the more polar structures. A rapid and pronounced uptake of lynestrenol in the brown adipose tissue of mice has actually been observed in the present study (Chapter 14).

Also plasma protein binding may be an important parameter in the relationship

Table 10.2 — The lipophilic character of the 19-norsteroids studied as expressed by their R_M values

	Mean R_M value	Inter-plate SE
Methyllynestrenol	> 1.3	—
Lynestrenol	0.755	0.023
Estrenol	0.683	0.031
Norethynodrel	- 0.128	0.034
d-Norgestrel	- 0.199	0.023
Norethindrone	- 0.319	0.035
Ethynodiol	- 0.329	0.035
Nortestosterone	- 0.401	0.025

between the dose and the drug concentration reached in the target tissue. The greater estrogenic activity of the 11-methoxy derivatives of estradiol and ethinyl-estradiol in spite of a stronger receptor binding of these latter compounds could be attributed to the comparatively small fraction of the derivatives bound to plasma proteins and concurrently higher uterine concentrations (Raynaud, 1973; Raynaud et al., 1973). Although we have no information about the binding of the nortestosterone derivatives to rat plasma proteins, a possible role for this parameter in the different *in vivo/in vitro* activities observed should also be taken into account.

Implications of the receptor interaction

The possible implications of the interaction of the progestins with the estrogen receptor for the biological activity of these compounds, viz. in addition to progestational activity an inherent estrogenicity or anti-estrogenic action, have already been outlined in a more general context in Chapter 1, to which we refer.

SUMMARY

Incubation of the 105,000xg supernatant of rat uterus homogenate with estradiol-^{3}H resulted in an estrogen specific binding of limited capacity to a

macromolecule sedimenting in the 8-9S region after density gradient centrifugation. The contraceptive progestins of the 19-nortestosterone series were able to interfere with estradiol binding in contrast to the hydroxyprogesterone derivatives chlormadinone, medroxyprogesterone and megestrol. The interaction appeared to be competitive. The strongest inhibition of estradiol binding was observed in the presence of ethynodiol, followed by norethynodrel, lynestrenol and norethindrone respectively. Norgestrel was almost inactive. Of the related structures tested estrenol displayed more, nortestosterone and ethisterone less activity than norethindrone, 6α-methyllynestrenol showed only border-line activity.

In comparison with norethynodrel and norethindrone, lynestrenol and estrenol appeared *in vitro* to be stronger competitors for estradiol than *in vivo* (this thesis, Chapter 9). This may be due to the great difference in lipophilic character which is reflected in the R_M values of these compounds.

From the results obtained it may be concluded, that the presence of a 17α-ethynyl substituent promotes receptor binding, whereas the introduction of methyl substituents in the positions 6, 10 and 18 causes the opposite effect. The relationship between the various ring A structures and the affinity to the receptor is discussed.

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AFFINITY OF THE CONTRACEPTIVE PROGESTINS TO THE CALF UTERINE RECEPTOR: BINDING STUDIES AS A TOOL IN DRUG RESEARCH*)

INTRODUCTION

In the preceding chapter we have shown, that the estrogenic activity of the 17α-ethynyl-19-nortestosterone derivatives used in contraception, often attributed to the formation of estrogenic metabolites (Fotherby & James, 1972), may be explained by the competitive interaction of these progestins with estradiol for its binding sites on the estradiol receptor. Furthermore, an insight was obtained in some structural requirements important for the occurrence of binding and the binding affinity of the compounds studied. For a more quantitative approach and a comparison, however, of this latter property within a series of compounds, the use of one and the same receptor preparation is to be preferred. The receptor content and the ratio of the specific and the non-specific binding sites vary with each preparation as is obvious from the binding experiments described in Chapter 10. Moreover, the rat uterus appeared us to be a rather laborious and expensive source of receptor material, particularly for large-scale work. For these reasons, we have directed our attention to the calf uterine receptor. The isolation of the estrogen binding protein from calf uteri and its characterization have been the subject of investigations in several laboratories (Baulieu et al., 1971; DeSombre et al., 1971; Puca et al., 1971; and others). We have studied the binding of estradiol to the calf uterine cytosol and determined the relative potency of the 19-nortestosterone derived progestins to displace estradiol from its specific binding sites. From the data obtained the binding parameters of these compounds could be calculated. The possibilities and limitations of this kind of studies as compared with the bio-assay methods in use for the evaluation of steroid hormone activity (Astwood, 1970; Dorfman, 1969; Emmens, 1969) will be discussed.

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Preparation of the calf uterine cytosol

The calf uteri were obtained from the slaughter-house, cooled in ice immediately after excision and processed the same day. The subsequent manipulations with the biological material were carried out at 1-4°C. After cutting the tissue with scissors the pieces were washed in buffer (0.01 M Tris-HCl with 0.001 M EDTA, pH 7.4) and homogenized in 0.5 volume of the same buffer using a Waring blendor. Homogenization was achieved by 5-10 pulses of 10 seconds duration with 2 minutes cooling inbetween. The homogenate was centrifuged for 10 minutes at 15,000xg, the pellet was discarded and the supernatant centrifuged again for 1 hour at 105,000xg in a Spinco L2-75B ultracentrifuge using a Spinco 50Ti rotor. The 105,000xg supernatant (cytosol) of successive runs were pooled, mixed thoroughly, divided into 1.4 ml fractions and stored deep-frozen at -80°C to preserve the full binding capacity. The protein content of the cytosol stock, determined according to Lowry et al. (1951), was 12.7 mg/ml.

Binding experiments

The procedures used in studying steroid-receptor binding have been described in detail in the preceding chapter and are discussed in Chapter 6. For the determination of the dissociation constant of estradiol binding to the receptor the cytosol was diluted 10 fold. The use of this dilution allowed measurements to be made between 20 and 80% saturation of the receptor binding sites, where the most accurate measurements of the dissociation constant and binding capacity can be done (Deranleau, 1969), and simultaneously over a concentration range of estradiol-^{3}H, where the ratios bound/unbound can be most accurately determined in the assay procedure applied.

Test compounds

Specifications of the steroids have been given in Chapter 3. For the determination of the binding inhibition curves the specific activity of the tritiated estradiol was reduced to 8.0 Ci/mmol by the addition of the non-labelled compound.

Assay of radioactivity

The radioactivity of incubation and gradient samples was measured by liquid scintillation counting as described (Chapter 5). The standard error of counting was 2.5% or less, the counting efficiency was about 45%.

RESULTS

Properties of the calf uterine receptor; effect of progestins on estradiol binding

The pattern of estradiol binding to the calf uterine cytosol in the presence of increasing concentrations (0.5-6 nM) of the tritiated hormone alone or together with a 500-fold excess of the synthetic non-steroid estrogen diethylstilbestrol (DES), which has a comparable affinity to the receptor binding sites, is shown in Fig. 11.1a. Two classes of binding sites can be discerned, one with high affinity and low capacity and the other one with much lower affinity and high capacity. The difference becomes manifest in the presence of excess DES, resulting in the displacement of estradiol from the saturable high affinity binding sites, but without influence on the high capacity binding of the hormone. By

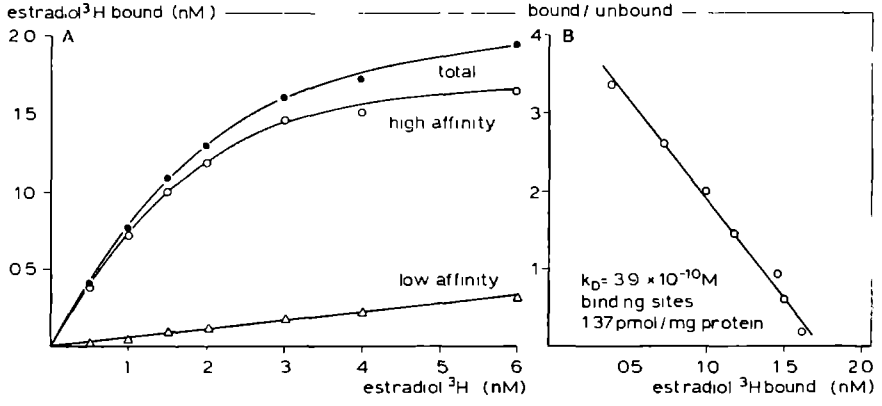

Fig. 11.1 — a. Binding curves of estradiol-^{3}H to calf uterine cytosol. Total binding and binding in the presence of a 500-fold excess of diethylstilbestrol were measured. High affinity binding was calculated as the difference of the total and low affinity binding. The points represent the mean of 3 experiments.
b. Scatchard plot of the high affinity binding curve.

subtracting the low affinity binding curve from the total binding curve a curve is obtained representing only high affinity binding. The Scatchard plot (Scatchard, 1949) corresponding to this latter binding curve is shown in Fig. 11.1b. For the high affinity binding sites a dissociation constant of 3.9×10^{-10} M can be calculated with a binding capacity of 1.4 pmol/mg protein.

The specificity of the high affinity binding sites for estrogenic compounds is apparent from the lack of influence of a 2000-fold excess of testosterone, progesterone and corticosterone on estradiol binding, whereas a 50-fold excess of ethinylestradiol or DES was able to abolish the high affinity binding completely (Fig. 11.2a,c). The peak of radioactivity in the gradient appeared to be localized in the 8S region (Fig. 11.2c).

Displacement of estradiol from its specific binding sites occurred to a varying degree in the presence of ethynodiol, norethynodrel, norethindrone (Fig. 11.2b) and lynestrenol (Fig. 11.2c). Norgestrel and the hydroxyprogesterone derivatives megestrol acetate, chlormadinone acetate and medroxyprogesterone acetate were devoid of activity in this respect. The increase of the inhibition of estradiol binding in the presence of an increasing excess of the inhibitor and the reversibility of lynestrenol binding to the estradiol receptor is shown in Fig. 11.2c and Fig. 11.2d respectively.

Binding affinity of the progestins

A quantitative insight in the potency of the competing steroids to displace estradiol from its binding sites on the receptor can be obtained by determining under defined conditions for each compound the amount necessary to reduce the specific binding of tritiated estradiol by 50%. The undiluted cytosol was incubated with 30 nM estradiol-^{3}H alone and in the presence of increasing amounts of the competing steroids. The total binding of estradiol thus measured was corrected for non-specific binding: the residual binding after incubation of the control samples with a 500-fold excess DES. It was observed, that the non-

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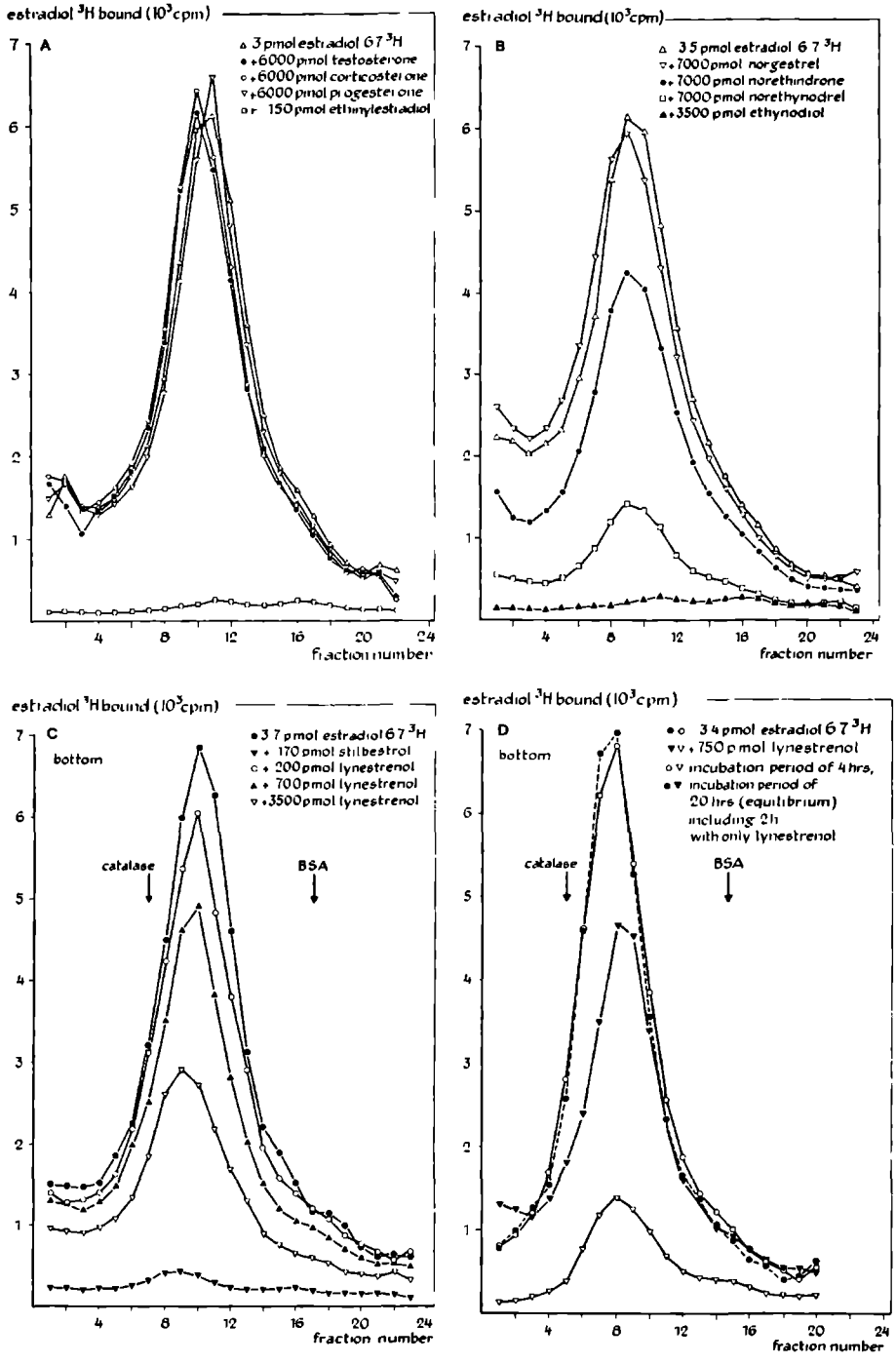
Fig. 11.2a-d — Sucrose density gradient patterns of 105,000xg calf uterine cytosol after incubation of 0.2 ml cytosol with estradiol-^{3}H alone or together with one of the unlabelled compounds in the amounts indicated. Incubations were carried out at 1-3°C for a period of 20 h, unless otherwise indicated (exp. d). Before centrifugation (7 h at 300,000xg) the unbound estradiol-^{3}H was removed with dextran-coated charcoal. BSA and beef liver catalase were used as standards to estimate the sedimentation coefficient (Martin & Ames, 1961).



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specific binding in the control samples (about 10% of the total binding) and in the samples with the progestins was the same. By plotting of the residual, specific, estradiol binding as a function of the molar ratio of the respective progestins and estradiol on a semi-log scale a series of almost parallel sigmoid-shaped curves was obtained (Fig. 11.3). The relative activity (RA) of the steroids is also shown in this figure and was calculated from the quotient of the molar ratio for estradiol itself (A) and the molar ratio of the respective progestin (B), corresponding to 50% residual binding of the tritiated hormone, according to the formula: RA = A/B (Korenman, 1969, 1970).

It can be observed from the inhibition curve of estradiol in Fig. 11.3, that a molar ratio of unity does not correspond with 50% reduction of binding. This can be explained by taking into consideration the shape of the binding curve in Fig. 11.1. Obviously, the occurrence and the extent of the reduction of estradiol binding, that will be observed, is a function not only of the molar ratio of the competing steroids, but also of the degree of receptor saturation. The concentration of estradiol-^{3}H used in the present experiments corresponded to approximately 83% receptor saturation in the control samples.

From the RA values of the competing steroids the ratio of the association

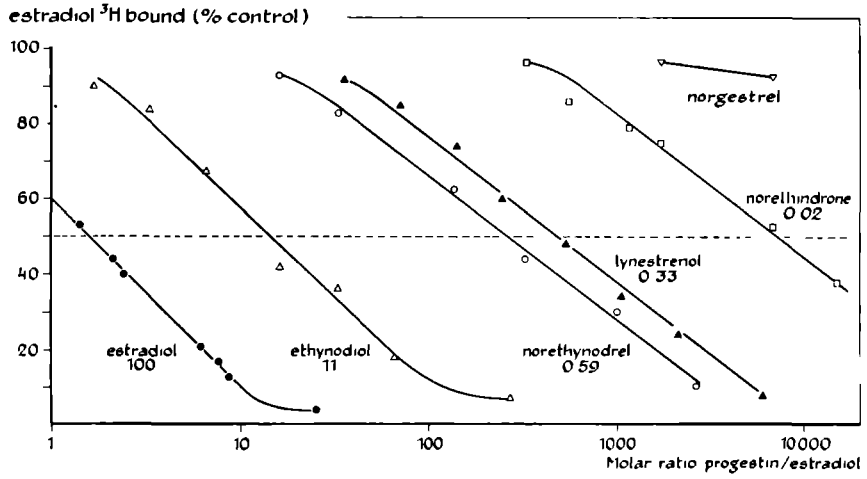

Fig. 11.3 — Binding inhibition curves of estradiol-^{3}H showing the inhibition of estradiol-^{3}H receptor binding in the presence of increasing excess of the non-labelled ligand and the competing nortestosterone derivatives. The relative activity (%) was calculated from the molar ratio progestin/estradiol-^{3}H corresponding to a residual estradiol binding of 50%. Each point in the curves represents the mean of 5 incubations.

Table 11.1 — Survey of RA, RAC and affinity constants of the nortestosterone derived progestins*)

COMPOUND	RA	RAC	K_A	K_D
Estradiol	100	100	2.6×10^{9}	3.9×10^{-10}
Ethynodiol	11	8.7	2.2×10^{8}	4.5×10^{-9}
Norethynodrel	0.59	0.45	1.2×10^{7}	8.7×10^{-8}
Lynestrenol	0.33	0.25	6.5×10^{6}	1.5×10^{-7}
Norethindrone	0.02	0.01	3.8×10^{5}	2.6×10^{-6}
d-Norgestrel	$\ll 0.02$	$\ll 0.01$	$\ll 10^{5}$	$\gg 10^{-6}$

*) Calculated from the data of Figs. 11.1 and 11.3.
Abbreviations and symbols: RA denotes relative activity in inhibiting estradiol receptor binding (%), RAC denotes ratio of association constants progestin/estradiol (%), K_A and K_D denote association (M^{-1}) and dissociation constant (M).

constants (RAC) can be calculated using the equation derived by Korenman (1970):

$$RAC = \frac{R \,.\, RA}{R + 1 - RA}$$

where $RAC = K_A^{\text{competitor}}/K_A^{\text{estradiol}}$ and R represents the ratio of free to specific-bound estradiol-^{3}H at 50% competition. Under the conditions of the present assay the ratio of free to specific-bound estradiol-^{3}H in the absence of competitor was 16/14 (see Fig. 11.1a), so $R = 3.3$. Like the RA value the RAC value is usually expressed in per cent (RAC x 100). Because the dissociation constant of estradiol to the cytosol receptor protein was determined in the same cytosol under similar experimental conditions, the affinity constants of the progestins could also be calculated. A survey of these values is given in Table 11.1.

DISCUSSION

The calf uterus as a source of receptor material

The present experiments have demonstrated, that the calf uterine cytosol contains a macromolecule with a high affinity and limited capacity for the

binding of estrogens. These properties, particularly specificity of binding, are considered to be characteristic for receptor molecules (Baulieu et al., 1971). The sedimentation pattern in the sucrose gradient, the dissociation constant and the binding capacity of the cytosol are consistent with previous reports concerning more extensive studies of the physicochemical properties of the calf uterine receptor (Baulieu et al., 1971; Puca et al., 1971). Comparative investigations of the estrogen receptors isolated from various mammalian species including the cow and the rat have shown a strong similarity even in immunochemical respect (Greene et al., 1977). The similarity of the receptors of the calf and rat uterus also with respect to their binding of compounds with less high affinity is supported by the results described in this and the preceding chapter. It seems justified, therefore, to conclude that with the calf uterus we have at our disposal an excellent alternative source of estrogen receptor material, that is easily to obtain and more economic and less laborious than the immature rat uterus and particularly suited for the preparation of larger quantities of cytosol.

Comparative binding affinity and biological activity

Because the action of estrogenic hormones in stimulating growth and function of female reproductive tissues takes place primarily, though perhaps not exclusively, through their interaction with the receptor protein present in the target tissues (Gorski & Gannon, 1976; Jensen & DeSombre, 1973; O'Malley & Means, 1974), the study of this interaction can be considered as an important instrument for obtaining a proper understanding of the biological activity of a particular drug and as a rational approach to the design and screening of potential drugs.

From the various assays of biological activity currently in use for the evaluation of sex hormone activity the relationship between structure and activity is difficult to assess (Grover & Odell, 1975; Raynaud et al., 1973). The activity measured is the final product of a chain of biochemical events in the responsive tissue and becomes manifest a relatively long period of time after the hormone-receptor interaction has occurred. As a consequence the information concerning the intrinsic activity of the compounds tested is confused very much by distribution and elimination processes occurring over the same time interval. No discrimination is possible between the effects of the compound administered and of its often multiple and unknown metabolites. Moreover, many of the synthetic compounds are not „pure", but show several types of activity, the particular spectrum displayed being characteristic for the substance. Estrogenic,

Table 11.2 — Comparative binding affinity and estrogenic potency

COMPOUND	RAC[2])	Estrogenic Activity (E.D. 75%, mg/kg)[1]) in the Allen-Doisy test		
		Subcutaneous	Oral	References
Estradiol	100	0.003		Desaulles & Krähenbühl, 1964
Ethinylestradiol	n.d. > estradiol	0.0025	0.050	Desaulles & Krähenbühl, 1964 Overbeek et al., 1962
Ethynodiol	8.7	active		Elton et al., 1962
Ethynodiol diacetate	n.d. ≪ ethynodiol	0.45		Elton & Nutting, 1961 Elton et al., 1962
Norethynodrel	0.45	1		Desaulles & Krähenbühl, 1964
Lynestrenol	0.25	9	5-10	Desaulles & Krähenbühl, 1964 Overbeek et al., 1962
Norethindrone	0.01	100		Desaulles & Krähenbühl, 1964
d-Norgestrel	≪ 0.01	inactive	inactive	Edgren et al., 1966; 1967
Medroxyprogesterone acetate	not binding	inactive	inactive	Edgren et al., 1967
Megestrol acetate	not binding		inactive	David et al., 1963
Chlormadinone acetate	not binding		inactive	Hecht-Lucari et al., 1965

[1]) The activities derived from Overbeek et al. (1962) and Elton et al. (1961; 1962) represent our own estimations based on the data as presented by these authors and assuming a body-weight of 200 g for the rats used in the assay.

[2]) RAC denotes the ratio of association constants progestin/estradiol (%).

androgenic, anabolic and progestational activities may occur simultaneously and interfere with each other. For this reason tests on compounds of mixed activity may be difficult to interpret (Astwood, 1970).

Binding studies offer the advantage of a direct insight in the relationship between chemical structure and receptor binding in the target tissue. The identity of the compound binding to the receptor is known and its binding affinity can be measured. Total absence of binding or a very low binding affinity preclude intrinsic estrogenic activity. Examples of this in the present investigations are norgestrel and the hydroxyprogesterone derivatives, which have been demonstrated to be devoid of estrogenic activity in the bio-assay (David et al., 1963; Edgren et al., 1966; Edgren et al., 1967; Hecht-Lucari et al., 1966). Binding affinity alone, however, does not imply estrogenic activity *in vivo* as it does not distinguish between estrogens and competitive inhibitors. This aspect has already been discussed in Chapter 1. For this reason the measurement of binding affinity can not be used as a substitute for the bio-assay.

In Table 11.2 the RAC values of the contraceptive steroids have been brought together with some data from the literature (David et al., 1963; Desaulles & Krähenbühl, 1964; Edgren et al., 1966; Edgren et al., 1967; Elton & Nutting, 1961; Elton et al., 1962; Hecht-Lucari et al., 1966; Overbeek et al., 1962) concerning their activity in the vagina cornification assay (Allen-Doisy test). Vagina cornification in the spayed rat is the most specific biological test available for the determination of the estrogenicity of a compound (Astwood, 1970). It can be observed from the results shown, that the ranking order of activity in the bio-assay and in the binding studies is the same. Furthermore, it is obvious that the relative binding affinity of the nortestosterone derivatives is higher than their relative potency *in vivo*. This may be explained by their rapid biotransformation (see Chapters 12, 15, 16), but other factors may be involved as has been discussed above and in Chapter 1. There is another reason, however, to be very cautious in drawing conclusions from relative activity values *in vivo:* the biological activity of the test compound as well as the activity of the reference compound may vary considerably and independently as illustrated in Table 11.2 by lynestrenol and ethinylestradiol. Quantitative structure-activity relationship studies of synthetic steroids should, therefore, not rely on the bio-assay alone, unless the pharmacokinetics of the compounds studied has been thoroughly explored in the species employed for testing activity.

The results presented in Section III illustrate, that the study of the interaction and affinity of the contraceptive steroids to the estrogen receptor may contribute to a better understanding of their biological activity and mode of action. Although it has to be realized, that apart from the binding affinity the

intrinsic activity of a compound is pertinent to the extent of its *in vivo* activity (Ariëns et al., 1964), the interaction with the receptor still has to be considered as a prerequisite for any action at all. In this connection, therefore, it can be concluded that drug-receptor interaction studies are a promising and multivalent tool in the design, screening and evaluation of compounds with sex hormone activity.

SUMMARY

Incubation of the 105,000xg supernatant of calf uterine homogenate with estradiol-^{3}H resulted in an estrogen-specific binding to a macromolecule with a sedimentation coefficient of 8-9S. The binding was furthermore characterized by a high affinity ($K_D = 3.9x10^{-10}$ M) and a limited capacity (1.4 pmol/mg protein). In contrast with chlormadinone, medroxyprogesterone and megestrol, the contraceptive progestins derived from 19-nortestosterone appeared to interfere with estradiol receptor binding. The inhibition of estradiol binding in the presence of the latter compounds was reversible and increased in the presence of increasing excess of the inhibitor as could be demonstrated by sucrose gradient centrifugation. The ratios of the equilibrium constants of association (RAC) of the competing steroids compared to estradiol were calculated from the molar excess of the competitor, which reduced specific estradiol-^{3}H binding to 50%. The following RAC values were obtained (estradiol = 100): ethynodiol 8.7, norethynodrel 0.45, lynestrenol 0.25, norethindrone 0.01 and *d*-norgestrel $\ll 0.01$. The corresponding association constants K_A are ($K_A^{\text{estradiol}} = 2.6x10^9$ M^{-1}): ethynodiol $2.2x10^8$ M^{-1}, norethynodrel $1.2x10^7$ M^{-1}, lynestrenol $6.5x10^6$ M^{-1} and norethindrone $3.8x10^5$ M^{-1}. The results demonstrate a close agreement between the binding characteristics of the calf and rat uterine receptor. *In vitro* binding of the contraceptive steroids and their estrogenic activity *in vivo* have been compared. The possibilities and limitations of both kinds of study for the design, screening and evaluation of compounds with sex hormone activity are discussed.

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SECTION IV

PHARMACOKINETICS OF LYNESTRENOL

ABSORPTION AND DISPOSITION OF LYNESTRENOL IN THE INTACT AND BILE DUCT CANNULATED RAT

INTRODUCTION

In spite of its wide-spread use as an oral contraceptive drug and progestin, little information is available about the absorption and elimination of lynestrenol in laboratory animals and man.

After the administration to humans (Kamyab et al., 1968; Murata, 1967) and rabbits (Yamamoto, 1968) lynestrenol appeared to be metabolised extensively to a variety of mostly unidentified compounds and was not excreted unchanged with the urine. Although renal excretion in man (Kamyab et al., 1968; Van der Molen et al., 1969) and rabbits (Yamamoto, 1968) accounted for only 30-60% and 35%, respectively, of the dose administered, no attention has been paid to the significance of the bile as a route of excretion for this compound. Other synthetic sex hormones or their biotransformation products have been shown to undergo biliary excretion to a varying degree in animals (Arai et al., 1962; Bolt & Remmer, 1972; Hanasono & Fischer, 1974; Honjo et al., 1976; Ishihara et al., 1975; Ishihara et al., 1976; Kamyab et al., 1967; Reed & Fotherby, 1975; Steinetz et al., 1967) and humans (Cargill et al., 1969; Layne et al., 1963). Recently, Coert et al. (1975) mentioned a high biliary excretion of radioactivity in the rat after the intravenous injection of lynestrenol-^{14}C.

In the light of the still scarce knowledge about its absorption and elimination, a more extensive study with regard to the fate of lynestrenol in the rat seemed worthwhile. In this chapter the results are described of investigations concerning the extent and rate of absorption of lynestrenol from the gut, its disposition and routes of excretion in the intact and bile duct cannulated rat. The nature of the conversion products has been studied in sofar this could be of help in the interpretation of the experimental results obtained in the present and in the next chapter of this thesis (Chapter 13).

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Animals and animal preparation

In all experiments female Wistar rats were used, weighing 180-220 gram (Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands). Some weeks before the start of an experiment the animals were placed periodically in the cages to be employed for the experiment in question to get acclimatized to the food and water supplies and to reduce stress in the experimental situation. Intact rats were housed in metabolism cages, which permitted a separate recovery of urine and faeces, whereas animals with urinary bladder and bile duct cannulas were maintained in a restraining cage after Bollman (1948) with slight modifications (Kilian, 1973). The rats were starved overnight before the administration of the test compound, afterwards they had free access to food and water or, in the case of bile duct cannulation, to saline instead of water to replace the salts lost with the bile.

In a number of experiments bile and urine were collected continuously from polyethylene (Intramedic PE 10, Clay-Adams Co., Parsipanny, USA) and polyvinyl chloride (C 2E tubing, Talas Comp., Ommen, The Netherlands) cannulas respectively. Surgery was performed under ether anaesthesia about 20 hours before lynestrenol administration. For the biliary fistula the upper part of the common bile duct was used so that the outflow of the pancreatic juice via the lower parts of this duct was not obstructed. The surgical procedure has been described in detail by Lambert (1965). The urinary bladder cannula was inserted via a small incision through the bladder wall. The urethra was tied off externally to exclude loss of fluid via this pathway. Because often a sort of sediment was formed in this cannula, tubing was used with an inner diameter of 1 mm to prevent obstruction of the urine flow. The volume of urine present in the bladder cannula during the experiment was 0.12 ml, the urine content of the bladder itself was approximately 0.05-0.10 ml. The urine and bile were collected in vessels positioned under the animal cage. At the conclusion of each experiment the rats were killed and the surgical procedure was verified.

Dosage

Specifications of lynestrenol-4-^{14}C, used in the present experiments, are given in Chapter 3. For intravenous and oral administration of lynestrenol, ethanol containing polysorbate 80 (OPG, Utrecht, The Netherlands) was added to the lynestrenol-^{14}C solution. The solvent was evaporated and the residue solubilized

in saline. The final polysorbate concentration was 2%. Of this mixture a volume of 0.2-0.5 ml was either injected in the tail vein or administered intragastrically via a stomach cannula.

The dosage form for intraduodenal administration in case of bile duct cannulated animals was prepared similarly, but here saline as the solvent was replaced by bile collected from the same rats after surgery had been performed.

Unless otherwise indicated, the rats received a dose of 50 μg lynestrenol corresponding to 1-2 μCi ^{14}C, depending on the kind of experiment to be performed.

Fractionation of radioactivity in urine, bile and faeces

1. Extraction

Urine and bile to be extracted were collected on ice and weighed, pooled, in sofar required, after removing an aliquot (not more than 10% of the sample) for total radioactivity assay, and stored at 3°C. The pH of these samples was controlled and held between pH 6.0 and 7.5. Within 2 days after collection extraction was performed after diluting 0.5 ml urine or 0.2 ml bile with water to a volume of 2.5 ml. When subsequently thin-layer chromatography (TLC) was to be carried out five-fold volumes were used and 25 μg lynestrenol and norethindrone, solved in 0.1 ml ethanol, were added for reference purposes. The samples were consecutively extracted three times with an equal volume n-hexane and likewise with ethylacetate. The joint hexane fractions and ethylacetate fractions were evaporated in a counting vial under a stream of air and assayed for radioactivity by liquid scintillation counting (LSC). When also TLC was to be performed, only an aliquot of the extracts was evaporated and used for LSC.

Faeces were suspended in water and after dispersion a volume of 1.0 ml was extracted and processed as described for urine and bile.

2. Enzymatic hydrolysis

An aliquot of urine and bile was diluted to 2.0 ml with water and acidified with acetic acid to pH 5.2. Then the solution was buffered by the addition of 0.5 ml 0.5 M sodium acetate buffer pH 5.2. Hydrolysis with 50 μl digestive juice of Helix pomatia (Boehringer, Mannheim, GFR), containing 5.2 U/ml β-glucuronidase and 2.6 U/ml arylsulphatase, was carried out by incubation overnight at 37°C. Under these conditions hydrolysis was proved to be complete as longer

incubation periods or the addition of more enzyme led to similar results. Subsequently, the solution was neutralized to pH 7.0 with sodium hydroxide and extracted three times with equal volumes of n-hexane and ethylacetate respectively. The whole extract or an aliquot was used for LSC. In the latter case the other part was used for TLC. The whole procedure was then modified with respect to the amounts used as described above (*1. Extraction*).

3. Chromatography and mass spectrometry

Thin-layer chromatography of extracts of urine and bile was carried out over silicagel-precoated glass plates (Kieselgel F_{254}, Merck) in the solvent system toluene/ethylacetate 80/20. The developed area (15 cm) was divided into 30 bands, and the silicagel of each band was scraped off with a razor blade and put into a vial for LSC. The co-chromatographed reference compounds lynestrenol and norethindrone were localized by exposing the plates to iodine vapour. The R_f value of lynestrenol in this system was 0.7. The relative R_f values (R_f/R_f lynestrenol) for ethynodiol and norethindrone were 0.35 and 0.38. Additionally, for the study of the metabolite with R_f 0.26 in the former TLC system use has been made of the solvent system toluene/ethanol 90/10 (R_f value for lynestrenol 0.7, relative R_f values for ethynodiol and norethindrone 0.47 and 0.71).

Gas chromatography was performed with a Hewlett-Packard 402 gas chromatograph fitted with a flame ionization detector. The 6 feet glass column, internal diameter 3 mm, was packed with 1% XE-60 on Gas-Chrom Q, 100-120 mesh (Applied Science Labs., USA). Nitrogen was used as carrier gas. Under the experimental conditions and a column temperature of 210°C the retention time of lynestrenol and norethindrone was 1.2 and 11.4 minutes respectively.

A VG Micromass 7070F double-focusing mass spectrometer combined with a Pye-Unicam 104 gas chromatograph was used for mass spectrometry and mass fragmentography. The apparatus was interfaced with a VG 2040 data system. Experimental conditions: column 1% SE-30 on Gas-Chrom Q, 80-100 mesh (Applied Science Labs., USA), length 6 feet; carrier gas helium, flow 20 ml/min; temperature column 185°C, separator 200 °C, ion source 200°C; electron impact 70 eV, ionisation current 100 μA, accelerating voltage 4 kV.

4. Assay of radioactivity

Radioactivity was measured by liquid scintillation counting as described in Chapter 5.

Aliquots of aqueous solutions and dried aliquots of organic solvent extracts were counted after the addition of 10 ml Instagel. In case of volumes larger than 0.2 ml the volume was made up to 5 ml with water prior to the addition of Instagel. The standard error of counting was 2.5% or less and the counting efficiency varied between 80 and 92%.

The radioactivity of faecal samples was determined after the preparation of a suspension in water. An aliquot of the suspension (0.05 - 0.15 g) was digested with 1.0 ml Soluene in a water bath at 50°C for 2 hours. The subsequent procedure is also outlined in Chapter 5. The standard error of counting was 2.5% or less, and the counting efficiency was more than 80%.

The silicagel samples obtained from thin-layer chromatograms were prepared for LSC by the addition of 5 ml water and 10 ml Instagel. The standard error of counting was 4.5% or less and the counting efficiency was about 85%.

RESULTS

Excretion pattern after intravenous and oral administration to the intact rat

In Table 12.1 the cumulative values of total radioactivity in urine and faeces are presented after intravenous and oral administration of lynestrenol-^{14}C.

Table 12.1 — Cumulative excretion of radioactivity after the administration of lynestrenol-4-^{14}C*)

	INTRAVENOUS ADMINISTRATION						
							Mean ± S.D.
Urine	18.9	18.6	14.2	19.4	12.0		16.6 ± 3.3
Faeces	71.4	71.1	68.2	67.0	76.5		70.8 ± 3.7
Total	90.3	89.7	82.4	86.4	88.5		87.5 ± 3.2
	ORAL ADMINISTRATION						
							Mean ± S.D.
Urine	33.8	19.3	22.4	19.9	31.0	15.7	23.7 ± 7.1
Faeces	54.7	66.7	69.9	65.6	66.1	67.8	65.1 ± 5.3
Total	88.5	86.0	92.3	85.5	97.1	83.5	88.8 ± 5.1

*) The individual values of 5 and 6 rats respectively at the end of a period of 5 days are presented, expressed in per cent of the dose administered (50 μg lynestrenol/rat).

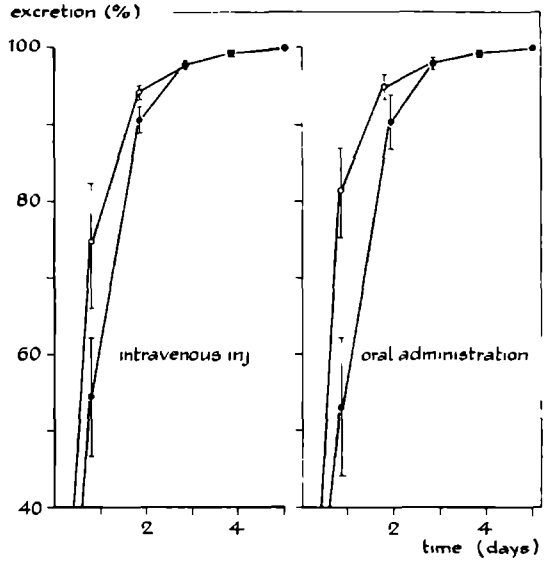

Fig. 12.1 — Cumulative excretion of radioactivity as a function of time after the administration of lynestrenol-^{14}C. The data points represent the mean ± S.D. in per cent of the total amount excreted with the urine (O——O) and faeces (●——●) over a five days period.

The total fraction of the dose recovered over a period of 5 days is about 90% and in this respect no difference seems to exist between the both routes of administration. In both experiments excretion with the faeces appeared to prevail widely over the renal pathway, the excretion ratio faeces/urine being somewhat higher in the injection experiment than in the case of oral administration. This difference, however, may be only accidentally, because it is principally created by the two high renal excretion values found in the oral experiment. As will be demonstrated in Chapter 13, the degree of renal excretion is also determined by factors not related with the route of administration and which might be involved in this experiment too.

The excretion of radioactivity with the urine and faeces during 5 successive 24-hour periods is shown in Fig. 12.1 and follows a similar pattern in both experiments. After 19 hours about 80% of the radioactivity to be excreted via the kidneys is present in the urine and this amounts to 95% 24 hours later. For the faeces these figures are about 50 and 90% respectively. After the third day the renal and faecal excretion curves coincide. Only minute amounts of

^{14}C were collected over the final sampling period in spite of the fact, that 10% and more of the dose in most cases still was to be excreted. The parallelism in the respective excretion patterns after oral and intravenous administration suggests analogous processes to be involved in both groups of animals and thus may be indicative for a high degree of absorption of the drug.

Excretion rate and absorption in the bile duct cannulated rat

1. Excretion

A representative example of the excretion rate and cumulative excretion of radioactivity in urine and bile after intravenous injection of lynestrenol-4-^{14}C is shown in Fig. 12.2. The values for bile and urine production demonstrate the steady condition of the rat during the measurements. The biliary excretion rate has reached its maximal value 15 minutes after injection, being followed by a strong decrease to about 1% of the maximal rate 8 hours later. The renal

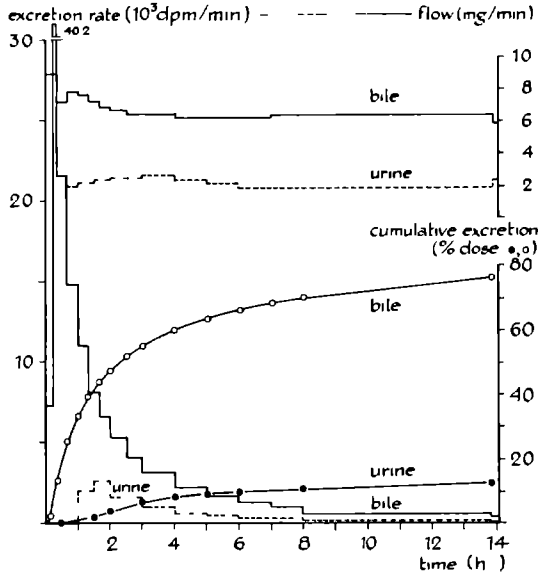

Fig. 12.2 — Cumulative excretion and excretion rate of radioactivity in urine and bile after intravenous injection of lynestrenol-^{14}C. Urine and bile flow are shown in the upper part of the figure.

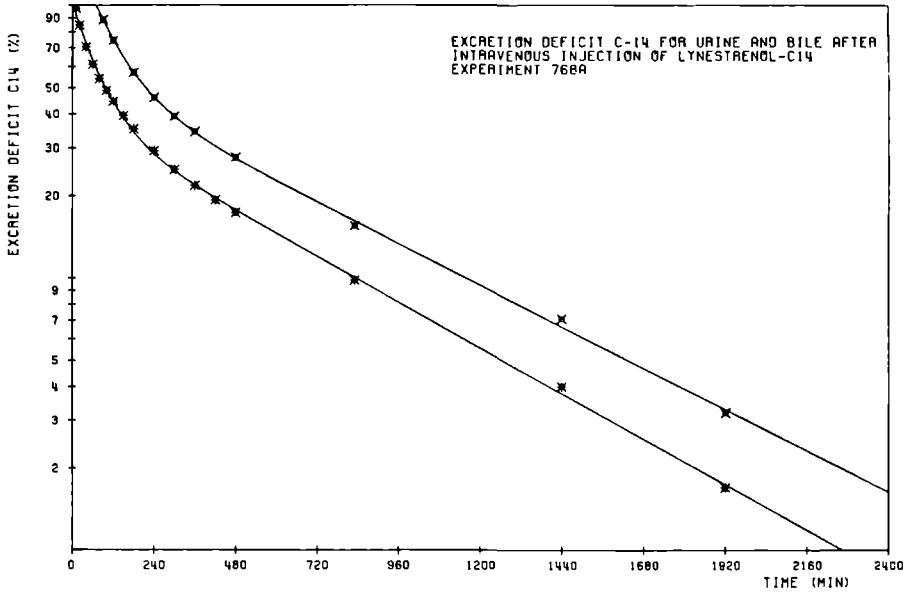

Fig. 12.3 — Computer-generated plot of the experimental data points and the corresponding best-fit curve of the renal (X——X) and hepatic (✱——✱) excretion deficit of radioactivity for the same experiment as shown in Fig. 12.2. The excretion deficit has been calculated from the values of cumulative excretion in bile and urine 48 hours after the injection of lynestrenol-^{14}C (see Table 12.3) and is expressed in per cent of the corresponding 48-hour value.

excretion rate initially displays a more gradual pattern, rising to its maximum over about 1.5 hour and also rapidly declining afterwards. The slow and late start of the renal excretion, however, can presumably be accounted for to a high extent by the void volume of the bladder cannula (0.12 ml), which corresponds to the urine production of a 60 minutes period in this experiment. It is clear from the curves of cumulative excretion, that more than 90% of the dose is excreted from the body within 14 hours after injection, the 50% level of excretion being already reached within only 2 hours.

From the cumulative excretion data in bile and urine the corresponding excretion-deficit was calculated and plotted as a function of time on a semi-logarithmic scale. The curves thus obtained are biphasic and can be described by a bi-exponential equation. The time constants of the respective curves and a computer-generated plot of the best fit were obtained with the help of the non-linear least squares curve fitting program FARMFIT (see Chapter 7). An

example is shown in Fig. 12.3 (same animal as in Fig. 12.2). The intercept of the renal excretion-deficit at zero time is exceeding the 100% level as is to be expected on account of the apparent delay in renal excretion occuring for reasons mentioned above. The times of half-life for the excretion of radioactivity in bile and urine are presented in Table 12.2.

The cumulative excretion in the bile, urine and faeces over the first and second 24 hour period after the injection of lynestrenol is shown in the upper half of Table 12.3. During the second day of the experiment the excretion of radioactivity is diminished to minute amounts. The fraction of the dose present in the bile after 48 hours is about 80%, 10% higher than was excreted with the faeces by the intact rat over a longer period of time (Table 12.1). Also the total excretion of radioactivity was smaller for the latter experiment.

2. *Absorption*

The absorption of lynestrenol has been studied by the determination of the excretion of radioactivity in bile, faeces and urine and by comparison of the biliary and renal excretion rate after the intravenous and intraduodenal administration of the compound. The latter route was preferred instead of oral administration to avoid the passage through the stomach as a possible source of variation in the absorption rate.

Table 12.2 — Half-life times for the time course of the hepatic and renal excretion deficit of ^{14}C in the rat after intravenous injection of lynestrenol-^{14}C*)

Experiment	Renal excretion		Hepatic excretion	
	$t^1/_2$,a	$t^1/_2$,b	$t^1/_2$,a	$t^1/_2$,b
762a	99	426	45	449
762b	69	297	61	284
762c	58	445	49	415
768a	64	474	52	430

*) The results are expressed in minutes. $t^1/_2$,a refers to the first and $t^1/_2$,b to the second phase of the respective curves. The excretion deficit has been calculated from the values of cumulative excretion in bile and urine 48 hours after the injection of lynestrenol-^{14}C (see Table 12.3).

Table 12.3 — Excretion of radioactivity in the bile duct cannulated rat after the administration of lynestrenol-4-^{14}C*)

	INTRAVENOUS ADMINISTRATION						
						Mean ± S.D.	
Bile	77.7/2.8	76.8/0.7	77.3/2.5	81.6/3.3		78.4 ± 2.2	2.3 ± 1.1
Urine	11.0/0.8	16.3/0.2	16.4/0.7	13.8/1.0		14.4 ± 2.6	0.68 ± 0.34
Faeces	< 0.1	< 0.1	< 0.1	0 /0.3		—	—
Total	88.7/3.6	93.1/0.9	93.7/3.2	95.4/4.6		92.7 ± 2.9	3.1 ± 1.6
	INTRADUODENAL ADMINISTRATION						
						Mean ± S.D.	
Bile	63.4/0.9	71.7/1.4	71.4/1.4	65.7/1.3	60.9/5.2	66.6 ± 4.8	2.0 ± 1.8
Urine	11.4/0.3	11.3/0.3	10.4/0.4	15.3/0.3	5.8/1.4	10.8 ± 3.4	0.54 ± 0.48
Faeces	13.9/1.5	5.9/0.4	2.1/0.9	9.6/1.6	10.1/2.4	8.3 ± 4.5	1.4 ± 0.8
Total	88.7/2.7	88.9/2.1	83.9/3.4	90.6/3.2	76.8/9.0	85.8 ± 5.6	4.1 ± 2.8

*) The figures represent individual excretion values over two successive 24-hour periods after lynestrenol dosage and express per cent of the dose administered (50 μg lynestrenol/rat).

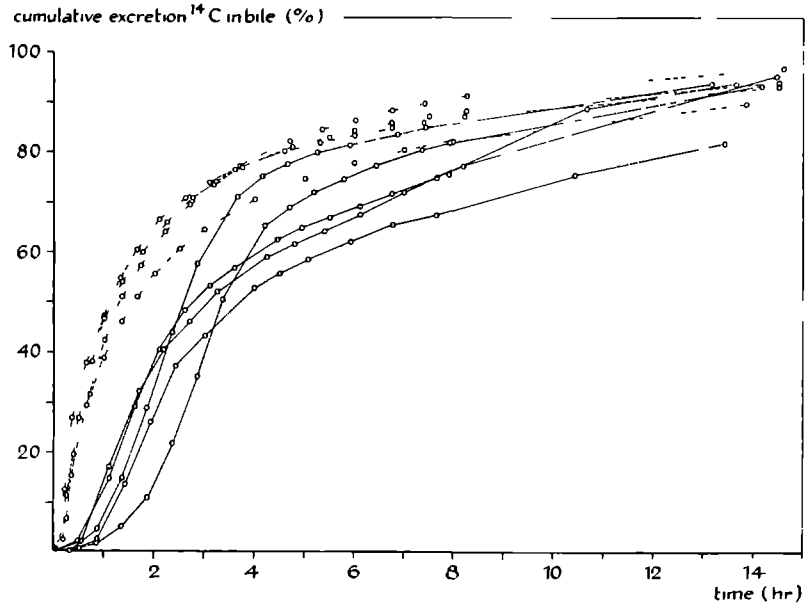

Fig 12.4 — Cumulative excretion of radioactivity in the bile as a function of time after the intravenous (- - - - - -) and intraduodenal (——) administration of lynestrenol-^{14}C. The data points express per cent of the total biliary excretion over 48 hours (see also Table 12 3)

Following intraduodenal administration of lynestrenol about 3-15% of the dose was excreted with the faeces (Table 12.3), so the absorption of lynestrenol may be supposed to be in the order of 90%. About 90% of the amount absorbed was excreted with the bile and urine after 48 hours, which is in close agreement with the results after intravenous injection (Table 12.3). Also in this experiment the total excretion with faeces and bile is higher than the faecal excretion observed in the intact rat after oral administration. If the fraction of the dose being absorbed may be assumed to be comparable in both experiments, in the intact rat the excretion with the faeces is smaller than is to be expected on account of the biliary excretion values. As will be shown in Chapter 13 this difference may be explained by the occurrence of an enterohepatic circulation of the lynestrenol metabolites.

If the absorption of lynestrenol is the rate-limiting step in the disposition of the compound, then the progress of the absorption process will be reflected in the respective curves of cumulative excretion. The results presented in Fig. 12.4 are consistent with this assumption. In contrast with the excretion pattern after

Table 12.4 — Results of fractionation of radioactivity present in excreta after lynestrenol-^{14}C administration[1])

	Route of administration	Number of samples	Sampling period	Extraction before hydrolysis		Extraction after hydrolysis[2])	
				hexane	ethylacetate	hexane	ethylacetate
Urine	oral	6	0-19 h	0.3 ± 0.2	25.1 ± 7.7	1.7 ± 0.4	47.0 ± 3.4
		6	19-44 h	0.4 ± 0.4	24.9 ± 2.8	1.4 ± 0.6	42.6 ± 3.5
Bile	intraduodenal	5	0- 8 h	0.8 ± 0.2	3.3 ± 1.3	18.0 ± 4.2	55.0 ± 3.4
	intravenous	4	0- 8 h	0.8 ± 0.1	2.2 ± 1.0	21.3 ± 3.5	51.5 ± 4.1
Faeces	oral	6	0-19 h	8.4 ± 1.5	41.4 ± 15.1	n.d.[3])	n.d.
		6	19-44 h	4.5 ± 0.8	44.4 ± 20.7	n.d.	n.d.
	intravenous	5	0-19 h	7.5 ± 2.1	54.9 ± 11.7	n.d.	n.d.
		5	19-44 h	4.0 ± 0.6	51.8 ± 12.1	n.d.	n.d.

[1]) The material analysed was part of the excreta collected during the experiments with the intact rats (urine and faeces) and bile duct cannulated animals as presented in foregoing Tables 12.1, 12.2 and 12.3. All results are expressed in per cent of the total radioactivity of the concerning sample ± S.D.

[2]) These results were obtained after the hydrolysis of unextracted urine.

[3]) n.d.: not determined.

injection, which has been described above, the biliary excretion after intraduodenal administration starts at a much slower rate, but continues to increase for a longer period of time, attaining its maximum only after 1 to 3 hours. The initially wide gap between the cumulative excretion of the respective routes of administration is then rapidly becoming smaller until about 10 hours after dosage there is no essential difference left with the intravenous excretion curves. It will be shown in Chapter 16 (Fig. 16.3) that the period of maximal biliary excretion coincides with the occurrence of the maximal blood concentrations of lynestrenol after the intraduodenal administration of the compound.

Fractionation of the radioactivity excreted

1. Urine and bile

To investigate the contribution of metabolism in the elimination of lynestrenol the radioactivity excreted in the urine and bile has been analysed by extraction before and after enzymatic hydrolysis. The results obtained are shown in Table 12.4. It is obvious that excretion of unchanged lynestrenol, that will be extracted with hexane for more than 90% from urine and bile under the conditions applied, cannot be considered of any significance in the total process of elimination of the compound. Furthermore it can be observed, that hexane-extractable steroids in conjugated form are present in the bile, but neither in free nor conjugated form in the urine. On the other hand a significant fraction of the steroids in the urine is freely extractable with ethylacetate. An explanation for these findings may be, that the more lipophilic and unconjugated steroids are likely to become reabsorbed from the ultrafiltrate in the kidneys in contrast to what can be expected for the more polar ethylacetate-extractable compounds. Another factor, however, which should be taken into account is a possible difference between the degree and character of plasma protein binding of the lipophilic and the more polar metabolites, which is especially of interest in the glomerular filtration process.

Thin-layer chromatography of the hexane extracts of the pooled urine and bile hydrolysates (Fig. 12.5) demonstrated only 1.7 and 2.4% of the respective radioactivity to migrate with the lynestrenol reference, so excretion of unchanged lynestrenol could be at best 0.5% of the dose. The chromatogram of the urine extract showed 70% of the radioactivity not to move at all, whereas for the bile 20% remained at the start. The other 80% of the radioactivity in the bile extract was found to be principally concentrated in one major peak at R_f 0.26. This chromatographic behaviour is compatible with the introduction of a second

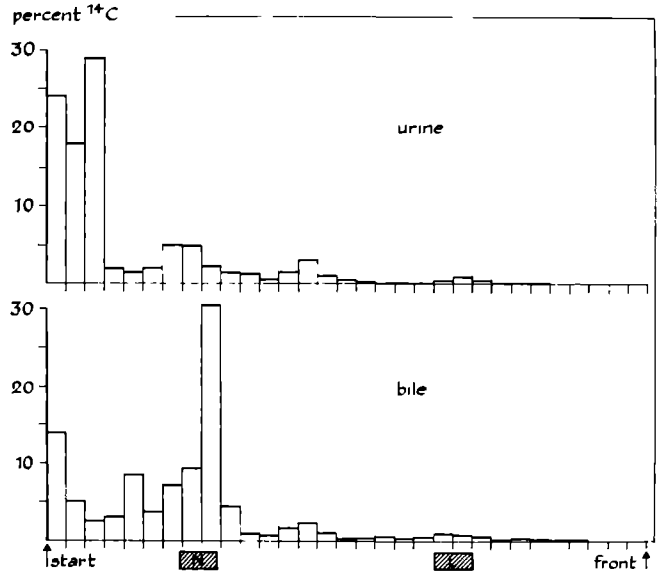

Fig. 12.5 — Thin-layer chromatograms of the hexane extracts of β-glucuronidase-hydrolysed urine and bile after oral, respectively intraduodenal administration of lynestrenol-^{14}C. Solvent system toluene/ethylacetate 80/20. The distribution of radioactivity over the chromatogram is shown as the percentage of the total amount ^{14}C recovered from the plate. Positions of co-chromatographed standards lynestrenol (L) and norethindrone (N) are indicated.

oxygen function in the lynestrenol molecule in view of R_f values of 0.23 and 0.26 for ethynodiol and norethindrone in this solvent system. After extraction of the radioactivity from the silicagel in this area and TLC of the extract in the solvent system toluene/ethanol 90/10 more than 95% of the radioactivity was recovered from a spot with R_f 0.40 compared with R_f 0.48 for norethindrone and 0.34 for ethynodiol. To gain an insight in the identity of this metabolite a bile duct cannulated rat was injected with 2 mg non-radioactive lynestrenol and 4 hours later, after changing the bile collecting vessel, again with the same amount of lynestrenol containing now 0.2 μCi lynestrenol-^{14}C. Bile collected from the same animal before the injection served for reference purposes. After hydrolysis and hexane extraction of both samples, as described before, 24% of the biliary radioactivity was present in the extract and 72% of this fraction could be recovered after chromatography in the system toluene/ethanol 90/10 at R_f 0.40. Gas chromatography of the corresponding non-radioactive extract

(Fig. 12.6) demonstrated the presence of a metabolite with a retention time of 4.2 min compared with 1.2 and 11.4 min for the lynestrenol and norethindrone standards. Mass fragmentography of the same extract was performed by repetitive scanning over the range m/e 20-400 with a speed of 1 decade/sec during the chromatographic run. The most abundant fragments with m/e > 200 present in the region of the fragmentogram and coinciding exactly with the elution profile of the metabolite peak were 201, 203, 215, 216, 284 and 302.

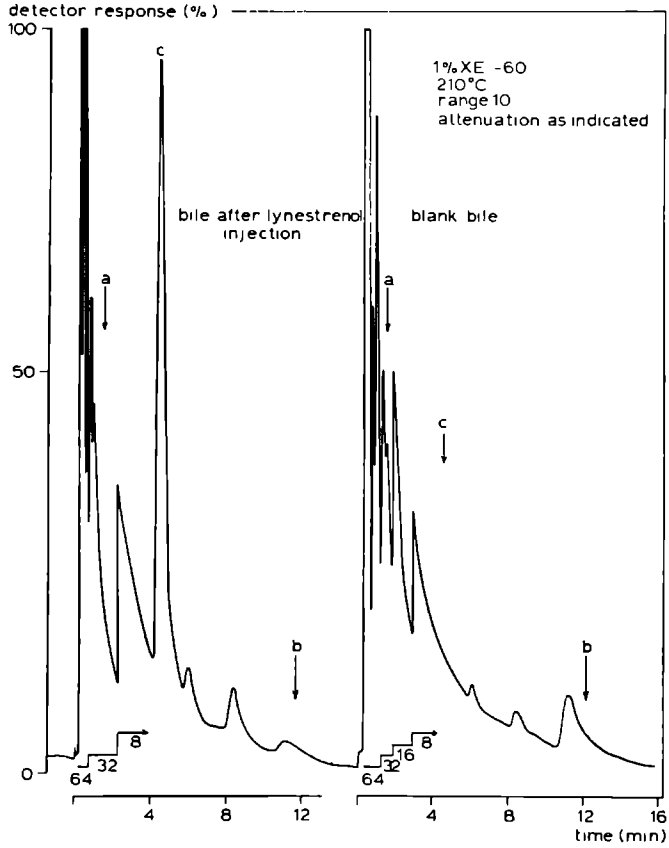

Fig. 12.6 — Gas chromatograms of the hexane extract of hydrolysed rat bile obtained from the same animal before and after lynestrenol injection. The attenuation factor of the detector signal was readjusted some times during chromatography as indicated in the figure in order to obtain maximal information. Notice the large metabolite peak c present in the bile after lynestrenol dosage and the absence of the parent compound a and norethindrone b.

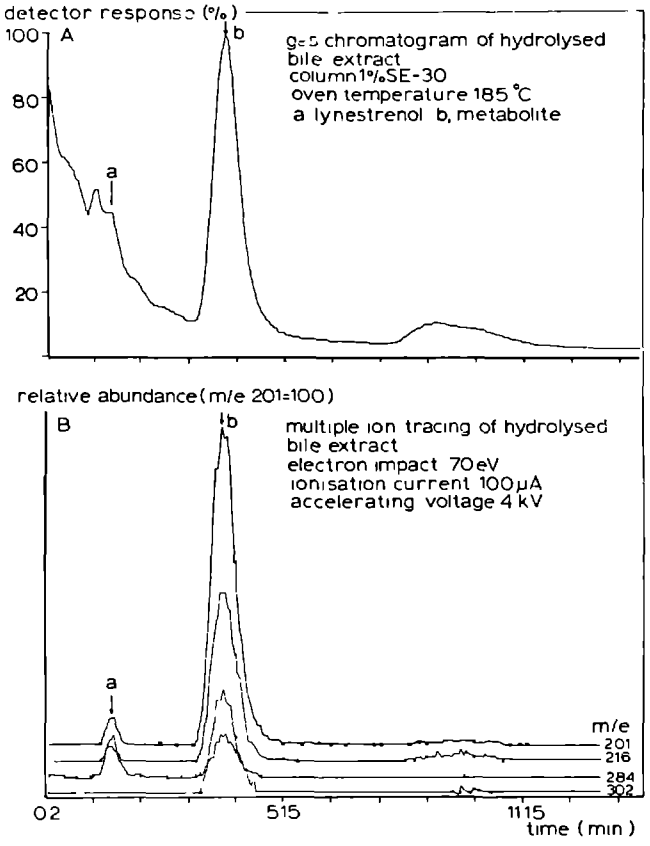

Fig. 12 7 — Gas chromatogram (a) and the corresponding multiple ion scan (b) of an hexane extract of hydrolysed bile Notice the homogeneity of the metabolite peak in the gas chromatogram as appearing from the multiple ion scan of the fragments m/e 201, 216, 284 and 302 The fragments m/e 201, 216 and 284 are abundantly present in the mass spectrum of lynestrenol (see Fig 12 8) The ion tracing is consistent with saturation of the 4,5 double bond and C_3-hydroxylation of lynestrenol. Another gas chromatogram of an aliquot of the same sample is shown in Fig 12 6.

No fragment could be detected with m/e > 302. The gas chromatogram and the corresponding multiple-ion trace of the fragments with m/e 201, 216, 284 and 302 are shown in Fig. 12.7. The former three fragments are also present in the mass fragmentogram at the retention time of lynestrenol, which is consistent with their high intensity in the mass spectrum of this compound (Fig. 12.8). Mass fragmentography also revealed the presence of fragments with m/e 282

and 290 at slightly increased retention times as compared with the fragments mentioned above. Because there was some overlap between the peaks of m/e 302, 290 and 282 in the mass fragmentogram and, therefore, the homogeneity of the metabolite peak in the gas chromatogram was questionable, a correct mass spectrum of the presumed metabolite could not be obtained from the bile extract studied.

Chromatography of the ethylacetate extracts revealed this material to consist largely of highly polar compounds, as more than 90% of the radioactivity was retained at the start following TLC.

2. *Faeces*

The faeces collected over the first two sample periods of the experiments described in the first section of this chapter have also been extracted with hexane and ethylacetate. In contrast with similar extractions of the bile, between 40 and 80% of the radioactivity appeared to be freely extractable without foregoing hydrolysis (Table 12.4). Apparently, a considerable fraction of the conjugated metabolites of lynestrenol is hydrolysed during the intestinal transport. This aspect will be further discussed in Chapter 13.

DISCUSSION

Absorption and bioavailability

Measurements of steroid absorption in humans (Schedl et al., 1965) and rats (Schedl & Clifton, 1961) as their disappearance rate from the intestine during

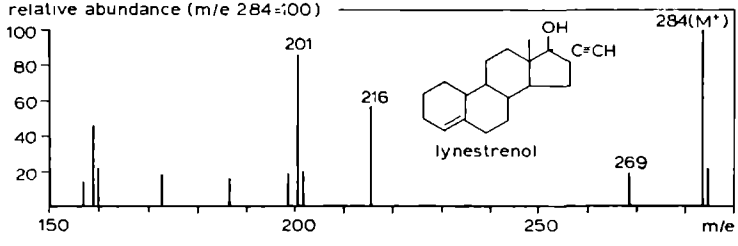

Fig. 12.8 — Mass spectrum of lynestrenol between m/e 150 and 284 (molecular ion). Most abundant fragments are m/e 201, 216 and 284. Conditions: electron impact 70 eV, accelerating voltage 4 kV, ionisation current 100 μA.

steady state perfusion through a transintestinal tube, revealed that absorption of these compounds correlated inversely with the extent of hydroxylation. Furthermore, evidence was provided that the absorption rate was directly proportional to the perfusate concentration. It is likely, therefore, that these compounds are absorbed predominantly by passive diffusion.

Little unequivocal information is available with respect to the degree of absorption of the contraceptive steroids. Schedl et al. (1965) observed with the help of their intestinal perfusion technique more than 95% absorption of norethindrone acetate in human subjects. Faecal excretion data after oral administration to bile duct cannulated animals or human subjects indicate an absorption of 70-80% of the dose for norethynodrel in rabbits (Arai et al., 1962) and humans (Layne et al., 1963) and of more than 95% of ethinylestradiol and its 3-cyclopentyl ether in humans (Cargill et al., 1969). Most other studies provide information of cumulative excretion in the urine or in urine and faeces of intact subjects, and do not allow conclusions about the extent of absorption of the respective compounds administered (for review: Fotherby, 1974; Thijssen, 1972). Comparison of the blood level profile and cumulative excretion of total radioactivity after the oral and intravenous administration of ethinylestradiol (Reed et al., 1972) and lynestrenol (Van der Molen et al., 1969) suggests a high degree of absorption for both compounds in humans. In view of the results presented here the conclusion seems justified, that in the rat lynestrenol absorption from the gastro-intestinal tract is almost complete within 14 hours, more than 80% of the dose being excreted after that time period in urine and bile. These findings are consistent with the good and rapid absorption, that is to be expected for a highly lipophilic compound like lynestrenol (this thesis, Chapters 8, 10).

Following intravenous injection of lynestrenol about 50% of the dose is metabolised and excreted in the urine and bile within 2 hours (Fig. 12.2). Coert et al. (1975) reported a biliary excretion in the rat of 40% over a period of 90 minutes after injection. In humans 15 minutes after the intravenous injection of lynestrenol-^{14}C less than half of the radioactivity present in the plasma appeared to be extractable with chloroform (Kamyab et al., 1968). Studying the hepatic clearance of lynestrenol in the isolated perfused rat liver we observed a complete extraction of the compound from the perfusion medium during its passage through the liver and the occurrence of an extensive biotransformation (Van Kordelaar et al., 1978; this thesis, Chapter 15). So the metabolic clearance rate of the compound in the rat *in vivo* is presumably rather high. After oral administration such a high clearance rate could result in the biotransformation of a considerable fraction of the dose in the liver before the lynestrenol could reach the systemic circulation. The lymphatic absorption of lynestrenol has

been demonstrated to be of little significance in comparison with absorption via the portal system (Kilian, 1973). So in spite of a complete absorption the bioavailability of lynestrenol itself, an important parameter in the dose-effect relationship (Ariëns, 1974), may be quite low. Experimental evidence for the validity of this assumption is provided in Chapter 16.

Elimination

About 70% of the conversion products of lynestrenol present in the bile appeared to be conjugated and could be hydrolysed with β-glucuronidase/aryl-sulphatase to aglycones much more polar than the parent compound. So like for the rabbit (Yamamoto, 1968) and man (Kamyab et al., 1968; Murata, 1967) hydroxylation of lynestrenol may be assumed to be a major metabolic pathway in the rat. The subsequent conjugation of the greater part of the conversion products will result in the formation of polar compounds of anionic nature with a molecular weight in the order of 400 to 500. According to the concept described by Smith (1971) with regard to the requirements to be fulfilled for a high biliary excretion, in rats such compounds are, for still unclarified reasons, likely to become eliminated with the bile to a high extent. This is in agreement with the present findings and previous investigations with norethindrone, norethynodrel, mestranol and chlormadinone acetate in the rat (Hanasono & Fischer, 1974).

Because with respect to the biliary excretion the threshold value for the molecular weight in rabbits and humans is estimated to be about 475 and 500 respectively versus 325 for the rat (Hirom et al., 1972; Millburn et al., 1967; Smith, 1974), in the former species the renal excretion may be of greater importance. As a matter of fact Kamyab et al. (1968) and Van der Molen et al. (1969) observed respectively 31-59% and 46-63% of the lynestrenol dose to be excreted in humans with the urine and in rabbit urine about 35% of the dose was recovered (Yamamoto, 1968). These authors, however, did not study the excretion with the faeces or bile, so it remains to be cleared, whether this higher renal excretion is resulting from a difference in biliary excretory mechanisms, a difference in the metabolic pathway of lynestrenol, leading to conversion products more readily disposed via the kidneys, enterohepatic circulation of the metabolites or a combination of these factors. That enterohepatic circulation can be the determining factor is demonstrated for lynestrenol in the next chapter. Also for norethynodrel elimination in intact rabbits appeared to occur for more than 50% via the kidneys, whereas in animals with bile fistulae this was reduced to about 20% of the dose (Arai et al., 1962). In patients with bile

fistulae renal excretion of radioactivity after intravenous administration of estradiol, estrone and estriol was largely reduced compared with the excretion in intact subjects (Sandberg & Slaunwhite, 1957, 1965).

Reconsidering the excretion process as characterized by the respective half-lives mentioned before (Table 12.2), it has to be realized, that the total excretion was measured of a variety of metabolites of lynestrenol. So the "excretion rate" in this connection is influenced by various parameters, including not only the generation rate of the metabolites and their physicochemical properties, but also the blood concentration-time course of lynestrenol and its apparent volume of distribution. In view of the half-life observed for the hepatic and renal excretion during the first hours after lynestrenol injection together with the fact, that the elimination of lynestrenol itself appeared to occur solely by metabolism, it can be concluded that the metabolic clearance capacity of the liver for this compound is high in the dose range studied. Indeed, in the isolated perfused rat liver the hepatic clearance of lynestrenol appeared to be independent of the perfusate concentration over a wide concentration range (Van Kordelaar et al., 1978; this thesis Chapter 15). For structurally close-related and extensively metabolised compounds like norethynodrel and norethindrone in the rat (Hanasono & Fischer, 1974) and ethynodiol diacetate in the baboon (Ishihara et al., 1975) comparable excretion rates in the bile and urine have been reported as for lynestrenol in the present study. A high metabolic clearance rate of lynestrenol in man, as already indicated by the findings of Kamyab et al. (1968), may therefore be expected. The final disposition, however, of this compound and its metabolites from the intact organism may be prolonged as a consequence of enterohepatic recycling, and will be discussed in the next chapter.

Metabolism

The fractionation of urine and bile revealed an extensive metabolisation of lynestrenol to occur. The results of chromatography indicate, that 7-10% of the parent compound is converted into a product less polar than ethynodiol and more polar than norethindrone. The results of mass fragmentography provide evidence for the formation of a metabolite with a molecular weight of 302, which is consistent with the saturation of the A-ring in lynestrenol and the introduction of a hydroxyl group. Recently, Coert et al. (1975) isolated a lynestrenol metabolite from rat bile with gas chromatographic properties similar to 17α-ethynyl-5α-estrane-3α,17β-diol. The structure of this compound is compatible with the present findings. The abundant fragments with m/e 284, 216 and 201 observed in the fragmentogram of the metabolite (Fig. 12.7) could

result from a fragmentation process involving the elimination of a molecule H_2O and a subsequent fragmentation as observed for lynestrenol (Fig. 12.8). Because the biotransformation involved may give rise to four isomers, viz. 3α,5α, 3α,5β, 3β,5β and 3β,5α, further investigations are necessary to establish the correct stereochemistry of this metabolite. On account of the present results it is not clear, whether the other fragments observed with m/e 282 and 290 originate from another conversion product of lynestrenol or from an endogenous compound.

In an *in vitro* study with rabbit liver homogenate Mazaheri et al. (1970) reported additionally the biotransformation of lynestrenol to norethindrone. This compound was also reported to be present in the blood and urine of rabbits (Yamamoto, 1968) and in human urine (Murata, 1967) after lynestrenol administration. The present study provided no indication for this conversion in the rat, although it could be present in the non-extractable fraction of the urine and bile. This fraction may contain non-phenolic steroid sulphates, which are not hydrolysed in our experimental procedure. The significance, however, of sulphate conjugation of these compounds in the rat may be limited, as Hanasono and Fischer (1974) could not demonstrate the presence of sulphates in the biliary and urinary excretion products of norethindrone and norethynodrel. Also conjugation products of glutathione and mercapturic acid may be present. It has been demonstrated by Elce (1972) and Elce and Harris (1971) that 2-hydroxy-17β-estradiol undergoes the complete mercapturic acid biosynthesis in the rat *in vivo*. Recently, Cook et al. (1974) isolated norethindrone-4,5-epoxide after the incubation of norethindrone with the 10,000xg supernatant of dog liver. Analogously, the formation of lynestrenol-4,5-epoxide as a precursor for mercapturic acid derivatives seems not unlikely, and norethindrone, if present as a metabolite of lynestrenol, may be involved, too.

Thin-layer chromatography of the ethylacetate extracts of urine and bile showed exclusively very polar compounds to be present in these fractions. Their chromatographic behaviour indicates the introduction of at least another two oxygen functions in the steroid moiety. The results obtained by Kamyab et al. (1968) with human urine point into the same direction. Conversion of norethindrone (Cook et al., 1974) and norethynodrel (Cook et al., 1972; Freudenthal et al., 1971) to poly-oxy-steroids has been demonstrated.

Taking into consideration on the one hand the extensive biotransformation of lynestrenol and the other progestins derived from 19-nortestosterone and on the other hand that the biotransformation routes for these compounds lead in part to identical intermediates and end-products (Fotherby, 1974; Thijssen, 1972), the elucidation of the structure and the biological activity of these conversion products should deserve further attention.

SUMMARY

After oral and intravenous administration of lynestrenol-4-^{14}C to intact female rats the excretion of radioactivity followed similar patterns. Within 24 hours 50% of the dose was recovered from faeces and urine, increasing to 80% 24 hours later. The final distribution ratio of radioactivity over urine and faeces was approximately 0.25. Elimination from animals prepared with bile fistulae did proceed more rapidly, about 90% of the dose being excreted after a 24-hours period. In these animals the contribution of renal excretion to the over-all elimination of radioactivity was decreased in favour of biliary excretion, indicating the possibility of enterohepatic circulation. After intravenous injection of lynestrenol-^{14}C the deficit for renal and hepatic excretion as a function of time could be described by a bi-exponential equation with similar time constants of approximately 1 hour for the first and rapid phase and 7 hours for the second phase. About 90% of the dose was absorbed from the intestines, most of it within 10 hours. The progress of the absorption process could be derived by comparison of the cumulative biliary and renal excretion of radioactivity after respectively intravenous and intraduodenal administration of lynestrenol. Fractionation of the excretion products present in the bile and urine revealed, that lynestrenol is eliminated by biotransformation to more polar compounds. The high excretion rate of these conversion products implies a high metabolic clearance rate for the parent compound and could result in a low bioavailability after oral administration in spite of the almost complete absorption. Without hydrolysis about 25% of the urinary radioactivity could be freely extracted with ethylacetate in contrast with only 3.5% for the bile. After treatment with Helix pomatia β-glucuronidase/arylsulphatase another 20% of urinary radioactivity could be extracted, whereas this amounted to 70% for the biliary excretion products. Thirty percent of the latter fraction was extractable with n-hexane and contained a metabolite tentatively identified as 17α-ethynyl-5ξ-estrane-3ξ,17β-diol. About 90% of the free and enzymatically liberated metabolites were much more polar than norethindrone and ethynodiol. Considerable hydrolysis of the biliary conjugates appeared to occur during intestinal transport, because half of the radioactivity present in the faeces could be extracted without foregoing hydrolysis.

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ENTEROHEPATIC CIRCULATION OF THE LYNESTRENOL METABOLITES AND THE INTERRELATIONSHIP OF HEPATIC AND RENAL EXCRETION

INTRODUCTION

In Chapter 12 it has been shown that biliary excretion is the major pathway of elimination for the metabolites of lynestrenol. The metabolites appeared in the bile predominantly in the form of glucuronides. During intestinal transport an extensive hydrolysis of these conjugates to the corresponding and less polar aglycones was observed. According to generally accepted theories of gastrointestinal absorption (Kurz, 1975; Parsons, 1975) the concomitant increase of lipophilicity may be assumed to favour absorption considerably as far as the process is determined by passive diffusion. So a large fraction of the metabolites is likely to become involved in an enterohepatic circulation. This possibility may have interesting consequences for the pharmacokinetics and time course of action of the recycling compounds, including the nature and incidence of side effects.

Enterohepatic circulation is known to play an important role in the disposition of endogenous steroidal sex hormones (Adlercreutz & Luukkainen, 1967; Long & Soyka, 1975; Sandberg & Slaunwhite, 1957, 1965) as well as of many drugs (Smith, 1971). Evidence for the absorption of the biliary excretion products of some contraceptive steroids in the rat has been presented by Steinetz et al. (1967) and recently by Hanasono & Fischer (1974). The decrease of the contribution of the renal elimination of the lynestrenol metabolites in the bile duct cannulated rat as compared with the elimination pattern shown by intact animals (see Chapter 12) seemed to justify further investigations into this direction. Therefore, a study has been made of the degree of absorption and the absorption rate of the lynestrenol metabolites present in the bile. Because, however, the demonstration of the possibility alone of reabsorption does not provide information about the significance of the enterohepatic circulation in a quantitative sense, it has been subsequently investigated to what extent

enterohepatic circulation could change the excretion pattern of the metabolites and whether such a change could serve as an indicator for the overall extensiveness of the recirculation. Furthermore, it was hoped, that the information thus obtained could throw some light upon the interrelationship of the renal and hepatic excretory pathways for the compounds in study.

MATERIALS AND METHODS

Most methods and materials used in the present investigations have already been described in detail in the preceding chapter. In this section, therefore, information about these aspects is only provided in sofar other procedures and techniques have been applied.

Ligation of the bile duct

Surgery of the animals was performed under ether anaesthesia. After exposure of the bile duct two ligatures were placed just below the junction of the hepatic duct. Subsequently, the bile duct was sectioned between the ligatures. In this way the outflow of the pancreatic juice via the distal segment of the bile duct remained undisturbed. An extensive description of the whole procedure, that was followed, is given by Lambert (1965).

About three hours after this operation and the insertion of the urinary bladder cannula lynestrenol-^{14}C was injected. This relatively short period of recovery of the rats was chosen on the one hand, because at that time the animals showed already their normal reactions and produced a steady flow of urine, and on the other hand in order to prevent as much as possible any interference of the developing jaundice with the metabolism and excretion processes in study.

At the end of the experiment, 48 hours after lynestrenol injection, the animals were sacrificed and opened for inspection of the position of the urinary bladder cannula and the bile duct. The proximal segment of the bile duct was always slightly distended. Not more than 0.05 ml volume of a clear and yellow fluid could be obtained with a syringe. Its content of radioactivity appeared to be negligible.

Dosage

For intravenous administration of lynestrenol, ethanol containing polysorbate 80 was added to the lynestrenol-^{14}C solution. The solvent was evaporated and

the residue solubilized in saline. The final polysorbate concentration was 2%. Of this mixture a volume of 0.2-0.5 ml was injected in the tail vein.

The enterohepatic circulation of the lynestrenol metabolites was studied with bile duct cannulated rats after the intraduodenal administration of radioactive bile, collected from donor rats during a period of 8 hours after the intravenous administration of lynestrenol-^{14}C, or of a combined hexane/ethylacetate extract of this bile obtained after enzymatic hydrolysis. The donor bile was collected on ice, pooled and an aliquot was analysed to establish the content of free and conjugated conversion products of lynestrenol. The results obtained were in accordance with the previous findings (Chapter 12, Table 12.4).

In case of administration of an extract of the donor bile, an aliquot of the same bile pool was buffered at pH 5.2 and hydrolysed with Helix pomatia β-glucuronidase/arylsulphatase as described in the preceding chapter. The hexane and ethylacetate extracts of the hydrolysed bile were combined and after evaporation of the solvents with a weak stream of air dissolved in bile. The donor bile and the extracts to be administered were made up to a volume of 1.0 ml/rat with non-radioactive bile.

The lynestrenol dose administered was 50 μg/rat, corresponding to 0.5 μCi ^{14}C for the animals with ligated bile duct and 1.0 μCi ^{14}C for the intacts rats. The radioactivity content of the donor bile and the donor bile extract administered was 0.5 μCi ^{14}C/rat.

RESULTS

Enterohepatic circulation

After the intraduodenal administration of radioactive bile obtained from rats injected with lynestrenol-^{14}C 10-35% of the dose was excreted with the faeces and 60-80% could be recovered from the urine and bile during the experiment (Table 13.1). So with respect to the elimination of the metabolites of lynestrenol a considerable degree of enterohepatic recycling has to be taken into account.

The pattern of the cumulative excretion of radioactivity in the bile is presented in Fig. 13.1 and demonstrates a considerable delay in the absorption and excretion of radioactivity compared with the intraduodenal administration of lynestrenol (cf. Chapter 12, Fig. 12.4). In view of the shape of the excretion curves it seems probable, that in addition to a slower absorption rate of the biliary metabolites of lynestrenol in comparison with the absorption rate of lynestrenol itself, another important process is involved, namely the hydrolysis of the glucuronides to the free steroids by β-glucuronidase of bacterial origin,

Table 13.1 — Excretion of radioactivity in the bile duct cannulated rat after the intraduodenal administration of radioactive bile or an extract of hydrolysed bile obtained from lynestrenol-^{14}C injected rats[1])

						day I	day II
			BILE				
						Mean ± S.D.	
Bile	61.2/ 5.9	67.1/0.4	66.2/ 1.7	55.8/0.4	57.0/0.1	61.5 ± 5.2	1.7 ± 2.4
Urine	9.2/ 1.6	9.0/0.2	10.5/ 0.7	7.0/0.1	5.8/0.2	8.3 ± 1.9	0.6 ± 0.6
Faeces	0.8/ 8.7	19.5/1.9	0.7/ 8.1	34.4/2.0	19.3/1.1	14.9 ± 14.3	4.4 ± 3.7
Total	71.2/16.2	95.6/2.5	77.4/10.5	97.2/2.5	82.1/1.4	84.7 ± 11.4	6.6 ± 6.5
			EXTRACT OF HYDROLYSED BILE				
						Mean ± S.D.	
Bile	65.4/ 2.0	81.2/0.1	82.6/ 0.1	67.2/3.9		74.1 ± 9.1	1.5 ± 1.8
Urine	8.2/ 1.1	11.1/0.1	10.3/ 0.1	9.1/1.6		9.7 ± 1.3	0.7 ± 0.8
Faeces	no sample/ 4.4	0.7/0 /0*	0.4/ 0 /0*	0 /0.5/0*		—	—
Total	73.6/ 7.5	93.0/0.2	93.3/ 0.2	76.3/6.0		84.1 ± 10.6	3.5 ± 3.8

[1]) The figures represent individual and mean excretion values over two and in some cases three (marked with *) successive 24-hour periods and are expressed in % of the dose. The bile extract was a combined hexane/ethylacetate extract of β-glucuronidase hydrolysed bile.

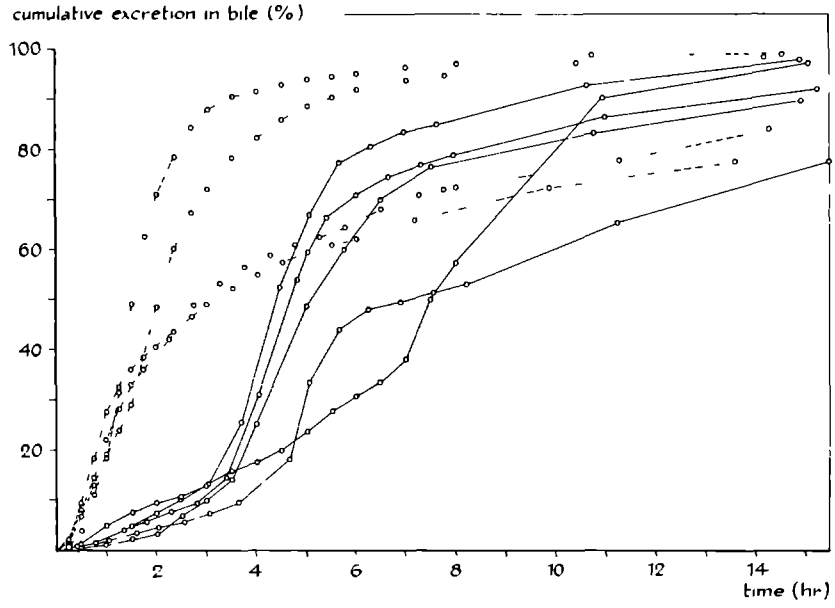

Fig. 13.1 — Cumulative excretion of radioactivity in the bile as a function of time after the intraduodenal administration of radioactive bile (——) and an extract of the same bile obtained after enzymatical hydrolysis (- - - - -) The data points are expressed in per cent of the total excretion in the bile over a period of 48 hours (see also Table 13.1).

which is present predominantly in lower parts of the intestine (Conchie & MacDonald, 1959; Kent et al., 1972; Marsh et al., 1952; Williams et al., 1970). Evidence for the hydrolysis of a large fraction of the biliary excretion products of lynestrenol in the intestine has already been provided in Chapter 12. The significance of this hydrolysis for the rate and degree of the absorption process can be derived from the cumulative excretion and excretion rate of radioactivity after the intraduodenal administration of a combined hexane/ethylacetate extract of β-glucuronidase treated bile, also shown in Table 13.1 and Fig. 13.1 respectively. The absorption of the free steroid fraction appeared to proceed with considerably less delay of time and to a high, almost complete, degree. The time lag observed for the absorption in case of administration of untreated bile is for this reason probably due to the additional time required for the transit of the substrate from the duodenum to the enzyme-rich caecum and colon (Conchie & MacDonald, 1959; Marsh et al., 1952). Because, however, in most rats the cumulative excretion with the faeces after the administration of radioactive bile appeared to be smaller than the non-hydrolysable fraction

of the biliary metabolites (cf. Chapter 12, Table 12.4), it is likely that part of this fraction may be absorbed, too.

After the administration of the bile extract in two animals biliary excretion did temporarily proceed even faster than after lynestrenol injection (Chapter 12). Such a phenomenon is conceivable under conditions of a high absorption rate and a volume of distribution smaller for the metabolites than for the parent compound. A smaller volume of distribution for the metabolites of lynestrenol is a reasonable assumption, since they are more polar and in part of ionic nature.

Influence of bile duct ligation and fasting on the excretion pattern

As is shown above the elimination of metabolites reabsorbed from the excreted bile takes place via the liver as well as via the kidneys. If the excretion ratio bile/urine after the reabsorption of the lynestrenol metabolites remains unchanged, then increasing frequency of enterohepatic recycling should ultimately result in an increase of the total renal excretion and thus in a decrease of the faeces/urine excretion ratio in the intact rat. In this connection the difference observed between the cumulative renal excretion in the intact rat and the rat with bile fistula on the one hand (Chapter 12) and the high degree of reabsorption of the biliary metabolites on the other hand seems to indicate an extensive but not frequent enterohepatic recycling. In order to get proof for the validity of this assumption more information was collected about the potential capacity of the rat kidney to excrete the lynestrenol metabolites. This capacity was assumed to become manifest in conditions of bile duct obstruction. Secondly it was tried by creating optimal conditions for reabsorption in the intact rat and thus for a possible increase in the frequency of recycling, whether this was reflected in a decrease of the faeces/urine excretion ratio.

1. Renal excretion in rats with bile duct ligation

That in certain conditions the kidneys may function as an equivalent alternative for the bile as the main route for excretion of the lynestrenol metabolites is obvious from the cumulative excretion data obtained after the intravenous administration of lynestrenol-^{14}C to bile duct ligated rats (Table 13.2). The time course for renal excretion followed a biphasic pattern and could be described by a bi-exponential equation. A survey of the time constants of the respective curves, calculated from the best fit of the experimental data with the help of the non-linear least squares curve fitting program FARMFIT (see

Table 13.2 — Renal excretion of radioactivity in the bile duct ligated rat after the intravenous administration of lynestrenol-^{14}C*)

						Mean ± S.D.
Day I	92.0	94.8	86.7	93.2	91.5	91.6 ± 3.0
Day II	5.3	5.2	6.7	5.0	1.9	4.8 ± 1.8
Total	97.3	100.0	93.4	98.2	93.4	96.5 ± 3.0

*) The figures represent individual and mean excretion values over two successive 24-hour periods and are expressed in % of the dose.

Chapter 7) is given in Table 13.3. A representative example of the good fit of the experimental data to the calculated curves is shown in Fig. 13.2. Like in the preceding chapter, the intercept of the renal excretion-deficit at zero time is exceeding the 100% level as a consequence of the void volume of the bladder cannula used in these experiments.

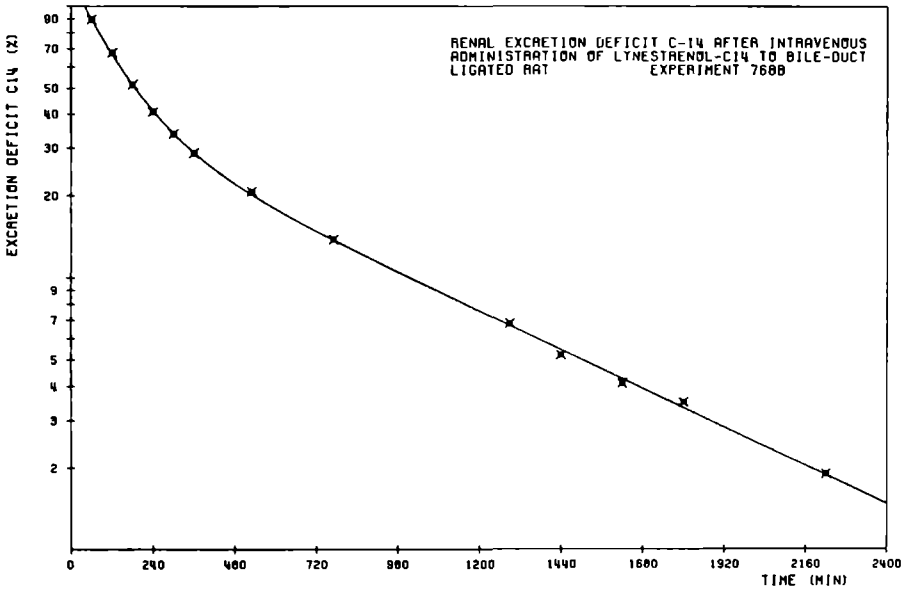

Fig. 13.2 — Computer-generated plot of the experimental data points and the corresponding best-fit curve of the renal excretion deficit of radioactivity (expressed in per cent of the total excretion values given in Table 13.2) after the intravenous administration of lynestrenol-^{14}C to a bile duct ligated rat.

Table 13.3 — Half-life times for the time course of the renal excretion deficit of radioactivity in the bile duct ligated rat after the intravenous injection of lynestrenol-^{14}C*)

$t^1/_2$,a	68.4	89.0	93.8	44.9	n.d.
$t^1/_2$,b	485	508	570	406	n.d.

*) The results are expressed in minutes for the half-life of the first ($t^1/_2$,a) and second ($t^1/_2$,b) phase of the respective curves. Same experiment as shown in Table 13.2. From one animal only the 24 and 48-hour samples were collected (n.d.). The excretion deficit has been calculated from the values of cumulative excretion 48 h after the injection of lynestrenol-^{14}C (see Table 13.2).

That under the conditions applied the biotransformation of lynestrenol is unaffected and that obstruction of the bile duct results in the renal excretion of compounds otherwise excreted largely with the bile, can be derived from the composition of the urinary radioactivity as shown in Table 13.4 and Fig. 13.3. Summation of the relative contribution of each metabolite fraction in the bile

Table 13.4 — Results of fractionation of radioactivity present in the urine of bile duct ligated rats after the intravenous administration of lynestrenol-^{14}C*)

	BEFORE HYDROLYSIS					
						Mean ± S.D.
Hexane extract	0.48	0.51	0.68	0.65	0.76	0.61 ± 0.12
Ethylacetate extract	7.9	10.1	10.2	9.8	7.0	9.0 ± 1.5
	AFTER HYDROLYSIS					
						Mean ± S.D.
Hexane extract	10.8	11.6	14.0	13.9	sample lost	12.6 ± 1.6
Ethylacetate extract	55.4	57.9	57.7	54.7	sample lost	56.4 ± 1.6

*) The urine samples of the first 24-hour period after injection were analysed (same experiment as shown in Tables 13.2 and 13.3). The results after hydrolysis were obtained with complete unextracted urine. The data represent % of the total radioactivity of the concerning sample.

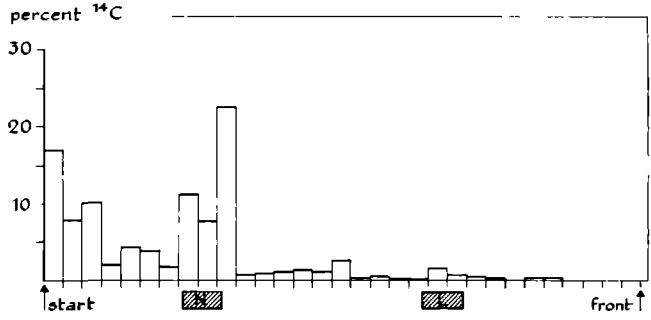

Fig. 13.3 — Thin-layer chromatogram of the combined hexane extracts of β-glucuronidase hydrolysed urine of bile duct ligated rats after the intravenous injection of lynestrenol-^{14}C. Solvent system toluene/ethylacetate 80/20. The distribution of radioactivity over the chromatogram is shown as the percentage of the total amount ^{14}C recovered from the plate. Positions of co-chromatographed standards lynestrenol (L) and norethindrone (N) are indicated.

and urine as mentioned in the preceding chapter (cf. Chapter 12, Table 12.4) demonstrates a close agreement with the corresponding fractions in Table 13.4. Also the relative increase of the non-polar fraction in the hexane extract of the hydrolysed urine (Fig. 13.3) indicates the presence of products normally excreted with the bile (cf. Chapter 12, Fig. 12.5). About 2% of the radioactivity in this extract migrated with the lynestrenol standard, providing evidence for the complete biotransformation of lynestrenol.

2. *Excretion pattern in fasting rats*

Assuming that the rate of elimination of radioactivity from the gut with the faeces is dependent upon the presence of food in the gut lumen and the food supply, and assuming furthermore, that the rate of fluid transport, i.c. bile, to the bacteria-rich parts of the gut is much less dependent upon these factors, empty bowels could result in an increase of the rate and frequency of enterohepatic recycling.

In Table 13.5 the excretion pattern of radioactivity is presented after the intravenous injection of lynestrenol to rats fasting from 24 hours before until 24 hours after lynestrenol dosage. From the results it is clearly visible that under these conditions the contribution of renal excretion in the over-all elimination process does indeed increase considerably. The cumulative excretion in urine and faeces during the 7 successive 24 hour periods is shown in Fig. 13.4 together

Table 13.5 — Cumulative excretion of radioactivity after the intravenous administration of lynestrenol-^{14}C to fasting rats*)

						Mean ± S.D.
Urine	27.3	40.6	43.3	25.5	39.7	35.3 ± 8.2
Faeces	55.7	47.8	48.7	73.6	57.0	56.6 ± 10.4
Total	83.0	88.4	92.0	99.1	96.7	91.8 ± 6.5

*) The individual and mean values of 5 rats at the end of 7 days are presented, expressed in % of the dose. The animals were deprived from food from 24 hours before until 24 hours after dosage.

with similar results obtained with non-starved intact rats, which have already been presented in Chapter 12. The change in the excretion pattern is obvious. The faecal excretion is delayed and reduced considerably over the first 48 hours after lynestrenol injection, whereas the renal excretion over the same period is greatly enhanced, indicating a considerably increased frequency of the enterohepatic circulation of the lynestrenol metabolites.

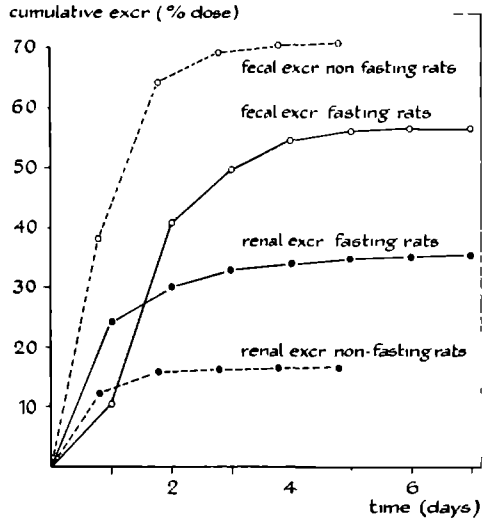

Fig. 13.4 — Cumulative excretion of radioactivity as a function of time after the intravenous administration of lynestrenol-^{14}C to fasting (n = 5) and non-fasting rats (n = 5). Fasting animals received no food from 24 hours before until 24 hours after lynestrenol dosage. The data points represent the mean ± S.D. of the excreted radioactivity in per cent of the dose.

Interrelationship of renal and hepatic excretion

The time course of the renal excretion as observed in the present study for the bile duct ligated rat is in very close agreement with the results obtained in animals with intact bile flow (see Chapter 12). So in spite of the fact, that in the intact rat biliary excretion appeared to be the major pathway for the elimination of the lynestrenol metabolites, in certain conditions the kidneys may take over their excretion with equal efficiency in time as well as in amount eliminated. On account of these observations the conclusion seems justified, that the contribution of plasma protein binding to the unequal distribution of the excretion products over the urine and bile is negligible and that the causal factor is localized within the liver. In the case of lynestrenol the liver, as the metabolic site, is the source of the products to be excreted. These conversion products will be in part secreted with the bile and will be in part released into the blood. If the structural requirements for an extensive excretion with the bile are fulfilled (Smith, 1971), it is conceivable that a large fraction of the metabolites does not reach the systemic circulation and as a consequence the kidneys. In the case of bile duct ligation, however, the total amount of metabolites is disposed with the blood and subsequently eliminated in the kidneys following a similar time course.

Enterohepatic circulation

The enterohepatic circulation of endogenous compounds as well as drugs or their metabolites is a not uncommon phenomenon in conditions, where these compounds appear in the bile in the form of polar conjugates such as glucuronides. Intestinal absorption of the glucuronides themselves is generally limited due to their strongly acidic character (Schanker, 1971). The lower parts of the intestines, however, have been demonstrated to contain large amounts of β-glucuronidase (Conchie & MacDonald, 1959; Marsh et al., 1952), mainly of bacterial origin (Kent et al., 1972; Williams et al., 1970). Liberation of the more lipophilic aglycones by this enzyme will result in their reabsorption and subsequent enterohepatic circulation (Smith, 1971).

The results reported here demonstrate, that considerable reabsorption of the biliary excretion products of lynestrenol occurs and that the effectuation of their enterohepatic recycling depends largely upon the rapid breakdown of their glucuronides in the gut. In this respect it is important to note, that after the

administration of the extract of hydrolysed bile the metabolites excreted afterwards with the bile proved to be hydrolysable with β-glucuronidase to the same extent as observed after lynestrenol administration. So on these grounds repetition of the process seems to be very likely.

Because with each succeeding enterohepatic circulation the absorbed compounds are lost in part by renal excretion, a progressively smaller fraction of the dose will be excreted with the bile and will be available for the next cycle. In spite of the demonstration of the possibility, that ultimately the excretion could proceed exclusively via the kidneys, faecal elimination did prevail even in the fasting animals. Assuming on account of the experimental results (Table 13.1; Chapter 12, Table 12.3), that in the course of each enterohepatic cycle about 80% of the biliary metabolites will become reabsorbed and subsequently excreted with the urine and bile in a ratio between 1 : 8 and 1 : 6, then a final distribution ratio over urine and faeces of 0.7 to 1.0 is to be expected after repetitive enterohepatic circulations. In the present study an urine/faeces excretion ratio of respectively 0.49, 0.85, 0.89, 0.35 and 0.70 was observed in the fasting rats (Table 13.5). Although these ratio's are considerably increased compared with urine/bile excretion ratio's of 0.12 to 0.18 in the bile duct cannulated animals and point to an extensive and repetitive recycling, a substantial interindividual variation can be observed. This variation is probably the reflection of the influence of intestinal factors, which affect the absorption rate, like the transit time through the small intestine, the interaction with faecal material, that may interfere with hydrolysis and absorption, and the β-glucuronidase activity of the gut contents.

For the intact non-fasting animals the excretion ratio urine/faeces varied between 0.2 and 0.3 with two exceptions of 0.47 and 0.62 after oral administration of lynestrenol (Chapter 12). On account of these findings and those mentioned above the conclusion seems permitted, that enterohepatic circulation of the lynestrenol metabolites occurs, but that in general the extensiveness of this process is limited, mainly as a result of the early loss of absorbable material with the faeces.

Implications

From a pharmacokinetic point of view the most important consequence of the enterohepatic circulation of the lynestrenol metabolites is the delaying effect this has upon their overall elimination from the body. If some of the recycling compounds, however, possess biological activity, this recycling phenomenon may also have an important bearing on their time course of action and the

nature and incidence of undesired effects. In this connection it should be realized, that in case of daily and long term administration of lynestrenol particularly the liver and bile ducts will be continuously exposed to relatively high concentrations of the metabolites. Little is known with regard to the biological activity of the lynestrenol metabolites identified thus far (Coert et al., 1975; Mazaheri et al., 1970; Murata, 1967; Yamamoto, 1968; this thesis, Chapter 12), with the exception of norethindrone. Apart from the progestational activity of the latter compound its reported conversion to the corresponding 4,5-epoxide (Cook et al., 1974) may be of interest. This epoxide has been demonstrated to bind irreversibly to microsomal free sulfhydryl groups containing proteins of rat liver and is converted by hepatic microsomal enzymes to another still unidentified compound, which can also react with proteins (Kappus & Bolt, 1976). Re-exposure of the excreted metabolites to the metabolic system of the liver as a consequence of enterohepatic circulation may give rise to further biotransformation via this kind of reactive intermediates.

Although enterohepatic circulation of other contraceptive steroids has been demonstrated previously in the rabbit (Arai et al., 1962) and rat (Hanasono & Fischer, 1974; Steinetz et al., 1967), investigations in man have not yet been reported. In the light of the present and previous findings and in view of the well-known enterohepatic circulation of endogenous sex hormones in humans (Long & Soyka, 1975; Sandberg & Slaunwhite, 1957, 1965) this process deserves further study also in the field of their synthetic counterparts.

SUMMARY

The excretion of radioactivity was studied in rats prepared with bile fistula after the intraduodenal administration of bile obtained from donor rats injected with lynestrenol-4-^{14}C. After 48 hours 9-36% of the dose was excreted with the faeces, 6-11% with the urine and 56-68% with the bile, demonstrating a high degree of absorption of the biliary excretion products of lynestrenol. In a similar experiment the cumulative excretion with urine and bile increased respectively to 9-11% and 67-83% of the dose, when an extract of enzymatically hydrolysed bile was administered. From the time course of cumulative biliary excretion in these experiments it could be derived, that the rate of absorption is largely dependent upon the hydrolysis of the conjugates in the lower parts of the alimentary tract. Renal excretion of the metabolites of lynestrenol appeared to be an alternative possibility equivalent to the biliary pathway, as in rats with ligated bile duct 93-100% of the dose was excreted via this route following a time course similar to that observed in animals with bile fistula. The delay of

faecal excretion in intact rats fasting from 24 hours before until 24 hours after the injection of lynestrenol-^{14}C resulted in a strong increase of the fraction excreted with the urine in comparison with intact non-fasting rats and rats with bile fistula. From the present and previous findings it may be concluded, that in the non-fasting intact rat extensive reabsorption of the biliary excretion products of lynestrenol will occur, but, subsequently, repetition of this process will generally be limited, mainly as a consequence of the early loss of absorbable material with the faeces.

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CHAPTER 14

DISTRIBUTION OF LYNESTRENOL IN PREGNANT AND NON-PREGNANT MICE

INTRODUCTION

Since the development of contact autoradiography for drug distribution studies, at first at the organ and tissue level (Ullberg, 1954; Ullberg, 1958), afterwards also at the cellular and subcellular level (Stumpf et al., 1971), the technique has proven to be a very useful tool in pharmacological and toxicological research (Conference on Autoradiography in Pharmacology and Toxicology, 1977). When care is taken to prevent translocation or removal of the drug or its metabolites, autoradiography of drugs in whole animals and tissues offers by far the best information on the localization of drugs in biological material, particularly in case of inhomogeneity of distribution within organs and tissues. On the other hand, quantitative analysis of the picture by densitometric methods suffers from inaccuracy, although recently in this respect progress has been made (Cross, 1977). If the drug under investigation is metabolised, combination of autoradiography with tissue extraction-separation procedures may give essential additional information.

In the present investigation the distribution of lynestrenol-^{14}C in mice has been studied by means of whole-body autoradiography after intravenous and oral administration. Pregnant mice were used for the study of the rate and extent of placental transfer of lynestrenol and its metabolites. Because of the extensive and rapid biotransformation of the drug observed in the rat (Chapters 12, 15, 16), separate experiments were undertaken to get information about the nature of the radioactive label as well as about the quantitative aspects of lynestrenol distribution with time in some selected organs and tissues.

MATERIALS AND METHODS

Animals

Adult female Swiss mice (Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands), body weight 18-20 g, and pregnant mice, two days before delivery,

body weight 42-45 g, were used. The animals were starved overnight before the experiment. Water was supplied ad libitum.

Radioactive lynestrenol

Autoradiography was performed after the administration of lynestrenol-4-^{14}C, impulse counting experiments were carried out separately after the administration of lynestrenol-^{3}H. Specifications of both compounds are given in Chapter 3. The synthesis of lynestrenol-^{3}H has been reported previously (Van Kordelaar et al., 1973) and is described in Chapter 4.

Dosage

For intravenous and oral administration of lynestrenol, ethanol containing polysorbate 80 (OPG, Utrecht, The Netherlands) was added to the radioactive lynestrenol solution. The solvent was evaporated with a weak stream of air and the residue was solubilized in saline. The final polysorbate concentration was 2%. A volume of about 0.1 ml was injected in the tail vein. For oral administration a volume of circa 0.25 ml was given through a stomach cannula. The dose injected was 17.5 μg lynestrenol/20 g body weight, corresponding to 2 μCi lynestrenol-^{14}C or 5 μCi lynestrenol-^{3}H. The tritiated material was diluted ten-fold with non-radioactive lynestrenol. In case of oral administration a dose of 3 μCi lynestrenol-^{14}C was given.

Autoradiography

Seven non-pregnant mice were sacrificed 2, 5, 10, 20, 60, 120, 180 min after lynestrenol-^{14}C injection. The three pregnant animals survived for 2, 10, 20 min. Oral dosage was supplied to seven animals with a survival period of respectively 5, 10, 20, 40, 60, 120, 240 min. At the appropriate time after lynestrenol administration the mice were anaesthetized with diethyl ether, stretched in a forceps and killed immediately by immersion in an isopentane bath cooled to about -80°C with solid carbon dioxide. After storage for one day at -20°C the fur was smoothened by moistening with water and the extremities were cut off. The frozen bodies were then placed on their side in a mould containing a 5% solution of carboxymethylcellulose (CMC) of medium viscosity (OPG, Utrecht, The Netherlands), precooled to 0°C. Subsequently,

the mould was cooled with solid carbon dioxide for about 10 minutes and stored at −20°C. For processing the sides of the mould were removed and the CMC block was mounted on the microtome (R. Jung A. G., Heidelberg, GFR) located in a chest-type freezer. Sagittal sections, 30 μm thick, of the whole body were cut at −15°C according to the autoradiographic technique of Ullberg (1954), by which each section is attached onto Scotch tape (no. 810, 3M, Leiden, The Netherlands). The tape mounted sections were transferred to card-boards as described by Van der Kleijn (1969a), then dried for at least 48 hours at −20°C, pressed against X-ray film (Structurix D7, Gevaert) and stored in a press at the same temperature. After an exposure time varying from 5 weeks to 20 weeks for the longest surviving animals the films were removed at room temperature and developed according to the manufacturer's advice.

Impulse counting

Groups of 4 female mice were injected with lynestrenol-^{3}H and sacrificed 2, 5, 10, 20, 40, 60, 120 and 180 min later by decapitation under light ether anaesthesia. Apart from blood, bile and urine, the following samples were taken for impulse counting: pituitary, cerebrum, liver, kidney, adrenal, heart, uterus, vagina, spleen, abdominal white fat, brown fat, lung, ovary, skeletal muscle. After removal the tissues were immediately cooled on ice. For each group of animals comparable parts of the respective tissues and fluids (about 50 mg/animal or the whole organ, i.c. pituitary, ovary, adrenal) were pooled in a pre-weighed counting vial, weighed and prepared for radioactivity assay.

Tissue extraction and chromatography

Simultaneously with the samples taken for the measurement of total radioactivity as described above aliquots of the same organs and tissues with the exception of the pituitary were collected and pooled for fractionation of the radioactivity by means of extraction and thin-layer chromatography. The samples were frozen immediately on dry ice and stored in a freezer at −25°C until processing. After thawing in the cold room at 3°C the tissues were homogenized in 1.0 ml cold water using a Teflon-glass Potter-Elvehjem type homogenizer. The homogenate was transferred to Teflon-stoppered conical glass tubes together with the 80% ethanol rinsing fluid of the homogenizer. After the addition of 4 ml ethanol containing 100 μg lynestrenol and standing of the suspension for half an hour under occasional swirling, the precipitate was spun

off and washed two times with 0.5 ml ethanol 80%. In general more than 95% of the radioactivity present in the tissue appeared to go into solution. The supernatant together with the washings was extracted three times with 5 ml n-hexane. The volume of the alcoholic phase was then made up to 10.0 ml with 80% ethanol and an aliquot of 1.0 ml was transferred into a counting vial for the assay of radioactivity. The joint hexane fractions were evaporated in a counting vial under a weak stream of air and radioactivity was measured similarly by liquid scintillation counting (LSC).

Blood samples were diluted with an equal volume of water and after the addition of 4 volumes of ethanol processed as described for the tissues. Urine and bile were diluted with water, neutralized to pH 6.5-7.5 and extracted likewise with hexane.

Part of the hexane extract of the blood and some tissues was also subjected to thin-layer chromatography over silicagel in order to determine the fraction of radioactivity representing lynestrenol. The procedure, which was followed, is described in Chapter 7.

The efficiency of the extraction was studied with blank tissue (heart, liver, cerebrum) and blood samples to which lynestrenol-^{3}H had been added. The mean recovery for each set of samples (n = 4) varied between 89 and 95%. The experimental values were corrected for incomplete recovery by multiplication by 1.09 (100/92). After thin-layer chromatography of these blank extracts as described in Chapter 7 more than 96% of the radioactivity could be recovered from the lynestrenol spot.

Assay of radioactivity

Radioactivity was measured by LSC as described in Chapter 5.

RESULTS

Autoradiography

1. Intravenous administration to non-pregnant mice (Fig. 14.1a,b)

After intravenous injection of lynestrenol a rapid but not uniform distribution of radioactivity could be observed over the whole body within some minutes. Already 2 min after injection blood radioactivity appeared to be less than that of the soft tissues with the exception of the thymus and white fat. In general,

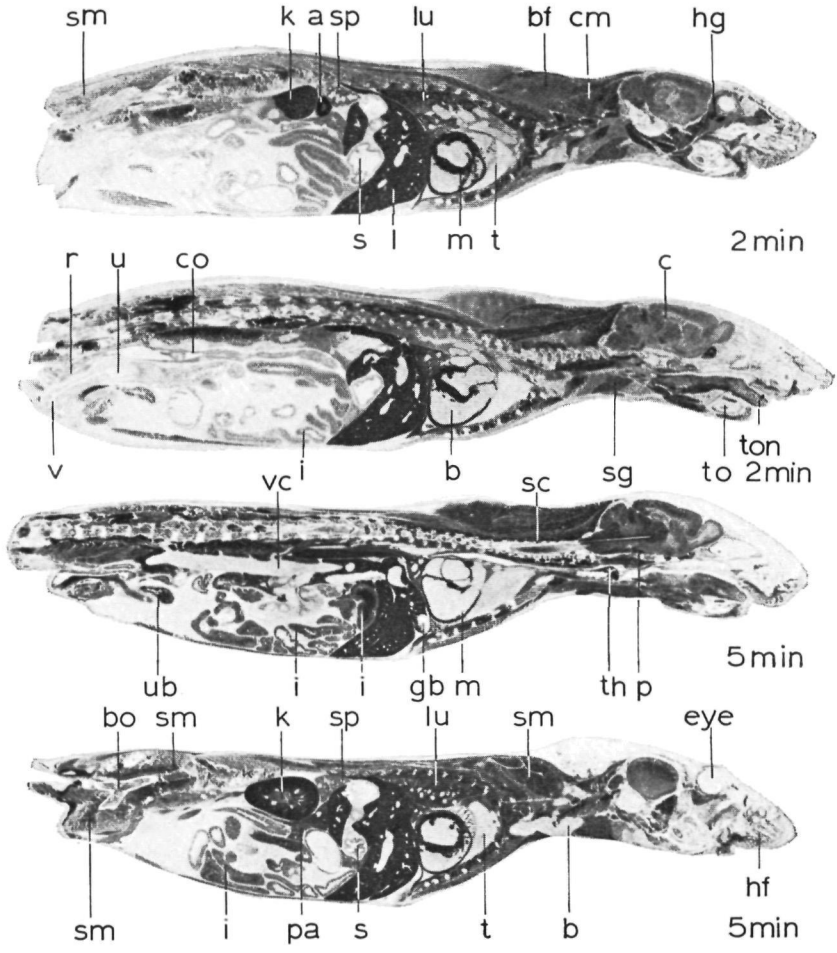

Fig. 14.1a — Autoradiograms of mice 2 and 5 min after the intravenous injection of lynestrenol-^{14}C. Note the rapid disappearance of radioactivity from the blood and the low uptake in the uterus, vagina and thymus in comparison with the other tissues. Excretion of radioactivity with the bile and urine is already proceeding. The key to the abbreviations is given in the Appendix (p. 182).

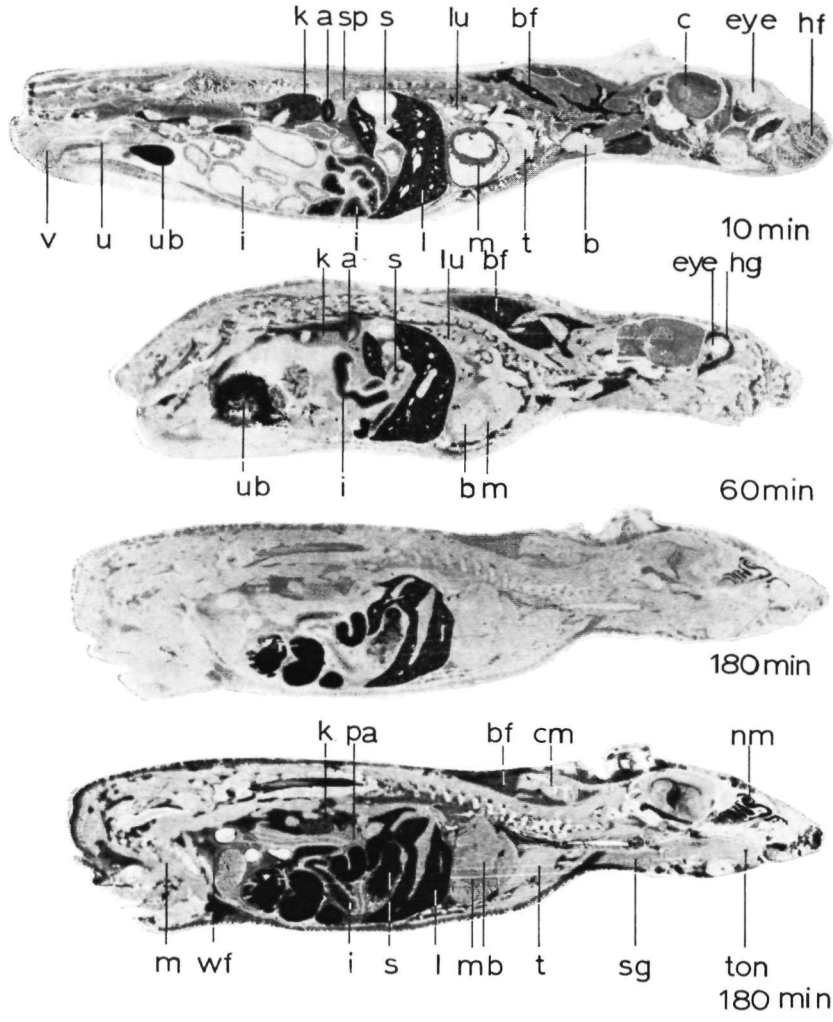

Fig. 14.1b — Autoradiograms of mice 10, 60 and 180 min after the intravenous injection of lynestrenol-^{14}C. The excretion of radioactivity in the urine and bile results in a considerable decrease of tissue radioactivity after 60 and 180 min. The 3rd and 4th picture (180 min) originate from the same animal, but the exposure time was increased from 5 to 20 weeks. Note the persistence of radioactivity in the liver and kidney, the adrenal cortex, the brown and white fat, Harder's gland, the corpus callosum and thalamic region in the brain, the trigeminal nerve, the nasal mucosa and the hair follicles. Labelling of the stomach contents is also apparent after 60 min. The key to the abbreviations is given in the Appendix (p. 182).

maximal levels of tissue radioactivity were reached within 5 min, uptake in the brown fat, however, continued for some time longer. High and very high levels of radioactivity were observed in the liver, kidneys, pituitary, adrenals, ovaries, lungs, central nervous system, myocard, thyroid, salivary glands, Harder's gland, brown fat and skeletal muscles. The rapid distribution was followed by rapid elimination as could be derived from increasing radioactivity levels in the urinary and gall bladder and the presence of radioactivity in the intestinal lumen from 5 minutes onwards. This coincided with a decrease of label in most organs and tissues. Retention of radioactivity over periods up to 3 hours after lynestrenol administration and longer was most pronounced in the liver, the adipose tissue and the white matter, furthermore relatively high levels of radioactivity persisted in the adrenal cortex, the kidney and Harder's gland. After the 3 hour interval the distribution pattern was almost uniform with regard to the other tissues. A more detailed description of the results is given below.

Cardiovascular system

As early as 2 min after injection the blood concentration was low compared with the radioactivity in the organs and remained so throughout the experiment. The myocard showed an extremely high uptake, which gradually decreased to the same level as the blood after about 60 min. No specific uptake could be observed in the blood vessel walls.

Digestive system

The uptake profile of the tongue was similar to that of the skeletal muscles reported below. Also the salivary glands showed initially a high labelling.

The glandular part of the stomach wall built up higher levels of radioactivity than the other part (Fig. 14.2), but this difference had been disappeared after 1 hour. Twenty minutes and later after drug administration the stomach lumen became progressively labelled.

Liver accumulation of radioactivity was very intense and persisted during the whole course of the experiment. Within 5 min following lynestrenol injection excretion with the bile had started, radioactivity being present in the gall bladder and the duodenal contents. In the later stages this spread to the lower parts of the intestines. The initially moderate uptake of radioactivity in the intestinal walls decreased fairly rapid to levels slightly above the blood concentration.

Moderate but persisting levels of radioactivity were also present in the pancreas.

Urogenital system

The kidneys showed very high labelling after 2 min, particularly in the

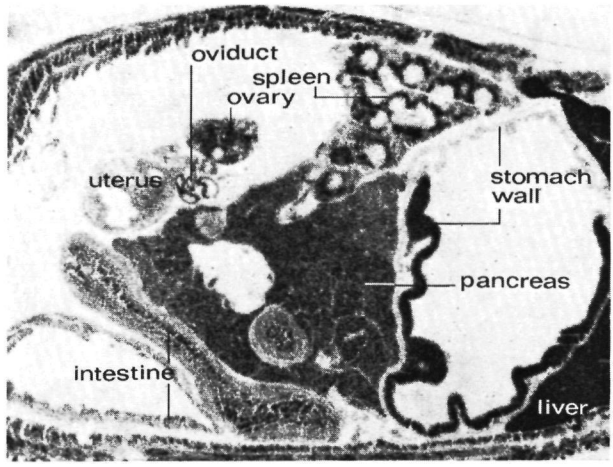

Fig. 14.2 — Detail of an autoradiogram 2 min after the intravenous injection of lynestrenol-^{14}C. Note the high uptake of radioactivity in the ovary and oviduct, in the secretory part of the stomach wall, and the accumulation of label at the interface between the red and white pulp of the spleen.

cortex, gradually decreasing during the next hour, but still distinct at the end of the experiment 3 hours later. At that time radioactivity was mainly concentrated in the pelvic region. The urinary bladder contained increasing amounts of radioactivity from 5 min onwards.

Intense labelling occurred also in the ovaries, particularly in the corpora lutea, follicle walls and oviduct (Figs. 14.2, 14.3). Blackening of the interstitial tissue was only moderate. A very slight accumulation was visible in the uterus and vagina.

Central nervous system

Drug penetration into the central nervous system did proceed easily and to a considerable extent, maximal levels being attained after 5 min. Initially the concentration of the gray matter exceeded the concentration of the white matter, but after about 60 min the picture was reversed with the highest labelling occurring in the white matter. The labelling of the spinal cord parallelled that of the brain. Three hours after lynestrenol administration the brain and blood levels were about equal. In the white matter, however, still distinct labelling could be noticed.

The respiratory system

A high amount of radioactivity was present in the parenchyma of the lungs

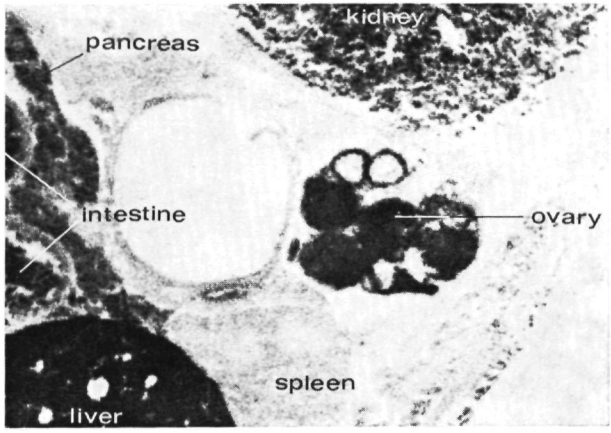

Fig. 14.3 — Autoradiogram of the ovary of a pregnant mouse 20 min after the intravenous injection of lynestrenol-^{14}C. Note the high uptake of radioactivity in the follicle walls and the corpora lutea.

during the first 5 min, this was followed by a gradual decrease to the blood level 3 hours later.

The nasal mucosa displayed increasing labelling after the 20 min interval. Accumulation and perhaps secretion of radioactivity were most pronounced after the 3 hour survival period.

Endocrine glands

A large difference was observed in distribution between the medulla and the cortex of the adrenal glands. The uptake in the latter tissue was very rapid and extremely high compared with the medulla of the organ as well as with the other organs including the liver. A slow decrease occurred with time, and after 3 hours the cortex radioactivity still exceeded the blood concentration.

Strong labelling of the pituitary was apparent during the first 20 min, afterwards no difference could be observed with the brain.

High uptake of radioactivity in the thyroid was observed 2 and 5 min after drug administration.

Lymphatic organs

In contrast with the other soft tissues radioactivity levels of the thymus, lymph nodes and the white pulp of the spleen did not exceed the blood levels even at the early stages of distribution. The red pulp of the spleen, however, showed slightly higher concentration than the blood during the first hour. Two

minutes after injection, pronounced accumulation of radioactivity was apparent at the interface between the red and white pulp in this organ (Fig. 14.2).

Skeletal muscles

A very high but heterogenous uptake of radioactivity took place in the skeletal muscles within some minutes, labelling, however, decreased rapidly to slight and low levels within 60 min. Particularly at the early survival periods the cervical muscle was stronger and more uniformly labelled than the other muscles.

Adipose tissue

For the adipose tissue the difference between the white and the brown fat appeared to be pronounced. Accumulation into the white fat did proceed slowly and only to a slight extent compared with the brown fat. In the latter tissue a high and increasing uptake could be observed during 10 min after injection. The decrease of the ^{14}C-levels occurred slowly and 3 hours after lynestrenol administration a distinct retention of label was apparent in the brown as well as in the white fat.

Skeleton, bone marrow, teeth, skin

No labelling of the hard tissues like bone and teeth could be detected during the experiment. A slight to moderate labelling of the bone marrow occurred. Small amounts of radioactivity were taken up by the skin. The hair follicles of the nose showed greater affinity, resulting in a long lasting and moderate accumulation of label.

2. *Oral administration to non-pregnant mice* (Fig. 14.4a,b)

Although during the entire observation period of 4 hours a considerable amount of labelling of the stomach contents could be seen, a large fraction of the small intestine was filled with radioactive material already within 5 min after lynestrenol administration. At that time the high concentration of label in the liver but not yet in the other tissues was indicative for the initiation of the absorption process. Ten minutes after drug administration the urinary and gall bladder were clearly labelled. Because the accumulation of label in the respective organs and tissues after oral administration is largely determined by the relationship between the rates of absorption and elimination, respectively excretion, for most tissues the maximal levels were reached only 20 to

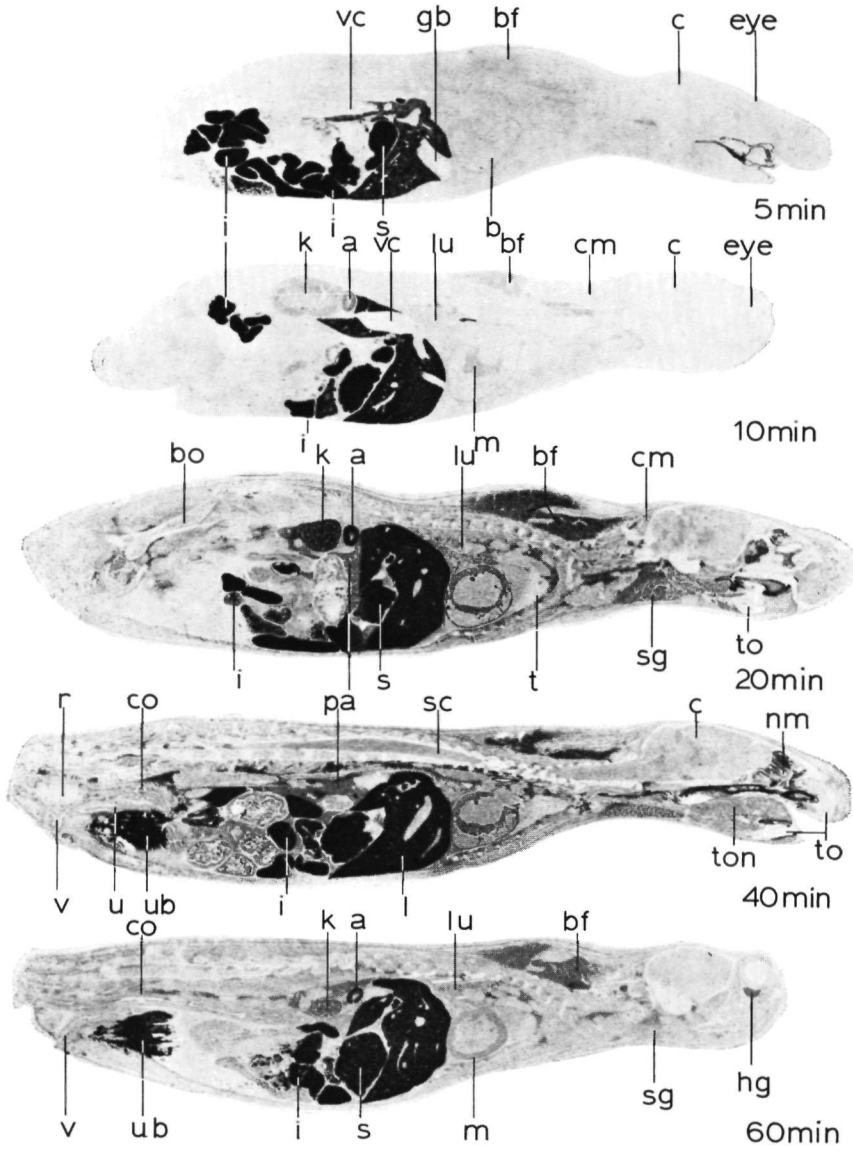

Fig. 14.4a — Autoradiograms of mice 5 to 60 min after oral administration of lynestrenol-^{14}C. Note the rapid absorption of the drug and the strong and persistent labelling of the liver, adrenal cortex, brown fat, kidney, Harder's gland and nasal mucosa. The key to the abbreviations is given in the Appendix (p. 182).

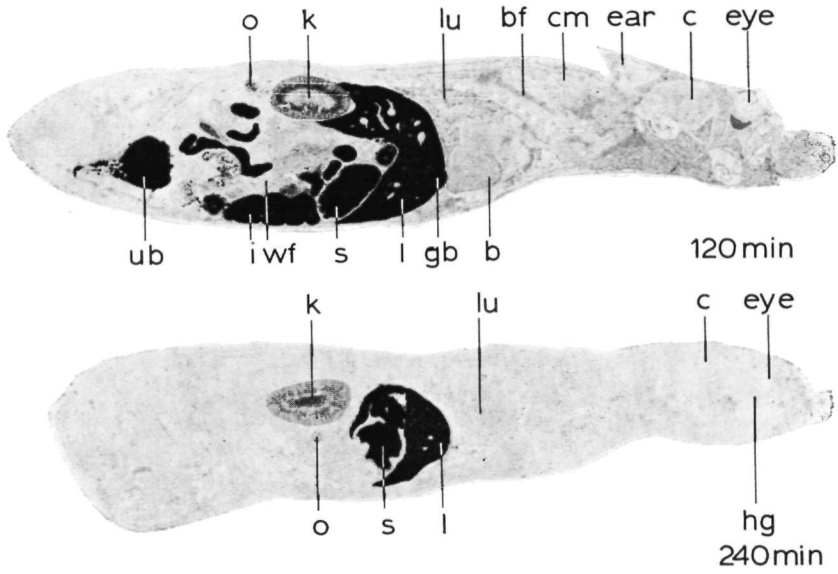

Fig. 14.4b — Autoradiograms of mice 2 and 4 hours after the oral administration of lynestrenol-^{14}C. Tissue radioactivity undergoes only small changes between 1 and 2 hours. A considerable loss of label occurs during the next 2 hours. Labelling of the ovary and Harder's gland is still clearly visible 4 hours after drug administration. The key to the abbreviations is given in the Appendix (p. 182).

40 min after drug ingestion. During the period of 20 to 120 min the distribution pattern was rather stabile in a qualitative and quantitative sense, probably reflecting a steady state of comparable absorption and excretion rates. In comparison with the intravenous route of administration no significant qualitative changes in the distribution pattern occurred: the tissues with a high uptake and/or retention of label were the same in both experiments. In general, however, the contrasts in the pictures were less sharp, probably because the maximal concentrations attained were lower after the oral route in spite of the higher dose. Four hours after lynestrenol administration the absorption rate of the compound was obviously declining. Still a distinct labelling of the various organs was visible, but the differences were negligible with an exception for the higher concentration of radioactivity in the liver and kidney, the adrenal cortex, the ovary and Harder's gland. In this stage no retention of label in the brown fat could be observed.

3. Distribution in pregnant mice after intravenous injection (Fig. 14.5)

The distribution over the maternal tissues was similar to that described above for the non-pregnant animals. The uptake in the brown fat, however, seemed to be somewhat retarded, and was initially uneven. Ten to twenty minutes after injection this difference has disappeared. In one of the animals the liver displayed a mottled pattern of labelling, possibly representing a centrilobular distribution of radioactivity. The passage through the placenta into the fetus was rather slow as compared with the transfer into the maternal organs. Two minutes after injection the presence of radioactivity was limited to the fetal membranes. Ten minutes after drug administration the placenta showed radioactivity levels above the maternal blood concentration. Also a slight but distinct uptake in some fetal tissues was apparent, particularly in the blood, liver, kidneys, adrenals, lungs and brown fat. The uptake process appeared to continue according to this same pattern for the next 10 min. After the 20 min interval a still faint distribution picture was obtained. At that time the intrafetal localization of radioactivity was most pronounced in the adrenal cortex, the liver and the lumen of the small intestine (Fig. 14.6a). Compared with the degree of labelling of the maternal organs the overall accumulation of radioactivity in the fetus remained fairly low.

Impulse counting and extraction/chromatography

The total and hexane-extractable radioactivity present in a number of tissues after various post-injection times is shown in Table 14.1. The total radioactivity levels are generally in good agreement with the autoradiographic pictures described above. Apparent exceptions are the comparatively moderate maximal levels observed in the cerebrum, ovary and skeletal muscles. In these tissues, however, the distribution of label appeared to be rather inhomogeneous, too.

The fraction of the total radioactivity, that could be extracted with hexane, is also shown in Table 14.1. An extremely rapid decrease of this fraction in the

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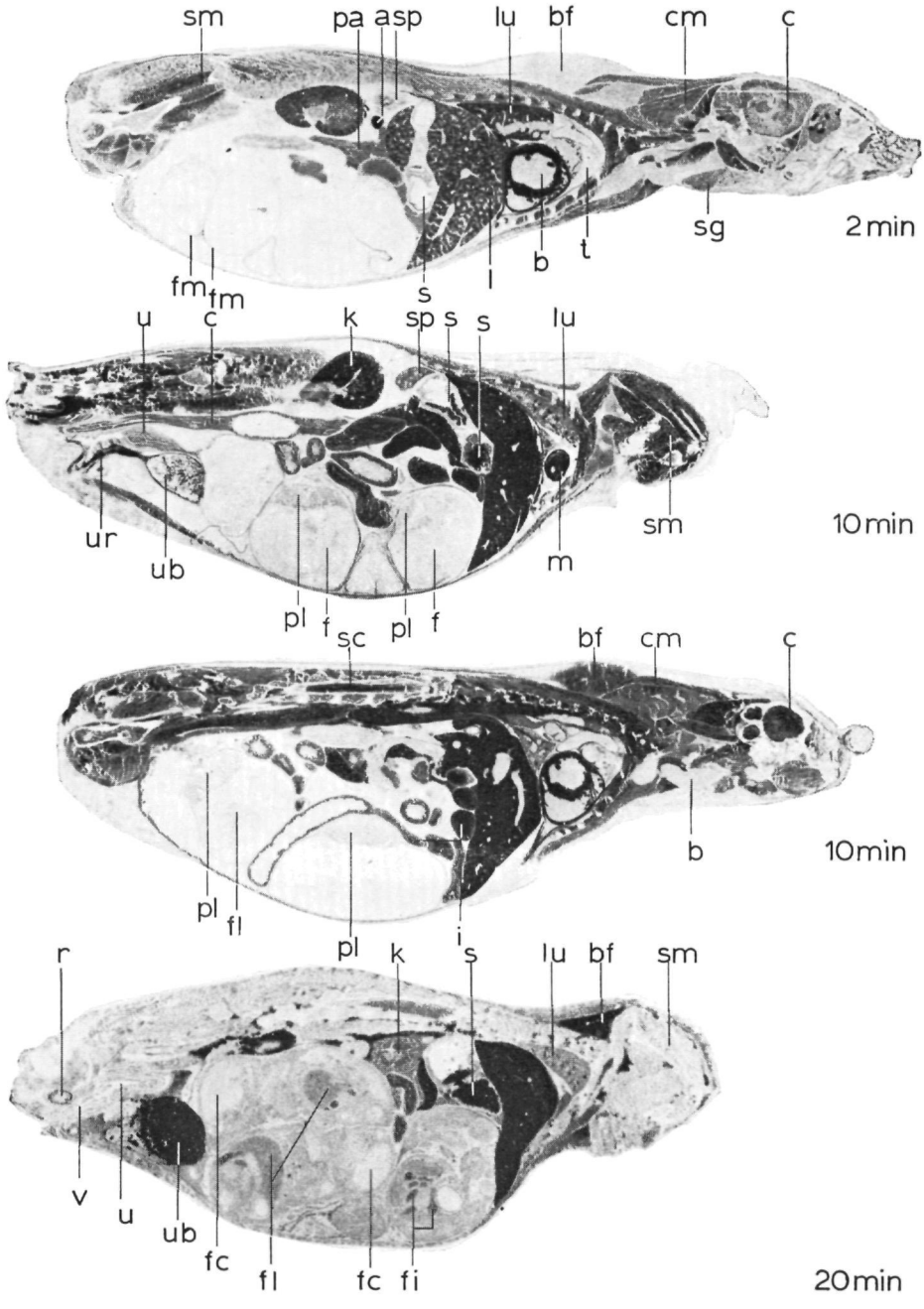
Fig. 14.5 — Autoradiograms of pregnant mice 2, 10 and 20 min after the intravenous injection of lynestrenol-^{14}C. Note the slow and limited uptake of label in the fetuses in comparison with the maternal levels of radioactivity. After 20 min labelling of the fetal liver, the intestinal contents and the adrenal cortex (not visible) is most pronounced. See also Fig. 14.6. The key to the abbreviations is given in the Appendix (p. 182).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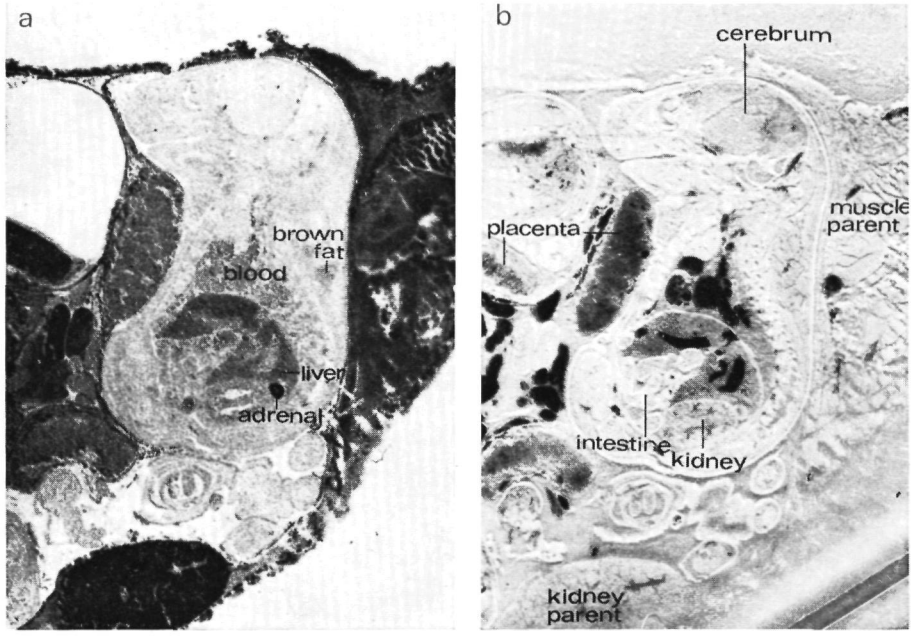

Fig. 14.6 — a. Detail from whole-body autoradiogram showing the distribution of radioactivity in a fetus 20 min after the intravenous administration of lynestrenol-^{14}C to the parent. Note the rather uniform distribution pattern. Apart from the very strong accumulation of radioactivity in the fetal adrenal cortex, the uptake even in those fetal tissues showing the highest concentrations of label (liver, kidney, blood, brown fat, intestinal contents) does not exceed the maternal blood level.
b. Photograph of the corresponding part of the section. Note the position of maternal and fetal blood vessels.

blood and liver can be observed, followed by a more gradual decrease in the other tissues. In the white and brown adipose tissue, showing moreover prolonged accumulation of radioactivity, as well as in the central nervous system, the non-polar fraction remained high during the entire experimental period. Furthermore it can be observed, that from the early start of the experiment only a very small fraction of the excretion products in the urine and bile could be extracted with hexane in contrast with the corresponding tissue and blood samples. Obviously, lynestrenol is not excreted unchanged. From the present experiment no information was obtained about the relative significance of the bile and urine as routes of excretion for lynestrenol and its metabolites.

The time course of the total radioactivity levels and the lynestrenol levels as derived from thin-layer chromatography of the hexane extracts of the blood

Table 14.1 — Total and hexane-extractable radioactivity in the blood and tissues of mice at different time intervals after the intravenous injection of lynestrenol-^{3}H*)

TIME (min)	2	5	10	20	40	60	120	180
Blood	397/ 194	346/ 160	282/ 69.1	193/ 28.2	181/ 21.4	135/ 11.1	112/ 5.0	86/ 3.2
Liver	1643/1104	1656/ 614	1627/ 397	1480/ 243	1304/287	898/154	1127/147	1160/ 47.6
Kidney	1593/n.d.	1246/1134	1072/ 844	708/ 460	435/238	278/124	260/ 82.9	212/ 82.3
Cerebrum	411/n.d.	386/ 338	438/ 355	390/ 351	365/317	262/236	231/188	169/140
Pituitary	1494/n.d.	1060/n.d.	913/n.d.	750/n.d.	451/n.d.	278/n.d.	227/n.d.	164/n.d.
Adrenal	3036/n.d.	2938/2409	2428/1651	1403/1010	1017/693	826/659	392/260	230/178
Ovary	339/n.d.	391/ 352	360/ 294	369/ 292	314/230	195/148	215/157	171/103
Uterus	254/n.d.	211/ 127	275/ 97.6	208/ 134	207/123	119/ 64.4	133/ 91.5	129/ 47.5
Vagina	147/n.d.	103/ 83.5	143/ 58.6	109/ 54.6	150/ 70.7	100/ 41.1	97/ 24.0	93/ 36.5
Lung	n.d./n.d.	1245/1134	875/ 760	655/ 540	530/402	309/200	280/192	216/143
Myocard	1863/n.d.	1159/1109	778/ 712	443/ 350	269/199	153/ 96.7	171/ 99.0	138/ 56.6
Muscle	667/n.d.	332/ 268	289/ 198	284/ 225	196/158	137/100	104/ 92.7	99/ 74.0
Brown fat	407/n.d.	657/ 569	1037/ 925	1162/1070	1022/876	899/810	438/388	202/166
White fat	82/n.d.	102/ 90.7	127/ 107	265/ 223	310/292	370/347	224/202	261/220
Spleen	328/n.d.	315/ 242	361/ 275	240/ 178	197/105	112/ 59.4	135/ 59.0	97/ 34.3
Urine	- /n.d.	- / 2.6	- /n.d.	- / 1.7	- / 2.2	- / 0.3	- / 0.2	- / 3.3
Bile	- /n.d.	- /n.d.	- / 2.8	- / 2.5	- /n.d.	- / 0.5	- / 0.5	- / 3.8

*) For each survival time the first figure represents the total radioactivity and the second one the hexane-extractable radioactivity, both expressed as desintegrations per minute (dpm) per mg wet tissue or blood. The results for the pituitary are expressed as dpm/pituitary (wet weight about 1 mg). The results for urine and bile are given in per cent of the total radioactivity. "n.d.": abbreviation for "not determined".

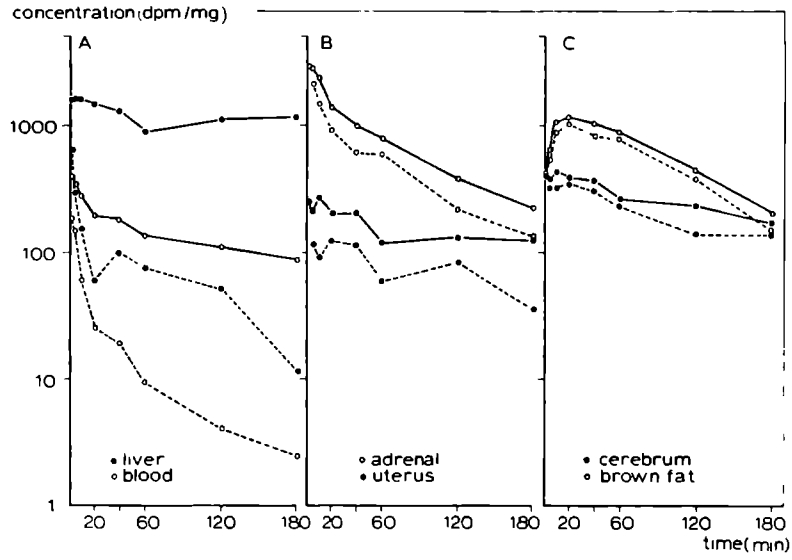

Fig. 14.7 — Concentrations of total radioactivity (closed line) and of lynestrenol (broken line) in the blood and liver (a), the adrenal and the uterus (b), and the cerebrum and brown fat (c) of mice at various times after the intravenous injection of lynestrenol-^{3}H. The concentrations are given as desintegrations per minute (dpm) per mg blood and tissue (log scale).

and liver, the uterus and adrenal, and the cerebrum and brown fat, are shown in Fig. 14.7a-c. The blood and liver curve for lynestrenol and, to a smaller extent, also the adrenal curve exhibit a biphasic decline, whereas the decline of lynestrenol in the brown fat, cerebrum and uterus proceeds monophasic. The time of half-life of lynestrenol as derived from these curves can be estimated to be in the order of 60 to 120 min. In the blood and in the liver a wide difference can be observed between the data of the total radioactivity and the lynestrenol concentrations, indicating a rapid biotransformation of the compound.

DISCUSSION

General considerations

Apart from the influence of the route of administration drug distribution and the time course of drug distribution are governed on the one hand by the

physicochemical properties of the drug molecule and on the other hand by the anatomical and physicochemical characteristics of the organism composed as it is from organs, tissues, cells and subcellular structures (Goldstein et al., 1974). Moreover, under conditions of non-equilibrium like e.g. immediately after intravenous injection the rate of drug delivery to the various organs and tissues is dependent on the blood flow and also this parameter should be considered as a determinant factor in the distribution process. Drug accumulation, therefore, in a certain organ or tissue is not necessarily a reflection of its *locus* of action, even under steady state conditions, but primarily an indication for a high affinity of that particular tissue for that particular drug. The mechanism responsible for the accumulation may be directly, somehow or not at all related to the biological activity of the drug or the biological function of the tissue (e.g. liver, kidney). For this reason, it is generally difficult to estimate the significance of drug accumulation in a certain organ or tissue without complete knowledge about its scala of biological activities and mechanism of action. On the other hand, it should be kept in mind that the time course of drug action may also be influenced by drug accumulation in and slow release from non-target tissues, especially in case of rapid biotransformation of the drug into inactive metabolites or in case of rapid excretion.

Pharmacokinetic aspects

On account of the generally accepted theories about the permeability of biological membranes the physicochemical characteristics of lynestrenol, namely its molecular weight of 284, its neutral character and high lipophilicity as illustrated by its partition coefficient heptane/water of about 5000 (Chapter 8), favour a rapid passage across these membranes as far as dependent on diffusion processes (Goldstein et al., 1974; Raaflaub, 1970). A rapid absorption and subsequent distribution over highly vascularized tissues and a rapid penetration into the central nervous system can therefore be expected and was actually observed. The significance of the blood supply in this respect is illustrated by the delayed uptake of label in the brown and white adipose tissue in spite of their obvious affinity for the drug. Drug accumulation in the latter tissue with its very poor blood supply remained less pronounced than the uptake in the better perfused brown fat, presumably as a consequence of the rapid biotransformation of the parent compound. On the other hand the uptake in these tissues of more polar conversion products of lynestrenol cannot be excluded, since 10-20% of their content of radioactivity could not be extracted with hexane. A strong accumulation and long retention in the body fat and brain of rats has been reported

previously for the cyclopentylether derivative of ethinylestradiol, quinestrol, by Meli et al. (1963, 1965, 1968). The concentration of this very lipophilic drug in the brown fat appeared to be about 3 times the concentration measured in the white fat 24 hours after its oral administration (Steinetz et al., 1967). Other steroids like pregnenolone (Appelgren, 1967), progesterone (Appelgren, 1967), mestranol (Appelgren, 1971) have also been shown to concentrate in the brown fat, but this accumulation was followed by a more rapid release of label than was observed in the present study.

The time course of total radioactivity and of lynestrenol in the blood and liver (Fig. 14.7a) point to a rapid biotransformation of the drug in the latter organ. Although the short duration of the experiment does not allow to draw final conclusions from the blood and tissue curves with respect to the time of half-life of lynestrenol (Fig. 14.7), in any case two phases can be discerned in the elimination of lynestrenol from the blood. The initial and steep decline occurring in the blood probably represents a decline caused primarily by distribution and metabolism, whereas the second slower phase may be governed by the transfer rate of lynestrenol from a second compartment to the central compartment consisting among others of blood and liver. This assumption is supported by the similar patterns of the blood and liver curve on the one hand and the monophasic decay observed for the other tissues with a slope comparable with the second phase of the blood curve on the other hand.

The rapid biotransformation of lynestrenol was followed by a rapid excretion of the metabolites with the urine and bile. An interesting observation in this connection was the appearance of increasing amounts of radioactive material in the stomach lumen 20 min after injection. Excretion of drugs by the stomach wall has previously been described for steroids like progesterone and pregnenolone (Appelgren, 1967), but also for synthetic compounds like rifampicin (Boman, 1975), diazepam and chlordiazepoxide in mice (Van der Kleijn, 1969) and zolimidine in rats (Ostrowski et al., 1976). In view of the neutral character of lynestrenol gastric excretion of the unchanged drug to the extent observed is difficult to explain on account of simple diffusion, because the blood radioactivity was already very low at that time. Neither is an explanation offered by the present knowledge about the conversion products of the drug (see Chapter 12) for the same reason. An active excretion process could be involved. Apart from gastric excretion, however, radioactive material originating from the lachrymal glands and the nasal mucosa may contribute to the labelling of the stomach. Finally, reflux of a very slight amount of the strongly labelled duodenal contents to the stomach would probably be sufficient to get a similar phenomenon.

In contrast with the easy and rapid passage of the blood-brain barrier the

diaplacental transfer of lynestrenol appeared to occur to a comparatively slight extent. To explain this apparent discrepancy it is necessary to discriminate between the permeability of these membranes for a compound on the one hand and the actually *in vivo* observed rate of permeation on the other hand (Villee, 1965). When the placental transfer of lynestrenol like for most drugs occurs by means of simple diffusion, then its placental permeation rate is a function of the concentration gradient between the maternal and fetal blood, of the surface area of the membrane, and inversely proportional to the thickness of the membrane. Particularly for readily penetrating drugs, including probably lynestrenol on account of its physicochemical properties (Goldstein et al., 1974), the blood flow to the placenta may be a rate limiting factor in the delivery of the drug to the fetal circulation. In addition to the blood supply also the short time of half-life of lynestrenol in the maternal blood will reduce the lynestrenol fraction available for transplacental diffusion very rapidly and, consequently, will decrease the maximal and equilibrium drug levels attained otherwise in the fetal blood (Goldstein et al., 1974).

Previous autoradiographic studies in mice with progesterone and the synthetic progestins norethindrone and ethynodiol diacetate have also revealed a placental transfer to the same degree as observed for lynestrenol (Appelgren, 1967; Freudenthal et al., 1972). On the contrary, after the administration of norethynodrel, differing from norethindrone only by the position of the double bond at $C_{5\text{-}10}$ instead of $C_{4\text{-}5}$, the fetal tissues were strongly labelled (Freudenthal et al., 1973). Because this compound, which is known to be metabolised rapidly and extensively at least in the rat and human (Cook et al., 1972; Hanasono & Fisher, 1974), was given orally and in the fetal tissues almost no unchanged drug could be detected, it remains to be studied, whether norethynodrel itself did actually pass the placenta.

Pharmacodynamic aspects

With respect to the biological activity of lynestrenol its concentration-time course at the sites of steroid hormone synthesis and action deserves special attention.

The synthesis of steroid hormones in the female is localized in the ovaries and the adrenal cortex. A pronounced uptake and retention of lynestrenol in these tissues could be observed in accordance with the behaviour of other contraceptive steroids like mestranol (Appelgren & Karlsson, 1971), norethindrone and ethynodiol diacetate (Freudenthal et al., 1972) and norethynodrel (Freudenthal et al., 1973) as well as the endogenous steroid hormone testo-

sterone (Appelgren, 1969). For progesterone, however, an initially high uptake in the adrenal cortex was followed by a rapid decrease within 10 min after injection (Appelgren, 1967). Accumulation in the adrenal cortex has also been demonstrated for corticosteroids (Hanngren et al., 1964) and estrogens (Ullberg & Bengtsson, 1963), whereas the uptake of these compounds in the corpora lutea occurred only to a slight extent. It is tempting to assume a relationship between the observed accumulation of lynestrenol and the other contraceptive steroids in the adrenal cortex and the corpora lutea on the one hand and the endocrine activity of these tissues and the steroids on the other hand. It has to be realized, however, that for still unclarified reasons a surprisingly large number of other substances has also been demonstrated to accumulate at these same sites, e.g. diazepam and chlordiazepoxide (Van der Kleijn, 1969b), delta-9-tetrahydrocannabinol (Kennedy & Waddell, 1972), diphenylhydantoin (Waddell & Mirkin, 1972), rifampicin (Boman, 1975), tofenacine (Hespe & Prins, 1969), zolimidine (Ostrowski et al., 1976), DDT and PCB (Brandt, 1977), dieldrin (Bäckström et al., 1965), thiourea (Slanina et al., 1973) etc.

In agreement with the distribution pattern of progesterone in mice (Appelgren, 1967) the total radioactivity levels in the uterus and vagina remained low during the entire experiment in comparison with the concentrations in the non-target tissues. Nevertheless, the uterine fraction representing lynestrenol exceeded the corresponding blood concentrations of lynestrenol by a factor of about 10 (Fig. 14.7). Binding of lynestrenol to the progesterone (Kontula et al., 1975) and to the estradiol receptors (Van Kordelaar et al., 1975a,b; this thesis, Section III) in the uterus has been demonstrated. It seems probable that at least part of the lynestrenol present in the uterus is bound to these receptors.

The easy penetration of lynestrenol into the central nervous system and the strong labelling of the pituitary are consistent with the current concepts about the mechanism of ovulation inhibition of the contraceptive steroids (Labhsetwar, 1973). Also the very high uptake of lynestrenol in the pituitary and the relatively low uptake in the uterus compare favourably with the stronger reduction of estradiol uptake in the former than in the latter organ of the rat after intravenous injection of lynestrenol (Chapter 9, Fig. 9.2). An explanation of the different dose-effect curves in the rat on account of distribution factors seems therefore most likely.

Comparing the potency of the contraceptive progestins to interfere with estradiol binding to the estrogen receptor *in vivo* and *in vitro,* a comparatively low activity has been observed by us for lynestrenol *in vivo* (Van Kordelaar et al., 1975a,b; this thesis, Section III). Apart from a rapid biotransformation of lynestrenol to less potent derivatives a greater volume of distribution through accumulation and storage in the adipose tissue as a consequence of

its greater lipophilicity could contribute to the difference observed. The present results show, that in any case the accumulation and retention of unchanged lynestrenol in the brown fat is pronounced and may prolong its physiological availability by protecting it from exposure to biotransformation and excretion. In spite of the relatively slow uptake in the adipose tissue compared with the rapid distribution over the organs the levels in the brown fat are already remarkably high within 10 min after lynestrenol injection. Taking into consideration, that maximal levels of estradiol in the rat uterus were only reached 60 min after intravenous injection (Chapter 9, Table 9.2), a change in the concentration-time course of lynestrenol during this period as a result of storage in the body fat may, at least for this organ, substantially contribute to the difference between the *in vivo* and *in vitro* activity of lynestrenol.

SUMMARY

The distribution of labelled lynestrenol in female mice has been studied by means of whole-body autoradiography and impulse counting methods combined with tissue extraction-separation procedures. Within 2 min after intravenous injection the compound was distributed over the whole body including the central nervous system. High and very high levels of radioactivity were observed in the liver and kidneys, the pituitary, thyroid, adrenals, ovaries, central nervous system, lungs, myocard, salivary glands, Harder's gland, brown fat and skeletal muscles. A prolonged accumulation could be observed in the adipose tissue with a much steeper increase for the uptake into the brown fat than into the white fat. After the post-injection redistribution phase and the early absorption phase the patterns of distribution following oral and intravenous administration were essentially the same. Comparatively high levels of radioactivity were maintained throughout the experiment in the liver and kidneys, in the adrenal cortex, the adipose tissue, the follicle walls and corpora lutea of the ovary, the white matter of the brain and Harder's gland. Uterine and vaginal levels of radioactivity remained relatively low. Tissue extraction with hexane and thin-layer chromatography of some of these extracts showed, that lynestrenol was metabolised rapidly in the liver and disappeared from the blood and tissues with a time of half-life of about 1-2 hours. The fraction of total radioactivity representing lynestrenol remained high in the lipid-rich tissues cerebrum and adipose tissue, probably as a consequence of the lipophilic character of the drug. The rapid biotransformation was followed by a rapid excretion of the conversion products with the bile and the urine. After the intravenous administration to pregnant animals the uptake of radioactivity in the fetuses

occurred only to a slight extent in comparison with the maternal tissue concentrations. Also in the fetus the accumulation of radioactivity in the adrenal cortex was very pronounced, a slight but distinct labelling being furthermore apparent in the blood, liver, kidneys, lungs, brown fat and the lumen of the small intestines.

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APPENDIX

KEY TO ABBREVIATIONS USED IN THE AUTORADIOGRAPHIC PICTURES SHOWN IN FIG. 14.1, 14.4 AND 14.5

a	adrenal	o	ovary
b	blood	p	pituitary
bf	brown fat	pa	pancreas
bo	bone	pl	placenta
c	cerebrum	r	rectum
cm	cervical muscle	s	stomach
co	colon	sc	spinal cord
f	foetus	sg	salivary gland
f(prefix)	foetal	sm	skeletal muscle
fm	foetal membrane	sp	spleen
gb	gall bladder	t	thymus
hf	hair follicle	th	thyroid
hg	Harder's gland	to	tooth
i	intestine	ton	tongue
k	kidney	u	uterus
l	liver	ub	urinary bladder
lu	lung	ur	urethra
m	myocard	v	vagina
nm	nasal mucosa	vc	vena cava
		wf	white fat

METABOLIC CLEARANCE OF LYNESTRENOL IN THE ISOLATED PERFUSED RAT LIVER

INTRODUCTION

In previous investigations (this thesis, Chapters 12, 13) we studied the absorption and disposition of lynestrenol and its metabolites in the rat by sampling of urine, faeces and bile after various routes of administration. The distribution of lynestrenol was studied in mice with the technique of whole-body autoradiography combined with impulse counting of selected tissue samples (this thesis, Chapter 14). From the results it could be concluded among others, that lynestrenol is metabolised rapidly in the liver to a variety of more polar metabolites, which become predominantly excreted with the bile. Excretion of the parent compound itself, if any, appeared to be negligible in a quantitative sense. In view of the experiments of Kamyab et al. (1968) rapid biotransformation of lynestrenol is also likely to occur in humans: within 15 minutes after the intravenous injection of lynestrenol-^{14}C less than 50% of the blood radioactivity appeared to become extracted with chloroform.

Taking into consideration on the one hand, that distribution and biotransformation of lynestrenol will govern its time course at the receptor sites in the target tissues to a high extent and considering on the other hand, that plasma protein binding of a drug may have an important bearing on its distribution into the tissues as well as on its metabolism (Dayton et al., 1973; Jusko & Gretch, 1976; Wilkinson & Shand, 1975), the present investigation was undertaken to gain an insight into these specific aspects of lynestrenol kinetics. This chapter deals with the hepatic clearance of lynestrenol studied *in vitro* using the isolated perfused rat liver preparation. In recent years this technique has been shown to be a very useful tool in pharmacokinetic research (e.g. Alvarez, 1971; Branch et al., 1973; Breimer, 1974; Nagashima et al., 1968; Rowland, 1972a; Shand et al., 1973, 1975, 1976; Von Bahr et al., 1970; Yih, 1976). Good correspondence has been demonstrated between drug clearance values *in vitro* and *in vivo* for

compounds eliminated mainly by metabolism (Alvarez, 1971; Breimer, 1974; Rowland, 1972a; Von Bahr et al., 1970; Yih, 1976).

Using the isolated perfused rat liver it could be established, that the hepatic clearance of lynestrenol is dependent on the perfusate flow rate through the liver. Extraction of the compound from the perfusate during liver passage appeared to be complete in spite of its high degree of binding to serum albumin. Under physiological flow rate conditions there was no apparent concentration dependence of the hepatic clearance of the drug over a wide concentration range.

MATERIALS AND METHODS

Lynestrenol-^3H

The synthesis and the specifications of the tritiated lynestrenol used in the perfusion experiments are described in Chapters 3 and 4. Each time before an experiment the radioactive material was diluted with non-radioactive lynestrenol to the specific activity required. The radioactive dose injected was about 50 μCi. The injection solution was prepared by adding 1.0 ml ethanol containing polysorbate 80 (OPG, Utrecht, The Netherlands) to an aliquot of the lynestrenol-^{3}H solution. The solvent was then evaporated with a weak stream of air and the residue was solubilized in 0.5 ml saline. The final polysorbate concentration was 2%.

Perfusion apparatus

The apparatus (Fig. 15.1) used for the isolated perfused liver experiments was based on the design of Miller et al. (1951) and Fisher & Kerly (1964). With the exception of the pump the whole system is housed in a Plexiglass cabinet (50 x 50 x 100 cm) with a sash window at the front. The semi-synthetic perfusion medium (Schimassek, 1962) is brought up from the collecting vessel via a filter, obtained from a disposable blood transfusion set, to the top of the oxygenator by means of a roller pump and flows down as a film along the oxygenator wall. Circumferential troughs in the wall of the oxygenator ensure good filming of the perfusate without rivulet formation. The hydrostatic reservoir at the bottom of the oxygenator is provided with a bypass outlet and supplies via a bubble trap the liver with oxygenated medium. The flow rate of the perfusate through the liver could be varied by using a screw clamp between the

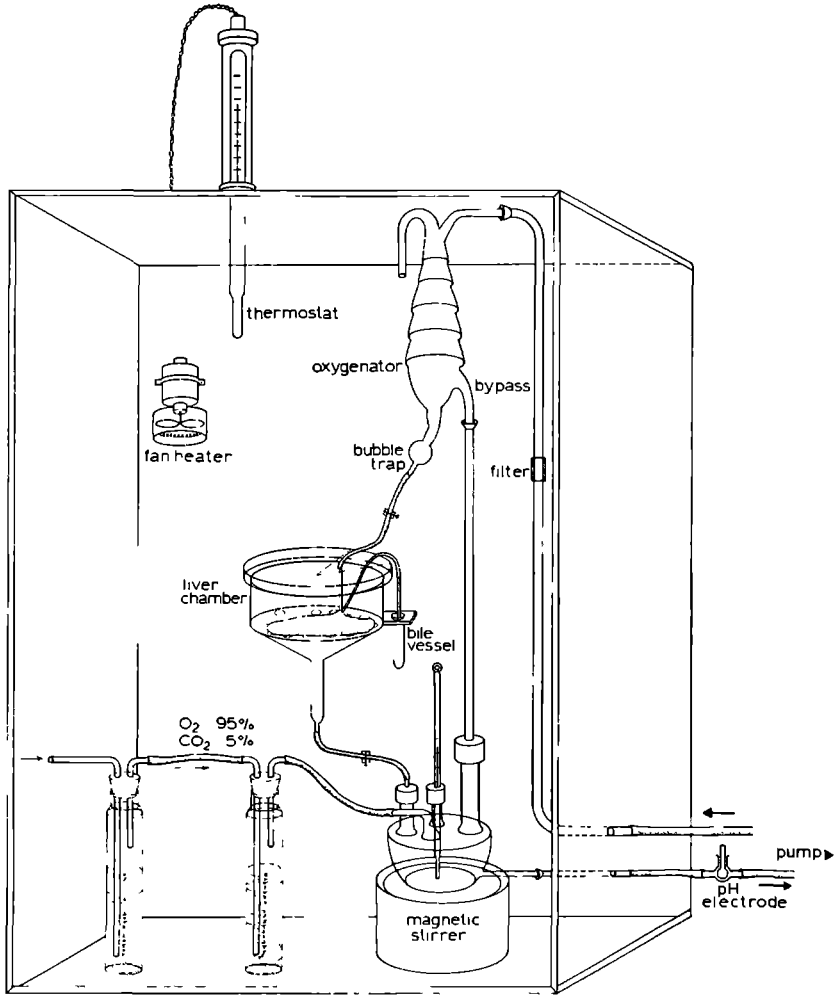

Fig. 15.1 — Schematic drawing of the apparatus used for the perfusion of the isolated rat liver.

portal cannula and the hydrostatic reservoir. When the clamp was removed a perfusion flow rate of about 60 ml/min was obtained with livers weighing about 8 gram and a perfusion pressure of 30 cm H_2O. In the organ chamber the liver is supported on a glass dish with perforations and the chamber is closed with a glass lid to protect the organ against drying. The liver flow rate was measured

at regular time intervals during each experiment by determining the time required for the filling of a calibrated tube into which the caval outlet flows. Caval and bypass perfusate return to the collecting vessel by gravity and are mixed with the contents of this vessel by a magnetic stirrer. Use of plastic tubing in the circuit was minimized and limited to the necessarily flexible connections between the oxygenator and the portal vein cannula, the liver chamber and the collecting vessel, and the tubing for the roller pump. The kind of tubing used had to be selected from a wide range of available materials because of the occurrence of a considerable loss of lynestrenol by adsorption to all but one (Flurane F 5000®, duMee, Sci. Instr., Soest, The Netherlands) of the materials tested. In control experiments without liver in the circulation system no loss of lynestrenol could be observed after the incorporation of this tubing. Several ball-and-socket joints were applied for glass-glass connections to facilitate cleaning and mounting of the components and to preserve some flexibility in the glass construction.

The perfusate was oxygenated with a counter-current stream of carbogen (95% oxygen/5% carbon dioxide). The gas is warmed and humidified by slow passage through two water traps, enters the collecting vessel, reaches the oxygenator via the bypass and escapes from a vent at the top. The oxygen pressure of the perfusate without liver in the system was 350-400 mm Hg. The pH of the circulating medium was monitored continuously by a glass electrode (Radiometer 22 pH meter, Copenhagen) and held at a value of 7.35-7.45 by the addition of 10% sodium bicarbonate solution. The temperature of the perfusate and cabinet was maintained at 38°C with the help of a thermostatically controlled fan heater. Cooling of the perfusate on its way through the pump outside the cabinet was reduced by heating this part of the circuit with a 100 W operation lamp. Samples of the perfusate were taken with a syringe from the medium leaving the collecting vessel.

The apparatus was prepared for use after cleaning and mounting by bringing into circulation 250 ml of a solution of 5% formaldehyde in 10% sodium bicarbonate. After 15 minutes this desinfection was succeeded by three 5 minutes washes with freshly distilled water and three washes with sterile saline, also during 5 minutes. The last portion saline was replaced by the perfusion medium.

Perfusion medium

In 60 ml Hank's Eagle solution, prepared by dissolving 1.51 g Hank's Bss dried (Difco Lab., Detroit, USA) in 150 ml distilled water and neutralized to

pH 7.40 by the addition of 10% sodium bicarbonate in distilled water (solution A), 400 mg glucose (Dextropur®, Corn Products Comp., Utrecht, The Netherlands) and 6.0 g bovine serum albumin (BSA, Povite, Amsterdam, The Netherlands) were dissolved (solution B). Packed red cells were obtained from freshly collected heparinized dog blood. After storage of the cells, under saline, and both solutions A and B overnight in a refrigerator, the next morning the medium was prepared about 1.5 hours before starting the perfusion. In a beaker 6.0 ml 10% sodium bicarbonate solution was mixed with 60 ml A and an equal volume B. If necessary, the pH was adjusted to pH 7.40 with sodium bicarbonate solution. Then 80 ml packed cells were added and 20 drops of Terramycin® (Pfizer Ltd., Sandwick, U.K.). The final volume of the perfusion medium was 220 ml because of the presence of some saline in the perfusion apparatus as a residue of the last saline wash. The haematocrite was measured with a micro-haematocrite centrifuge (Hawksley, Lancing, U.K.) and varied between 27.0-28.5%.

Surgical procedure

Female Wistar rats (Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands), 220-240 g body weight, served as liver donors. The animals were maintained on a standard laboratory diet (Hope Farms, Woerden, The Netherlands) and had free access to food and water. The animals were starved overnight before the experiment. The surgical procedure, that was followed, has been described in detail by Fisher & Kerly (1964). The animal was anaesthetized with diethylether, the common bile duct was cannulated with polyethylene tubing (Intramedic PE 10, Clay-Adams Co., Parsipanny, USA) and a Braunula® 2 R cannula (Braun AG, Melsungen, GFR) was used for the portal vein. After the insertion the portal cannula usually fills with blood, but when this did not happen, sufficient saline was added to prevent the admission and inclusion of air bubbles in the portal circulation. Similar precautions were taken in connecting the cannula to the perfusion circuit. Immediately after the transfer of the liver to the organ chamber the perfusion was started with the previously warmed and oxygenated perfusate. Subsequently the position of the liver and the portal cannula were adjusted, if necessary, to increase the perfusate flow to its maximal value of about 60 ml/min. The bile duct cannula was led into the down-side closed cylinder of a 1 ml syringe and the organ chamber closed. The entire operation was finished in 15-20 minutes and not more than 2 minutes elapsed between the cannulation of the portal vein and the start of the perfusion.

Performance of the liver

Assessment of the liver function was based on the macroscopic appearance of the liver, the perfusate flow rate through the organ, the oxygen consumption and the bile production. The liver should have a uniform red-brown colour without ischaemic spots during the entire experiment. About 2 min after starting the perfusion the unrestricted flow rate, with a value of at least 50 ml/min at a perfusion pressure of 30 cm water, was adjusted to the value desired (40-20-10 ml/min) and should require only minor readjustment during the experiment. The pO_2 deficit of the caval perfusate was measured (Radiometer 27/ Gasmonitor PHA 927, Radiometer, Copenhagen, Denmark) 20 min after the adjustment of the flow and at the end of the experiment 0.5 to 2 h later. Both values were almost equal and appeared to be at least 250 mm Hg at a liver flow rate of 10 ml/min. The bile flow started immediately at a rate of about 0.4 ml/hour resulting in a total production of 0.6 to 0.9 ml after 2 hours.

Injection and sampling procedure

The very rapid clearance of lynestrenol from the perfusion medium observed in preliminary experiments would result in a disproportionate loss of drug during its first-pass through the liver especially at the higher flow rates, if the distribution of the drug over the medium was not complete before the first liver passage. For this reason the perfusate flow to the oxygenator was temporarily reversed into the opposite direction just before the drug was injected into the collecting vessel, thus allowing a rapid distribution of the drug over the entire medium with the exception of the volume present in the hydrostatic reservoir and in the liver (5-10% of the total volume). After a decrease of the volume in the hydrostatic reservoir to about 5 ml, the perfusate flow to the oxygenator was restored. Time was started at the moment the lynestrenol containing medium reached the hydrostatic reservoir.

Samples of the perfusion medium were withdrawn from the medium just leaving the collecting vessel by use of a disposable syringe. Sampling was continued over a period of 4-5 times the expected time of half-life, according to the schedule $2^1/_2$, 5, $7^1/_2$, 10, $12^1/_2$, 15, $17^1/_2$, 20, $22^1/_2$ min post-injection in case of a liver perfusate flow rate of more than 30 ml/min; 5, 10, 15, 20, 25, 30, 40, 50 min post-injection (flow rate 20 ml/min) and 5, 10, 20, 30, 40, 50, 60, 80, 100 min post-injection (flow rate 10 ml/min). Total radioactivity and the lynestrenol concentration of the perfusion medium as well as of the perfu-

sate plasma were determined. The total amount of perfusate withdrawn during an experiment, excluding the last sample, did not exceed a volume of 10 ml, less than 5% of the circulating volume.

Assay of lynestrenol

Immediately after collection the samples were cooled in ice and processed during the same day. A volume of 0.1 ml was diluted with an equal volume of water. After lysis of the erythrocytes the albumin was precipitated by the addition of 0.8 ml ethanol, containing 100 μg lynestrenol/ml as a carrier and marker for chromatography. The solution obtained was extracted consecutively with 2, 1 and 1 ml n-hexane. The combined extracts were evaporated in a counting vial with a weak stream of air and radioactivity was measured by liquid scintillation counting.

The lynestrenol content of the hexane extracts was authenticated and corrected accordingly by means of thin-layer chromatography of a duplicate series of extracts. The sample volumes used for the extraction were increased to 0.2 ml and in case of the last two samples to 0.4 and 1.0 ml respectively. The volumes of the other solvents were correspondingly adapted. The chromatographic procedure and the measurement of the distribution of radioactivity over the developed plate is outlined in Chapter 7. The fraction of the total plate radioactivity measured in the lynestrenol spot varied for the first sample between 97.9 and 99.2% for the perfusate and 96.8 and 98.9% for the perfusate plasma. For the last sample the corresponding values were 89.2 and 97.8% for the perfusate and 86.5 and 92.2% for the perfusate plasma.

For each experiment the efficiency of the extraction was determined by extracting 4 blank perfusate samples to which a known amount of lynestrenol-^{3}H had been added. The efficiencies thus measured appeared to vary between 88 and 94% for the perfusate (mean relative error 2.4%) and between 92 and 95% for the perfusate plasma (mean relative error 2.1%).

Assay of radioactivity

Radioactivity was measured by liquid scintillation counting as described in Chapter 5. Except for some silicagel samples, the standard error of counting of the samples did not exceed 1.0%.

Viability of the isolated liver preparation

The viability of the liver was assessed with the criteria mentioned in the experimental part. The perfusate flow rate through the liver, the bile flow and the oxygen uptake by the organ are listed in Table 15.1a-d. The perfusate flow rate required only minor readjustment during the experiments. The smallest flow rate used was in the physiological range of 1.2-1.5 ml/min/g liver observed in the intact rat (Dobson & Jones, 1952; Grayson & Mendel, 1965). In view of the inflow pO_2 of at least 300 mm Hg at this flow rate and the haematocrite value of about 28% the oxygen supply to the liver can be considered to meet the requirements for maintenance of the vital processes as well as for metabolism (Cumming & Mannering, 1970). The rates of bile production observed in this study compare well with those described by other investigators (Fisher & Kerly, 1964; Hems et al., 1966; Nagashima et al., 1968; Yih, 1976), the rates being also in the range obtained with intact rats (Byers & Friedman, 1952; Fisher & Kerly, 1964; this thesis, Chapter 12). The continuing viability of the liver preparations during the experimental period is evident also from the drug elimination data described in the subsequent paragraph.

Pharmacokinetics of lynestrenol elimination

The elimination of lynestrenol by the isolated perfused rat liver under flow rate conditions approaching the physiological value of about 1.2-1.5 ml blood/min/g liver (Dobson & Jones, 1952; Grayson & Mendel, 1965) is shown in Fig. 15.2. The concentration-time curves of lynestrenol in the perfusate and in the perfusate plasma display the same pattern, but the concentration of lynestrenol in the erythrocytes is obviously much higher than the plasma concentration. Both curves can be described by a mono-exponential equation:

$$C_p = A.e^{-t/\tau_{el}}$$

where C_p is the concentration of lynestrenol in the perfusate respectively perfusate plasma at time t, A is this concentration at zero-time and τ_{el} is the elimination time constant. The elimination of lynestrenol can therefore be considered as a clearance from a single compartment with a volume V, where V = dose/A. From the experimental curve the time of half-life, $t^1/_2$, and A can be easily deduced and from these parameters the elimination time constant

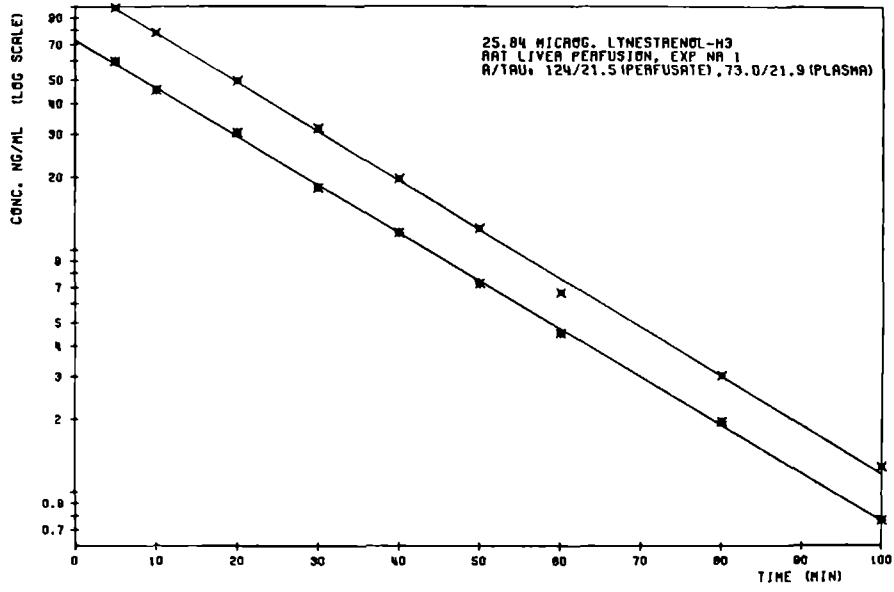

Fig. 15.2 — Time course of the lynestrenol concentration on semi-logarithmic scale in the perfusate (—X—X—) and in the perfusate plasma (—✱—✱—) showing the elimination of the compound by the isolated perfused rat liver. The curve is the best fit of the experimental data according to a one compartment open model. The figure is a reproduction of a direct computer plot. See text for explanation of symbols.

and the clearance constant, k_{Cel}, can be calculated with the help of the following formulas (Van Rossum, 1971):

$$t^1/_2 = 0.69\ \tau_{el} \qquad k_{Cel} = V/\tau_{el}$$

The best fit of the experimental data and the corresponding parameters have been obtained with the help of the non-linear least squares curve fitting program FARMFIT, previously outlined by Breimer (1974). A survey of the pharmacokinetic parameters of the experiment shown in Fig. 15.2 and other similar experiments is given in Table 15.1a and Table 15.2. The clearance of lynestrenol from the perfusion medium appeared to be equal to the perfusate flow rate through the liver, resulting in a time of half-life of about 14 minutes. In these experiments as well as in those described below less than 0.3% of the biliary excretion products, representing up to 80% of the dose administered, could be extracted with n-hexane, so the elimination of lynestrenol seemed to proceed exclusively after biotransformation.

Table 15.1 — Experimental conditions and pharmacokinetic parameters of lynestrenol in the isolated perfused rat liver[1])

a. Perfusion rate about 10 ml/min, dose 20-25 μg lynestrenol-^{3}H

Rat number	1	2	3
Liver weight (g)	6.6	9.1	8.6
Bile flow (mg/min)			
before injection[2])	5.2	8.8	2.4
after injection[3])	5.0	8.1	n.d.[8])
pO_2 deficit (mm Hg)[4])			
before injection[5])	266	285	305
after sampling[6])	247	289	308
Haematocrite (%)	28.0	30.0	27.5
Perfusion rate (ml/min)	10.1	10.7	11.0
Dose (μg)	25.84	23.04	22.22
A (ng/ml)	123.8 (3.7%)	110.5 (3.0%)	101.3 (4.9%)
τ_{el} (min)	21.5 (1.5%)	19.7 (1.1%)	19.3 (1.8%)
V (ml)	209	208	219
k_{Cel} (ml/min)	9.7	10.6	11.3
k_{Cel}/flow	0.96	0.99	1.03
Cum. excr. (% dose)[7])	71.5/100	79.4/100	19.2/80-85[9])

[1]) The parameters A and τ_{el} as well as their standard error (in parentheses) are calculated from the best fit of the lynestrenol concentrations measured in the perfusate with the help of the curve fitting program FARMFIT (Breimer, 1974).

[2]) Mean value measured over 30 to 60 minutes prior to injection of lynestrenol.

[3]) Mean value measured over the entire sampling period.

[4]) The difference of the oxygen pressure of the perfusate before and beyond the liver.

[5]) The value measured after the equilibration of the liver under the experimental conditions, but prior to injection of lynestrenol.

[6]) The value measured after taking the last sample.

[7]) The cumulative biliary excretion (first figure) over the indicated time interval in minutes (second figure).

[8]) n.d.: "not determined".

[9]) Bile flow stopped between 80 and 85 minutes post-injection.

Table 15.1b — Perfusion rate about 10 ml/min, dose about 1 mg lynestrenol-^{3}H

Rat number	4	5	6
Liver weight (g)	6.7	8.3	7.3
Bile flow (mg/min)			
before injection[2])	7.8	8.2	7.6
after injection[3])	7.7	7.9	4.0
pO_2 deficit (mm Hg)[4])			
before injection[5])	287	250	255
after sampling[6])	280	260	259
Haematocrite (%)	28.5	27.5	30.0
Perfusion rate (ml/min)	11.0	10.9	10.5
Dose (μg)	983	947	950
A (ng/ml)	4713 (4.0%)	4542 (4.1%)	4314 (4.0%)
τ_{el} (min)	19.8 (1.5%)	19.6 (1.5%)	22.3 (1.7%)
V (ml)	209	209	219
k_{Cel} (ml/min)	10.5	10.6	9.8
k_{Cel}/flow	0.96	0.97	0.93
Cum. excr. (% dose)[7])	63.5/104	64.6/105	15.9/104

[2-7]) see Table 15.1a.

A 40-fold increase of the lynestrenol dose to about 1 mg did not affect the clearance (Table 15.1b). Doubling of the perfusate flow rate through the liver was accompanied by a corresponding increase of the clearance (Table 15.1c). A still further increase, however, of the perfusate flow rate to 3 and 4 times the physiological value was followed by an increase of the clearance in an absolute sense, but a decrease of the extraction ratio k_{Cel}/flow rate (Table 15.1d).

A representative example of the time course in the perfusate and in the perfusate plasma of lynestrenol, of the total radioactivity and of the metabolites together with the cumulative excretion of radioactivity in the bile is shown in Fig. 15.3a. The distribution of lynestrenol and its metabolites between the perfusate, respectively the red cells, and the perfusate plasma displayed opposite patterns. A high uptake of lynestrenol in the red cells was accompanied by a very low uptake of its metabolites and correspondingly high plasma concen-

Table 15.1c — Perfusion rate about 20 ml/min, dose 20-25 μg lynestrenol-^{3}H

Rat number	7	8	9
Liver weight (g)	9.4	8.9	9.4
Bile flow (mg/min)			
before injection[2])	9.4	7.4	8.1
after injection[3])	n.d.[8])	n.d.[8])	n.d.[8])
pO_2 deficit (mm Hg)[4])			
before injection[5])	207	205	215
after sampling[6])	n.d.[8])	210	225
Haematocrite (%)	27.0	27.5	27.5
Perfusion rate (ml/min)	22.2	21.2	22.1
Dose (μg)	23.69	21.91	22.66
A (ng/ml)	106 (7.6%)	104 (5.2%)	99.7 (4.9%)
τ_{el} (min)	9.9 (2.5%)	10.4 (1.9%)	10.4 (1.8%)
V (ml)	224	211	228
k_{Cel} (ml/min)	22.5	20.3	21.9
k_{Cel}/flow	1.01	0.96	0.99
Cum. excr. (% dose)[7])	57.2/56	45.6/54	48.1/55

[2-8]) see Table 15.1a.

trations. In Fig. 15.3b similar curves are shown for experiment 3, where a low and untimely stop of the bile production during the experiment resulted in a poor excretion of the metabolites. As a consequence the accumulation of the conversion products in the plasma is still proceeding at the end of the experiment. The slope, however, of the lynestrenol decay curves was apparently not affected.

For a number of experiments, where in addition to the lynestrenol levels in the perfusate the plasma concentrations were measured, the pharmacokinetic parameters of the plasma curves were also calculated (Table 15.2). In spite of the high uptake of lynestrenol in the erythrocytes (Table 15.2, last column) resulting in only about 45% of the lynestrenol being present in the plasma fraction of the perfusate, the clearance of the compound from the perfusate appeared to be limited by the flow rate of the perfusate through the liver

Table 15.1d — Perfusion rate > 30 ml/min, dose about 25 μg lynestrenol-^{3}H

Rat number	10	11	12
Liver weight (g)	8.2	8.1	8.0
Bile flow (mg/min)			
before injection[2])	7.2	6.3	7.7
after injection[3])	6.8	5.9	7.2
pO_2 deficit (mm Hg)[4])			
before injection[5])	145	n.d.[8])	110
after sampling[6])	160	n.d.[8])	113
Haematocrite (%)	27.5	n.d.[8])	28.5
Perfusion rate (ml/min)	33.2	31.3	44.3
Dose (μg)	24.19	27.41	18.33
A (ng/ml)	118 (3.8%)	123 (4.1%)	84.7 (5.0%)
τ_{el} (min)	8.7 (2.4%)	10.3 (3.0%)	7.9 (4.0%)
V (ml)	205	223	216
k_{Cel} (ml/min)	23.6	21.7	27.3
k_{Cel}/flow	0.71	0.69	0.62
Cum. excr. (% dose)[7])	53.9/40	17.5/41	n.d.[8])

[2-8]) see Table 15.1a.

(Table 15.1). This implies a very rapid exchange of lynestrenol between the red cells and the plasma. In this light it is not surprising, that the elimination time constants derived from the plasma concentration measurements are equal to the elimination time constants derived from the lynestrenol time course in the perfusate. Another consequence of the high partition-coefficient and rapid movement of lynestrenol from the cells to the plasma is, that a plasma clearance constant is calculated from the plasma concentrations, which exceeds by far the flow rate of the perfusate plasma through the liver (Rowland, 1972b).

DISCUSSION

From the present investigations lynestrenol appears as a most interesting and rather unique drug. In spite of an albumin bound fraction of more than 97%

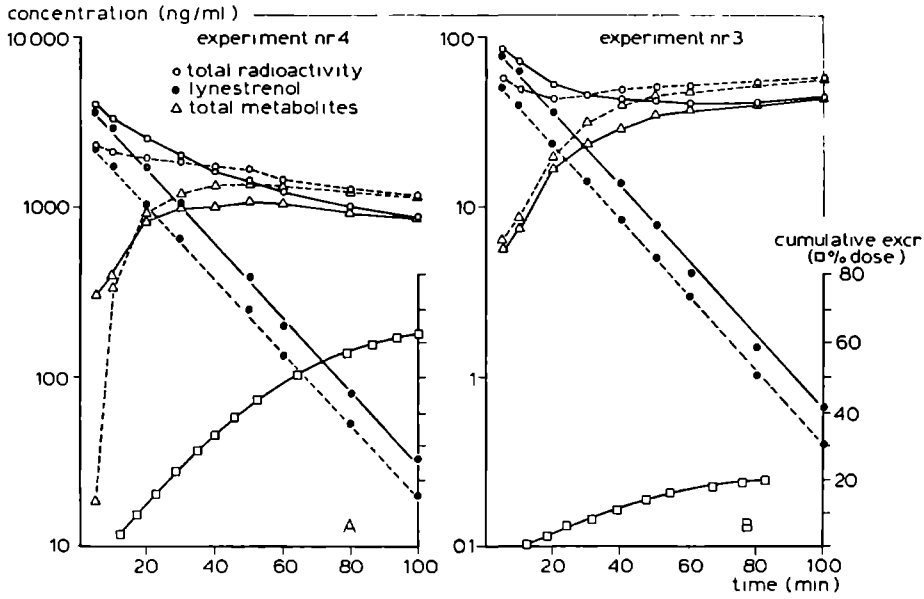

Fig. 15.3 — Time course of the concentrations of lynestrenol, total radioactivity and metabolites on a semi-logarithmic scale in the perfusate (closed lines) and the perfusate plasma (broken lines) during perfusion of the isolated rat liver. All concentrations, measured as dpm/ml, were converted into ng/ml using the molecular weight and specific activity of lynestrenol-^{3}H. The concentrations of the metabolites were derived from the experimental data of total radioactivity and lynestrenol. On a linear scale the cumulative excretion of radioactivity in the bile is also shown.

(see Chapter 8), the total plasma concentration of the drug is less than one third of the concentration in the erythrocytes, resulting in only 45% of the total amount of lynestrenol being present in the plasma. At the same time the transfer rate of the compound from the red cells to the plasma is so rapid, that the decay curves of lynestrenol in the perfusate and in the perfusate plasma remain parallel under conditions of the highest clearance being theoretically possible. Conversely, the observation of the highest possible clearance under physiological flow conditions as observed in the present study implies a complete extraction of lynestrenol from the plasma as well as from the red cells during liver passage and consequently an elimination process, limited only by the perfusate flow rate through the liver and independent of the extent of protein binding. Because of the latter property the elimination of lynestrenol is of the non-restrictive type (Shand et al., 1976; Wilkinson & Shand, 1975).

Table 15.2 — Pharmacokinetic parameters of lynestrenol in the isolated perfused rat liver as derived from concentrations in the perfusate plasma[1])

Rat no.[2])	Perfusion rate (ml/min)	Haematocrite (%)	A (ng/ml)	τ_{el} (min)	V (ml)	k_{Cel} (ml/min)	Ratio ery/plasma[3])
1	10.1	28.0	73.0 (1.8%)	21.9 (0.7%)	353	16.1	3.50
3	11.0	27.5	64.4 (1.4%)	19.6 (0.5%)	345	17.6	3.07
4	11.0	28.5	2830 (2.3%)	20.1 (0.9%)	347	17.3	3.33
7	22.2	27.0	69.0 (6.5%)	10.2 (2.2%)	343	33.5	2.99
8	21.2	27.5	63.9 (2.2%)	10.5 (0.8%)	343	32.7	3.29
10	33.2	27.5	68.7 (2.4%)	8.8 (1.6%)	352	40.1	3.61

[1]) The parameters A en τ_{el} and their standard errors (in parentheses) are calculated from the best fit of the lynestrenol concentrations measured in the perfusate plasma with the help of the curve fitting program FARMFIT (Breimer, 1974).

[2]) Corresponding numbers in this and the preceding table refer to the same experiment.

[3]) The ratio ery/plasma is the ratio of the respective lynestrenol concentrations at zero time. The lynestrenol concentration in the erythrocytes has been calculated from the concentrations in the perfusate and the perfusate plasma with the help of the haematocrite value.

Under flow rate conditions exceeding physiological values it was observed (Table 15.1d) that the clearance of lynestrenol is determined by some other factor than the perfusate flow rate through the liver. The hepatic clearance is a function of liver blood flow on the one hand and the ability of the organ to extract the drug as it perfuses through the hepatic capillaries on the other hand (Nagashima et al., 1968). It is conceivable that under conditions of an increased liver flow rate this latter parameter, which is a function of the activity of the drug metabolising enzyme system in the liver cells, may become rate-limiting in the elimination of lynestrenol. Another explanation may be provided by the assumption, that at high perfusate flow rates the conditions for the exchange of drug between the perfusate and the liver become less favourable. Drug transfer from the perfusate to the liver may then become rate-limiting or by-pass of the perfusate through some larger liver vessels could occur. Then the perfusate flow rate measured changes into an apparent flow rate greater than the one, which actually determines the exchange process in the liver.

The rapid biotransformation of lynestrenol, the rapid excretion of the conversion products and the absence of the parent compound in the bile are in every respect in good agreement with the results obtained in the rat *in vivo* (Chapter 12). As already could be derived from the renal and hepatic excretion patterns of radioactivity in the rat with and without bile duct ligation, the biotransformation rate of lynestrenol appeared not to be disturbed in case of insufficiency of the hepatic excretory mechanisms. Instead of elimination with the bile the conversion products are then released into the perfusate (Fig. 15.3b).

The low biological availability of lynestrenol after oral administration *in vivo,* which is to be expected on account of the rapid metabolic clearance observed in the present experiments, has been validated in the rat *in vivo* (this thesis, Chapter 16). No correlation, however, could be demonstrated between the metabolic clearance rate *in vitro* and the clearance of the compound *in vivo.* This lack of correspondence is discussed in Chapter 16.

SUMMARY

The hepatic clearance of lynestrenol has been studied with the isolated perfused rat liver preparation. Lynestrenol disappearance followed a mono-exponential decline. In spite of a 3-fold difference between the erythrocyte and plasma concentration of lynestrenol the elimination time constants for the perfusate and the perfusate plasma were similar. At perfusate liver flow rates between 1.2 and 2.4 ml/min/g liver the extraction of the drug from the perfusate during liver passage was complete. In view of the high plasma protein binding

of 97.4% the elimination of lynestrenol is of the non-restrictive type. There was no apparent concentration dependence of the clearance over a wide concentration range. At perfusate flow rates of 4-5 ml/min/g liver the extraction ratio in the liver decreased to about 0.7. Over a period of about 100 minutes after injection 65-80% of the dose was excreted in the bile. Less than 0.3% of the excretion products could be extracted with n-hexane. On account of the present findings a considerable pre-systemic elimination of lynestrenol after oral administration can be expected.

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PHARMACOKINETICS AND BIOLOGICAL AVAILABILITY OF LYNESTRENOL IN THE RAT

INTRODUCTION

With respect to the pharmacokinetics of oral contraceptive steroids most attention has been given in the past to the study of their biotransformation in the liver and the nature of the excretion products isolated from the urine and sometimes from the bile. The study of blood concentrations has been hampered by the lack of analytical methods of sufficient sensitivity and specificity. Radioactive methods cannot be considered for pharmacokinetic investigations in humans unless in a few selected cases. The progress being made in recent years in the development of radio-immunological methods (Abraham, 1974; James & Jeffcoate, 1974) and computerized gas chromatography-mass spectrometry (Carrington & Frigerio, 1977) has already been shown to provide the analytical tools allowing the measurement of blood concentrations of drugs administered in milligram and sub-milligram amounts per day, including the oral contraceptive steroids (Adlercreutz et al., 1974; Braselton et al., 1977; De la Pena et al., 1975; Hiroi et al., 1975; Nieuweboer & Lübke, 1977; Nygren et al., 1974; Saxena et al., 1977; Stanczyk et al., 1975; Victor et al., 1977; Warren & Fotherby, 1974).

Blood concentrations after the administration of lynestrenol have not been reported thus far except by Kamyab et al. (1968) and Van der Molen et al. (1969). In the latter report only total radioactivity levels were measured after the administration of lynestrenol-4-^{14}C and no discrimination was made between the rapid decline of chloroform-extractable radioactivity and the much slower decrease of total radioactivity as apparent from the study of Kamyab et al. (1968). Apart from the lack of specificity of the assay employed in the latter investigation the very limited number of data presented by these authors does not allow a meaningful pharmacokinetic analysis to be made.

The rapid hepatic clearance of lynestrenol as observed by us in the isolated perfused rat liver preparation (this thesis, Chapter 15; Van Kordelaar et al.,

1978) and the extensive and rapid biotransformation of the steroid also in the rat *in vivo* (this thesis, Chapters 12, 13) made an extension of the investigations to its blood level profile in the rat interesting and desirable. In the present chapter we provide information about the pharmacokinetics and biological availability of lynestrenol in the rat following intra-arterial and intraduodenal administration.

MATERIALS AND METHODS

Animals

In all experiments female Wistar rats, age 3-5 months, were used (Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands). The animals were maintained on a standard laboratory diet (Hope Farms, Woerden, The Netherlands) and tap water. During the experiments the animals were housed in a restraining cage after Bollman (1948) with small modifications (Kilian, 1973). Some weeks before the experiment the animals were placed periodically in these cages in order to acclimatize and to reduce stress in the experimental situation. The rats were starved overnight before the administration of the test compound, afterwards they had free access to food and water or, in the case of bile duct cannulation, to saline instead of water to replace the salts lost with the bile. For the biliary fistula a polyethylene cannula (Intramedic PE 10, Clay-Adams Co., Parsipanny, USA) was inserted in the upper part of the common bile duct the day before the experiment. Surgery was performed under ether anaesthesia according to the procedure described by Lambert (1965). The collection of bile allowed the determination of the fraction of the dose absorbed after intraduodenal administration of lynestrenol and the measurement of the cumulative excretion of radioactivity in the bile.

Blood sampling

In each experiment 13 blood samples were taken over a period of 23-28 h after drug administration. Samples of 0.15-1 ml volume were collected using a heparinized syringe (Sherwood Medical Industries Ltd., Crawley, U.K.) fitted with a hypodermic needle. Sampling was accomplished via a polyethylene cannula (Intramedic PE 10, Clay-Adams Co., Parsipanny, USA) introduced in the aorta through the carotid artery the day before the experiment. This method of sampling combines a number of features especially of advantage in pharmaco-

kinetic investigations using radiolabelled compounds. The technique is simple and rapid, clean and does not disturb the animal. Moreover, by using a syringe of 1 ml the volume of the sample can be accurately restricted to the quantity desired so that the small blood volume available for sampling (see Chapter 7) can be actually used with the greatest economy.

Dosage

Specifications of the administered drug, lynestrenol-^{3}H, have been given in Chapter 3. For intra-arterial administration of lynestrenol ethanol, containing polysorbate 80 (OPG, Utrecht, The Netherlands), was added to the lynestrenol-^{3}H solution. The solvent was evaporated with a weak stream of air and the residue solubilized in saline. The final polysorbate concentration was 2%. A volume of about 0.3 ml was injected via the carotid artery cannula.

The dosage form for intraduodenal administration was prepared similarly, but here saline as the solvent was replaced by 1.0 ml bile collected from the same rat after surgery had been performed.

The rats received a dose of about 50 μg lynestrenol-^{3}H, corresponding to 125 μCi.

Sample analysis and presentation of results

A detailed description of the analytical procedure applied has been given in Chapter 7. As pointed out, a complete analysis of each sample, including thin-layer chromatography of the hexane extracts, was not feasible for several reasons. The results, however, obtained with chromatography of the blood extracts in the course of the investigations revealed that the fraction of these extracts representing lynestrenol varied always between 85 and 95%. We thought it, therefore, justified to extrapolate these results to the extracts not subjected to chromatography and to calculate the lynestrenol content of these extracts by multiplication by 0.9. Although for this reason the present results may differ from the pharmacokinetic data of lynestrenol, deviations of more than 5% in the calculated concentrations and in the fitted parameters are not likely.

No corrections have been applied to the values of hexane-extractable radioactivity measured following intraduodenal administration of lynestrenol, because in these experiments chromatography was performed with only one sample and the lynestrenol content of the blood extracts seemed to be less uniform. The

results of these experiments have been presented, therefore, as concentrations of hexane-extractable radioactivity instead of lynestrenol concentrations.

Assay of radioactivity

The measurement of radioactivity by means of liquid scintillation counting and the preparation of the samples has been described in Chapter 5. For the measurement of the blood levels of total radioactivity 10-20 mg blood was used.

RESULTS

Intra-arterial administration

The blood or plasma concentration of lynestrenol was measured over a period of about 24 hours after injection. After an initial rapid fall of the concentration primarily as a result of the distribution of the drug into the tissues a more gradual decline of the blood and plasma levels was observed. About 4 hours after lynestrenol administration a third phase became apparent in the semi-logarithmic concentration-time curve (Fig. 16.1). The entire curve can be described mathematically as a sum of three exponentials according to the three-compartment open model:

$$C_1 = A_1 . e^{-t/\tau_1} + A_2 . e^{-t/\tau_2} + A_3 . e^{-t/\tau_3}$$

where C_1 is the lynestrenol concentration (ng/ml) at time t, τ_1, τ_2 and τ_3 are the time constants (min) and A_1, A_2 and A_3 are the coefficients (ng/ml). From the linear part of the semi-logarithmic concentration-time curve τ_3 and A_3 can be deduced, representing the slope and the intercept of the extrapolated curve at zero-time respectively. Similarly A_1 and A_2 and τ_1 and τ_2 may be obtained from the intercepts and slopes of the lines found by stripping of the original curve using the subtraction method or by a non-linear regression analysis of a three-term exponential equation.

The pharmacokinetic parameters of the parenteral experiments are summarized in Table 16.1 and have been derived from the best fit of the experimental curves according to the equation shown above. Curve-fitting and calculations have been accomplished with the help of the computer program FARMFIT in use in our department (see Chapter 7). The volume of the central compartment

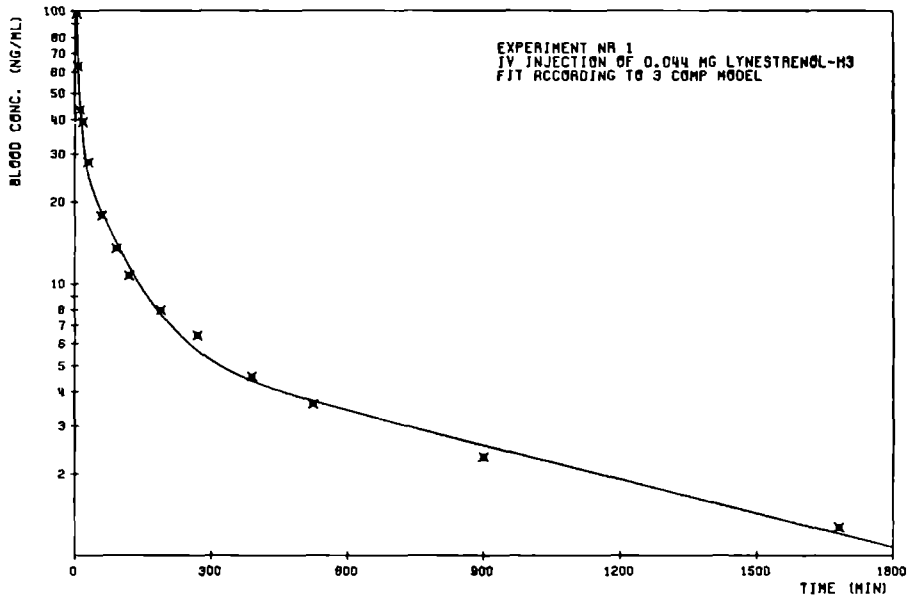

Fig. 16.1 — Concentration-time course of lynestrenol in rat blood after intra-arterial injection of the compound. The curve is the best fit of the experimental data according to a three-compartment open model. The pharmacokinetic parameters are given in Table 16.1. The figure is a reproduction of a direct computer plot.

(V_1), the area under the curve (AUC) and the apparent volume of distribution (V_d) have been calculated by means of the following equations:

$$V_1 = \frac{\text{dose}}{A_1 + A_2 + A_3} \qquad AUC = A_1 \cdot \tau_1 + A_2 \cdot \tau_2 + A_3 \cdot \tau_3$$

$$V_d = \frac{\text{dose}\,(A_1 \cdot \tau_1^2 + A_2 \cdot \tau_2^2 + A_3 \cdot \tau_3^2)}{(A_1 \cdot \tau_1 + A_2 \cdot \tau_2 + A_3 \cdot \tau_3)^2}$$

The biological half-life ($t^1/_2$) has been calculated from the largest time constant ($t^1/_2 = 0.69\,\tau_3$) and the elimination clearance constant (k_{Cel}) by dividing the dose (D) by the area under the curve ($k_{Cel} = D/AUC$).

Table 16.1 — Fitted and calculated pharmacokinetic parameters for lynestrenol after intra-arterial administration of the compound to 6 rats[1])

EXPERIMENT	1		2		3		4		5		6[2])	
Body weight (g)	190		185		241		239		227		175	
Dose (μg)	44.1		47.4		48.3		45.4		51.1		52.0	
Dose/100 g (μg)	23.2		25.6		20.0		19.0		22.5		29.7	
Cum. excr. (%)[3])	68.8		67.3		—		—		71.2		78.3	
τ_1 (min)	6.9	(23.3%)	5.6	(29.5%)	7.6	(18.5%)	2.8	(35.7%)	5.9	(15.8%)	18.2	(11.2%)
τ_2 (min)	84	(17.7%)	94	(15.2%)	91	(18.5%)	52	(19.2%)	61	(8.2%)	136	(7.9%)
τ_3 (min)	1040	(11.4%)	910	(10.7%)	1840	(25.7%)	1218	(16.0%)	1076	(7.2%)	995	(7.3%)
$t^1/_2$ (min)	723		631		1274		844		746		689	
A_1 (ng/ml)	101	(20.6%)	153	(34.9%)	212	(20.5%)	375	(74.6%)	230	(17.9%)	126	(5.8%)
A_2 (ng/ml)	25.8	(16.7%)	41.5	(11.7%)	28.1	(14.9%)	29.3	(18.5%)	46.6	(9.9%)	62.9	(7.0%)
A_3 (ng/ml)	6.03	(11.5%)	7.55	(14.7%)	7.00	(13.1%)	6.78	(10.3%)	7.07	(5.7%)	12.8	(9.9%)
V_1 (ml)	333		234		196		111		180		258	
V_f (l)	3.56		2.32		3.98		3.92		3.06		1.30	
k_{Cel} (ml/min)	4.8		4.1		2.8		4.2		4.3		2.2	
AUC (ng.min/ml)	9143		11,640		17,030		10,830		11,820		23,590	
AUC/μg/100 g	394		455		852		570		525		794	

[1]) The parameters were derived from the best fit of the blood (experiment 1-5) or plasma (experiment 6) concentration-time curve of lynestrenol according to a three-compartment open model. The figures in parentheses denote the relative errors (%) of the fitted parameters. For pharmacokinetic symbols and calculations see text.

[2]) The parameters of experiment 6 have been derived from plasma concentrations instead of blood concentrations and cannot be considered to be equivalent to the parameters derived from experiments 1-5 (Gibaldi & Perrier, 1974; Rowland, 1972).

[3]) Cumulative biliary excretion of radioactivity in rats with bile fistula (experiments 1, 2, 5 and 6).

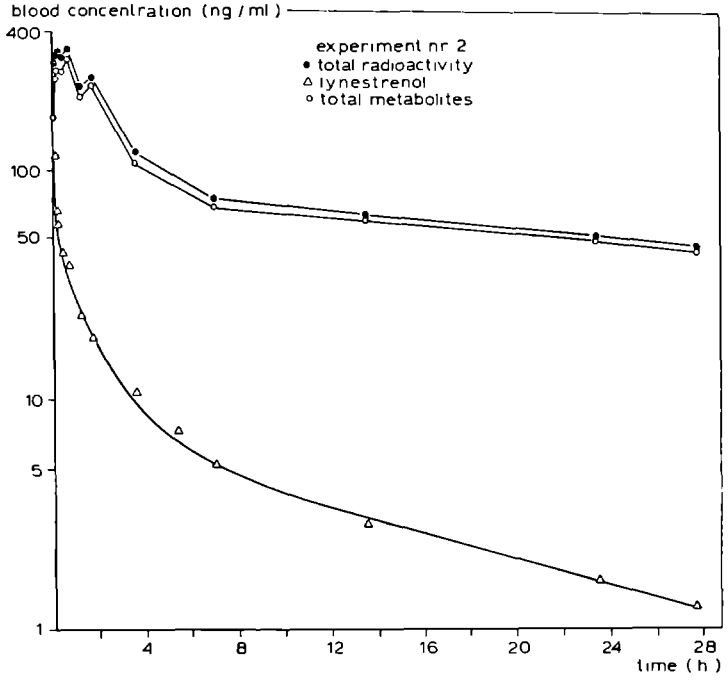

Fig. 16.2 — Time course of the blood concentration of total radioactivity, of lynestrenol and of the metabolites after intra-arterial injection of lynestrenol-^{3}H. All concentrations, measured as dpm/ml, were converted into ng/ml using the molecular weight and specific activity of lynestrenol-^{3}H. The concentration of the metabolites was derived from the experimental data of total radioactivity and of lynestrenol.

The simultaneous measurement of the blood levels of total radioactivity showed a large difference with the blood levels of lynestrenol (Fig. 16.2) in spite of the rapid excretion of the metabolites with the bile, that is to be expected on account of previous findings (see Chapter 12). Generally, the peak level of the metabolite curve was already attained before the first sample was taken. Its relatively late appearance in the experiment shown (Fig. 16.2) can be attributed to some delay in the biliary excretion of radioactivity observed during the first 15 minutes after injection. The final slope of the metabolite curve indicated in all experiments a substantial increase of the time of half-life of the metabolites as compared with the parent compound (30-90 h versus about 12 h). The longest half-life was observed in the intact rats, namely 75 h and 88 h, and a contribution of enterohepatic recycling of the metabolites to the slow elimination in these animals seems likely. It should be realized, however, that

this half-life as measured in the present study does not provide conclusive information about the real disappearance rate of the lynestrenol metabolites, because in the course of the biotransformation of lynestrenol-^{3}H tritiated water may be formed. The slow elimination and the small volume of distribution of this tritiated water could influence the final slope of the blood level-time curve of the total radioactivity to a high extent. With respect to the time course of the metabolites more reliable information can be obtained when a metabolically indifferent radiolabel is used like is present in lynestrenol-4-^{14}C.

Intraduodenal administration

After the intraduodenal administration of lynestrenol-^{3}H dissolved in bile rapid absorption occurred as can be derived from the peak level times of the various blood concentration-time curves as well as from the rapid excretion of radioactivity in the bile (Fig. 16.3). Although the blood level profile of the hexane-extractable radioactivity during the absorption phase was rather irregular, beyond the peak level time a biphasic decline appeared to occur. The first phase observed after injection of the steroid is not visible after intraduodenal administration, probably because in these experiments the first-phase distribution and the absorption process coincide. In spite of the rapid absorption the maximal blood concentration of hexane-extractable radioactivity remained considerably below the lynestrenol levels observed after parenteral administration (Figs. 16.1, 16.2). For this and other reasons mentioned in Chapter 7 only the sample taken at time 90 min was subjected to thin-layer chromatography. The results of chromatography are also shown in Fig. 16.3. With one exception the fraction of the hexane extract representing lynestrenol varied between 80 and 90%. The hexane-extractable radioactivity, on the contrary, represented only 10-20% of the total radioactivity levels (not shown) during the first two hours after drug administration and decreased afterwards progressively below 5%.

The fraction of the dose absorbed was determined by measurement of the amount of radioactivity present in the faeces and gut contents after 24 h and appeared to be more than 90% (Table 16.2). The biological availability (F) of lynestrenol was determined by comparing the area under the blood concentration-time curve after intraduodenal (AUC_{id}) and intra-arterial (AUC_{ia}) administration with the proper dose correction for the body weight and for the unabsorbed fraction in the intraduodenal experiments included, using the formula:

$$F\,(\%) = \frac{AUC_{id} \cdot D_{ia}}{AUC_{ia} \cdot D_{id}} \times 100$$

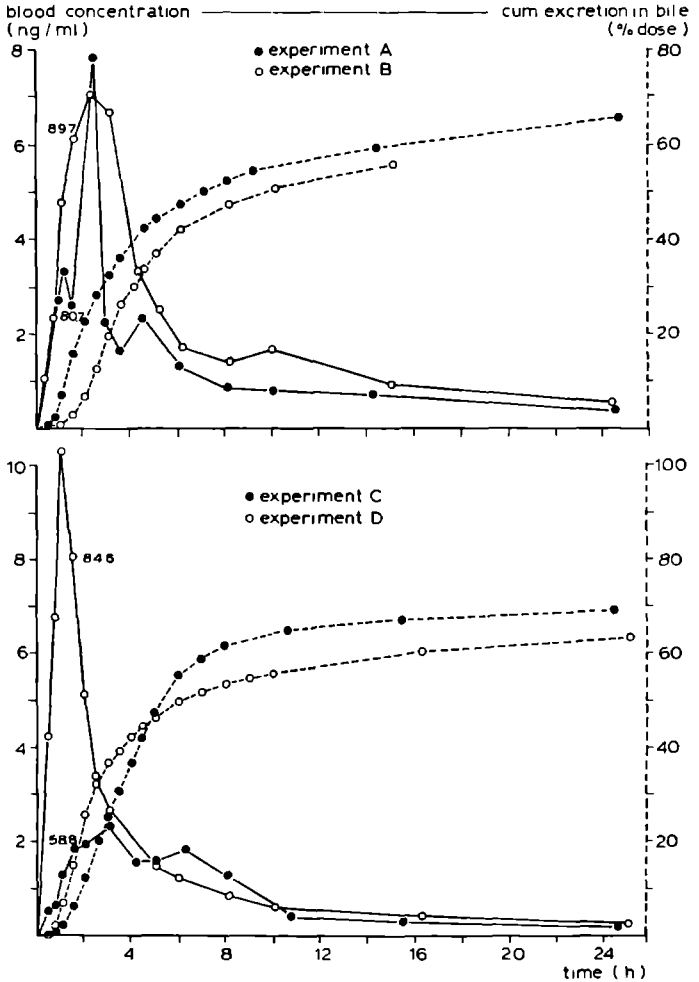

Fig. 16.3 — Time course of hexane-extractable radioactivity in rat blood following the intraduodenal administration of about 50 µg lynestrenol-^{3}H to four rats with bile fistula. The concentrations, measured as dpm/ml blood, were converted into ng/ml using the molecular weight and the specific activity of lynestrenol-^{3}H. The cumulative excretion of radioactivity in the bile is also shown in the figure. For the 90 min samples the percentage of the hexane-extractable radioactivity representing lynestrenol is indicated. Notice the rapid fall of the blood concentration beyond the peak level time and the overall low concentrations in comparison with the parenteral route of administration (cf. Figs. 16.1 and 16.2).

Table 16.2 — Absorption and biological availability after the intraduodenal administration of lynestrenol-^{3}H to 4 rats[1])

EXPERIMENT	A	B	C	D
Body weight (g)	240	228	229	235
Dose (μg)	58.9	55.9	45.0	64.5
Absorption (%)[2])	94.1	94.6	89.1	92.7
Dose abs./100 g (μg)	23.1	23.2	17.6	25.4
C_t (ng/ml)[3])	0.49	0.67	0.12	0.24
τ_{el} (min)[4])	1728	1339	691	994
AUC 0-t (ng.min/ml)	1801	2769	1063	1803
AUC t - ∞ (ng.min/ml)	846	897	83	238
AUC 0- ∞ (ng.min/ml)	2647	3666	1146	2041
AUC 0-∞/dose abs./100 g	115	158	65.1	80.4
F (%)[5])	18.5	25.4	10.5	12.9
	(12.1,26.2)	(16.7,36.0)	(6.9,14.8)	(8.5,18.3)

[1]) The individual blood concentration-time curves of these experiments are shown in Fig. 16.3. For symbols and calculations see text.

[2]) The fraction absorbed (% dose) was calculated as the difference between the radioactive dose administered and the quantity of radioactivity recovered from the faeces and gut contents about 24 h later at the end of the experiment.

[3]) The last blood concentration measured.

[4]) The elimination time constant derived from the ultimate slope of the semi-logarithmic blood concentration-time curve.

[5]) The biological availability of hexane-extractable radioactivity after intraduodenal administration of lynestrenol-^{3}H calculated by using the mean or, in parentheses, the maximal and minimal value respectively of the area under the blood concentration-time curve of hexane-extractable radioactivity following intra-arterial injection (AUC_{ia} as shown in Table 16.1 and multiplied by 1.11).

The areas under the curve, AUC_{id} and AUC_{ia}, were calculated for the hexane-extractable radioactivity fraction of the blood level-time curve. Following intra-arterial injection the best fit of the respective curves was used for the calculations. In case of intraduodenal administration the AUC_{id} for the portion between zero-time and the time of the last sample was determined by comparing its weight relative to a standard area. If at the last data point, 24 h after drug

administration, absorption is complete, and this condition is obviously fulfilled in our experimental set-up, the area beyond this point can be determined using Dost's law of corresponding areas. The following equation applies for the area from zero-time up to infinite time (Dost, 1968):

$$AUC = \int_0^{\infty} C_1 \, dt = \int_0^{t} C_1 \, dt + \int_t^{\infty} C_1 \, dt = AUC\ 0\text{-}t + C_t \cdot \tau_{el}$$

where C_t is the last blood concentration determined and τ_{el} is the terminal elimination time constant. So the residual area can be calculated by multiplying C_t by τ_{el}. The latter parameter was derived from the terminal phase of the semi-logarithmic blood concentration-time curves.

Because for all experiments different rats were used no comparison was possible of parenteral and intraduodenal curves for one and the same animal. This implies, that the biological availability obtained by comparing the AUC_{id} and AUC_{ia} after drug administration to different animals is influenced also by the value of the total body clearance of the respective rats. The clearance constants, however, observed after intra-arterial injection of lynestrenol varied within rather narrow limits (Table 16.1). For this reason interindividual variation of this parameter is probably not a complicating factor for the biological availability as measured in the present study. Moreover, it has to be realized that as a result of interindividual variations in the clearance constant the biological availability will always have only absolute significance for one individual and under well-defined experimental conditions. In Table 16.2 the extreme values of the biological availability have been presented for each rat as derived from the ratio of the AUC_{id} and the minimal as well as the maximal AUC_{ia} observed in the former experiments (Table 16.1).

DISCUSSION

Physiological and pharmacological meaning of the pharmacokinetic data

The present results emphasize that measurement of the blood levels of total radioactivity does provide hardly any information about the time course of lynestrenol in the blood. The three-term exponential decline of the blood concentration indicates the distribution of the drug over a central compartment comprising the blood and those tissues and fluids, which are instantaneously accessible to the drug, and two more slowly accessible peripheral tissue com-

partments. It is generally assumed that the liver, where the elimination of lynestrenol takes place, belongs to the central compartment. The peripheral compartments will include among others the target tissues of lynestrenol and the adipose tissue. Accumulation of the very lipophilic lynestrenol (this thesis, Chapter 8) in the latter tissue may explain at least in part the remarkably large volume of distribution of the drug. On the other hand, the slow release of lynestrenol from the poorly perfused body fat may govern the shallow slope of the terminal phase of the blood concentration-time curve. Retention, however, of the steroid in the target tissues as a result of its binding to the estradiol and progesterone receptor molecules will certainly not be reflected in the blood profile of the compound as measured in the present study. Current knowledge of the total binding capacity of the estrogen receptor (Eisenfeld et al., 1977; Ginsburg et al., 1975; Korach & Muldoon, 1974a,b; Lee & Jacobson, 1971; Sen & Menon, 1978) and the receptor for progesterone (Booth & Colas, 1977; Kato & Onouchi, 1977; Walters & Clark, 1977) in the rat indicate that the total binding capacity of these receptors does not exceed an amount of 50 pmol/animal. Taking the double value it can be easily calculated that the instantaneous distribution of 100 pmol lynestrenol over the smallest volume of distribution of the central compartment found in the present study (111 ml) would result in a blood concentration of only 0.25 ng lynestrenol/ml.

In Chapter 9 the inhibition of estradiol-^{3}H receptor binding by lynestrenol was studied in the rat *in vivo* using the intravenous route of administration. A significant decrease of estradiol-^{3}H binding in the pituitary and preoptic area could be observed following the injection of a 250-fold excess of lynestrenol over estradiol-^{3}H (see Chapter 9, Fig. 9.2). The physiological blood levels of estradiol in the rat vary between 20 and 90 pg/ml (Butcher et al., 1974). Assuming that the ratios of the blood/target tissue concentration for estradiol and lynestrenol are about equal, it follows that blood concentrations of 5-20 ng lynestrenol/ml and higher will interfere with the binding of estradiol to the estrogen receptor. Taking into account the results presented in Fig. 16.3 it may be inferred that inhibition of estradiol receptor binding will also be of significance following oral administration of lynestrenol, the more so because the anti-ovulatory dose range of this compound for the oral route is in the order of 1-5 mg/rat (Overbeek et al., 1962).

Absorption and biological availability

The high percentage of lynestrenol found to be absorbed after intraduodenal administration of the drug is in good agreement with the estimation on basis

of the cumulative excretion data in the urine, bile and faeces in the intact and bile duct cannulated rat (this thesis, Chapter 12). Kilian (1973) reported an "apparent" absorption of lynestrenol-^{14}C in the rat after oral administration of only 45.9 and 73.6% of the dose. These values were calculated as in the present study but instead of rats with bile fistula animals with intact bile ducts were used. Because 24 h after lynestrenol administration about 65% of the lynestrenol dose is excreted with the bile (Fig. 16.3 and Chapter 12) and is included by Kilian in the unabsorbed fraction, the "apparent" absorption in his study does not reflect the actually occurring absorption, which is considerably greater. On the other hand the lymphatic absorption of lynestrenol of 3.2% and 6.2% of the dose observed by Kilian (1973), although representing a minor portion of the total absorbed amount, may be nevertheless of importance for the biological activity of lynestrenol in view of its poor biological availability of about 15%.

The measurement of the biological availability, using the concentrations of hexane-extractable radioactivity instead of lynestrenol concentrations, may be considered to provide strong evidence for a considerable first-pass elimination of lynestrenol in the liver and a concurrently low fraction of the oral dose reaching the general circulation. Although the data calculated represent an apparent rather than a true value for the biological availability, an overestimation of this parameter in the present investigation is more likely to occur than an underestimation. Underestimation of the biological availability in this study would imply, that the lynestrenol fraction in the hexane extract should be almost permanently smaller after parenteral than after enteral administration. Such an assumption is very unlikely in the light of the rapid metabolism of lynestrenol as observed in the isolated perfused rat liver (this thesis, Chapter 15). In fact, the lynestrenol fraction in the hexane extract showed a tendency to decrease rather than to increase in case of intraduodenal administration.

The limited bioavailability of lynestrenol raises the question, whether and to what extent the biological activity of this steroid is an intrinsic property of the drug itself or is derived from its bioactivation in the liver. In the first case it seems worthwhile to study the possibility of increasing the biological availability by using another route of administration which avoids the first passage through the liver, e.g. buccal administration. Also an increase of the fraction absorbed via the intestinal lymphatics under the influence of biopharmaceutical modifications in the dosage form may serve this purpose as has been shown by Kilian (1973) for DDT, by Coert et al. (1975) for testosterone undecanoate and by De Visser & Van der Vies (1977) for estradiol decanoate. A concurrent reduction of the therapeutic dose would be advantageous with regard to the incidence of side effects, which accompany the use of oral contraceptives. In the second

case, however, when activity resides in a metabolite of lynestrenol, and it has been suggested that lynestrenol might be the pro-drug of norethindrone (Fotherby, 1972; Kontula et al., 1975; Mazaheri et al., 1970), it seems logical to replace lynestrenol by this conversion product.

In vivo and in vitro clearance of lynestrenol

On account of the flow-limited metabolic clearance rate of lynestrenol in the isolated perfused rat liver as well as the low biological availability of the compound observed in the present study a higher elimination clearance constant was expected than we actually observed after injection. When the liver is an integral part of the central compartment, when furthermore elimination occurs solely by hepatic metabolism and when the metabolic clearance rate is independent of the route of administration, the following relationship applies (Gibaldi et al., 1971; Rowland, 1972):

$$F = 1 - \frac{k_{Cel}}{Q}$$

Using this equation the value of the apparent hepatic blood flow (Q) can be calculated to be about 5 ml/min for $F = 0.15$ and $k_{Cel} = 4$ ml/min (see Tables 16.1 and 16.2). According to Dobson & Jones (1952) and Grayson & Mendel (1965) the physiological blood flow through the rat liver is 1.2-1.5 ml/min/g liver, which should result in a hepatic blood flow of 9-12 ml/min in the present study. So, the apparent hepatic blood flow is about half of the physiological value. The lack of correlation between the first-pass metabolism and *in vivo* clearance on the one hand and between *in vivo* and *in vitro* clearance on the other hand are indications that the pharmacokinetics of lynestrenol *in vivo* may be more complex than the model experiments with the isolated rat liver suggested. Although the possibility should not be neglected that the liver blood flow in the experimental animals was actually smaller than the normal value, some other possible complicating factors deserve consideration.

The metabolic clearance rate was studied in the isolated liver using instead of rat blood a semi-synthetic medium consisting of dog erythrocytes and an electrolyte solution containing bovine serum albumin (BSA) as the only protein (this thesis, Chapter 15). That lynestrenol behaves differently in rat blood than in the perfusion medium under the experimental conditions may be derived from experiment 6 (Table 16.1), where plasma concentrations of lynestrenol have been measured instead of blood concentrations. In this experiment a strong

reduction of the calculated apparent volume of distribution was found. So, it seems likely that the plasma concentration of lynestrenol is much higher than the blood concentration, whereas an opposite relationship appeared to exist in the perfusion medium (see Chapter 15, Tables 15.1 and 15.2).

An explanation which could reconcile the *in vivo* and *in vitro* results, but which awaits experimental proof, may be found in binding of lynestrenol to some fraction of the rat plasma proteins with a higher affinity than the binding to BSA. Then the non-restrictive elimination of lynestrenol observed *in vitro* may be restrictive of character *in vivo*. Consequently, the clearance *in vivo* may become dependent upon the capacity of those hypothetical binding sites. The presence and significance of such high affinity binding sites in the globulin fraction of the plasma proteins and their influence on the metabolic clearance rate, distribution and activity of endogenous steroid hormones (Anderson, 1974; Burke & Anderson, 1972; Vermeulen et al., 1969) as well as of synthetic derivatives (Chan & Slaunwhite, 1977; Raynaud, 1973), including some of the contraceptive progestins (Murugesan & Laumas, 1975; Uniyal & Laumas, 1976; Victor et al., 1977) has been demonstrated. The low biological availability of lynestrenol after oral administration may then be explained by saturation of the high affinity binding sites in the portal blood entering the liver during the absorption process and a concurrently non-restrictive elimination of the free and more loosely bound lynestrenol fraction. Similarly, when lynestrenol levels are falling after injection of the steroid, the significance of the restrictive elimination will gradually increase and the metabolic clearance rate will slow down.

Apart from the involvement of the hepatic blood flow and plasma protein binding the metabolic clearance rate of lynestrenol *in vivo* could also be reduced as a result of competition of the compound with other substrates for the metabolic sites on the liver enzymes. In this case the low biological availability may be caused by the occurrence of significant pre-hepatic metabolism, e.g. in the gut wall. Biotransformation of orally administered progesterone and testosterone in the gut wall has been reported to occur in the dog (Harri, 1970a,b; Niensted et al., 1969).

Further investigations are needed to gain an insight, whether one or more of the complicating factors touched in the present discussion are pertinent to the pharmacokinetics of lynestrenol. Moreover, studies in humans are to be considered an indispensable sequel to the present work and should be initiated now suitable analytical tools have recently become available. Extrapolation of the results obtained in the rat to the human is hazardous in view of the possibly significant species differences with regard to the metabolic clearance rate in the rat and in man. No information is available at present with respect to the biological availability of the contraceptive steroids in animals and humans. Com-

parison, however, of the anti-ovulatory activity of these compounds showed a good correlation to exist between the effective subcutaneous dose in the rat and the oral dose in humans, whereas a bad correlation was found between the effective dose after oral administration to both species (Neumann et al., 1977). It seems possible that pharmacokinetic investigations could provide the key to this and other, still open, questions.

SUMMARY

The pharmacokinetics and biological availability of lynestrenol have been studied in the rat after the administration of 50 μg lynestrenol-^{3}H. Blood and plasma concentrations were measured over a period of about 24 h. The analytical procedure allowed discrimination between concentrations of total radioactivity, hexane-extractable radioactivity and lynestrenol. Within 1 h after injection lynestrenol represented less than 10% of the total radioactivity levels. The time course of the drug in the blood and plasma could be described by a three-term exponential equation according to the three-compartment open model. The time of half-life varied between 10-21 h (mean value 13.6 h, 6 animals). The large apparent volume of distribution V_d (12-19 l/kg, mean value 15.5 l/kg) and the high lipophilicity of the compound indicate the occurrence of accumulation in the adipose tissue. The decreased V_d of 7.4 l/kg when derived from the plasma curve (one animal) indicates an uneven distribution of lynestrenol between the plasma and the erythrocytes. The total body clearance constant varied between 2.8-4.8 ml/min (mean value 4.0 ml/min, 5 animals). Following intraduodenal administration to animals prepared with bile fistula 89-95% of the dose (mean value 92.6%, 4 animals) was absorbed and 65-70% was excreted with the bile during the experiment. Maximal concentrations of hexane-extractable radioactivity were measured 1-2 h after lynestrenol ingestion. The blood concentrations remained considerably below the levels observed after injection. The biological availability varied between 11-25% (mean value 16.8%). Neither was a correlation found between the *in vivo* clearance of lynestrenol and its clearance in the isolated perfused rat liver (this thesis, Chapter 15) nor between the *in vivo* clearance and the biological availability. Possible explanations for this apparent discrepancy are discussed. The present findings, however, provide evidence that interference of lynestrenol with estradiol receptor binding in the rat *in vivo* (this thesis, Chapter 9) may also be of significance after oral administration of this progestin.

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Het doel van het farmacologisch onderzoek beschreven in dit proefschrift is tweeledig. Enerzijds is gepoogd meer inzicht te krijgen in de relatie tussen *chemische structuur* en *werking* van de meest gebruikte synthetische progestativa, terwijl anderzijds de *farmacokinetiek* van een van deze stoffen, het lynestrenol, diepgaand is bestudeerd. Beide aspecten van het onderzoek vullen elkaar aan, omdat de biologische activiteit van een stof niet los gezien kan worden van haar structuur en daarnaast mede bepaald dan wel gemoduleerd wordt door haar absorptie, distributie, metabolisme en excretie.

Voor wat betreft de relatie tussen *structuur* en *werking* is de aandacht gericht op de estrogene component, aanwezig in het werkingsspectrum van de progestativa afgeleid van 19-nortestosteron, te weten het lynestrenol, ethynodiol, norethynodrel en norethindron. Al sedert de introductie van deze stoffen was het een twistpunt, of de estrogene (bij)werking een intrinsieke eigenschap is van de stof dan wel het gevolg is van een omzetting *in vivo* in estrogene metabolieten. Onderzocht is door ons of, en zo ja in welke mate, progestativa met en zonder estrogene activiteit de binding van estradiol-^{3}H aan de receptor moleculen in de estrogene target weefsels van de rat kunnen beïnvloeden. Weliswaar is binding aan de receptor niet synoniem met biologische activiteit, aangezien behalve de affiniteit ook de intrinsieke activiteit een rol speelt, maar anderzijds is het optreden van binding een absolute voorwaarde wil er van activiteit sprake kúnnen zijn. Daarnaast is gepoogd door onderzoek aan het bindingsgedrag van deze en een aantal andere, medisch niet toegepaste, steroïden een inzicht te krijgen in de relatie tussen chemische structuur en bindingsaffiniteit. Dit laatste aspect is bestudeerd *in vitro* met behulp van de 105000xg supernatant van kalfs- en ratte-uterus homogenaten. Deze preparaten zijn relatief zeer rijk aan receptor moleculen en bieden het voordeel, dat invloeden van distributie, metabolisme en excretie worden vermeden.

Het *farmacokinetisch* gedeelte van het onderzoek is toegespitst op lynestrenol. Vanwege het feit dat geen methode beschikbaar is, die zich leent voor de bepaling van de uiterst lage bloedconcentraties van deze stof bij de mens, moest gebruik worden gemaakt van de radioactief gemerkte verbinding, waardoor het onderzoek beperkt bleef tot de rat en de muis. Het verdelingspatroon van lynestrenol en de passage door de placenta is bestudeerd bij de muis door middel van autoradiografie in combinatie met analytisch-chemische methoden.

De metabole klaring van lynestrenol is onderzocht met de techniek van de geïsoleerde doorstroomde rattelever. Voor de studie van absorptie, eliminatie en excretie van lynestrenol en zijn metabolieten is gebruik gemaakt van de intacte rat en de rat met gecannuleerde urineblaas en galbuis. Daarnaast werd gemeten de verdelingscoëfficiënt van lynestrenol en met behulp van evenwichtsdialyse de binding aan serum albumine.

Het proefschrift is verdeeld in 4 secties:

In *Sectie I, Hoofdstuk 1,* wordt een korte schets gegeven van een aantal fundamentele begrippen uit de farmacologie, die de interactie tussen het farmacon en het biosysteem in kwalitatieve en kwantitatieve zin bepalen. Daarnaast geeft dit hoofdstuk ter inleiding op Sectie III een literatuuroverzicht van de grote vorderingen gedurende de laatste 10 jaar op het gebied van het werkingsmechanisme van estrogene hormonen alsmede een beschouwing over de farmacologische implicaties daarvan.

Hoofdstuk 2 bevat een korte beschrijving van de historie, medische toepassing en farmacologie van de orale anticonceptiva, waaraan in het kader van het onderhavige onderzoek aandacht is besteed. De doelstelling van het onderzoek wordt aangegeven.

Sectie II, Hoofdstukken 3-8, omvat het algemeen experimenteel gedeelte.

Hoofdstuk 3 behandelt de controle op zuiverheid en de zuiveringsprocedure van de gebruikte radioactief gemerkte steroïden, te weten estradiol-6,7-^{3}H, lynestrenol-4-^{14}C en lynestrenol-^{3}H. Verder worden hier de bronnen van deze en de overige, radioïnerte, steroïden vermeld.

Aangezien het voor de farmacokinetische onderzoekingen benodigde lynestrenol-^{3}H niet voorhanden was, moest hierin zelf worden voorzien. De synthese van deze stof is beschreven in *Hoofdstuk 4.*

Het principe van de radioactiviteitsmeting door vloeistof-scintillatietelling, de monstervoorbereiding en een evaluatie van deze techniek vormen het onderwerp van *Hoofdstuk 5.*

De interactie van de anticonceptieve steroïden met de estradiol receptor *in vitro* is gemeten met behulp van een verdringingsmethode. *Hoofdstuk 6* bevat een beschrijving en verantwoording van de gevolgde experimentele procedure alsmede de toetsing van deze techniek op haar bruikbaarheid in het kader van de vraagstelling van dit onderzoek. Het principe van de bepalingsmethode is, dat de mate, waarin een competerende niet-radioactieve verbinding in staat is het radioactief gemerkte estradiol uit zijn bindingsplaatsen op de receptor moleculen te verdrijven, een graadmeter is voor de affiniteit van deze stof voor de receptor. Een voorwaarde is wel, dat er sprake dient te zijn van een reversibele

interactie en er gemeten wordt onder evenwichtscondities. Indien men twee van de drie variabelen, te weten de totale estradiol concentratie in de oplossing, de receptor-gebonden en de vrije estradiolfractie kan bepalen, is de affiniteit van het competerende steroïd te kwantificeren. In de door ons toegepaste procedure wordt het receptor-gebonden estradiol gescheiden van de vrije fractie door adsorptie van deze laatste fractie aan adsorberende kool. Het gebonden estradiol en de totale estradiol concentratie worden gemeten door vloeistof-scintillatie telling.

De beperkende factoren, die een rol spelen bij bio-analyses ten behoeve van farmacokinetisch onderzoek, in het bijzonder van laag gedoseerde geneesmiddelen zoals orale anticonceptiva, komen aan de orde in *Hoofdstuk 7*. Een verantwoording wordt gegeven van de toegepaste bepalingsmethode voor lynestrenol in het bloed van de rat *in vivo*, resp. het perfusiemedium van de geïsoleerde doorstroomde rattelever *in vitro*. De analyse berust op een extractie van het radioactief gemerkte lynestrenol uit het monster met hexaan en een dunne-laag chromatografische scheiding van de moederstof en meegeëxtraheerde omzettingsprodukten.

De bepaling van de verdelingscoëfficiënt van lynestrenol over heptaan/buffer en de binding van de stof aan runder serum albumine wordt beschreven in *Hoofdstuk 8*. Het lynestrenol is zeer lipofiel getuige de waarde van circa 5000 voor de verdelingscoëfficiënt. De eitwitgebonden fractie is eveneens hoog (97,4%) en constant over een breed concentratiegebied van 2-1700 ng/ml, de associatieconstante bedraagt $8{,}9 \times 10^4$ M^{-1}.

In *Sectie III (Hoofdstukken 9, 10, 11)* wordt verslag gedaan van het onderzoek naar de binding van de anticonceptieve progestativa aan de estradiol receptor van de rat en het kalf.

Het verdelingspatroon van estradiol-^{3}H in de rat na intraveneuze injectie *(Hoofdstuk 9)* laat een groot verschil zien tussen het verloop van de concentratie in enerzijds de zogenaamde target weefsels en anderzijds het bloedplasma en de andere organen. In de target weefsels zoals uterus, vagina, hypofyse-voorkwab, preoptisch gebied en hypothalamus, vindt een sterke accumulatie en retentie plaats van estradiol ten gevolge van de binding van het hormoon aan de estradiol receptoren in deze weefsels. Deze binding is specifiek voor estrogene stoffen en verzadigbaar: gelijktijdige toediening van testosteron naast het estradiol heeft geen invloed op het distributiepatroon, bij gelijktijdige injectie van een overmaat (d.w.z. een niet-fysiologische dosis) van niet-radioactief estradiol of een andere estrogene stof daarentegen gaat het verschil in opname van estradiol-^{3}H tussen de target organen en de andere weefsels verloren. Het blijkt, dat de steroïden afgeleid van 19-nortesto-

steron de binding van estradiol-^{3}H in alle target weefsels, zowel perifeer als in het centrale zenuwstelsel, in aanzienlijke mate kunnen remmen. Enkele van deze target weefsels, zoals de hypothalamus, het preoptisch gebied en de hypofyse, spelen een sleutelrol bij de regulering van het ovulatieproces. Het meest effectief is ethynodiol, gevolgd door resp. norethynodrel, lynestrenol en norethindron. Ook 19-nortestosteron vertoont enige activiteit, terwijl het marginale effect van norgestrel beperkt blijft tot de hypofyse en het preoptisch gebied in de hersenen. Het effect van de medisch eveneens veel toegepaste acetaatesters van deze stoffen komt overeen met dat van de moederverbindingen. De orale anticonceptiva afgeleid van hydroxyprogesteron, namelijk medroxyprogesteron, chlormadinon en megestrol en hun acetaten, hebben geen invloed op de receptorbinding van estradiol. Voor lynestrenol is bovendien nagegaan de relatie tussen de dosis, resp. het tijdstip van toediening ten opzichte van estradiol, en het effect. Het blijkt, dat de remming van de receptorbinding in de hypofyse sterker is dan in de uterus. Dit is waarschijnlijk een gevolg van het verdelingsproces van lynestrenol in het organisme. De betekenis van deze resultaten wordt besproken in het licht van andere, onderling strijdige, onderzoekingen en het werkingsmechanisme van deze stoffen bij de ovulatieremming.

De receptor moleculen van estradiol zijn eiwitmoleculen, die gelocaliseerd zijn in het cytoplasma van de target cellen. De eigenschappen van de receptor en de bindingsparameters van estradiol kan men bestuderen door onderzoek van het bindingsgedrag van getritieerd estradiol onder diverse experimentele condities (*Hoofdstuk 10, 11*). Zo sedimenteert het estradiol-receptor complex bij centrifugeren door een sucrose dichtheidsgradiënt in het 8-9S gebied en wordt het bindingsgedrag van estradiol niet beïnvloed door de aanwezigheid van een 2000-voudige overmaat van niet-estrogene steroïde hormonen. De dissociatieconstante van het estradiol-receptor complex is zeer laag (K_D $3x10^{-10}$ M). De bestudering van de invloed van de anticonceptiva en structuur-verwante steroïden op de receptorbinding van estradiol *in vitro* heeft het voordeel, dat behalve de mogelijke invloed van biotransformatie ook die van distributie en excretie processen op de resultaten wordt voorkomen, zodat kwantificering van de interactie *in vitro* direct gerelateerd kan worden aan de chemische structuur. De 19-nortestosteron derivaten blijken ook *in vitro* de binding van estradiol te remmen, de acetaatesters zijn *in vitro* echter veel minder actief dan in het *in vivo* experiment *(Hoofdstuk 10)*. Vermoedelijk wordt de acetaatgroep *in vivo* zeer snel afgekoppeld. De hydroxyprogesteron derivaten en norgestrel vertonen *in vitro* evenmin activiteit. De remming van de receptorbinding van estradiol door lynestrenol is dosis-afhankelijk en competitief (K_i $1,1x10^{-7}$ M). Blijkbaar binden estradiol en lynestrenol op dezelfde plaats aan het receptor molecuul. De relatie tussen de remming van de receptorbinding van estradiol door de orale

anticonceptiva en andere onderzochte stoffen en hun chemische structuur wordt uitvoerig besproken en vergeleken met gegevens uit ander onderzoek.

In *Hoofdstuk 11* wordt gewezen op de voordelen van het gebruik van de kalfs-uterus als alternatieve bron van receptor materiaal ten behoeve van bindingsstudies. De cytosol van kalfs-uterus homogenaat is gebruikt voor verdringingsexperimenten, waarmee de verhouding van de associatieconstanten van het competerende steroïd en estradiol kon worden bepaald en daaruit, na meting van de associatieconstante van het estradiol-receptor complex, de associatieconstante van de inhiberende stof. De volgende waarden zijn gevonden voor de anticonceptieve steroïden met activiteit (K_A estradiol $2{,}6 \times 10^9$ M^{-1}): ethynodiol $2{,}2 \times 10^8$, norethynodrel $1{,}2 \times 10^7$, lynestrenol $6{,}5 \times 10^6$, norethindrone $3{,}8 \times 10^5$. Tenslotte worden in dit hoofdstuk de resultaten van de bindingsstudies vergeleken met de activiteit van deze stoffen in de biologische test voor estrogene werking (Allen-Doisy test) zoals bekend uit ander onderzoek, een en ander in samenhang met de invloed van farmacokinetische factoren.

De resultaten van het onderzoek beschreven in Sectie III pleiten voor de opvatting, dat de estrogene werking van de bovengenoemde stoffen een direct gevolg is van hun binding aan de estradiol receptor. Een mogelijke bijdrage hierin van estrogene omzettingsprodukten kan echter niet worden uitgesloten. De gemeten bindingsaffiniteiten zijn behalve voor het ethynodiol weliswaar niet hoog, maar daarbij dient bedacht te worden, dat deze progestativa in de medische praktijk veelal ca. 100-voudig worden gedoseerd in vergelijking met estrogene hormonen. Onze onderzoeksresultaten illustreren verder de mogelijkheden, die de bestudering van de receptorbinding biedt naast de klassieke onderzoekmethoden ten aanzien van de evaluering van het werkingsspectrum van de reeds toegepaste steroïde hormonen alsmede ten aanzien van de ontwikkeling van nieuwe steroïden met een smaller werkingsspectrum. Vooral dit laatste aspect zal voor de praktische therapie van betekenis zijn, ervan uitgaande, dat idealiter gestreefd dient te worden naar een therapeutisch arsenaal van farmaca met één bepaalde farmacologische werking en zonder bijwerkingen.

Sectie IV, Hoofdstukken 12-16, is gewijd aan het onderzoek naar de farmacokinetiek van lynestrenol in de rat en de muis.

De excretie van lynestrenol-^{14}C en zijn metabolieten na orale en intraveneuze toediening aan de intacte vrouwelijke rat vertoont hetzelfde beeld (*Hoofdstuk 12).* Binnen een tijdsbestek van 24 uur wordt 50% van de dosis uitgescheiden met de faeces en de urine, en dit neemt toe tot 80% 24 uur later. Ongeveer 4/5 van de totale excretie verloopt via de faeces. Bij ratten voorzien van een galfistel verloopt de excretie sneller, terwijl het aandeel van de galexcretie in de totale excretie toeneemt in vergelijking met de intacte rat. Dit laatste

aspect doet het bestaan van een enterohepatische kringloop vermoeden van de excretieprodukten aanwezig in de gal. Informatie omtrent de absorptie van lynestrenol is verkregen door vergelijking van de cumulatieve excretie van ^{14}C in de gal en urine na intraveneuze en intraduodenale toediening van lynestrenol-^{14}C. Ongeveer 90% van de dosis blijkt te worden geabsorbeerd (zie ook Hoofdstuk 16). Een indruk van de absorptiesnelheid is verkregen door de excretie in de gal als functie van de tijd te vervolgen na intraveneuze en intraduodenale toediening. De absorptie verloopt snel en na ongeveer 10 uur is er geen verschil meer in de cumulatieve excretie voor beide applicatieroutes. Fractionering van het uitgescheiden radioactief gemerkte materiaal in gal, urine en faeces door extractie met hexaan en ethylacetaat voor en na enzymatische hydrolyse en dunne-laag chromatografie van deze extracten laat zien, dat lynestrenol volledig wordt gebiotransformeerd tot polaire verbindingen. Met behulp van gas-chromatografie en massa-spectrometrie werd de identiteit van de kwantitatief belangrijkste metaboliet in het hexaanextract van de gal nader onderzocht. De verkregen gegevens duiden op het optreden van een monohydroxylering en hydrogenering van lynestrenol in de A-ring en naar analogie van de biotransformatie van andere 19-nortestosteron derivaten is het gevormde produkt vermoedelijk 17α-ethynyl-5ξ-estrane-3ξ,17β-diol. De hoge excretiesnelheid van de omzettingsprodukten impliceert een hoge metabole klaringssnelheid en een vermoedelijk lage biologische beschikbaarheid van het lynestrenol ondanks de bijna volledige absorptie.

Hoofdstuk 13 beschrijft de excretie van ^{14}C bij ratten met een galfistel na de intraduodenale toediening van gal verkregen van donor-ratten, ingespoten met lynestrenol-4-^{14}C. Er treedt een aanzienlijke absorptie op van de galmetabolieten. De geabsorbeerde fractie is groter, indien een extract van vooraf langs enzymatische weg gesplitste gal wordt toegediend. Uit het verloop van de ^{14}C excretie in de gal als functie van de tijd kan worden afgeleid, dat de absorptiesnelheid grotendeels bepaald wordt door het enzymatische splitsingsproces van de geconjugeerde galmetabolieten in de lagere delen van de darm. Het optreden van deze hydrolyse in de darm van de intacte rat blijkt uit het feit, dat de zonder voorafgaande hydrolytische splitsing extraheerbare fractie van de metabolieten veel groter is in de faeces dan in de gal. De conjugaten lijken als zodanig niet of slechts langzaam te worden geabsorbeerd. De betekenis van de enterohepatische kringloop voor het excretieproces neemt verder toe, indien de excretie met de faeces wordt vertraagd of verminderd en de condities voor absorptie worden begunstigd. Zo gaat een tragere eliminatie van ^{14}C via de faeces bij vastende ratten gepaard met een grote stijging van de renale excretie. Tenslotte is gezocht naar een antwoord op de vraag, of het grote overwicht van de hepatische over de renale excretie bij de uitscheiding van de lynestrenol metabo-

lieten bepaald wordt door structurele verschillen tussen het excretieproces in de lever en in de nier, alsmede door binding aan de plasma eiwitten, dan wel door andere factoren. Een volledige excretie van de lynestrenol metabolieten met de urine blijkt plaats te vinden in ratten met geobstrueerde galbuis. Het metabolisme en het excretieproces volgen in deze ratten overigens hetzelfde patroon als is waargenomen bij de intacte rat. De verdeling van de excretieprodukten over urine en gal in de verhouding van circa 1 : 6 kan derhalve verklaard worden door aan te nemen dat een groot deel van de biotransformatieprodukten in de lever direct in de gal overgaat en derhalve niet meer beschikbaar komt voor de renale excretieroute, tenzij via de genoemde enterohepatische kringloop.

De verdeling van lynestrenol en zijn metabolieten over de diverse organen en weefsels en de passage door de placenta is bestudeerd bij de vrouwelijke muis door middel van autoradiografie en analytisch-chemische methoden (*Hoofdstuk 14*). De stof blijkt zich binnen enkele minuten na intraveneuze injectie te verdelen over alle weefsels en organen, inclusief de hersenen. Gedurende deze verdelingsfase lijkt de doorbloeding van de organen bepalend te zijn voor het verdelingspatroon. Na de redistributiefase en na orale toediening van lynestrenol blijven gedurende enige uren relatief hoge concentraties aanwezig in de organen betrokken bij de eliminatie (lever en nier), in vetrijk weefsel (bruin en wit vet, haarfollikels en klieren van Harder) en sommige endocrinologisch actieve weefsels (ovarium en bijnierschors). Opvallend is de naar verhouding geringe opname in de vagina en uterus. Extractie van bloed en weefselhomogenaten met hexaan en dunne-laag chromatografie van enige van deze extracten toont aan, dat lynestrenol zeer snel wordt omgezet in de lever en gedurende de eerste 3 uur na injectie uit bloed en weefsels wordt geëlimineerd met een halfwaardetijd van 1 tot 2 uur. De lynestrenolfractie blijft gedurende deze periode relatief hoog in de vetrijke weefsels zoals wit en bruin vet en cerebrum, waarschijnlijk ten gevolge van de grote vetoplosbaarheid van de stof. De placenta passage en de opname van de stof in het foetale compartiment is kwantitatief van geringe betekenis. Ophoping van radioactiviteit is het meest uitgesproken in de foetale bijnier, maar is ook zichtbaar in lever, nier, long, bruin vet en lumen van de darm.

In *Hoofdstuk 15* zijn de resultaten beschreven van het onderzoek naar de metabole klaringssnelheid van lynestrenol met behulp van de geïsoleerde doorstroomde rattelever. Lynestrenol wordt tijdens de leverpassage volledig geëxtraheerd uit het perfusiemedium en gemetaboliseerd, ondanks de hoge binding aan het serum albumine aanwezig in het semi-synthetische perfusiemedium. De afname van de lynestrenolconcentratie in het perfusiemedium als functie van de tijd verloopt mono-exponentieel bij fysiologische (10 en 20 ml/min) en

hogere (35 tot 45 ml/min) perfusiesnelheden door het orgaan. De klaringssnelheid is onafhankelijk van de lynestrenolconcentratie over een groot concentratiegebied. De stof hoopt zich in het perfusiemedium op in de rode bloedcellen, maar de uitwisseling met het "plasma" verloopt zo snel, dat voor de "plasma"concentratie-tijd curve dezelfde eliminatie tijdconstante wordt gevonden als voor de "bloed"concentratie-tijd curve.

De absorptie en de biologische beschikbaarheid van lynestrenol zijn bestudeerd in de rat na intra-arteriële en intraduodenale toediening van 50 µg lynestrenol-^{3}H *(Hoofdstuk 16)*. Behalve de meting van de totale radioactiviteit in bloed en plasma als funktie van de tijd gedurende een periode van ongeveer 24 uur is de concentratie bepaald van hexaan-extraheerbare radioactiviteit (ongeveer voor 90% bestaande uit lynestrenol) en van lynestrenol, de laatstgenoemde concentratie alleen na injectie van de stof. Binnen 1 uur na injectie blijkt nog slechts 10% van de radioactiviteit in het bloed aanwezig in de vorm van lynestrenol. Het verloop van de semilogarithmische bloed, respectievelijk plasmaconcentratie-tijd curve wordt bepaald door 3 tijdconstanten (drie-compartimenten model). De biologische halfwaarde-tijd varieert van 10 tot 21 uur (gemiddelde 13,6 uur, 6 dieren). Het schijnbare verdelingsvolume van lynestrenol is erg groot, 12 tot 19 l/kg (gemiddelde 15,5 l/kg). Mede in aanmerking genomen de grote lipofiliteit van de stof kan het grote verdelingsvolume waarschijnlijk worden verklaard door stapeling van het lynestrenol in het lichaamsvet. In tegenstelling tot de accumulatie van lynestrenol in de erythrocyten van het perfusiemedium (zie Hoofdstuk 15) wijst het schijnbare verdelingsvolume van 7,4 l/kg berekend uit de plasmacurve (één experiment) op een tegenovergestelde verdeling van de stof over plasma en rode cellen *in vivo*. De totale lichaamsklaring bedraagt ongeveer 2,8-4,8 ml/min (gemiddelde 4,0 ml/min, 5 dieren). Na intraduodenale toediening van lynestrenol aan 4 ratten met een galfistel wordt 89-95% van de dosis geabsorbeerd (gemiddelde 92,6%) en 65-70% uitgescheiden met de gal over een periode van 24 uur. De bloedconcentraties zijn maximaal 1-2 uur na toediening. De concentraties blijven aanzienlijk lager dan de concentraties na injectie. De biologische beschikbaarheid varieert van 11 tot 25% (gemiddelde 16,8%). Deze waarde is kleiner dan verwacht op basis van de klaring gemeten na injectie. Er wordt geen correlatie gevonden tussen de klaring *in vivo* en de klaring gemeten in de experimenten met de geïsoleerde lever (zie Hoofdstuk 15). Mogelijke oorzaken van deze schijnbare tegenstrijdigheden worden besproken. Op grond van de bloedconcentraties van lynestrenol kan verwacht worden, dat de remming van de receptor binding van estradiol na intraveneuze injectie van lynestrenol (zie Hoofdstuk 9) ook van betekenis zal zijn na orale toediening van de stof.

Zoals veel onderzoekingen roept ook het onderzoek beschreven in Sectie IV

van dit proefschrift nieuwe vragen op. Van de aspecten, die stellig nadere bestudering verdienen, kunnen genoemd worden in het bijzonder de binding van het lynestrenol aan de plasma-eiwitten, de klaring van de stof in de met ratte-bloed doorstroomde geïsoleerde lever, de biologische beschikbaarheid na intraperitoneale toediening en het metabolisme. Naast dit dier-experimentele onderzoek dient de bestudering van de farmacokinetiek van het lynestrenol bij de mens ter hand te worden genomen. De hiertoe benodigde analytische technologie is de laatste jaren beschikbaar gekomen. Optimalisering en rationalisering van de therapie met orale anticonceptiva op basis van nog ontbrekende farmacokinetische gegevens lijkt nog zeer wel mogelijk ondanks of misschien juist dank zij de ruime klinische ervaring, die de afgelopen 15 jaar met deze stoffen is opgedaan. Zo zou een lage biologische beschikbaarheid bij de mens als in dit onderzoek waargenomen voor lynestrenol bij de rat, kunnen leiden tot een verdere verlaging van de effectieve dosis door de keuze van een andere toedieningsweg, bijv. de sublinguale route. Een en ander zou leiden tot een geringere belasting van de lever en galwegen met stoffen, waarvan identiteit en werking niet vast staan. Zou echter de omzetting van bijv. lynestrenol in de lever juist noodzakelijk blijken voor de biologische werkzaamheid (bioactivering), en er zijn gegevens, die dit vermoeden wettigen, dan lijkt nadere bezinning op de plaats van deze stof in de therapie met de orale anticonceptiva op zijn plaats.

CURRICULUM VITAE

Johannes Mathieu Gerardus van Kordelaar werd geboren op 4 december 1942 te Brunssum. Hij bezocht het St. Bernardinuscollege te Heerlen en behaalde aldaar in 1961 het diploma Gymnasium-β. In hetzelfde jaar begon hij zijn studie in de farmacie aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen werd afgelegd in 1964, het doctoraalexamen met bijvak klinische chemie in 1968. De apothekersexamens I en II werden afgelegd in december 1968 en juli 1969.

In oktober 1969 trad hij voor een periode van 5 jaar in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO) te 's Gravenhage. Gedurende dit dienstverband en daarna, verrichtte hij binnen het kader van de Werkgroep Farmacokinetiek onder leiding van Prof. Dr. J. M. van Rossum in het Farmacologisch Laboratorium van de Katholieke Universiteit te Nijmegen onderzoek naar de analyse, de farmacokinetiek en receptorbinding van orale anticonceptiva, in het bijzonder lynestrenol. In 1971 werd een aanvang gemaakt met de onderzoekingen beschreven in dit proefschrift.

In november 1974 vestigde hij zich als apotheker te Cuijk.

Uit gezamenlijk onderzoek zijn de volgende publikaties voortgekomen:

J. M. van Rossum, D. D. Breimer, C. A. M. van Ginneken, J. M. G. van Kordelaar en T. B. Vree:
Gas-liquid chromatography in pharmacology and toxicology: pharmacokinetic analysis limited by the sensitivity of the analytical technique.
Clinica Chimica Acta 34, 311-319 (1971).

J. M. G. van Kordelaar, J. S. Favier en J. P. Kitcher:
Synthesis of the contraceptive progestin lynestrenol-^3H from tritiated estr-4-en-3-one.
Journal of Labelled Compounds 9, 635-642 (1973).

J. M. G. van Kordelaar, M. M. M. Broekman en J. M. van Rossum:
Interaction of contraceptive progestins and related compounds with the oestrogen receptor. Part I: Effect on oestradiol-^3H distribution pattern in the ovariectomized rat.
Acta endocrinologica 78, 145-164 (1975).

J. M. G. van Kordelaar, A. J. M. Vermorken, C. J. M. de Weerd en J. M. van Rossum:
Interaction of contraceptive progestins and related compounds with the oestrogen receptor. Part II: Effect on oestradiol-^3H binding to the rat uterine receptor in vitro.
Acta endocrinologica 78, 165-179 (1975).

J. M. G. van Kordelaar, M. M. M. Broekman, G. J. T. Grutters en J. M. van Rossum:
Pharmacokinetics of lynestrenol in the isolated perfused rat liver.
Submitted for publication.

STELLINGEN

I

De bewering van Braselton et al. (1977), dat de estrogene activiteit van norethindron en het ontbreken van deze werking in megestrol, zoals waargenomen door McPherson et al. (1974), de aromatizering van de eerstgenoemde stof impliceert, is onvoldoende gefundeerd.

Braselton, W. E., Lin, T. J., Mills, T. M., Ellegood, J. O. & Mahesh, V. B., *J. Steroid Biochem. 8,* 9 (1977).
McPherson, J. C., Costoff, A., Eldridge, J. C. & Mahesh, V. B., *Fertil. Steril. 25,* 1063 (1974).
Dit proefschrift.

II

Het is aan twijfel onderhevig, of de K_i waarden van ethynodiol, ethynodiol diacetaat, norethindron, norethindron acetaat en lynestrenol zoals gemeten door Tamaya et al. (1977), een maat zijn voor de affiniteit van deze steroïden tot de estradiol en progesteron receptor.

Tamaya, T., Nioka, S., Furuta, N., Shimura, T., Takano, N. & Okada, H., *Endocrinology 100,* 1579 (1977).
Bouton, M. M. & Raynaud, J. P., *J. Steroid Biochem. 9,* 9 (1978).
Dit proefschrift.

III

De gewoonte om bij klinisch onderzoek naar de toxiciteit van orale anticonceptiva binnen de onderzochte populatie slechts onderscheid te maken tussen een groep van gebruiksters en een groep van niet-gebruiksters, zonder de kwalitatieve en kwantitatieve verschillen in samenstelling van de gebruikte preparaten in aanmerking te nemen, geeft blijk van een onderschatting van de verschillen in de farmacologie van de samenstellende actieve componenten.

Briggs, M. H. & Briggs, M., *NZ Med. J. 83,* 257 (1976).
Edgren, R. A., Jones, R. C. & Peterson, D. L., *Fertil. Steril. 18,* 238 (1967).
Fotherby, K., *Acta endocr. (Kbh.) Suppl. 185,* 119 (1974).
Kontula, K., Jänne, O., Vihko, R., De Jager, E., De Visser, J. & Zeelen, F., *Acta endocr. (Kbh.) 78,* 574 (1975).
Neumann, F., Elger, W., Nishino, Y. & Steinbeck, H., *Arzneim.-Forsch. 27 (I),* 296 (1977).
Victor, A., Weiner, E. & Johansson, E. D. B., *J. Clin. Endocr. Metab. 43,* 244 (1976).
Dit proefschrift.

IV

In onderzoekingen met betrekking tot de farmacokinetiek van orale anticonceptiva is tot heden, zeer ten onrechte, geen aandacht geschonken aan de biologische beschikbaarheid.

Dit proefschrift.

V

De demonstratie van ethinylestradiol in de urine na toediening van norethindron laat geen conclusie toe ten aanzien van de aromatizering van deze stof *in vivo* op basis van de door Braselton et al. (1977) gevolgde werkwijze.

Braselton, W. E., Lin, T. J., Mills, T. M., Ellegood, J. O. & Mahesh, V. B., *J. Steroid Biochem. 8*, 9 (197).
Sisenwine, S. F., Liu, A. L., Kimmel, H. B. & Ruelius, H. W., *Acta endocr. (Kbh.) 76*, 789 (1974).

VI

De omschrijving van het begrip relatieve activiteit (RA) door Sanborn et al. (1976) en de definiëring van de factor R door Katzenellenbogen et al. (1973) zoals voorkomend in de door hen toegepaste formule van Korenman (1970):

$$RAC = \frac{R \cdot RA}{R + 1 - RA}$$

is onjuist.

Katzenellenbogen, J. A., Johnson, H. J. & Myers, H. N., *Biochemistry 12*, 4085 (1973).
Sanborn, B. M., Held, B. & Kuo, H. S., *J. Steroid Biochem. 7*, 665 (1976).
Korenman, S. G., *Endocrinology 87*, 1119 (1970).

VII

De afleiding van de dissociatieconstante door Fillion et al. (1978) voor de binding van serotonine en lysergide aan synaptosomale membranen is onjuist.

Fillion, G. M. B., Rousselle, J.-C., Fillion, M.-P., Beaudoin, D. M., Goiny, M. R., Deniau, J.-M. & Jacob, J. J., *Molec. Pharmacol. 14*, 50 (1978).
Buller, R. E., Schrader, W. T. & O'Malley, B. W., *J. Steroid Biochem. 7*, 321 (1976).

VIII

De inhoud gegeven door Lan & Katzenellenbogen (1976) en Mayer & Komarek (1978) aan het begrip "pharmacodynamie" is aan bedenkingen onderhevig.

Lan, N. C. & Katzenellenbogen, B. S., *Endocrinology 98*, 220 (1976).
Mayer, W. & Komarek, A., *Arzneim.-Forsch. 28 (I)*, 680 (1978).

IX

De risico's en de bijverschijnselen van pijnbestrijding met behulp van anaesthetica en narcotische analgetica in aanmerking genomen, dient het wetenschappelijk onderzoek naar het mechanisme en de praktische mogelijkheden van de analgesie door middel van acupunctuur te worden gestimuleerd.

Mayer, D. J., Price, D. D. & Rafii, A., *Brain Research 121*, 368 (1977).
Pomeranz, B. & Chiu, D., *Life Sci. 19*, 1757 (1976).
Sjölund, B., Terenius, L. & Eriksson, M., *Acta physiol. scand. 100*, 382 (1977).

X

Gezien het belang en de toenemende betekenis van de adviserende taak van de apotheker terzake van een optimale geneesmiddelkeuze door arts en patiënt is een volledige decommercialisering van de apotheek gewenst. Een belangrijke stap in deze richting zou zijn de honorering van de apotheker op basis van een verrichtingensysteem, zoals reeds van toepassing in de ziekenfondspraktijk, ook door te voeren in de tariefvorming voor de particulier verzekerde.

XI

Discussies over de zogenaamde thuis- en ziekenhuisbevallingen zouden moeten uitgaan van gegevens over perinatale mortaliteit en morbiditeit en minder van emoties.

XII

De gewoonte farmacotherapeutica te betitelen als "genees"middel wekt in het bijzonder ten aanzien van de groep der psychofarmaca te hoge verwachtingen bij de patiënt en werkt mede een overconsumptie van deze middelen in de hand.

Nijmegen, 9 juni 1978 J. M. G. van Kordelaar