CYTOCHROME P-450-SUBSTRATE INTERACTION AND HEPATIC DRUG METABOLISM IN THE MOUSE

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VI. Sex and strain differences in the kinetic constants of ethylmorphine demethylation and type I binding to hepatic microsomal cytochrome P-450 in mice. The influence of castration and testosterone pretreatment.
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IX. The relation between the sex-dependency of type I binding of ethylmorphine and the 1-butanol-induced spectral change in mouse liver microsomes.
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X. The use of competitive inhibition of substrate-binding to cytochrome P-450 in the determination of spectral dissociation constants for substrates with multiple types of binding, as illustrated with 1-butanol.
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CHAPTER I. INTRODUCTION.

A drug which has entered the circulation, may be eliminated as such by the kidney and excreted in the urine. This depends on the lipid-solubility (lipophilicity) of the drug. In the kidney, lipid-soluble drugs are reabsorbed by the tubules, whereas water-soluble (hydrophilic) compounds cannot cross the tubular membrane. Upon passing through the liver some lipophilic drugs can be modified by conjugation with polar molecules, in order to be excreted in the bile or the urine, for example in the form of glucuronides. A large number of drugs, however, do not possess reactive groups for conjugation. They might tend to remain in the body, which is, in many cases, undesirable. In the liver, however, an enzyme system is present, which is capable of metabolizing these compounds by introducing polar groups with the aid of molecular oxygen. The metabolites produced may be excreted either directly or after subsequent conjugation.

In 1958 Klingenberg (1) and Garfinkel (2) discovered a carbon-monoxide binding pigment in the endoplasmatic reticulum of liver cells. This pigment was further characterized by Omura and Sato (3,4) in 1964, and found to be a heme-protein, which they named cytochrome P-450. Thereafter, an increasing number of publications reported that cytochrome P-450-containing enzyme systems occur almost universally and have many different functions. High concentrations of cytochrome P-450 appeared to be present in the livers of mammals, where this heme-protein was found to catalyze the hydroxylation of various compounds, such as steroids, fatty acids and xenobiotics (compounds foreign to the body). Especially interesting is its ability to hydroxylate drugs, and it is because of this property that the hepatic cytochrome P-450 system is often referred to as the drug metabolizing enzyme system.

The magnitude and duration of the therapeutic effect of a drug is determined by various factors, apart from the properties of the drug itself. Important are, for example, the route of administration, and the pathway the drug follows through the body. From the above it is evident that the degree to which the drug is attacked by the cytochrome P-450 system is of great importance. This implies that factors which affect this system, may also influence the drug effect. Such factors are, for example, the nutritional status (5), disease (6), genetic variations (7), and, in some species, sex (8). Substances which are deleterious to the enzyme system, or the presence of other drugs, which compete for the same metabolizing enzyme, may lower the

rate of metabolism and potentiate the effect of a drug. Conversely, metabolic activity may be considerably elevated by potent inducers of the enzyme system, such as barbiturates. This leads to a rapid inactivation of the drug, making higher doses necessary.

As stated above, the cytochrome P-450 system not only metabolizes drugs, but a wide variety of compounds, many of which are harmful to the organism. The enzymes are, therefore, often called 'detoxifying'. This is a very misleading term, as it has been discovered that the metabolism of relatively harmless compounds sometimes yields extremely agressive intermediates. Some of these have been identified as the inducers of cancer (9-12).

A study of the mechanisms of drug metabolism may, therefore, be of clinical importance. The unusual properties of the cytochrome P-450 molecule, the intricate assembly of the whole enzyme system, and its action on steroids, drugs and many other compounds, in fact, form an interesting research area to scientists from many disciplines, including biophysics, biochemistry, pharmacology, endocrinology, clinical medicine, and toxicology.

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CHAPTER II. OBJECT AND FRAMEWORK OF THE INVESTIGATIONS.

The main purpose of the studies decribed in this thesis was to investigate the relationships between the binding of substrates to cytochrome P-450, the reduction of the complex formed in this way by NADPH-cytochrome P-450 reductase, and the *in vitro* metabolism of these substrates in mouse liver.

Substrate binding was determined using spectrophotometric techniques, since the formation of characteristic difference spectra (type I spectra) is thought to reflect cytochrome P-450-substrate interaction, which may represent the formation of an enzyme-substrate complex (Chapter III,4.2). Ethylmorphine and, in some experiments, hexobarbital were used as model substrates, because they produce well-defined spectral changes.

It was of particular interest to establish whether the reduction of cytochrome P-450-substrate complex is a rate-limiting step in the reaction sequence, which has been suggested to be the case in rats (Chapter III,4.3). Such information is important, since any influence on the rate of drug metabolism is exerted primarily at the stage of the rate-limiting step. Furthermore, the measurement of the rate-determining factor might, in principle, serve as a test of the functional capacity of the drug metabolizing enzyme system, which could be of clinical importance.

In previous investigations in our laboratory mice have been shown to exhibit a sex difference in the duration of hexobarbital-induced narcosis and in the *in vitro* metabolism of hexobarbital (Chapter III,4.6). In this study sex differences were used as a tool to investigate the relationships between metabolism and components of the reaction chain. The effects of testosterone pretreatment and castration as well as the development of the enzyme system were studied to establish whether sex-dependent parameters are modified in parallel. A second tool was formed by the use of different substrates. Especially interesting in this respect is the metabolism of optical antipodes, such as d- and l-hexobarbital.

Chapters V-VIII concentrate on sex- and strain-dependent differences in the metabolism of ethylmorphine and hexobarbital and the relation of metabolism to type I binding and NADPH-cytochrome P-450 reductase activities. In Chapters IX-XI a closer examination of the type I binding capacity of mouse liver cytochrome P-450 is presented, thereby dealing with the role of tightly binding endogenous substrates and heterogeneity of cytochrome P-450.

CHAPTER III. CYTOCHROME P-450-DEPENDENT MONOOXYGENATION.

In this chapter an introduction to the field of cytochrome P-450-catalyzed reactions is presented. General information on cytochrome P-450, its occurrence, and its role in monooxygenase reactions is contained in sections 1-3. In section 4 the liver microsomal cytochrome P-450 system is described in more detail. Only those topics which are relevant to the investigations described in the following chapters are treated. Particular attention is paid to the organization of the enzyme system, cytochrome P-450-substrate interaction, NADPH-cytochrome P-450 reductase, the kinetics of the monooxygenase reaction, and sex differences in drug metabolism.

1. DEFINITION AND NOMENCLATURE.

The cytochrome P-450-dependent enzyme systems belong to a class of enzymes which catalyze oxidative reactions. Biological oxidations may involve the insertion of oxygen into the substrate molecule. This oxygen may be derived from water, as in the case of β-oxidation of fatty acids (1):

$$-CH_2-CH_2- \xrightarrow{-2H} -CH=CH- \xrightarrow{+H_2O} -CH_2-\underset{\displaystyle OH}{\overset{\displaystyle |}{C}}H-$$

For many substrates, such as apolar aliphatic or aromatic compounds, however, this route is energetically unfavourable. These compounds may be oxidized by molecular oxygen with the aid of enzymes called oxygenases (2-5).

When the reaction requires the incorporation of both atoms of the O_2-molecule, the enzymes are called dioxygenases. The overall reaction is represented by

$$X + O_2 \rightarrow XO_2$$

In many cases only one oxygen atom is utilized, whereas the other is reduced to water. Then the enzyme system is defined as monooxygenase, mixed-function oxygenase, or mixed-function oxidase. An example of such a reaction is

$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O$$

Sometimes the term hydroxylase is used instead of monooxygenase, but this is not ideal, since several other oxidation reactions, such as epoxidations, are performed by these enzymes. The general equation is

$$X + \tfrac{1}{2}O_2 \rightarrow XO$$

The mixed-function oxidase systems are characterized by their terminal oxidase. This is the component which binds and activates molecular oxygen, often a heme-protein. The reduction equivalents are provided by NADPH or NADH via electron transport chains, containing flavoproteins and, in some cases, non-heme iron proteins. An important group of monooxygenases contain cytochrome P-450 as a terminal oxidase.

2. CYTOCHROME P-450.

Cytochrome P-450 is a heme-protein, i.e. a protein with a porphyrin as a prosthetic group. The porphyrin ring contains six-coordinated iron. Four coordination bonds are used up by the porphyrin itself. The nature of the remaining two (axial) ligands is uncertain, and subject to considerable debate (5,6-17). One of them is probably an SH-group, belonging to a cysteinyl residue from the protein part of the molecule. The other ligand may be a nitrogenous group from the protein or an external ligand, such as H_2O, or it may not exist at all. If it exists, it is apparently easily displaced. In the course of the monooxygenase reaction oxygen binds to the iron in this position. The potent inhibitory action of carbon monoxide on monooxygenase activity is due to binding of CO at this site. Also compounds containing nucleophilic groups (e.g. basic amines) bind to the heme in this position.

Cytochrome P-450 is a very unusual cytochrome in that the absorption spectrum of the CO-complex of the reduced form (Fe^{2+}) displays a characteristic peak at 450 nm (18,19). Its name has been derived from this property. This absorbance peak is used for quantitation of the cytochrome. The inhibition by CO can be used as a criterion for the involvement of cytochrome P-450 in an oxidative reaction, since this inhibition can be reversed by irradiation with light of 450 nm, which dissociates CO from the cytochrome (20-22). Cytochrome P-450 is easily inactivated by factors such as heat, salts, detergents, and converted into cytochrome P-420, a form which has its reduced CO absorbance peak at 420 nm (18,19,23-27).

3. CYTOCHROME P-450-DEPENDENT MONOOXYGENASES.

3.1. General features.

Cytochrome P-450-catalyzed reactions have been detected in so many species that they may be assumed to occur universally (2,5,28-31). Apart from mammals (including humans (32)) they have been found in birds (33,34), fish (35,36), insects (37-39), fungi (40), yeasts (41), bacteria (42,43) and higher plants (44,45) (citations refer to reviews or recent examples). The enzyme systems of mammals and bacteria are the ones most extensively studied, the latter because of the relative ease to obtain a pure cytochrome P-450 preparation.

Within the mammal cytochrome P-450 has been found (30) in such diverse organs or tissues as the adrenal cortex, liver, kidney cortex, lung (46), testis, spleen, intestinal mucosa, skin (47), pancreas, placenta, and recently in blood platelets (48) and aorta (49). The first reports on cytochrome P-450-catalyzed reactions concerned C-21 hydroxylation of steroids in adrenal cortex microsomes (20,21). The detection of high cytochrome P-450 activity in the endoplasmic reticulum of liver parenchymal cells opened the way to the discovery of the many functions of cytochrome P-450.

The composition of monooxygenase systems with cytochrome P-450 as the terminal oxidase varies to a certain extent depending on the source. Very schematically, cytochrome P-450-catalyzed oxidations follow the pathway

$$\begin{array}{ccccccc} & \overset{S}{\downarrow} & & & \overset{O_2}{\downarrow} & & \\ P\text{-}450(Fe^{3+}) & \rightarrow & P\text{-}450(Fe^{3+})\text{-}S & \rightarrow & P\text{-}450(Fe^{2+})\text{-}S & \rightarrow & \left(\begin{smallmatrix}\text{inter-}\\ \text{mediates}\end{smallmatrix}\right) \rightarrow P\text{-}450(Fe^{3+}) \\ & & & \overset{e^-}{\uparrow}\ (I) & & \overset{e^-}{\uparrow}\ (II) & \quad + SOH + H_2O \end{array}$$

The major variations are observed in the donation of the electrons via the electron transport chains. In the microorganism Pseudomonas putida both electrons are delivered according to the pathway

$$\text{NADH} \rightarrow \text{flavoprotein} \rightarrow \underset{\text{(putidaredoxin)}}{\text{iron-sulfur protein}} \rightarrow \text{P-450}$$

In adrenal cortex mitochondria both electrons follow the pathway

$$\text{NADPH} \rightarrow \text{flavoprotein} \rightarrow \underset{\text{(adrenodoxin)}}{\text{iron-sulfur protein}} \rightarrow \text{P-450}$$

In liver microsomes the situation is more complicated, as apparently two different electron transport chains are involved. The first electron is donated via

$$\text{NADPH} \rightarrow \text{flavoprotein} \rightarrow \text{'X' ?} \rightarrow \text{P-450}$$

and the second presumably via

$$\text{NADH} \rightarrow \text{flavoprotein} \rightarrow \text{cytochrome } b_5 \rightarrow \text{P-450}$$

The Pseudomonas putida system ($P\text{-}450_{cam}$) has been studied with respect to its activity towards camphor (2). Its components have been obtained in a pure form (50). Much of the known chemistry of cytochrome P-450 has been derived from this system (7).

The reaction mechanisms of the adrenal cortex and liver systems are still far from clear, mainly as a consequence of the membrane-bound nature of these systems, which makes it difficult to obtain the components in a pure form. The system present in adrenal cortex mitochondria is mainly involved in steroidogenesis (51), and appears to be different from other systems with respect to the electron transport chain (52-55). Also cytochrome P-450 may be different: a subunit structure has been demonstrated, which is not known to exist in the liver (56-59). Interestingly, the cytochrome P-450 system present in adrenocortical microsomes bears much resemblance to that in liver microsomes. The flavoproteins involved in the donation of the first electron appear to be immunologically similar, and can replace each other to reconstitute enzymatic activity (52-55). This might indicate that the microsomal and mitochondrial cytochrome P-450 systems form separate classes.

In this thesis the investigations have been confined to the liver microsomal system, which will be described in section 4 of this chapter. It should be kept in mind that the results cannot be simply extrapolated to other cytochrome P-450 systems.

3.2. Substrate specificity, induction, and heterogeneity of cytochrome P-450.

Cytochrome P-450 catalyzes the oxygenation of a large number of different substrates. Many different types of reactions can be distinguished, for example, aliphatic and aromatic hydroxylations, demethylations (N- and O-), epoxidations, etc. (28). The substrates used in this thesis present examples of direct hydroxylation at a C-atom of a cyclohexenyl-ring (hexobarbital),

and of N-demethylation (ethylmorphine). In the latter reaction the $\rangle N\text{-}CH_3$ group is converted into $\rangle N\text{-}H$ by the removal of the methyl group in the form of HCHO.

The idea that the metabolism of each (type of) substrate would require a particular cytochrome P-450 was not too appealing at first. But there is increasing evidence that several types of cytochrome P-450 exist. In the adrenal cortex, for example, cytochrome P-450 is mainly involved in steroid hydroxylation and cholesterol side-chain cleavage (2,51). Cytochromes P-450 have been isolated, which show different specificities with regard to these reactions (56-58,60-62).

A greater diversity of cytochromes P-450 is observed in the liver. Liver microsomal cytochrome P-450 has been known for some time to exist in two forms. Treatment of animals with either phenobarbital or 3-methylcholanthrene both appeared to elevate the cytochrome P-450 content considerably, but the metabolic enzymes of the 3-methylcholanthrene-induced animals exhibited a preference for polycyclic aromatic hydrocarbons. Furthermore, the absorbance peak of the reduced cytochrome P-450-CO complex had shifted to 448 nm. Hence, a different type of cytochrome P-450, called cytochrome P-448 (or sometimes P_1-450 or P-446), was postulated (63-65). This was confirmed with purified preparations (66-68).

Several other cytochromes P-450 have now been characterized, either from animals pretreated with inducers or untreated animals. These cytochromes have been classified according to their immunological properties, gel-electrophoretic mobility or catalytic specificity (69-82). The specificity is not confined to the nature of the substrate, but may involve the introduction of hydroxyl-groups at different positions of a steroid molecule (82). Thus the concept of one cytochrome P-450 for each type of substrate gains ground again. Nevertheless, it is hard to visualize why the organism would possess specific enzymes for the oxygenation of exogenous compounds for which there are no readily recognizable endogenous analogues. The answer might lie in the striking inducibility and adaptivity of cytochrome P-450, as indicated by the following considerations.

Apart from the well-known inducers phenobarbital and 3-methylcholanthrene a great variety of other compounds have been demonstrated to elevate the level of the cytochrome, and in many instances a change in specificity is noticed (83). An extreme case of adaptivity is shown by a microorganism (the yeast Candida tropicalis), which can be forced to grow on alkanes (41,84). A cytochrome P-450 is developed, which attacks C-H bonds in order to transform

the alkanes into useful compounds. It has been speculated that cytochrome P-450 may be one of the oldest heme-proteins developed during evolution, and that it originally functioned as a scavenger of oxygen, a substance which was highly toxic in the primordial anaerobic milieu. Later this protein was retained because of its usefulness as a hydroxylating agent (85). This may explain the versatility of the cytochrome. During the long evolutionary pathway it must have been exposed to various hydroxylatable substances, which may have led to the development of many slightly different heme-proteins and to a mechanism which enables the rapid induction of one specific type.

It is evident that in analyzing drug metabolism data, one has to guard against misinterpretation due to a possible involvement of multiple forms of cytochrome P-450. Also from the investigations presented in this thesis evidence has been obtained for a multiplicity of cytochrome P-450.

4. THE LIVER MICROSOMAL CYTOCHROME P-450 SYSTEM.

4.1. Organization of the enzyme system.

The liver has a large capacity for the metabolism of xenobiotics. Cytochrome P-450, in fact, may constitute about 2% of the total liver protein. In the early fifties the oxidative conversion of drugs had already been recognized, but the major knowledge about the enzymatic process involved has developed during the past ten years. In 1968 Lu et al. (86) succeeded in the solubilization and separation of the enzyme system into three components, which could be reconstituted to regain ω-hydroxylation activity. Further work on the resolution of the system, which also appeared to hydroxylate steroids and xenobiotics, has been comprehensively reviewed by Lu and Levin (87). The essential factors appeared to be cytochrome P-450, a flavoprotein, and a lipid fraction. The flavoprotein was identified with NADPH-cytochrome c reductase, an enzyme already known to be present in the endoplasmic reticulum. The active component of the lipid fraction was demonstrated to be phosphatidylcholine (88). Cytochrome b_5 and NADH-cytochrome b_5 reductase, which have been postulated to function in drug metabolism, were not necessary to reconstitute activity. Therefore, these factors may not be obligatory, but only facilitative.

In recent years purification of cytochrome (89,90) and reductase (91-93) preparations to apparent homogeneity has been achieved, despite severe

difficulties due to the membrane-bound nature of the enzyme system (94). Initially an intermediate electron carrier 'X' had been postulated to explain some discrepancies observed (95), such by analogy to the mitochondrial and bacterial enzyme systems, which contain iron-sulfur proteins. No direct evidence for the existence of this intermediate, however, was obtained. The lipid fraction cannot function as such, as phosphatidylcholine does not possess electron-carrying properties. The function of the lipid may rather be of structural nature, by providing the right mode of association between the component proteins. Although the reconstitution of enzyme activity does not seem to involve the formation of membrane-like aggregates (96), a role of the membrane is conceivable, and is, in fact, supported by many observations (94,97-106).

A few recent studies have provided some indirect evidence, which renewed the discussion about the possible intermediate carrier. (1) Mull et al. (107) solubilized a cytochrome P-450 system from mouse liver microsomes and observed an unknown factor. (2) Careful titration of a purified cytochrome P-450 preparation with the chemical reductant sodium dithionite indicated that two electron equivalents were needed to reduce the preparation, thus indicating the presence of an additional electron acceptor (108,109). A similar suggestion was made by Estabrook et al. (110). (3) Some evidence has been obtained for the existence of an iron-sulfur centre in rat liver microsomes (111,112), indicating the presence of an iron-sulfur protein, as observed in the bacterial and adrenocortical cytochrome P-450 systems.

4.2. Spectral interaction of substrates with cytochrome P-450.

Substrates of the microsomal mixed-function oxygenase (as well as many other compounds not yet known to be metabolized) cause characteristic spectral changes when added to a microsomal suspension (113-115). This has been identified with shifts of the absorbance peak of the oxidized form of cytochrome P-450 (116). The spectral changes are measured by means of difference spectroscopy (Chapter IV,2). The magnitude of these spectral changes depends on the concentration of cytochrome P-450 as well as of the substrate, and has been attributed to binding of the substrate to the cytochrome (115). Several types of difference spectra can be observed, which are classified according to the position of the absorbance maxima and minima (Fig. III,1).

A type I spectrum is elicited by a great variety of compounds, including fatty acids, steroids, and drugs (e.g. hexobarbital, ethylmorphine, amino-

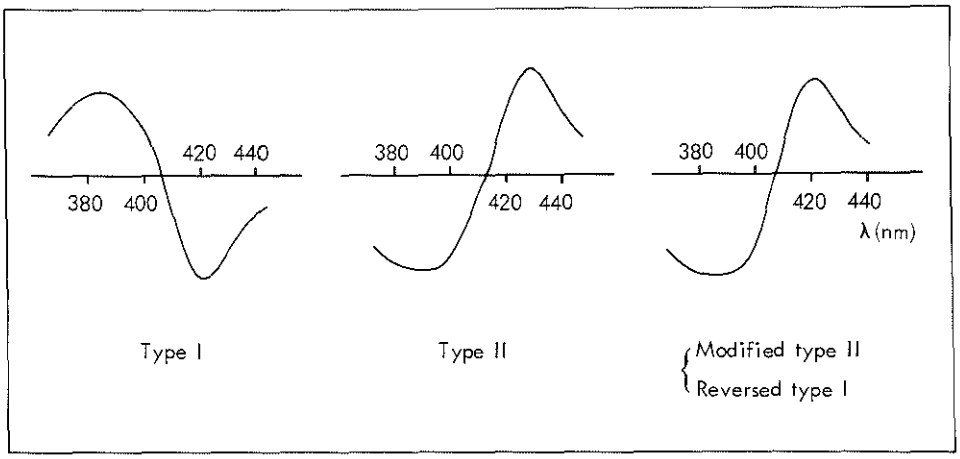

Fig. III,1. Main types of difference spectra.

pyrine). These compounds do not seem to have anything in common, except that they are lipophilic and that they are metabolized (with a few exceptions (117)). The spectrum is presumed to reflect binding to a hydrophobic part of the cytochrome, which induces a conformational change affecting the physical properties of the heme (118,119). There may be a certain degree of binding specificity, due to the existence of different types of cytochrome P-450. It had already been recognized by Diehl et al. (120) that only a fraction of the total amount of cytochrome P-450 present is involved in type I binding. Grasdalen et al. (72) showed that different type I substrates may bind to different classes of type I binding sites. Also in this thesis evidence is presented for this (Chapter XI). Type I spectra have also been observed with solubilized cytochrome P-450 (121). Results with purified preparations suggested that the lipid fraction is not essential for type I binding (87). Recent experiments with extensively purified cytochrome P-450, however, have demonstrated that lipid is needed to obtain maximal binding (102). This emphasizes the importance of the lipophilicity of compounds which show type I binding.

There exists some controversy as to whether the type I spectral change is a prerequisite for a substrate to be metabolized. Two observations seem to contradict this proposition. First, some substrates are metabolized despite the appearance of different types of spectral change. It is probable, however, that a type I component is hidden in such spectra (122-126). Second, treatment

of microsomes with phospholipase C seems to destroy type I binding, whereas metabolism is only partly inhibited (127). This, also, may be only an apparent discrepancy. Phopholipase C treatment has been shown to eventually result in the release of free fatty acids, which competitively inhibit the metabolism of other type I substrates (128,129). Since they themselves elicit the type I spectral change, type I binding of other substrates will be hardly detectable in preparations containing fatty acids, despite the competitive nature of the inhibition.

A type II spectrum is observed with compounds which have a nucleophilic group, such as basic amines (115). They react directly with the heme at the position of the sixth ligand, thereby interfering with oxygen binding. The display of such a spectral change does not preclude the metabolism of the substrate concerned, as examplified by aniline. As it is hard to understand how such substrates can be metabolized at a site where they inhibit the binding of oxygen, it is believed that they additionally bind to the type I site. A type I component may, in fact, be observed in several of these spectra (122-126).

A third type of difference spectrum resembles the type II spectrum to a certain extent, and has, therefore, been defined as 'modified type II' (115). With some substrates (phenacetin, lower alcohols) the spectrum seems to be the reverse of the type I spectrum (reverse type I or RI spectrum (123)). A suitable division into subclasses, however, is not readily made (also definitions such as IIA and IIB have been used (130)). The nature of this third class of binding sites is subject to controversy. It has been proposed that the spectral change merely reflects the release of endogenously bound type I substrates (120) (this is discussed more extensively in Chapter IX). On the other hand, the sometimes anomalous shape of the spectrum suggested that it might be the result of superimposition of type I and type II spectra (118). Evidence is now accumulating that the spectra indeed resemble the type II spectra in that they are elicited by the interaction of nucleophilic substrates with the heme, and that a varying nucleophilicity is the cause of the variation in the absorbance maxima and minima (17,130,131). The fact that such substrates bind directly to the heme implies that the maximal magnitude of the spectral change is related to the total cytochrome P-450 content, which is in contrast to the type I spectrum.

4.3. NADPH-cytochrome P-450 reductase.

The donation of the first electron to cytochrome P-450 (see 3.1) is an intriguing feature of the monooxygenase reaction chain. This electron is derived from NADPH and is transferred via the flavoprotein NADPH-cytochrome c reductase. An additional redox component may be involved, but for this no conclusive evidence is available yet (4.1). The activity of the reductase is usually assayed with respect to cytochrome c, which is apparently an artificial substrate, since it does not occur naturally in the endoplasmic reticulum. The involvement of this enzyme in the reduction of cytochrome P-450 was evidenced by (1) the inhibition of drug metabolizing activity by cytochrome c or specific antibodies against the flavoprotein (52,132,133), and (2) the reconstitution of enzyme activity using purified flavoprotein preparations (87). NADPH-cytochrome c reductase does not show heterogeneity, as cytochrome P-450 does (134-136). Induction with phenobarbital or 3-methylcholanthrene did not alter its specificity or immunological and gel-electrophoretical properties.

The reduction of cytochrome P-450 is studied under anaerobic conditions in a CO-atmosphere. The addition of NADPH to the microsomal preparation causes the rapid appearance of the reduced cytochrome P-450-CO complex, which can be followed spectrophotometrically (Chapter IV,3). The kinetics of this reaction are intricate and not yet fully resolved. The initial rate of the reduction appeared to be stimulated by the presence of type I substrates (type II substrates cause a decrease in reduction rate, which is mechanistically interesting, but which is not discussed in this thesis)(138). Moreover, a relationship between the magnitude of this stimulation and the rate of ethylmorphine demethylation was observed. This led to the hypothesis that the reduction might represent a rate-limiting reaction (137,138).

The reaction proceeds in two phases, a rapid and a slow phase, which occur simultaneously. The relationship between reductase stimulation and ethylmorphine demethylation suggested that the observed fast phase does not participate in the metabolic reaction. The stimulation of reductase activity by type I substrates would be due to an activation of the reduction of cytochrome P-450 which is reduced in the slow phase in the absence of substrate (138). If this endogenous rate of the slow phase is negligible with respect to the rate observed in the presence of substrate, the reductase stimulation approximates the actual rate of cytochrome P-450 reduction. Later it was suggested that the fast phase occurring in the absence of substrate involved

the reduction of cytochrome P-450 bound to endogenous substrates, and that the increase in initial rate observed after addition of type I substrate was due to an increase in the amount of cytochrome P-450 being reduced in this fast phase as the result of the formation of a cytochrome P-450-substrate complex (120). Other observations, however, have indicated that the substrate-induced stimulation of reductase activity may be the result of an increase in the rate constant of the fast phase rather than of the amount of cytochrome P-450 being involved in this phase (139).

According to the most recent theory on the kinetics of cytochrome P-450 reduction the two phases relate to cytochrome P-450 molecules which are differently located in the microsomal membrane. The fast phase would represent the reduction of cytochrome P-450 molecules arranged in a cluster around a central flavoprotein molecule, whereas the slow phase reflects the reduction of cytochrome P-450 molecules which are not associated with the cluster (106). Similar cluster models had been suggested before for the functioning of the cytochrome P-450 system as a whole (98,100,105).

Several investigations have demonstrated a relationship between type I binding, reductase stimulation and metabolic activity. Especially in the case of ethylmorphine a relationship between reductase stimulation and demethylation in rats is consistently observed (138,140-142). This relationship appears to be stoichiometrical. This strongly suggests that a cytochrome P-450, which is reduced at a negligible rate, is activated to take part in the demethylation reaction. Reductase stimulation might, therefore, be a measure of the reduction of cytochrome P-450-substrate complex. If the stimulation is only due to an increase in the rate constant of the fast phase, this result must be fortuitous.

The role of NADPH-cytochrome P-450 reductase is discussed further in Chapter XII, in particular with regard to the question, which parameter of reductase activity adequately reflects the reduction of cytochrome P-450-substrate complex.

4.4. Cytochrome b_5-dependent electron transport.

Microsomes contain an electron transport chain consisting of the hemeprotein cytochrome b_5 and NADH-cytochrome b_5 reductase, which is active in fatty acid desaturation (143,144). Some observations, such as the synergistic effect of NADH on the NADPH-driven monooxygenase reaction (145-147), have led to the hypothesis that the second electron, which is needed to activate the

oxygen, might be provided via this system. On the other hand, cytochrome b_5 and the reductase did not appear to be essential to reconstitute an active enzyme system (see 4.1). The possible roles of cytochrome b_5 have been reviewed by Schenkman (148), and an extensive discussion of this problem is beyond the scope of this thesis.

4.5. Reaction scheme and kinetic behaviour.

The cytochrome P-450-catalyzed monooxygenase reaction (depicted in Fig. III,2) has long been considered to follow simple Michaelis-Menten kinetics, according to the equation

$$v = \frac{V_{max}.[S]}{[S] + K_m} ,$$

in which K_m would reflect the dissociation constant K_s of the enzyme-substrate complex. The formation of this complex is thought to be represented by the type I spectral change, and is determined according to the equation

$$\Delta A = \frac{\Delta A_{max}.[S]}{[S] + K_s}$$

The similarity between K_m and K_s for some substrates led to the suggestion that the reaction step following the formation of the enzyme-substrate complex is rate-limiting. This becomes evident from the following scheme (E = enzyme or cytochrome P-450, S = substrate, P = product, k = rate constant):

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} ES_{reduced} \rightarrow \ldots\ldots\ldots \rightarrow E + P$$

If the steps beyond k_3 are very fast, K_m is approximated by

$$K_m = \frac{k_2 + k_3}{k_1} \qquad \text{with} \qquad \frac{k_2}{k_1} = K_s$$

k_3 is sometimes low, as compared with k_1 and k_2. In that case K_m is equal to K_s, otherwise K_m will exceed K_s to a certain extent.

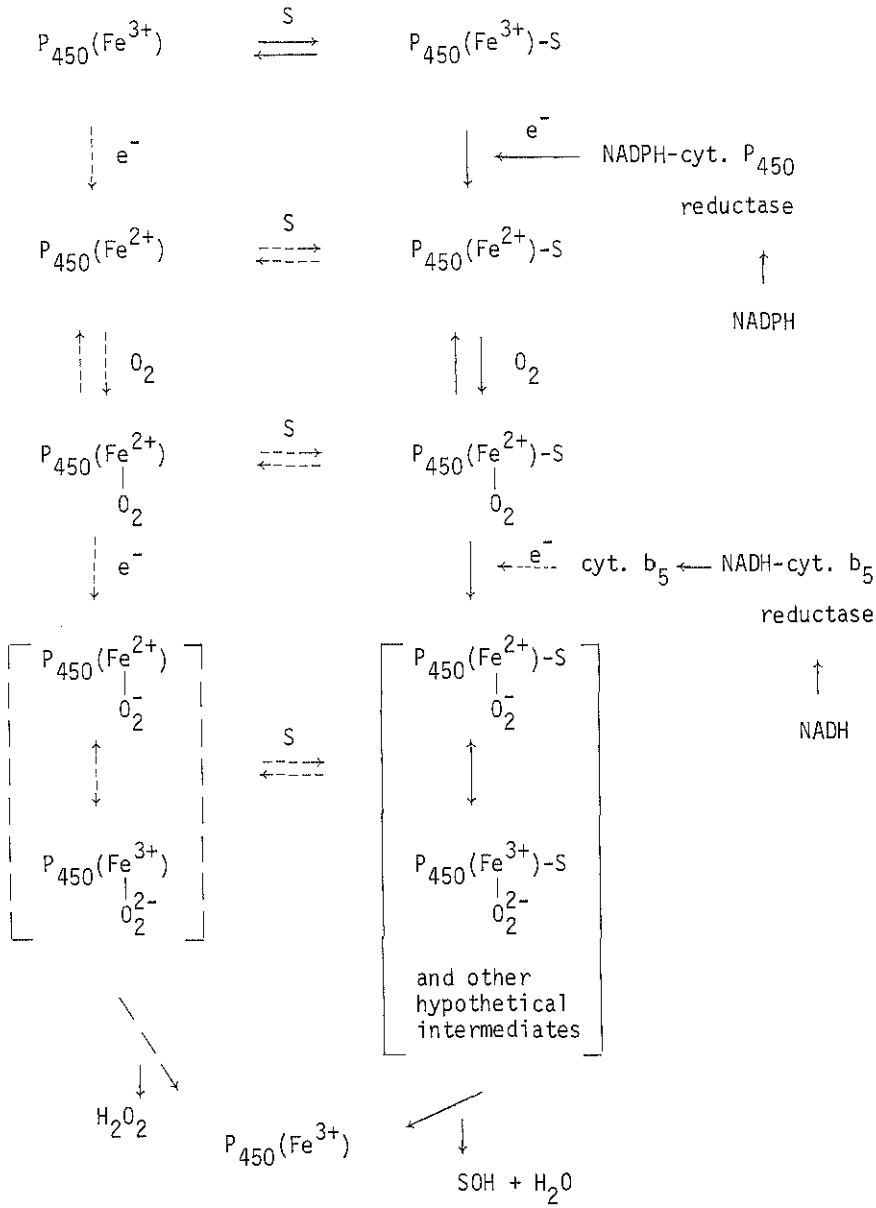

Fig. III,2. Reaction scheme of cytochrome P-450-catalyzed monooxygenation, including a hypothetical cycle not involving substrate.

The reduction may be considered to be a rate-limiting step, if variations in reduction rate correlate with variations in the overall metabolic reaction.

The observation that double reciprocal plots of 1/v vs. 1/S are usually straight lines, seems to indicate that the kinetic behaviour of the oxidation reaction is simple. Hayes et al. (149) observed a concentration dependent K_m and V_{max} of ethylmorphine demethylation in rats, but this was attributed to the presence of two separate demethylases. Pederson and Aust (150) observed non-linear kinetics with aminopyrine demethylation, but this may be attributed to the fact that aminopyrine is demethylated at two positions.

Some observations, however, suggest that the reaction mechanism should be more complicated. The finding that after phenobarbital treatment an oxygenated intermediate is detectable in rat liver microsomes, could imply that at least one other slow step occurs in the reaction sequence (110). Also the measurement of steady-state levels of oxidized cytochrome P-450 suggested the presence of a certain amount of intermediate forms (132). Furthermore, cytochrome P-450 is reduced endogenously, i.e. without the addition of substrate. This may be attributed to the presence of endogenous substrates, but this is not definitively proven. Reversible substrate complex formation with intermediate forms of cytochrome P-450 is difficult to establish. It has been suggested that this may occur, but it may be of less importance.

Gillette et al. (132) have extensively discussed the implications of these considerations for the kinetic behaviour of the monooxygenase reaction. They demonstrated that in certain limiting cases the rate equations may yield linear Lineweaver-Burk plots. This linearity will be observed, if the substrate does not equilibrate with any of the intermediate forms of cytochrome P-450. And in case these equilibria do occur, it may still be noticed, when the rates of the reaction steps in the absence of substrate are zero or equal to those in the presence of substrate. It is not unrealistic to assume that one of these conditions is fulfilled, at least with regard to the reduction by NADPH-cytochrome P-450 reductase, i.e. either the reduction rate of unbound cytochrome P-450 is negligible, or the rate of reduction of substrate-bound cytochrome P-450 is comparable to that observed with the unbound form. This becomes apparent from the following considerations.

As discussed in section 4.3, the initial rate of reduction observed without added substrate (which is approximately the rate of the fast phase), may represent (1) the reduction of cytochrome P-450 which is not active in metabolism, (2) the reduction of cytochrome P-450 bound to endogenous substrates, or (3) the genuine rate of reduction of unbound cytochrome P-450. In cases

(1) and (2) the unbound form of (catalytically active) cytochrome P-450 is reduced in the slow phase of reduction, and thus its reduction rate is negligible. In case (3) the reduction rates of the bound and unbound forms do not differ greatly, since the stimulation observed after addition of substrate is relatively small. On the basis of these considerations Gillette et al. (132) explained the fact that often linear Lineweaver-Burk plots are observed. Nevertheless, it should be recognized that the curvature in such plots may be small and difficult to detect.

It can be calculated that in all limiting cases K_m should reflect K_s, if the first reduction is rate-limiting, except when the reaction sequences in the presence and absence of substrate have the same rate constants and, in addition, the intermediate forms are in equilibrium with each other. In that case K_m reflects the dissociation constant of the active oxygen-cytochrome P-450-substrate complex.

4.6. Sex differences.

Sex-dependent drug metabolism has been reported to occur only in rats and mice, and not, for example, in guinea pigs, hamsters or rabbits (151). The sex-dependency in mice is considered to be small (151) and is not observed consistently (strain-dependent) (152-155). The origin of sex differences has been studied in detail in rats only.

The high activity of the drug metabolzing enzyme system in male rats as compared with females has been attributed to a stimulatory action of androgen, which becomes apparent during sexual maturation (141,151,156-161). This action is reversible, as demonstrated by castration or testosterone treatment (151,159,161). Estrogens do not seem to have a direct effect, but merely impair the action of androgen (151). The response to androgen during puberty seems to be imprinted by neonatal androgen (161,162). The degree of responsiveness, however, seems to vary with the substrate studied.

A great deal of the knowledge about these hormonal mechanisms has been obtained from studies on the pattern of steroid metabolism (163-165). In many of these reactions, in particular the hydroxylations, cytochrome P-450 is involved, so that the results may be of significance for drug metabolism as well. Recent investigations have indicated that the mechanisms which regulate the responses to androgen in rats, may be extremely complicated. The hypophysis, the hypothalamus, as well as androgen receptors in liver cytosol, have been proposed to be involved in these processes (166-170).

The sex differences which we have observed in mice, are the reverse of those observed in rats. Yet they seem to arise through the action of androgens, which in this case is inhibitory (153-155). Schriefers et al. (171) have indicated that the responses to androgen might be species-specific. In this thesis the origin of these sex differences is discussed in the light of our findings, although we realize that other studies would have to be performed to provide a full answer.

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CHAPTER IV. METHODOLOGICAL ASPECTS.

1. ASSAY SYSTEMS FOR DRUG METABOLIZING ENZYMES.

In order to study the properties of the drug metabolizing enzyme system it is of advantage to study isolated enzymic function, thus making the metabolic activity independent of factors such as blood-flow, drug uptake, drug transport, cofactor availability, etc.. This is accomplished by using cell homogenates, homogenate subfractions, or purified enzymes.

Cell homogenates are readily prepared, but are somewhat artificial, since in these systems cell constituents are brought together, which are separated in the intact cell. Under carefully controlled conditions homogenates may be useful, especially when the amounts of tissue available are too low to allow fractionation (liver biopsy samples) (1). They have the disadvantage that spectral measurements are difficult to perform, because of the high turbidity and the presence of mitochondrial pigments. Nevertheless, a reliable measurement of cytochrome P-450 contents seems to be possible (1).

The fraction obtained after centrifugation of a homogenate at 9000 g for 20 minutes is devoid of cell debris, nuclei, cell membranes and mitochondria. This system, simply called 9000 g supernatant, is often used for the assay of drug metabolic activity. Spectral measurements may be perturbed by the presence of pigments from ruptured mitochondria or hemoglobin (if the liver has not been perfused before homogenization).

After further centrifugation of this supernatant at 75,000 g for 90 min. (or, for example, 60 min. at 105,000 g) the microsomal fraction is obtained in the form of a pellet. This fraction mainly represents the endoplasmic reticulum, which containes the drug metabolizing enzymes. A suspension of this pellet is the most widely used assay system for the characterization of drug metabolizing enzymes. Although (after adequate supply of cofactors) the metabolic rates obtained with this fraction sometimes equal those observed with the 9000 g supernatant (2), the reaction rate is often lower. We have observed this with the hydroxylation of hexobarbital. It has been suggested that this might be due to a soluble protein which is lost during centrifugation (3).

The constituent enzymes may be solubilized by means of treatment with detergents, separated and further purified. The availability of pure enzymes is essential for the establishment of their chemical properties and the

understanding of the modes of interaction. It may also provide insight into the specificity of substrate-binding. Its usefulness, however, should not be overestimated. The cytochrome P-450 system is embedded in the microsomal membrane, and its activity has been shown to be lipid-dependent. It is conceivable that the membrane structure determines enzyme activity to a certain degree (Chapter III,4.1). The use of a relatively crude preparation, such as the microsomal fraction, for determining enzyme characteristics is, therefore, justified, provided the limitations are recognized (4-6).

The availability of basic knowledge about the enzymic reactions makes it possible to study the influence of the factors mentioned above (blood flow, drug transport, etc.) on the in vivo rate of metabolism. The use of isolated liver cells has yielded promising results in this respect (7-10). Furthermore, recent studies with the isolated perfused liver have demonstrated that the hepatic extraction ratio may be predicted from K_m and V_{max} values determined in vitro (11).

2. THE MEASUREMENT OF DRUG-INDUCED SPECTRAL CHANGES.

Changes in the absolute spectrum of cytochrome P-450 cannot be measured directly using microsomal suspensions. The large background turbidity as well as the magnitude of the absolute spectrum of cytochrome P-450 itself hamper the accurate recording of relatively small absorbance changes induced by drug binding. This problem can be overcome by employing the technique of difference spectroscopy using a spectrophotometer which is able to record the difference in absorbance between a sample and a reference cuvette containing turbid samples (12). A baseline of zero absorbance is established with the microsomal suspension divided between both cuvettes and then the substrate is added to the sample cuvette. A shift in the absorbance peak from wavelength λ_1 to λ_2 is then visualized as a decrease in absorbance at λ_1 and an increase at λ_2 (Fig. IV,1). The magnitude of the spectral change may be defined as either the peak-to-trough difference or the height of the peak (or trough) relative to an isosbestic point. An isosbestic point occurs at a wavelength at which the absorbance remains constant when varying concentrations of the drug are added. We have adopted the first method, which, in fact, is the one most commonly used in drug-binding studies. Differences in turbidity and changes in the cytochrome P-450 concentration between sample and reference cuvettes must be avoided, as they may give rise to spectral artifacts. This

is accomplished by the addition of the substrate in very small volumes, or by adding equivalent volumes of buffer to the reference cuvette and equally stirring the contents of both cuvettes after each addition.

A second method of measuring absorbance changes can be used if one is familiar with the type of spectrum to be expected, and only interested in the

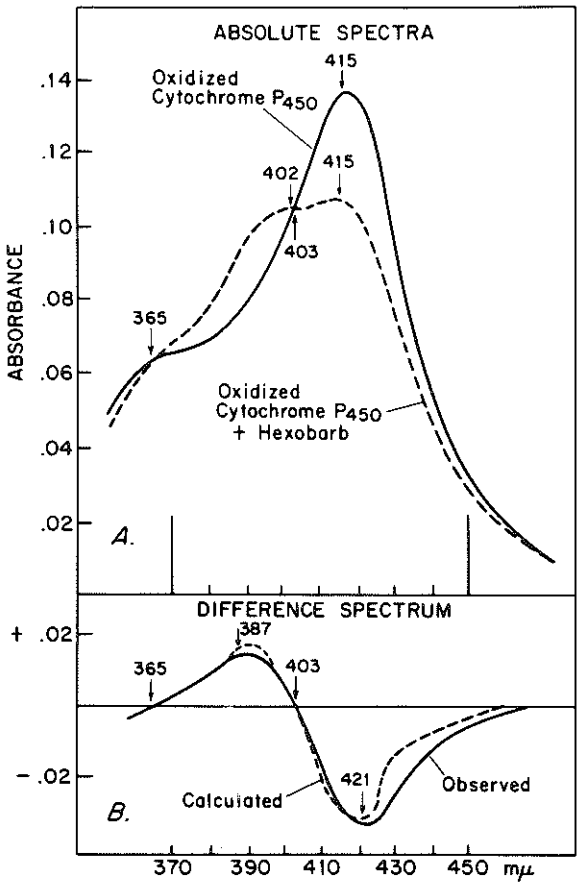

Fig. IV,1. Type I spectrum after addition of hexobarbital (10^{-4} M) to the microsomal preparations. Lower part of the picture: ——— difference spectrum of a microsomal suspension (∿2 mg protein/ml) from livers of phenobarbital-treated rats in sample and control cuvette, + 10^{-4} M hexobarbital in sample cuvette. - - - Calculated curve from the upper part of the figure between the spectrum of oxidized cyt. P-450 and oxidized cyt. P-450 + hexobarbital (Reproduced from Remmer et al. (13)).

magnitude of the spectral change. A spectrophotometer which is designed for dual wavelength measurements is employed. Two beams of light of different wavelengths are directed together through a single cuvette. The wavelengths are adjusted to the absorbance peak and trough (or isosbestic point). After compensating for the absorbance difference between these two wavelengths the substrate is added and the absorbance recorded. In this way rapid titrations can be performed. Changes in turbidity and cytochrome P-450 concentration during the measurements, however, may cause serious pitfalls in this case, for example, as a consequence of settling of the microsomes, or simply by dilution of the sample. The absorbance due to turbidity increases exponentially with increasing wavelength, which effect becomes prominent in the region of 400 nm. It is our experience that the recording of type I spectral changes (385 vs. 420 nm) may easily yield artifacts.

The binding of the drug to cytochrome P-450 can be quantitated using the laws of enzyme-substrate or drug-receptor interaction:

$$\Delta A = \frac{\Delta A_{max} \cdot [S]}{[S] + K_s}$$

K_s represents the dissociation constant of the drug, or the concentration at which half of the binding sites are occupied. ΔA_{max} is the maximum spectral change, and is a measure of the total number of binding sites. These constants

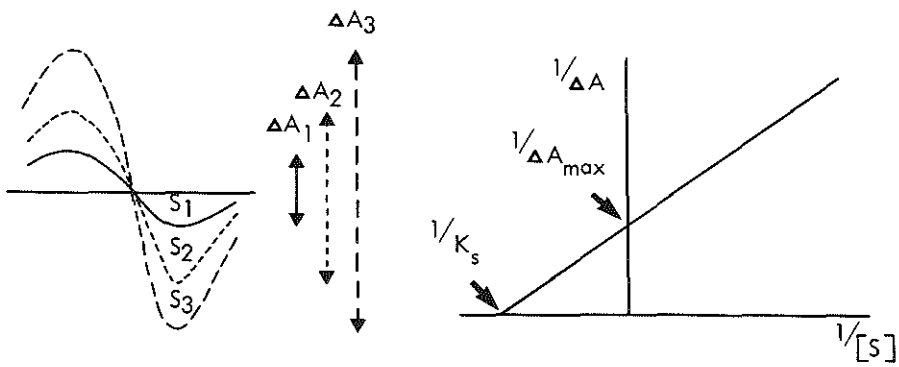

Fig. IV,2. Determination of binding constants (spectral dissociation constant K_s, and maximum spectral change ΔA_{max}), using a double reciprocal plot.

can be calculated from a double reciprocal plot (Fig. IV,2) or by using the method of Wilkinson (14), which fits the data directly to the equation of a hyperbole after a provisional estimation of the constants by a weighted linear regression analysis. We have used the latter method, as it puts less emphasis on the less accurate data points (at the lower substrate concentrations).

3. THE ASSAY OF NADPH-CYTOCHROME P-450 REDUCTASE ACTIVITY.

The assay of the reduction of cytochrome P-450 by NADPH in liver microsomes has been described by Gigon et al. (15). This reduction should be examined under anaerobic conditions in a carbon monoxide atmosphere. The assay is based on the characteristic absorbance peak of the reduced cytochrome P-450-CO complex (Chapter III,2). Since CO binds only to the reduced form of cytochrome P-450 it is essential that the rate of CO-binding does not interfere with the rate of reduction. Several investigations have indicated that CO-binding is indeed a very fast reaction (15-19), and that the binding affinity is high (20,21).

The microsomal suspension may be made anaerobic by bubbling with CO, which has been passed through an alkaline dithionite solution (0.5% sodium dithionite and 0.05% sodium anthraquinone-2-sulfonate in 0.1 N NaOH) to remove traces of oxygen. For some studies an additional treatment with either a glucose oxidase/glucose/catalase system is needed, or alternatively a treatment with electron transport particles (beef heart mitochondria) supplemented with succinate (in the absence of carbon monoxide). It has been demonstrated, however, that the initial rate of reduction was not affected by traces of oxygen (15,22). Therefore, bubbling with CO for 5 minutes was considered to be sufficient for the reductase assay (prolonged treatment did not yield any improvement). An instrumentation analogous to the Aminco anaerobic cuvette (A1-65085) was used as a reaction vessel (Fig. IV,3).

Bubbling was performed in the cuvette, which was placed in a waterbath, thermostatically controlled at 30°C. The stopcock-plunger assembly containing the NADPH-solution as a starter was mounted on the cuvette and CO was led over the suspension for an additional one minute. Then the stopcock was turned, which closed the vessel. The cuvette was then placed in the thermostatically controlled (30°C) spectrophotometer (Aminco DW/2) and allowed to equilibrate for 1 minute. A level of zero absorbance was established. Then

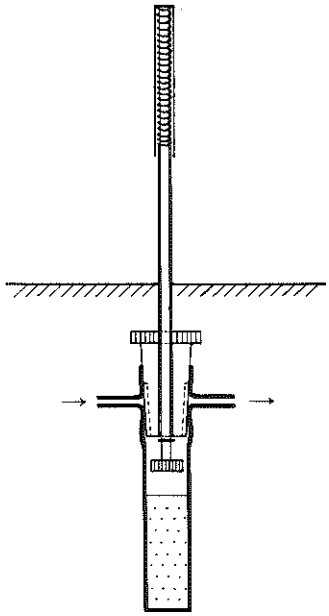

Fig. IV,3. Anaerobic cuvette. Further description in the text.

the plunger was rapidly depressed and the reaction started. Settling of microsomes during the time of equilibration did not introduce a mixing artifact. This was verified by the observation that stirring (with a magnetic stirring rod) did not affect the reaction profile.

The best estimate of the initial reduction rate was obtained by determining the absorption at a fixed time interval after the depression of the plunger (indicated by the rapid deviation from the line of zero absorbance). It was observed that when running a blank with only buffer in the plunger the absorbance arrived at a constant level after approx. 1.2 sec. The absorbance at 1.5 sec after mixing was, therefore, taken as the most reliable estimate of the initial rate. This value was corrected for the small change in absorbance caused by dilution of the preparation with 50 µl buffer, which is the volume in which NADPH was added.

When the reductase activity was measured in the presence of a type I substrate, which stimulates the rate of reduction, this substrate was added to the microsomes prior to the bubbling with CO. There has been one study which indicated that the addition of ethylmorphine together with NADPH

resulted in a much smaller stimulation as compared with prior addition to the microsomes (23). We did not observe this effect, which is, in fact, hard to understand, since the combination of type I substrates with oxidized cytochrome P-450 occurs very rapidly. Half-times of 15 msec (for hexobarbital at 4°C) (24) and 35 msec (for benzphetamine at 7°C) (25) have been reported.

The stimulation of reductase activity (Δ_{red}) was simply defined as the difference between the initial rates in the presence and absence of substrate.

The NADPH concentrations used, as described in the literature, show considerable variation, and the same is observed with the protein concentrations. A concentration of 0.48 mM NADPH was routinely used in our experiments. Table IV,1 and Fig. IV,4 show that this leaves a safe margin, since the protein concentration was always approx. 1 mg/ml (∿1 nmol P-450/mg protein). Some authors use NADPH-generating systems, but Gigon et al. (1) demonstrated that there is no essential difference between these two methods.

Table IV,1. Effect of NADPH-concentration on the initial rate of reduction of cytochrome P-450 by NADPH-cytochrome P-450 reductase (female mice).

NADPH concentration (mM)	basal reduction rate *	reduction rate in the presence of 1 mM ethylmorphine *
0.48	9.67 ± 0.13[a]	16.01 ± 0.13[b]
0.24	9.41 ± 0.06	15.85 ± 0.14[c]
0.096	9.02 ± 0.06[a]	15.35 ± 0.08[b,c]

* Values are expressed as nmol reduced/nmol P-450, and represent means ± SE of 3 experiments.
a,b,c Significant differences, $p<0.05$ (Student's t-test).

It is necessary to keep factors, such as temperature, ionic strength, pH, and buffer controlled, since considerable variation in reductase activity might occur (26,27). As an example we have observed that the use of Tris or phosphate buffer influences the stimulation produced by hexobarbital (Table IV,2). Interestingly, the basal activity was not affected. Although this is not of practical importance, this phenomenon might deserve further investigation.

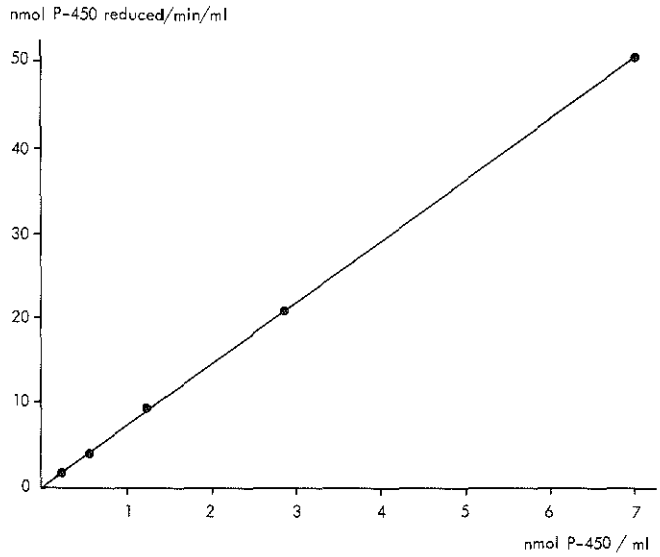

Fig. IV,4. Linearity of the initial rate of cytochrome P-450 reduction with the concentration of microsomes (marked by their cytochrome P-450 content). Microsomes from male mice were used. Data points represent means of duplicate determinations.

Table IV,2. Effect of buffer on the initial rate of reduction of cytochrome P-450 by NADPH-cytochrome P-450 reductase (female mice).

buffer	basal reduction rate *	reduction rate in the presence of 1 mM hexobarbital *	reductase stimulation *
Tris (0.05 M, pH 7.5)	8.32 ± 0.15	13.26 ± 0.25[a]	4.94
Phosphate (0.1 M, pH 7.4)	8.29 ± 0.15	12.07 ± 0.20[a]	3.78

* Values are expressed as nmol reduced/nmol P-450, and represent means ± SE of 4 or 3 experiments, in the absence or presence of hexobarbital respectively.
a: Significantly different from each other, $p<0.05$ (Student's t-test).

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CHAPTER V. SEX- AND STRAIN-DEPENDENT HEPATIC MICROSOMAL ETHYLMORPHINE N-DEMETHYLATION IN MICE: THE ROLES OF TYPE I BINDING AND NADPH-CYTOCHROME P-450 REDUCTASE.

SUMMARY.

The roles of type I binding and NADPH-cytochrome P-450 reductase in ethylmorphine demethylation were investigated in two strains of mice, using sex differences in these activities as a tool. In the CPB-SE strain, females metabolize ethylmorphine faster than males. Sex differences in cytochrome P-450 content and endogenous NADPH-cytochrome P-450 reductase activity were too small to account for this. On the other hand, the differences in the magnitudes of type I spectra and ethylmorphine-induced enhancement of cytochrome P-450 reduction were considerably larger than those in the rates of demethylation. All parameters, except endogenous cytochrome P-450 reduction, were modified in a similar way by testosterone pretreatment: in females they were depressed to the male level, whereas in males they remained unchanged. Castration had no effect in females and enhanced the activities in males. The CPB-V strain exhibited little or no sex differences in ethylmorphine demethylation, cytochrome P-450 content and endogenous cytochrome P-450 reduction. Testosterone pretreatment had little or no influence on these activities. Type I binding and reductase stimulation, however, showed sex differences, comparable to those observed in the CPB-SE strain, which were also abolished by testosterone. A relationship between reductase stimulation and type I binding was observed, which was, apparently, independent of sex or strain. It is concluded that androgen primarily influences the amount of cytochrome P-450-substrate complex formed, but that the reduction of this complex is not rate-limiting in the demethylation of ethylmorphine.

INTRODUCTION.

The activity of the hepatic microsomal cytochrome P-450-dependent drug metabolizing enzyme system is sex-dependent with regard to type I substrates (1) in rats and mice. This sex-dependency has been extensively studied in rats (see 2 for review), and seems to be the result of a stimulatory action of androgen, which elevates the activity in males. In mice, sex differences

are not consistently observed (2-6) and appear to be strain-dependent (7,8). We have directed our attention to mice, since this species is seldomly studied and the sex difference appears to be opposite to that observed in rats, i.e. female mice metabolize hexobarbital and ethylmorphine faster than males (8-11). We have suggested, on the basis of the effects of testosterone pretreatment and castration, that this paradox in mice may be attributed to an inhibitory action of androgen (8).

In this study the N-demethylation of ethylmorphine in mice was further investigated. According to the hypothetical reaction scheme (e.g. 12) the initial reaction steps involve the binding of substrate to cytochrome P-450 and the subsequent reduction of the cytochrome P-450-substrate complex by NADPH-cytochrome P-450 reductase. The spectral change observed upon the addition of the substrate to microsomes is presumed to reflect the formation of this complex (1). The reductase step has long been considered to be rate-determining in rats, after Gigon et al. (13) had observed a parallel between the stimulation of reductase activity produced by type I substrates and their rate of metabolism. This stimulation was presumed to represent the rate of reduction of the cytochrome P-450-substrate complex.

In a previous investigation in our laboratory the cytochrome P-450 content, NADPH-cytochrome c reductase activity and magnitude of type I spectral interaction were shown to be unrelated to the rate of hydroxylation of hexobarbital in mice of the CPB-SE strain (8). Hexobarbital hydroxylation, however, was measured in 9000 g supernatant, whereas the other parameters were determined in microsomes. Furthermore, the assay of NADPH-cytochrome P-450 reductase was not included.

In the present study the relationships between NADPH-cytochrome P-450 reductase activity, type I binding, cytochrome P-450 content and ethylmorphine N-demethylation were investigated in liver microsomes from mice of the CPB-SE strain. For this purpose the existence of sex differences is a very useful tool. The comparison of sex differences alone, however, is not sufficient. It is necessary to demonstrate that different reaction steps can be modified in parallel. Therefore, the effects of testosterone pretreatment and castration were also examined.

Previously, we have observed that the CPB-V strain, in contrast to the CPB-SE strain, does not exhibit a sex difference in ethylmorphine demethylation (8), but, nevertheless, a sex difference in type I binding is apparent. On the basis of this interesting finding the CPB-V strain was also investigated further. We describe here the observations that both type I binding

and ethylmorphine-induced stimulation of NADPH-cytochrome P-450 reductase activity are sex-dependent in both strains. The significance of these findings with regard to the rate-limiting step in the demethylation of ethylmorphine is discussed. In addition, the effects of androgen are further specified.

MATERIALS AND METHODS.

Chemicals.

Ethylmorphine was obtained from Brocacef; $NADP^+$ (grade I), NADPH (grade I), glucose-6-phosphate (disodium salt), and glucose-6-phosphate dehydrogenase (grade I) were obtained from Boehringer, and bovine serum albumin from Poviet. Testosterone propionate was a generous gift from Organon. All other chemicals used were at least reagent grade.

Animals.

10 Week old male and female mice from both CPB-SE and CPB-V strain were obtained from the Central Animal Breeding Station TNO, Zeist, The Netherlands. They were kept in Makrolon cages with pinewood shavings, received standard food pellets (Hope Farms) and water ad lib., and were used within a few weeks.

Treatments.

Testosterone pretreatment was carried out by subcutaneous injection of testosterone propionate (2.5 mg in 0.1 ml arachis oil/animal) 14, 10, 7 and 3 days before the experiments. Control animals received an equivalent volume of oil. Castration (orchectomy and ovariectomy) was performed under light ether anesthesia at least 4 weeks in advance. Control animals were sham operated.

Preparation of microsomes.

After starving overnight, the animals were killed by a blow on the head. Livers were homogenized in 3 volumes of 0.1 M phosphate buffer (pH 7.4), using glass Potter tubes with a Teflon pestle. The homogenate was centrifuged for 20 minutes at 9000 g and the microsomal fraction was sedimented from the resulting supernatant by centrifuging at 75,000 g for 90 minutes. The pellet was suspended in 0.1 M phosphate buffer (pH 7.4). For each determination the

livers of several animals were pooled.

Enzyme assays.

Ethylmorphine demethylation was assayed by measuring the formation of formaldehyde. Incubations (10 minutes) were carried out at 37°C, with shaking, air being freely admitted. The incubation mixture contained ethylmorphine (SE-strain, 3 µmol; V-strain 1.5 µmol), $NADP^+$ (0.75 µmol), glucose-6-phosphate (12.5 µmol), glucose-6-phosphate dehydrogenase (1 IU), semicarbazide (12.5 µmol), nicotinamide (5 µmol), $MgCl_2$ (12.5 µmol), and microsomes , in a total volume of 3 ml. The reaction was initiated by the addition of 0.5 ml of microsomal suspension (10 mg protein/ml) and stopped by subsequent additions of 0.5 ml 40% $ZnSO_4$ and 1 ml saturated $Ba(OH)_2$. After centrifugation, 2 ml of the supernatant were mixed with 1 ml double strength Nash reagent (14), and incubated at 60°C for 30 minutes. The amount of HCHO formed was estimated by determining the absorbance at 415 nm relative to that at 500 nm, and subtracting the amount of Nash-positive material formed in the appropriate blank.

Difference spectra were recorded on an Aminco-Chance dual wavelength spectrophotometer in the split beam mode. Cuvettes contained 3 ml of microsomal suspension (3 mg prot./ml). After establishing a baseline of equal absorbance, ethylmorphine (3 µmol) was added to the sample cuvette and the difference spectrum was recorded. The peak-to-trough difference (385-420 nm) was taken as the magnitude of spectral interaction, and expressed in absorbance units.

NADPH-cytochrome P-450 reductase was assayed essentially as described by Gigon et al. (13). 2.5 ml of a microsomal suspension, containing about 1 mg protein/ml, were bubbled with carbon monoxide for 5 minutes at 30°C. The CO was passed through an alkaline dithionite solution to remove traces of oxygen. A stopcock-plunger assembly provided with 50 µl of NADPH-solution (20 mg/ml) was fitted to the cuvette and CO was led over the suspension for an additional 1 minute. The cuvette was then closed and placed in an Aminco DW-2 UV-VIS spectrophotometer, used in the dual wavelength mode. The cuvette-holder was thermostatically controlled at 30°C. A baseline of zero absorbance was established. After 1 minute the plunger was quickly depressed and the 450 minus 490 nm absorbance change recorded at a chart speed of 2 inch/sec. The initial rate of reduction was estimated by determining ΔA at 1.5 sec after mixing and expressed as nmol cytochrome P-450 reduced/min/mg protein using 91 $cm^{-1}mM^{-1}$ as the value of the absorption coefficient (15). Reductase

stimulation (Δ_{red}) represents the difference between the initial rates of reduction, with and without added ethylmorphine (2.5 µmol), which were determined in duplicate and triplicate respectively. The total amount of cytochrome P-450 present was determined by completely reducing the cuvette contents by adding a few crystals of sodium dithionite and allowing the reaction to proceed for at least 1 minute.

Microsomal protein was assayed according to the method of Lowry et al. (16), using crystalline bovine serum albumin as a standard.

Statistics.

Student's t-test (two-tailed) was used for statistical analysis.

RESULTS.

1. CPB-SE strain.

1.1. No treatment.

In Fig. V,1 a sex difference in ethylmorphine N-demethylation is demonstrated. Cytochrome P-450 content and magnitude of type I spectral interaction showed a similar sex-dependency, although to different extents, the difference being smaller for cytochrome P-450 and larger for type I binding. NADPH-cytochrome P-450 reductase was also more active in females than in males, the sex difference being comparable to that found with cytochrome P-450 content. Addition of ethylmorphine, however, caused a profound stimulation of reductase activity in females. The stimulation was fourfold greater than that observed in males. The sex differences in all parameters were qualitatively similar.

1.2. Testosterone pretreatment.

Treatment with testosterone has been shown to abolish the sex differences in hexobarbital hydroxylation, by depressing female values to the male level, leaving male values unchanged (8). The present results indicate that ethylmorphine demethylation is affected in a similar way (Fig. V,2). Cytochrome P-450 content, type I binding and reductase stimulation were also diminished in females and remained unaltered in males. There was no significant sex difference between the resulting values. No significant change was seen in basal reductase activity. These results strengthen the proposition that androgen plays a role in regulating microsomal drug metabolism in mice.

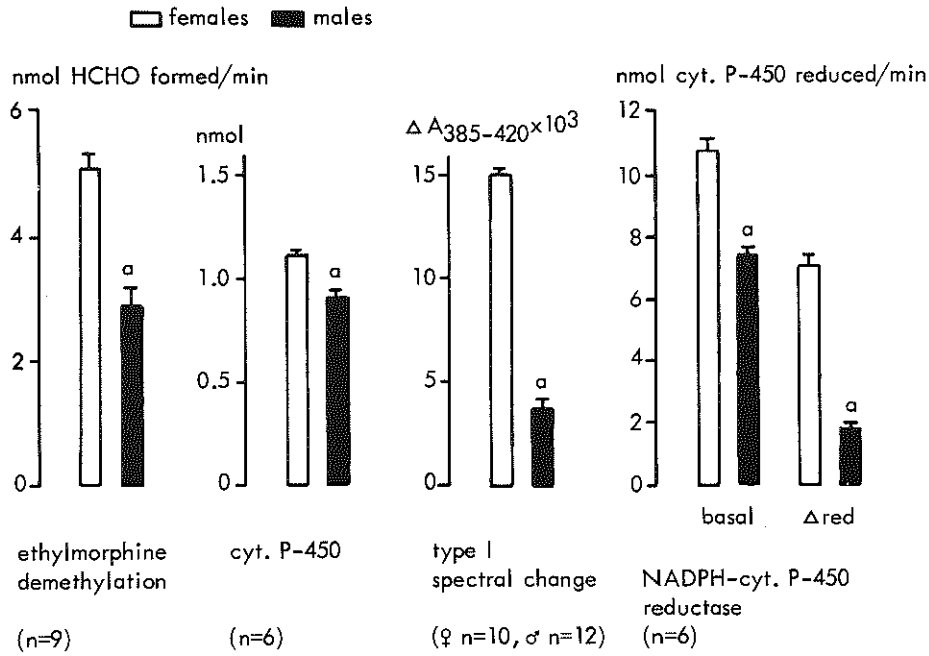

Fig. V,1. Ethylmorphine N-demethylation and components of the mixed-function oxidase system in hepatic microsomes from male and female mice of the CPB-SE strain. All values are related to mg microsomal protein, each representing the mean ± SE of the number of experiments indicated. a. Significant sex difference ($p<0.001$).

1.3. Castration.

Castration abolished the sex difference in demethylation activity by enhancing the rate of metabolism in males to the level observed in females (Fig. V,3). In females a slight, but statistically significant decrease was noticed. This had not been observed in the case of hexobarbital metabolism (8). In males, cytochrome P-450 content, type I binding and reductase stimulation underwent parallel changes as a consequence of the treatment. In contrast, basal reductase was not affected. Castration of males did not in all cases lead to complete feminization of enzyme activities. Statistically significant sex differences remained in basal reductase ($p<0.02$) and in

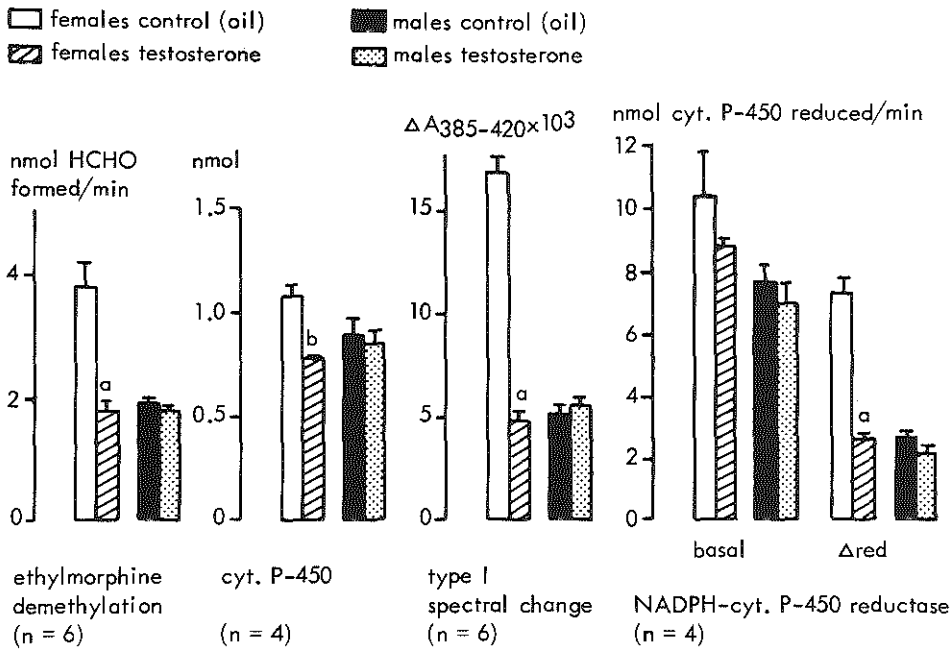

Fig. V,2. Effects of testosterone pretreatment on ethylmorphine N-demethylation and on components of the mixed-function oxidase system in hepatic microsomes from male and female mice of the CPB-SE strain. All values are related to mg microsomal protein, each representing the mean ± SE of the number of experiments indicated. a. Significant effect of treatment ($p<0.001$), b. id. ($p<0.002$).

reductase stimulation ($p<0.05$).

In order to examine the possible relationships between the various parameters more thoroughly, sex differences and the effects of treatments were expressed as percentages, which are shown in Table V,1. A suitable candidate for a rate-controlling factor should exhibit values similar to those obtained for ethylmorphine demethylation. The total amount of cytochrome P-450 as well as the endogenous rate of cytochrome P-450 reduction show much less variation than the demethylation rate. A striking correlation between type I binding and reductase stimulation is revealed, suggesting that these phenomena are

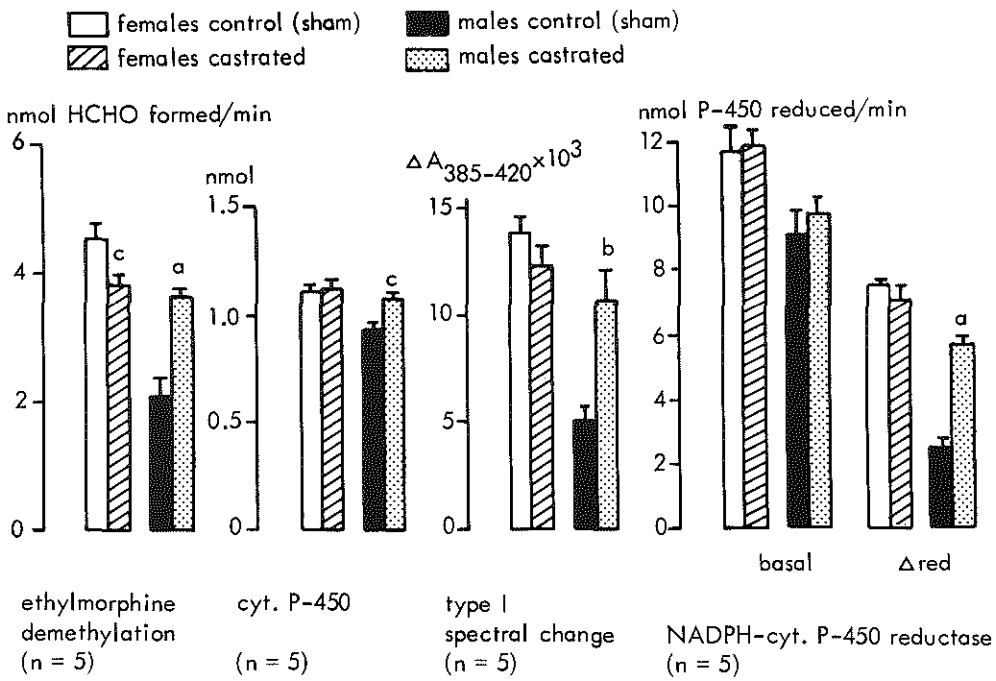

Fig. V,3. Effects of castration on ethylmorphine N-demethylation and on components of the mixed-function oxidase system in hepatic microsomes from male and female mice of the CPB-SE strain.
All values are related to mg microsomal protein, each representing the mean ± SE of the number of experiments indicated. a. Significant effect of treatment ($p<0.001$), b. id. ($p<0.01$), c. id. ($p<0.05$).

intimately associated. In view of several other reports (13,17,18) it is tempting to interpret this correlation as the expression of enzyme-substrate binding and subsequent reduction of enzyme-substrate complex. The fact that these parameters were more sensitive to androgen than the rate of demethylation implies that the reduction is not solely rate-limiting.

Table V,1. Comparison of the variations due to sex, testosterone pretreatment, and castration in ethylmorphine N-demethylation and components of the mixed-function oxidase system in hepatic microsomes from mice of the CPB-SE and CPB-V strain.

Sex differences and effects of treatments are expressed as males/females x 100% and treated/untreated x 100%, respectively. Values were calculated from Figs. V,1-5.

strain	treatment		ethyl-morphine demeth.	P-450	type I spectrum	P-450 reduction	
						basal	Δ_{red}
CPB-SE	none	M/F	57	81	25	70	26
	testosterone	M contr./F contr.	50	83	30	74	38
		F test./F contr.	46	72	29	85	36
	castration	M sham/F sham	46	84	35	78	33
		M castr./M sham	175	115	218	107	220
CPB-V	none	M/F	107	118	47	108	49
	testosterone	M contr./F contr.	94	120	50	116	54
		F test./F contr.	106	113	47	120	56

2. CPB-V strain.

2.1. No treatment.

Fig. V,4 shows the lack of a sex difference in ethylmorphine demethylation in the V-strain. Male liver microsomes were slightly richer in cytochrome P-450. Basal reductase activities were not significantly different. A sex difference was present in type I binding, although less profound than that observed in the SE-strain. A similar pattern was found for reductase stimulation. The absence of a sex difference in ethylmorphine demethylation suggests that in the V-strain this reaction is insensitive to androgen. Nevertheless, type I binding and reductase stimulation do show sex differ-

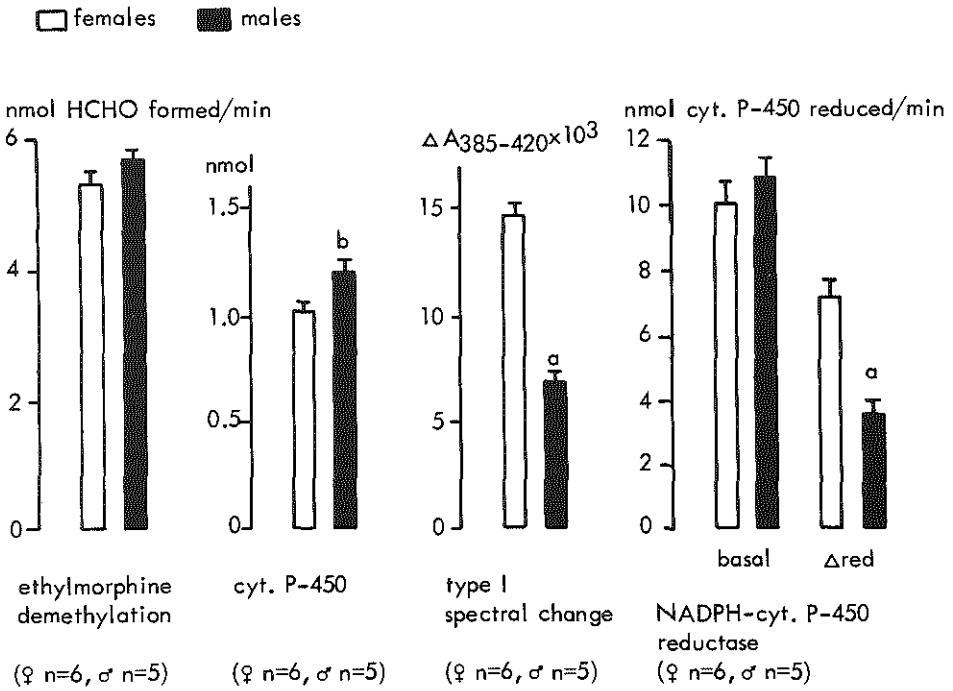

Fig. V,4. Ethylmorphine N-demethylation and components of the mixed-function oxidase system in hepatic microsomes from male and female mice of the CPB-V strain. All values are related to mg microsomal protein, each representing the mean ± SE of the number of experiments indicated. a. Significant sex difference ($p<0.001$), b. id. ($p<0.05$).

ences, which indicates that they are regulated by sex hormones. It was, therefore, of interest to see whether testosterone pretreatment would abolish the sex differences in these parameters, or at least affect them in the same way.

2.2. Testosterone pretreatment.

As expected, testosterone did not produce any changes in ethylmorphine demethylation (Fig. V,5). In females, type I binding and reductase stimulation were depressed to male levels. Cytochrome P-450 content was slightly increased in females, which is also in accordance with the slight sex difference in this strain. With basal reductase a similar effect was observed.

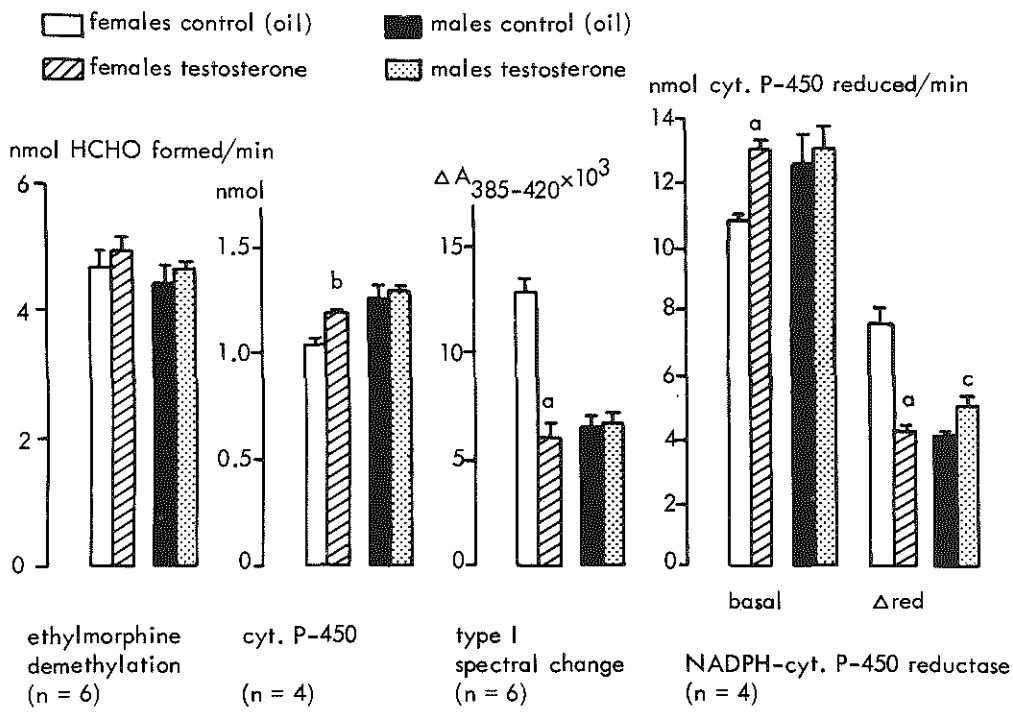

Fig. V,5. Effects of testosterone pretreatment on ethylmorphine N-demethylation and on components of the mixed-function oxidase system in hepatic microsomes from male and female mice of the CPB-V strain. All values are related to mg microsomal protein, each representing the mean ± SE of the number of experiments indicated. a. Significant effect of treatment ($p<0.001$), b. id ($p<0.002$), c. id. ($p<0.02$).

No significant alterations occurred in males, except that reductase stimulation was somewhat enhanced. It is not clear what significance this might have.

Examination of the relative sex differences and effects of treatment reveals a parallel between reductase stimulation and type I binding, as was observed in the SE-strain (Table V,1). However, demethylation rates do not reflect changes in either parameter. This indicates that reductase stimulation and type I binding are not rate-controlling.

The results with both the SE- and the V-strain suggest that a relationship between reductase stimulation and type I binding exists, which is independent of sex or strain. In order to further substantiate this, a constant ratio $\Delta_{red}/\Delta A$ should be demonstrated, besides concurrent variations in Δ_{red} and ΔA. In Table V,2 these ratios are listed for all groups of animals used throughout this study. They appear to be in reasonable agreement, as the relative standard errors of these ratios amount to approx. 5-10%. Three values in the testosterone treatment group of the V-strain are rather high. These might be related to the corresponding basal reduction rates, which are the highest measured in this study.

Table V,2. Relationship between ethylmorphine-induced enhancement of NADPH-cytochrome P-450 reductase activity and ethylmorphine binding in hepatic microsomes from male and female mice of the CPB-SE and CPB-V strain.

strain	treatment	$\Delta_{red}/\Delta A$ *			
		females control	females treated	males control	males treated
CPB-SE	none	0.47		0.49	
	testosterone	0.43	0.55	0.54	0.41
	castration	0.54	0.58	0.51	0.51
CPB-V	none	0.50		0.51	
	testosterone	0.59	0.70	0.63	0.75

* Values from Figs. V,1, V,2, V,3, V,4 and V,5 were used to calculate reductase stimulation (Δ_{red}) vs. type I spectral change (ΔA) ratios.

DISCUSSION.

The correlation between type I binding and reductase stimulation in mice strongly suggests that substrate binding to the type I binding site is directly responsible for reductase stimulation, although discrepancies between these parameters have been reported in rats (17,19). As it is unlikely that the reductase step is bypassed in the demethylation reaction, this observation emphasizes the importance of type I binding for this reaction. This enforces the general belief that type I binding represents the formation of an enzyme-substrate complex (1,20). The lack of agreement between the sex-dependent differences in the magnitude of the type I spectral interaction and the demethylation of ethylmorphine, therefore, indicates that the reduction of this complex by NADPH-cytochrome P-450 reductase may not control the rate of demethylation. The rate-limiting character of the reductase step was originally proposed by Gigon et al. (13). This concept has found considerable support (17-19,21), but has also been challenged (22-24).

A measurement of NADPH-cytochrome P-450 reductase activity can be obtained from the endogenous (basal) rate of reduction, the reduction rate in the presence of substrate (total activity), or the difference between these two (reductase stimulation, Δ_{red}). The endogenous rate of reduction can hardly be expected to control the rate of substrate oxidation, since rates of drug metabolism within a single enzyme preparation vary widely, depending on the substrate. Table V,1, in fact, demonstrates that the basal reduction rate is not related to the demethylation rate, which is in agreement with data obtained by Davies et al. (5).

Cytochrome P-450 reduction proceeds via two superimposed phases, a rapid phase and a slow phase (13,18,24-26). Type I substrates enhance the initial rate of reduction, probably by binding to cytochrome P-450. If this enhancement is the result of an increase in the amount of cytochrome P-450 which is reduced in the rapid phase (13,18), Δ_{red} represents the rate of reduction of the cytochrome P-450-substrate complex which is formed. Significant correlations between Δ_{red} and substrate oxidation have been observed repeatedly (13,17-19,21). Our results show that Δ_{red} is not a rate-limiting factor in ethylmorphine demethylation in both the SE- and the V-strain. If reductase stimulation is due to an effect on the rate constant of the rapid phase (25), the measurement of the rate of reduction of the cytochrome P-450-substrate complex is not well-defined. The heterogeneity of cytochrome P-450 (27-32),

and especially the heterogeneity of type I binding sites (28), implies that not all the reducible cytochrome P-450 is involved in the binding of a single substrate. The actual rate of complex reduction will thus be lower than the total reduction rate, but higher than the value of Δ_{red}. As the relative sex differences in complex reduction will be less marked than in Δ_{red}, a rate-limiting function of the reductase in the SE-strain thus cannot be fully rejected. Other work on the SE-strain, however, has revealed further discrepancies (33).

The effects of androgen on the cytochrome P-450 system in mice are inhibitory (in contrast to the stimulatory effects observed in rats (2)). The sex differences in some parameters of the enzyme system, however, mainly arise from increases occurring in females during sexual maturation (33). This suggests that androgen reversibly impairs developmental changes. Basal cytochrome P-450 reductase activity appeared to have a more permanent sex difference, which explains the present finding that this activity was not affected by testosterone pretreatment and castration.

The main site of androgen interaction appears to reside in the cytochrome P-450 fraction, resulting in a discrepancy between cytochrome P-450 content and extent of type I binding. Testosterone might cause a decrease in the number of a specific type of binding sites for ethylmorphine. Alternatively, it might introduce a high level of tightly-binding endogenous substrates, such as steroids, which are themselves metabolized by the cytochrome P-450 system (34), and which possess a high affinity for cytochrome P-450 (35). We have obtained data which suggest that the first possibility is more likely (33, and Van den Berg et al., to be published (Chapters IX and X)).

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CHAPTER VI. SEX DIFFERENCES IN THE KINETIC CONSTANTS OF ETHYLMORPHINE DEMETHYLATION AND TYPE I BINDING TO HEPATIC MICROSOMAL CYTOCHROME P-450 IN MICE. THE INFLUENCE OF CASTRATION AND TESTOSTERONE.

SUMMARY.

1. In the CPB-SE mouse strain sex differences were observed in the K_m and V_{max} of ethylmorphine demethylation and in the ΔA_{max} of its type I binding to cytochrome P-450.

2. In the CPB-V strain a small sex difference in the V_{max} of the demethylation was found, whereas K_s and ΔA_{max} of type I binding differed considerably.

3. Testosterone pretreatment of female CPB-SE mice abolished all sex differences, and castration of males all except that in V_{max}, which was partially decreased.

4. In the CPB-V strain testosterone pretreatment of females abolished sex differences in type I binding but had no effect on ethylmorphine demethylation.

5. K_m values exceeded the corresponding K_s in all cases and sex differences in ΔA_{max} far exceeded those in V_{max}.

6. It is concluded that the K_m is not only determined by the K_s of type I binding and the reduction rate of the type I complex between ethylmorphine and cytochrome P-450.

7. The larger sex differences in ΔA_{max} as compared with V_{max} may be attributable to type I binding of ethylmorphine to cytochrome P-450 subspecies not involved in its demethylation.

INTRODUCTION.

Type I binding of substrates to hepatic microsomal cytochrome P-450 may be regarded as an interaction with the active site of the enzyme or, at least, as a facilitation of the metabolic reactions catalyzed by this cytochrome (1). The dissociation constants (K_s) have been compared, under various conditions, with the Michaelis-Menten constants (K_m) of the metabolic reaction (see review by Mannering (2)). Although, in many cases, a large discrepancy was found between these constants, K_m values usually exceeding K_s, in some

instances no differences were found (3,4). Our investigations, using the enantiomers of hexobarbital in mice and rats, revealed that, in mice, K_m and K_s do not differ, whereas, in rats, K_m was about five to six times higher than K_s (5). This could be explained by the observation that differences in the rates of metabolism of the enantiomers in rats were not paralleled by differences in the substrate-induced enhancement of NADPH-cytochrome P-450 reductase, which is regarded as the expression of the reduction rate of the type I complex between the substrate and the cytochrome (6). If this reduction is not rate-limiting for the whole reaction, the kinetic constants of type I binding and metabolism are not necessarily similar. In mice, however, a parallel between metabolism and enhancement of cytochrome P-450 reduction was observed which explained the similarity between K_m and K_s for hexobarbital in this species.

We previously reported a sex difference in the rate of metabolism, in mice, of various type I substrates including hexobarbital, ethylmorphine and aminopyrine (5,7). Using a single substrate concentration, we demonstrated that this sex difference is abolished by either castration of males or testosterone pretreatment of females (7). A relation between type I binding and metabolism was suggested by the fact that only type I substrates and, to a lesser extent, substrates binding to both type I and type II sites are metabolized faster by female mice than by males, whereas for the type II substrate aniline, no such sex difference could be demonstrated (5).

However, the relationship might be complicated by the heterogeneity of type I binding sites in rats (8,9) a phenomenon which may also occur in mice.

In the present work we studied the kinetics of ethylmorphine demethylation in male and female CPB-SE mice. In this strain, males exhibit a higher K_m and lower V_{max} for the reaction than females. Analogous sex differences were observed with K_s and ΔA_{max} for type I binding to cytochrome P-450, K_s being higher and ΔA_{max} lower in males (5).

By determining the effect of castration and testosterone pretreatment on these parameters other possible parallels between K_s and ΔA_{max} on the one hand and K_m and V_{max} on the other could be investigated. We further studied these constants in the CPB-V strain, which has previously been reported to lack a sex difference in hexobarbital metabolism (7). The absence of this sex difference was further clarified by determining the effect of testosterone pretreatment on ethylmorphine demethylation and by measuring plasma testosterone levels.

MATERIALS AND METHODS.

Animals.

Male and female mice of the CPB-SE and CPB-V strain (TNO,Zeist) were used, which were at least 10 weeks old. They were housed in Makrolon cages on pine-wood shavings and allowed free access to Hope Farm food pellets and tap water.

Treatments.

Castration (orchectomy or ovariectomy) was carried out under light ether anesthesia, four weeks before the experiments. Control animals were sham-operated. Testosterone pretreatment consisted of 4 subcutaneous injections of 2.5 mg testosterone propionate in 0.1 ml arachis oil 14, 10, 7 and 3 days before the experiments. Controls received arachis oil only.

Preparation of microsomes.

Animals, starved overnight, were killed by a blow on the head. After removal of the gall bladder, livers were rapidly excised and homogenized in 3 volumes of ice-cold 0.1 M phosphate buffer, pH 7.4, using glass Potter tubes with a Teflon pestle. The homogenate was centrifuged at 9000 g for 20 minutes. The microsomal fraction was sedimented from the supernatant by 90 minutes centrifugation at 75,000 g. The pellet was resuspended in the same buffer to a final concentration of 10 mg protein/ml. Protein was determined with the Folin reagent (10) using bovine serum albumin as a standard.

Ethylmorphine demethylation.

Ethylmorphine demethylation was determined by measuring the formation of formaldehyde. Incubations were carried out at 37°C for 10 minutes in a shaking incubator under air. The incubation mixture contained $NADP^+$ (0.75 µmol), glucose-6-phosphate (12.5 µmol), glucose-6-phosphate dehydrogenase (1 IU), semicarbazide (12.5 µmol), nicotinamide (5 µmol), $MgCl_2$ (12.5 µmol) and ethylmorphine in concentrations of 0.4, 0.5, 0.67, 1 and 2 mM in a total volume of 2.5 ml 0.1 M phosphate buffer, pH 7.4. The reaction was started by addition of 0.5 ml microsomal suspension and stopped 10 minutes afterwards by addition of 0.5 ml 40% $ZnSO_4$ and 1 ml saturated $Ba(OH)_2$, respectively. After centrifugation, 2 ml of the supernatant were mixed with 1 ml double strength Nash reagent (11) and incubated for 30 minutes at 60°C. The amount

of formaldehyde formed was estimated from the difference in absorbance between 415 and 500 nm after subtraction of Nash-positive material formed in incubates without added substrate.

Spectral studies.

Difference spectra due to the addition of ethylmorphine to microsomal suspensions (3 mg protein/ml), were recorded in an Aminco-Chance dual wavelength spectrophotometer in the split-beam mode. The magnitude of the spectral interactions is given as $\Delta A_{390-420}$.

Testosterone determination.

Testosterone was determined in plasma by radioimmunoassay, as described by Verjans et al. (12). Blood was collected by means of orbital puncture in tubes containing heparin.

Calculations and statistical methods.

Kinetic constants for ethylmorphine demethylation (K_m, V_{max}) and type I binding (K_s, ΔA_{max}) were calculated using the method of Wilkinson (13). Differences between groups of data were tested using Student's t-test (two-sided).

Chemicals.

Ethylmorphine-HCl was obtained from Brocacef; $NADP^+$ (grade I) and glucose 6-phosphate dehydrogenase (grade I, from yeast) from Boehringer and crystalline bovine serum albumin from Poviet. Testosterone propionate was a generous gift from Organon. All other chemicals used were of reagent grade purity.

RESULTS.

Kinetic constants in male and female CPB-SE mice.

Table VI,1 shows the kinetic constants of ethylmorphine demethylation and type I binding to cytochrome P-450 in male and female CPB-SE mice. It appears that, in both male and female animals, the K_m for metabolism exceeds the K_s for type I binding. Comparing the sexes, it is evident that the values for K_m differ far more than K_s values, which either do not differ, as also observed

by Van den Berg et al. (14), or differ slightly (5). The ΔA_{max} values also differ much more than do V_{max} values.

Table VI,1. Kinetic constants of demethylation and type I binding of ethylmorphine in liver microsomes of male and female CPB-SE mice.

	K_m (mM)	K_s (mM)	V_{max} (nmol/min/mg prot)	ΔA_{max} ($\Delta A_{390-420}$/mg prot)
Males	1.27 ± 0.15 (9)	0.20 ± 0.02 (9)	6.50 ± 0.34 (9)	4.7 ± 0.5 (9)
Females	0.69 ± 0.07 (9)**	0.22 ± 0.01 (10)	9.18 ± 0.72 (9)**	18.5 ± 0.4 (10)*

Values represent means ± SE of the number of experiments indicated in parentheses. * Significant sex difference, $p<0.001$; ** id., $p<0.01$.

The influence of castration.

Table VI,2 summarizes the kinetic constants for ethylmorphine demethylation and type I binding in liver microsomes from orchectomized male and ovariectomized female CPB-SE mice and their sham-operated controls. Again, in all groups studied, K_m values exceeded those of K_s, particularly in males. In females, castration did not significantly influence any kinetic parameter of demethylation or type I binding. In males, however, K_m for demethylation was significantly decreased, though V_{max} was not influenced, despite a significant sex difference ($p<0.02$) in this parameter. On the other hand, the V_{max} in castrated males did not differ from the V_{max} in females. The ΔA_{max} for type I binding was significantly increased by castration of male animals, although these values did not completely achieve female levels. K_s was not influenced by castration, which is in accordance with the absence of a sex difference in this parameter in sham-operated animals.

Castration, thus, abolishes sex differences in the kinetic constants of ethylmorphine demethylation, but does not completely abolish those of type I binding. Moreover, K_m, but not K_s, is decreased by castration of males. Therefore, the effects of castration on the kinetic parameters of demethylation and type I binding of ethylmorphine are not parallel.

Effects of testosterone pretreatment.

Table VI,3 shows the kinetic constants for ethylmorphine demethylation

Table VI,2. The influence of castration on the kinetic constants of demethylation and type I binding of ethylmorphine in liver microsomes of male and female CPB-SE mice.

		K_m (mM)	K_s (mM)	V_{max} (nmol/min/mg prot.)	ΔA_{max} ($\Delta A_{390-420}$/mg prot.)
Males	sham	2.15 ± 0.33 (12)	0.28 ± 0.04 (9)	4.41 ± 0.58 (12)	6.2 ± 0.6 (9)
	castr.	1.08 ± 0.10 (12)*	0.20 ± 0.02 (7)	5.36 ± 0.48 (12)	11.6 ± 1.5 (7)*
Females	sham	0.79 ± 0.05 (12)	0.30 ± 0.02 (5)	5.84 ± 0.18 (12)	18.7 ± 0.9 (5)
	castr.	0.81 ± 0.08 (12)	0.27 ± 0.04 (5)	5.40 ± 0.28 (12)	15.7 ± 0.8 (5)

Values represent means ± SE of the number of experiments indicated in parentheses.
* Significant effect of treatment, $p<0.01$.

Table VI,3. The influence of testosterone pretreatment on the kinetic constants of demethylation and type I binding of ethylmorphine in liver microsomes of male and female CPB-SE mice.

		K_m (mM)	K_s (mM)	V_{max} (nmol/min/mg prot.)	ΔA_{max} ($\Delta A_{390-420}$/mg prot.)
Males	contr.	1.16 ± 0.10 (10)	0.36 ± 0.04 (6)	4.24 ± 0.34 (10)	7.1 ± 0.6 (6)
	test.	1.21 ± 0.10 (10)	0.35 ± 0.07 (5)	3.76 ± 0.18 (10)	8.2 ± 0.6 (5)
Females	contr.	0.75 ± 0.05 (10)	0.23 ± 0.02 (6)	7.24 ± 0.48 (10)	21.0 ± 1.0 (6)
	test.	1.16 ± 0.08 (10)*	0.54 ± 0.07 (5)**	4.46 ± 0.24 (10)*	8.0 ± 0.4 (5)*

Values represent means ± SE of the number of experiments indicated in parentheses.
* Significant effect of treatment, $p<0.001$; ** id., $p<0.01$.

and type I binding in testosterone-pretreated and control CPB-SE mice. Contrary to the results in intact and sham-operated mice, a sex difference in K_s was observed ($p<0.02$) in the arachis oil-pretreated controls. The reason for this apparent discrepancy is not clear, although the oil pretreatment in itself might have exerted some influence. Testosterone had no effect on any kinetic parameter in male mice. In females, K_m was increased and V_{max} decreased. Parallel changes were observed in K_s and ΔA_{max} respectively. Moreover, testosterone changed the female values to the male level. Values of K_m, as in the other groups, exceeded K_s values.

Kinetic constants in male and female CPB-V mice.

In the CPB-V strain, a previous study revealed that there was no sex difference in the rate of hexobarbital metabolism, using a single substrate concentration (7). This finding was repeated for ethylmorphine in the present investigation. Kinetic constants for ethylmorphine demethylation in this mouse strain are given in Table VI,4. No sex difference in K_m and a small sex difference in V_{max} were found. Surprisingly, however, kinetics of type I binding of ethylmorphine in CPB-V mice proved to be different in males and females. In males ΔA_{max} was significantly lower and K_s higher than in females (Table VI,4). In both males and females K_m exceeded K_s. These observations clearly show the discrepancy between the K_s for type I binding and the K_m for demethylation. It seems that the CPB-V strain either does not produce sufficient quantities of androgen or the demethylating enzymes are relatively insensitive to testosterone.

Table VI,4. Kinetic constants of demethylation and type I binding of ethylmorphine in liver microsomes of male and female CPB-V mice.

	K_m (mM)	K_s (mM)	V_{max} (nmol/min/mg prot.)	ΔA_{max} ($\Delta A_{390-420}$/mg prot)
Males	0.76 ± 0.05 (5)	0.41 ± 0.02 (6)	13.24 ± 0.26 (5)	8.6 ± 0.7 (6)
Females	0.90 ± 0.07 (6)	0.23 ± 0.02 (6)*	15.68 ± 0.44 (6)**	16.2 ± 0.9 (6)*

Values represent means ± SE of the number of experiments indicated in parentheses. * Significant sex difference, $p<0.001$; ** id., $p<0.01$.

Effects of testosterone on demethylation and type I binding in CPB-V mice.

As can be seen in Table VI,5, testosterone pretreatment of female CPB-V mice significantly increases K_s for type I binding and decreases ΔA_{max}, whereas in males no effect was observed. Testosterone pretreatment also failed to influence ethylmorphine demethylation (15). Therefore, it seems justified to conclude that the activity of the demethylating enzyme system is insensitive to testosterone in CPB-V mice.

Table VI,5. The influence of testosterone pretreatment on the kinetic constants of ethylmorphine type I binding in liver microsomes of male and female CPB-V mice.

		K_s (mM)	ΔA_{max} ($\Delta A_{390-420}$/mg prot.)
Males	contr.	0.35 ± 0.05	9.2 ± 0.5
	test.	0.42 ± 0.05	9.9 ± 0.5
Females	contr.	0.23 ± 0.02	16.2 ± 0.9
	test.	0.41 ± 0.02 *	8.6 ± 0.7 *

Values represent means ± SE of 6 experiments.
* Significant effect of treatment, $p<0.001$.

Plasma levels of testosterone in male and female CPB-SE and CPB-V mice.

Testosterone levels in the plasma of male and female CPB-SE and CPB-V mice are given in Table VI,6. In both strains a sex difference was apparent. Testosterone levels in male CPB-SE mice were significantly higher ($p<0.01$) than in male CPB-V animals.

Table VI,6. Plasma testosterone levels (µg/100 ml) in male and female CBP-SE and CPB-V mice.

	Males	Females
CPB-SE	0.60 ± 0.05	0.24 ± 0.02
CPB-V	0.35 ± 0.03	0.26 ± 0.03

Values represent means ± SE of 4 experiments.

DISCUSSION.

The comparison of the kinetic constants of ethylmorphine demethylation and type I binding in mice of the CPB-SE and CPB-V strain revealed discrepancies between these constants themselves as well as between the strains. As we used the same ethylmorphine concentrations for both determinations, influences of the concentration range on the kinetic constants should not play a role (16). The apparent Michaelis-Menten constant K_m, however, does not necessarily equal the dissociation constant K_s, since K_m may be determined by the velocity of subsequent reaction steps and the values of the dissociation constants of possible intermediate (oxygenated) cytochrome P-450-substrate complexes (17). All K_m values observed in this study were, in fact, higher than the corresponding K_s values. The sex-dependent modifications of K_m, however, were not paralleled by modifications of K_s in the SE-strain, and the converse was observed in the V-strain. This apparent dissociation of K_s and K_m may indicate a more complex reaction mechanism or conformational changes in one of the intermediate forms of the complex due to hormone action.

The discrepancy between the sex differences in V_{max} and the maximum spectral change (ΔA_{max}) is more difficult to explain. The sex differences in ΔA_{max} were much larger than in V_{max} in both strains. The effects of testosterone treatment and castration were also more pronounced on ΔA_{max}, but this is evident, if the treatments merely abolish the sex differences. V_{max} is determined by the total amount of enzyme-substrate complex, and by one or more rate constants. The small effects on V_{max} with respect to ΔA_{max} may, therefore, be attributable to compensatory effects on the rate constant of the rate-limiting step. This cannot be accounted for by effects on NADPH-cytochrome P-450 reductase, since the basal reduction rate was shown in a previous study to have a slight sex difference in favour of females and to be unaffected by testosterone pretreatment and castration, whereas the stimulation of the reduction rate by ethylmorphine was directly proportional to the magnitude of the spectral change (15). A more realistic possibility was suggested by a study of the development of the ethylmorphine demethylase in the CPB-SE strain (14). Different forms of cytochrome P-450 may exist, which have diverging properties with respect to type I binding and demethylation. A heterogeneity of cytochrome P-450 has, in fact, been observed in mice (18,19). Grasdalen et al. (8) even observed a heterogeneity of type I binding sites in rats.

The origin of the observed sex differences in ethylmorphine demethylation

and type I binding in the CPB-SE strain is obviously testicular testosterone, which exerts an inhibitory action, in contrast to the stimulatory effects observed in rats (20). This confirms our previously published conclusion for the hydroxylation of hexobarbital (7). The primary role of androgens is best illustrated by the effect of castration in male animals and the lack of effect in females. The lack of effect of castration on the V_{max} suggests that the sex difference in this parameter is irreversible and not due to testicular androgen. This is supported by our observation that V_{max} for ethylmorphine demethylation also differs in immature male and female animals (14). Castration of males did not convert ΔA_{max} completely to the female values. We found no sex difference in ΔA_{max} in immature animals (14), which indicates that this sex difference is, in part, irreversible, although of androgenic origin, since testosterone pretreatment reduced ΔA_{max} to the male level in females.

Apparently, the degree of sex-dependency of type I binding and ethylmorphine demethylation is strain-dependent. In the V-strain type I binding of ethylmorphine is androgen-dependent, but the sex difference is smaller than in the SE-strain, due to higher ΔA_{max} values in V-males. This is not likely to be simply the effect of the lower testosterone level observed in male V-mice as compared with SE-mice, since testosterone pretreatment did not further decrease ΔA_{max} in males. The demethylation reaction did not seem to be sex-dependent at all, despite the fact that males of the V-strain do produce testosterone. Only a very small sex difference in V_{max} was observed, but testosterone pretreatment did not affect ethylmorphine demethylation in males or in females, when measured at a single substrate concentration (15). These results suggest that in mice of the V-strain the responsiveness to androgen is smaller than in the SE-strain. The degree of responsiveness might be neonatally imprinted, as observed in rats (21). In this respect it is interesting that in males of both the CPB-V and CPB-FT strain, which lack a sex difference in hexobarbital metabolism, the 'major urinary protein', the hepatic production of which is stimulated by testosterone (22,23) is virtually absent in the serum (24).

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CHAPTER VII. THE DEVELOPMENT OF SEX DIFFERENCES IN THE DEMETHYLATION OF ETHYLMORPHINE AND IN ITS INTERACTION WITH COMPONENTS OF THE HEPATIC MICROSOMAL CYTOCHROME P-450 SYSTEM IN MICE.

SUMMARY.

The development of sex differences in ethylmorphine N-demethylation and several components of the reaction chain were studied in hepatic microsomes from mice of the CPB-SE strain between 3 and 11 weeks of age.

Sex-specific changes were observed in demethylation rate, type I spectral interaction, cytochrome P-450 content, and ethylmorphine-induced stimulation of NADPH-cytochrome P-450 reductase activity. These changes occurred mainly between the third and seventh weeks and were confined to females. It is concluded that the development of the cytochrome P-450 system is repressed by androgen during sexual maturation.

The kinetic constants of demethylation developed differently from the ethylmorphine binding constants. Changes in demethylase were mainly restricted to K_m, whereas the changes in type I binding only involved the maximum spectral change. In combination with differences observed between the developmental patterns of demethylation rate and cytochrome P-450 reductase activities, this demonstrated that the reduction of cytochrome P-450-substrate complex is not rate-limiting in ethylmorphine demethylation.

The type I spectral change was correlated with the amount of cytochrome P-450 only when a large portion of the cytochrome was considered inactive in ethylmorphine binding. It is suggested that immature animals possess a low basal level of ethylmorphine binding type I sites, which is elevated selectively in females during sexual maturation.

INTRODUCTION.

The development of hepatic microsomal drug metabolizing enzyme activity (cytochrome P-450 system) varies with species and substrate. Nevertheless, monooxygenase activity is usually hardly detectable during fetal life (with the exception of primates) and increases postnatally to reach a plateau at adulthood. Results of investigations in this area have recently been reviewed by several authors (1-3).

Some substrates (type I substrates, defined by their spectral interaction with cytochrome P-450 (4)) show sex-dependent metabolism. These sex differences appear to occur primarily in rats (see (5) for review). In this species, males exhibit higher hydroxylating activity than females. On the basis of the results of castration and testosterone treatment, this was attributed to an androgenic action (5-7). The development of the sex difference in demethylation was found to run parallel with the onset of sexual maturity, and was mainly the result of an increase in enzyme activity in the male between approximately 3 and 7 weeks of age. This was observed for aminopyrine (8,9) as well as ethylmorphine (10,11). Studies which included only male rats also revealed sudden increases in ethylmorphine demethylation (12,13).

Some strains of mice also exhibit a sex difference in the metabolism of type I substrates (14), which has been the subject of a number of studies in our laboratory (15-19). Interestingly, in the CPB-SE strain, metabolism is faster in females than in males. Although this difference is opposite to that found in rats, it appeared to be abolished by treatment of females with testosterone and castration of males, thus suggesting that this sex-dependency is also due to androgenic action. Pomp et al. (20) demonstrated that mice were not able to O- or N-demethylate p-nitroanisol or monomethyl-p-nitraniline, respectively, at birth. The development of these activities was followed for a period of 4 weeks thereafter and found to be completed at approximately 3 weeks of age. No specification was given as to the sex of the animals. It was therefore of interest to study the demethylation of ethylmorphine in the CPB-SE strain during sexual development.

In a previous investigation (19) the roles of type I binding and NADPH-cytochrome P-450 reductase were examined in two strains of mice. It had been proposed by others (21-23) that type I binding might reflect the formation of enzyme-substrate complex, whereas the substrate-induced stimulation of NADPH-cytochrome P-450 reductase activity would represent the reduction of this complex by the reductase, which probably constitutes the rate-limiting step. Both mouse strains exhibited considerable sex differences in type I binding and reductase stimulation, these parameters being highly correlated. As demethylation was not sex-dependent in the CPB-V strain, the reduction of cytochrome P-450-substrate complex can not be rate-limiting, but this could not be excluded in the CPB-SE strain. In order to further elucidate the nature of this sex difference the development of NADPH-cytochrome P-450 reductase activities and cytochrome P-450 were studied as well as the kinetic constants of ethylmorphine demethylation and type I binding.

MATERIALS AND METHODS.

Animals.

Immature male and female mice of the CPB-SE strain (2 weeks old) were obtained from the Central Animal Breeding Station TNO, Zeist, The Netherlands. The mice of each sex were randomly divided into 5 groups, which were sacrificed when the animals reached the age of 3, 5, 7, 9, and 11 weeks respectively. All animals were kept under similar conditions in Makrolon cages, with pinewood shavings, and received standard food pellets (Hope Farms) and water *ad lib*.

Chemicals.

Ethylmorphine was obtained from Brocacef; $NADP^+$ (grade I), NADPH (grade I), glucose-6-phosphate (disodium salt), and glucose-6-phosphate dehydrogenase (grade I) were obtained from Boehringer, and bovine serum albumin from Poviet. All other chemicals used were at least reagent grade.

Preparation of microsomes.

After starving overnight, the animals were killed by a blow on the head. Livers were homogenized in 3 volumes of 0.1 M phosphate buffer (pH 7.4), using glass Potter tubes with a Teflon pestle. The homogenate was centrifuged for 20 minutes at 9000 g and the microsomal fraction was sedimented from the resulting supernatant by centrifuging at 75,000 g for 90 minutes. The pellet was suspended in 0.1 M phosphate buffer (pH 7.4). For each determination the livers of several animals were pooled.

Enzyme assays.

Ethylmorphine N-demethylation was assayed by measuring the formation of formaldehyde. Microsomes were incubated with ethylmorphine (0.4, 0.5, 0.67, 1.0, and 2.0 mM) for 10 minutes at 37°C, with shaking, air being freely admitted. The incubation mixture further contained $NADP^+$ (0.75 µmol), glucose-6-phosphate (12.5 µmol), glucose-6-phosphate dehydrogenase (1 IU), semicarbazide (12.5 µmol), nicotinamide (5 µmol), $MgCl_2$ (12.5 µmol), and microsomes, in a total volume of 3 ml. The reaction was initiated by the addition of 0.5 ml of microsomal suspension (10 mg protein/ml) and stopped by consecutive additions of 0.5 ml 40% $ZnSO_4$ and 1 ml saturated $Ba(OH)_2$. After

centrifugation, 2 ml of the supernatant were mixed with 1 ml double strength Nash reagent (24), and incubated at 60°C for 30 minutes. The amount of HCHO formed was estimated by determining the absorbance at 415 nm relative to that at 500 nm, and subtracting the amount of Nash-positive material formed in the appropriate blank. The apparent Michaelis-Menten constant (K_m) and maximum velocity of demethylation (V_{max}) were calculated according to the method of Wilkinson (25).

Difference spectra were recorded on an Aminco-Chance dual wavelength spectrophotometer in the split-beam mode. Cuvettes contained 3 ml of microsomal suspension (3 mg protein/ml). After establishing a baseline of equal absorbance, ethylmorphine (0.33, 0.4, 0.5, 0.67, 1.0, and 2.0 mM) was added to the sample cuvette and the difference spectrum was recorded. The peak-to-trough difference (385-420 nm) was taken as the magnitude of spectral interaction, and expressed in absorbance units. The spectral dissociation constant (K_s) and maximum spectral change (ΔA_{max}) were calculated according to the method of Wilkinson (25).

NADPH-cytochrome P-450 reductase was assayed under anaerobic conditions in a carbon monoxide atmosphere at 30°C essentially as described by Gigon et al. (13). The formation of the reduced cytochrome P-450-CO complex was recorded as the absorbance change at 450 nm relative to 490 nm, using an Aminco DW-2 UV-VIS spectrophotometer in the dual wavelength mode. The reaction was initiated by the addition of 50 µl of NADPH-solution (20 mg/ml) to 2.5 ml of a microsomal suspension containing about 1 mg protein/ml. The initial rate of reduction was expressed as nmol cytochrome P-450 reduced/min/mg protein using 91 $cm^{-1}mM^{-1}$ (26) as the value of the absorption coefficient. Reductase stimulation (Δ_{red}) represents the difference between the rates of reduction in the presence and in the absence of ethylmorphine (endogenous or basal reduction rate), which were determined in duplicate and triplicate, respectively. Total cytochrome P-450 content was determined by completely reducing the cuvette contents with sodium dithionite.

Microsomal protein was assayed according to the method of Lowry et al. (27), using crystalline bovine serum albumin as a standard.

Statistics.

Two-way analysis of variance was employed to analyze all data for effects of age, sex, and interaction (age × sex), the latter representing the degree of dissimilarity between the developmental patterns. In the case of significant effects of sex, the sex differences at all ages were tested separately

against the residual variance. K_m values could not be analyzed this way, because of a significant difference between the male and female population variances. Sex differences were, therefore, tested by Student's t-test or Wilcoxon test where appropriate. $P<0.05$ was taken as the lowest level of significance. Additionally, the significance of differences at the $p<0.01$ level has been indicated. The number of experiments was 4, except in the case of 3 week old females, where this number was 3.

RESULTS.

Kinetics of ethylmorphine demethylation.

The maximum rate of demethylation (V_{max}) decreased between 3 and 5 weeks in both sexes, and then remained constant (Fig. VII,1). The developmental patterns were not significantly different. However, taking all ages together, males had a significantly lower (about 20%) V_{max} than females ($p<0.01$).

The apparent K_m decreased by a factor of 3, in females, whereas no statistically significant change was observed in males. Age-dependent changes in K_m have also been observed in rats (7,12,28). The sex-dependency of K_m was rather complicated. At 3 weeks, females had a higher K_m, whereas the opposite was observed at 11 weeks, when normal, adult values were reached (18,29). These developmental patterns of V_{max} and K_m imply that an increasing sex difference is apparent when measured at low substrate concentrations, which might be of significance in the *in vivo* situation.

Type I binding of ethylmorphine.

The development of the maximum spectral change (ΔA_{max}) was highly sex-dependent (Fig. VII,2). In females, a dramatic increase was observed, whereas ΔA_{max} remained at a constant level in males. At 3 weeks no sex difference existed, but at 11 weeks females exhibited a three-fold higher ΔA_{max} than males. ΔA_{max} does not correlate with the demethylation rate (Fig. VII,1), which appears to disagree with the concept that the spectral interaction reflects the formation of enzyme-substrate complex (21).

There was no significant effect of age on K_s, neither in females nor in males (Fig. VII,2), nor were the developmental patterns significantly different. A significant sex difference was found at 9 weeks only. In other experiments we have sometimes noticed that adult animals do show a small

sex difference in K_s, females having lower values (18). In this study, also, a significant sex difference was found when K_s was tested at all ages simultaneously ($p<0.05$). Nevertheless, a large discrepancy between the developmental patterns of K_m and K_s is apparent.

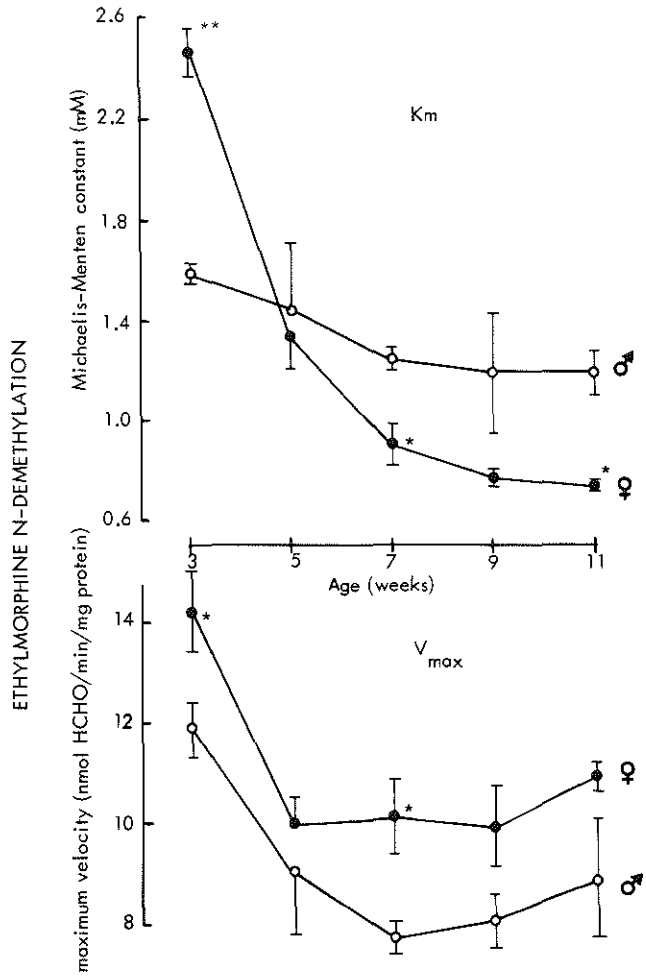

Fig. VII,1. Effects of age on the kinetics of ethylmorphine demethylation in liver microsomes from male and female mice. * indicates a significant sex difference, $p<0.05$; ** id. $p<0.01$. Values represent means $\pm$ SE.

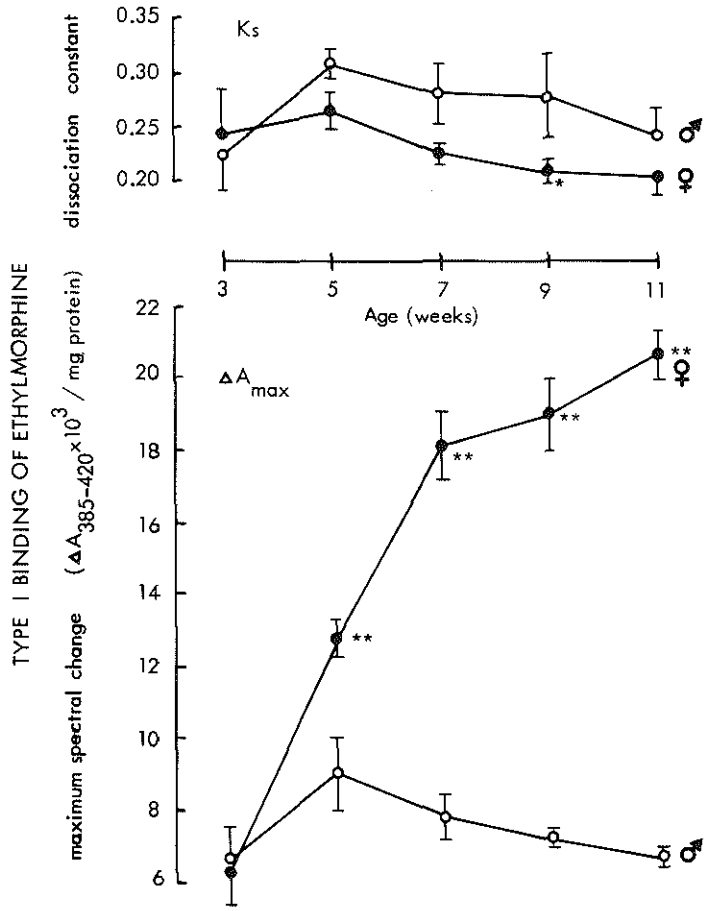

Fig. VII,2. Effects of age on the kinetics of type I binding of ethylmorphine in liver microsomes from male and female mice. * indicates a significant sex difference, $p<0.05$; ** id. $p<0.01$. Values represent means ± SE.

Cytochrome P-450 content.

The sex difference in cytochrome P-450 content in adult animals appeared to be mainly the result of developmental changes occurring in females (Fig. VII,3). Between 3 and 11 weeks an increase of 41% was observed. A significant sex difference in favour of females did not arise until the age of 7 weeks. At 3 weeks, even an opposite sex difference was noticed. The cyto-

chrome P-450 content does not seem to be related to the rate of demethylation, which is in agreement with our previous findings, as well as with those obtained in rats by many other authors (9-12,22,30,31).

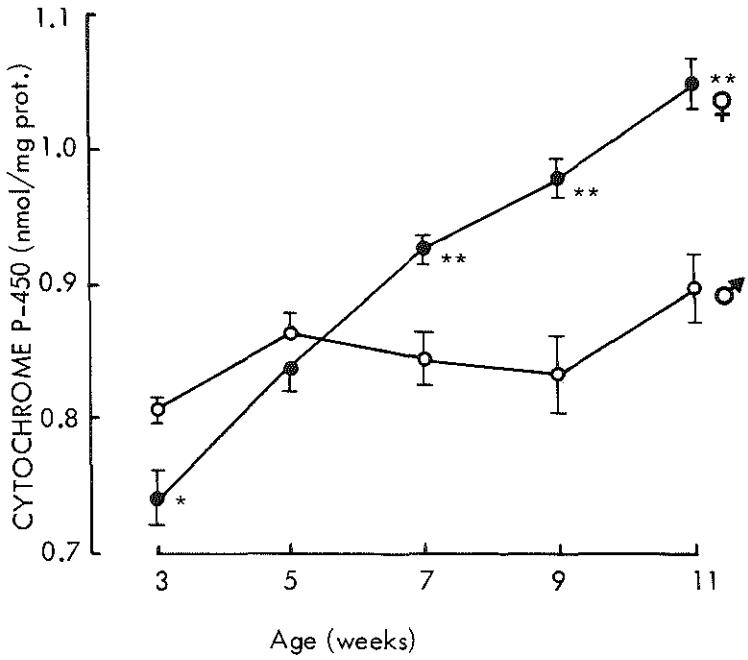

Fig. VII,3. Effects of age on the cytochrome P-450 content of liver microsomes from male and female mice. * indicates a significant sex difference, $p<0.05$; ** id. $p<0.01$. Values represent means ± SE.

Relationship between ΔA_{max} and cytochrome P-450.

The development of sex differences in ΔA_{max} and cytochrome P-450 content is mainly the result of age-dependent increases occurring in females (Figs. VII,2 and VII,3). The maximum spectral change is an expression of the total amount of binding sites involved in ethylmorphine binding. If all cytochrome P-450 is involved in this binding, then ΔA_{max} and cytochrome P-450 content should be related. The data presented, however, show that the relative changes in cytochrome P-450 content are much smaller than those in ΔA_{max}. A better proportionality can be obtained when the increments in female values

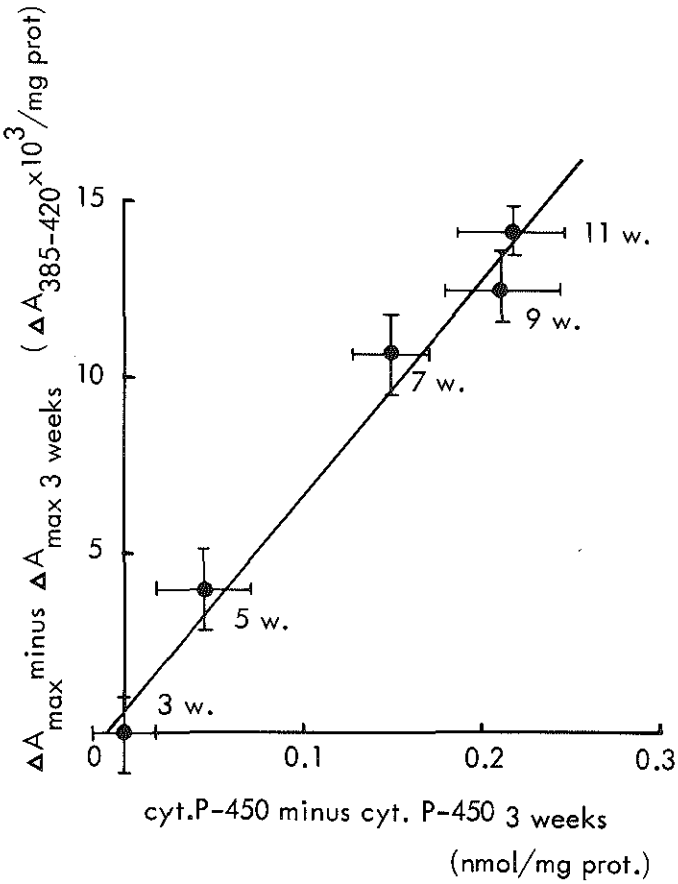

Fig. VII,4. Relationship between the sex-specific increases in maximum spectral change (ΔA_{max}) and cytochrome P-450 content in liver microsomes from female mice between 3 and 11 weeks of age. Values were calculated from the data presented in Figs. VII,1 and VII,3, by taking the differences between the male and female values at all ages and defining the value at 3 weeks as zero level. Standard errors were obtained using the weighted mean variances of the male and female series of experiments. The line shown was obtained by linear regression analysis.

are calculated at all time intervals, with the situation at 3 weeks as a reference level. Furthermore, when the female values are corrected for fluctuations occurring in the male, a linear proportionality between ΔA_{max} and ΔP-450 is observed (Fig. VII,4). This suggests that, until the age of 3 weeks, a low basal level of type I binding activity has developed in both

sexes, which does not involve the total amount of cytochrome P-450. Thereafter, an additional ethylmorphine binding capacity develops selectively in females. This might be due to synthesis of a cytochrome P-450 fraction with high specific binding activity, which can be calculated (using Fig. VII,4) to be about 7 times higher than that of the cytochrome already present.

NADPH-cytochrome P-450 reductase activity.

High basal activities of NADPH-cytochrome P-450 reductase were found at 3 weeks, which subsequently decreased to the same degree in males and females, 37 and 36% respectively (Fig. VII,5). A sex difference was already apparent at 3 weeks and remained constant up to 11 weeks (male values approximately 85% of those in females). The development of basal reductase activity resembled, to a certain extent, that of V_{max} of ethylmorphine demethylation. The decrease which appeared between 5 and 7 weeks, however, was not observed with V_{max}.

The sex difference in ethylmorphine-induced stimulation of NADPH-cytochrome P-450 reductase activity, observed previously (19), seems to arise from developmental changes in both sexes. Reductase stimulation increased by 95% in females, whereas a gradual 43% decrease was noticed in males. There appears to be no correlation with V_{max} values. The concentration of ethylmorphine used (1 mM), however, did not produce maximal stimulation. The use of still higher concentrations is hampered by the fact that the initial rate of reduction becomes too fast to be measured accurately. When the demethylation rate is examined at 1 mM ethylmorphine a developmental increase of approx. 50% is observed in females (due to the decreasing K_m), whereas hardly any change is noticed in males. Thus reductase stimulation does not correlate with the demethylation rate, in contrast to the results obtained with rats (11,22,23,32).

Relationship between reductase stimulation and type I binding.

The magnitude of spectral interaction in males did not change between 3 and 11 weeks, whereas a substantial decrease in reductase stimulation was noticed (Figs. VII,2 and VII,5). Furthermore, the increases in these parameters in females were parallel. Interestingly, however, the sex differences developed simultaneously and to the same extent (Table VII,1). This suggests that Δ_{red} and ΔA are intrinsically related, which would confirm our previous observations (19), but it indicates that this relationship is masked by

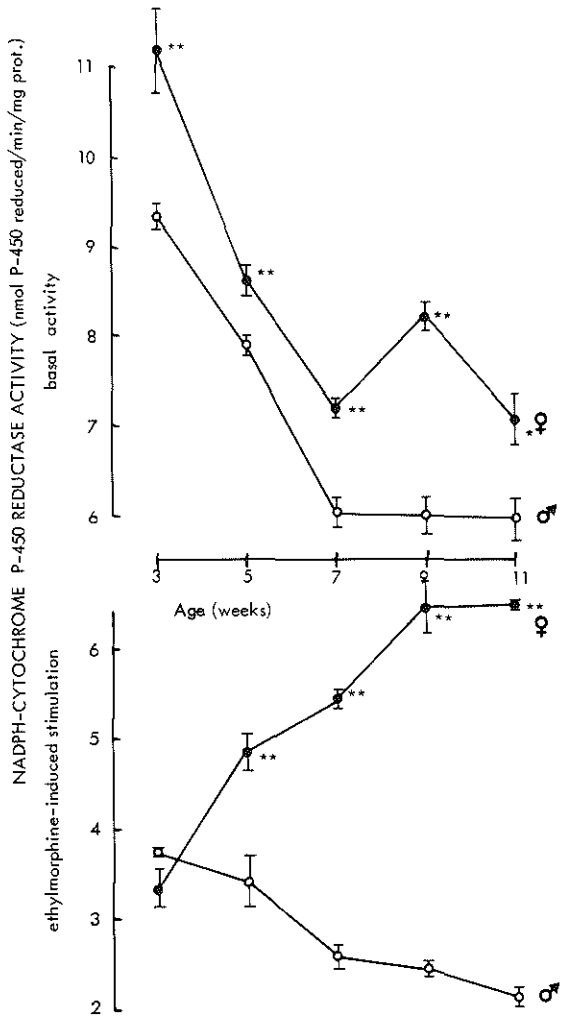

Fig. VII,5. Effects of age on basal NADPH-cytochrome P-450 reductase activity (upper part) and on ethylmorphine (1 mM)-induced stimulation of NADPH-cytochrome P-450 reductase activity (lower part) in male and female mice. * indicates a significant sex difference, $p<0.05$; ** id. $p<0.01$. Values represent means ± SE.

a factor which affects one of these parameters in both sexes to the same degree. This points to the decline in basal NADPH-cytochrome P-450 reductase activity, which amounted to 36% in males and 37% in females (Fig. VII,5). This decline is proportional to the decline in Δ_{red} values in males. The assumption that Δ_{red} was similarly affected in females satisfactorily explains the discrepancy between the increases in Δ_{red} and ΔA in this sex, and at the same time the highly-correlated development of sex differences in these parameters.

Table VII,1. The development of sex differences in ethylmorphine-induced stimulation of NADPH-cytochrome P-450 reductase activity and magnitude of type I spectral interaction.

age (weeks)	reductase stimulation* ($\frac{\text{males}}{\text{females}} \times 100\%$)	type I spectrum* ($\frac{\text{males}}{\text{females}} \times 100\%$)
3	112	106
5	71	68
7	48	40
9	38	35
11	33	31

* Values were calculated from the data shown in Figs. VII,1 and VII,5.

DISCUSSION.

The present study demonstrates a sex-dependency in the development of ethylmorphine N-demethylation, ethylmorphine binding to cytochrome P-450, cytochrome P-450 content and ethylmorphine-induced stimulation of NADPH-cytochrome P-450 reductase activity in mice. Adult values are reached at about 11 weeks of age, thus coinciding with sexual maturation. The time course of this development is similar to that found in rats, but the sex differences tend towards the opposite direction. We have demonstrated that,

in mice, castration of males and testosterone-treatment of females abolish the sex differences (17,19). These findings indicate an inhibitory effect of androgen, whereas, in rats, the androgen effect is presumed to be stimulatory (5). Androgen apparently prevents the stimulation of enzyme activity seen in female mice, which is probably the basic developmental pattern in this species. The responses to androgen might be enzyme-specific (33-35) as well as species-specific (36), as observed with some enzymes involved in steroid metabolism.

The different developmental patterns of the kinetic constants of the demethylation reaction and type I binding present a problem concerning the mechanism of the reaction. The concept that type I binding represents the formation of enzyme-substrate complex, which is subsequently reduced by NADPH-cytochrome P-450 reductase in a rate-limiting reaction (21,37), is apparently not applicable. In a previous study we observed that, in mice, the stimulation of cytochrome P-450 reductase activity by type I substrates was correlated with the magnitude of spectral interaction, thus confirming results obtained with rats (22,23), although certain discrepancies have been reported (11). The results presented in Figs. VII,1 and VII,5 apparently contradict the earlier findings. The data given in Table VII,1 and Fig. VII,5, however, indicate that this paradox may be explained on the basis of developmental changes in basal reductase activities. These changes might reflect changes in the (pseudo first order) rate constant of cytochrome P-450 reduction, which alter the absolute value of Δ_{red} independently from ΔA. The correlation between reductase stimulation and type I binding suggests that type I binding represents the formation of a functional cytochrome P-450-substrate complex.

Several authors have observed significant correlations between Δ_{red} and substrate oxidation in rats (11,22,23,32), but other reports have indicated that the reductase does not control the rate of substrate oxidation (38-40). Previously, we could not exclude this possibility, as any one of the accessible and routinely measured parameters (endogenous or basal reduction rate, substrate-induced or total reduction rate, or reductase stimulation) might not be totally representative of the rate of reduction of the cytochrome P-450-substrate complex, formed by type I binding (19). From the present results it is evident that cytochrome P-450 reduction is not correlated with ethylmorphine demethylation, whichever parameter of reductase activity is used.

If the reductase step is not rate-limiting, changes in K_m do not necess-

arily reflect changes in K_s. In the case of a reaction mechanism which is not completely ordered (as discussed by Gillette et al. (41)) K_m may approximate the dissociation constant of the oxygenated or the active oxygen cytochrome P-450-substrate complex rather than K_s. A changing K_m may be indicative of conformational changes or of a shift in the rate-limiting step. According to the most plausible models, however, V_{max} should be related to ΔA_{max}. If the intermediate forms of cytochrome P-450 reversibly interact with the substrate and if the unbound forms of the cytochrome are reduced and oxygenated at rates which differ considerably from those of the bound intermediates, plots of 1/v vs. 1/S will be curves. This curvature may not be detected and may lead to V_{max} and K_m values which are dependent on the substrate range chosen. Although in that case these parameters would be ill-defined, it is unlikely that this explains the highly diverging ΔA_{max} and V_{max} observed during development. Hayes et al. (42) have suggested the existence of two demethylases with different kinetic constants (in rats). This might introduce a discrepancy between ΔA_{max} and V_{max}. Our results may indicate the presence of two demethylases in female mice, one of which operates at a negligible rate, although they are characterized by cytochromes P-450 which have a similar ability to form type I complexes and to activate the reductase. This is suggestive of ethylmorphine functioning as a (partial) uncoupler of monooxygenase activity (43-45). Further work is needed to resolve these questions.

An increasing body of evidence suggests the existence of multiple forms of cytochrome P-450, which have distinct (but sometimes overlapping) specificities with regard to substrate or ligand interaction, and substrate hydroxylation (46-53). The demonstration of different classes of type I binding sites is of particular interest (48). Our study indicated that type I binding of ethylmorphine is restricted to a limited amount of cytochrome P-450. This was deduced from the data presented in Figs. VII,3 and VII,4, which demonstrate a correlation between the sex-specific increases in the extent of spectral interaction (ΔA_{max}) and cytochrome P-450, and not between the absolute values of these parameters. This correlation might indicate either specific synthesis of the ethylmorphine-binding cytochrome P-450 already present in small amounts in immature animals, or generalized synthesis of cytochrome P-450 together with a process of differentiation. The latter possibility is supported by the work of Levin et al. (54), who observed a sex-dependent differentiation of rat liver cytochrome P-450 into two fractions with different rates of turnover. It might be speculated that, in female mice, a type of ethylmorphine-binding cytochrome P-450 develops,

which is different with respect to functions other than type I binding (for example at the stage of the oxygenated complex), thus accounting for the kinetic anomalies discussed above.

Different levels of tightly-binding endogenous substrates may explain differences in type I binding (23,55). We have obtained evidence that this is not the case with the observed sex difference (Van den Berg et al., to be published) (Chapters IX and X).

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CHAPTER VIII. METABOLISM OF HEXOBARBITAL ENANTIOMERS AND INTERACTION WITH CYTOCHROME P-450 IN MALE AND FEMALE MICE AND RATS.

SUMMARY.

The metabolism of hexobarbital enantiomers by 9000 g liver supernatant was determined in mice and rats. Both male and female mice and rats metabolize hexobarbital stereospecifically. In mice the l-enantiomer is metabolized faster, whereas the opposite was observed in rats. The kinetics of metabolism and type I binding to cytochrome P-450 of the enantiomers was compared in female mice and male rats. In mice K_m equaled K_s for type I binding, but in rats K_m exceeded K_s. This suggests that in mice the type I binding site is the active site of the enzyme, whereas in rats this cannot be proved. In female rats the ΔA_{max} for type I binding of the enantiomers differs, indicating a binding to different cytochrome P-450 subspecies. Comparison of the enhancement of NADPH-cytochrome P-450 reductase activity by the enantiomers with their rate of metabolism at the same concentration revealed that in rats the reduction of the cytochrome P-450-substrate complex cannot be rate-limiting for the total reaction, whereas in mice this cannot be disproved. This would explain the apparent discrepancy between the kinetics of type I binding and metabolism in rats, which was not found in mice.

INTRODUCTION.

Comparison of the kinetic parameters of metabolism and type I binding of substrates to cytochrome P-450 can be used to investigate the possibility that the type I binding site is the catalytic site of the enzyme. This method is valid only if the reduction of the substrate complex is rate-limiting for the total reaction. Several authors have compared these parameters in different species and sexes (1,2,3). Although in some studies a similarity between K_m of metabolism and K_s of substrate binding was observed, in many others large differences were reported (4). Investigations conducted in this department, using a sex difference in mice for the metabolism of type I substrates (5,6), also revealed some discrepancies (7).

Instead of species and sex differences the use of different substrates may further clarify this problem. The ideal compounds for such studies are

the enantiomers of chiral substrates, many of which are metabolized stereospecifically (8). Up till now investigations with benzphetamine (9) and other demethylated substrates (10) failed to give consistent parallels between the kinetic constants of metabolism and type I binding. This may be due to cytochrome P-450-catalyzed metabolism of the substrate along pathways other than demethylation, which makes demethylation and substrate binding incomparable.

For our studies we chose hexobarbital as a substrate. In mice (11) and rats (12) this drug is converted almost exclusively into its 3-hydroxy metabolite, which is further dehydrogenated to 3-keto-hexobarbital. In unpublished experiments we found that in 9000 g mouse liver supernatant less than 2% of hexobarbital metabolism is due to demethylation. It appears, therefore, that only one reaction determines hexobarbital metabolism. Stereoselective metabolism of hexobarbital was demonstrated in rats (13-16), mice (15) and humans (17), but not in guinea-pigs (18). The kinetics of metabolism and type I binding of the enantiomers in untreated animals were compared only in guinea-pigs, in which no correlation was found (18).

In the present study the kinetics of metabolism and type I binding of hexobarbital enantiomers are compared in male and female mice and rats. In addition, the hexobarbital-induced enhancement of NADPH-cytochrome P-450 reductase activity was investigated. The formation of a cytochrome P-450 type I substrate complex is thought to be responsible for this enhancement, which is, therefore, suggested to be the expression of the reduction of this complex (19,20). If this reduction is rate-limiting in the hydroxylation reaction, differences in reduction enhancement by the enantiomers should be accompanied by similar differences in hydroxylation rate. Furthermore, a relationship between reductase stimulation and the magnitude of the type I spectral interaction is to be expected. This was also investigated.

MATERIALS AND METHODS.

Animals.

Male and female Swiss (CPB-SE) mice, at least 10 weeks old, and male and female Wistar (WAG) rats (TNO, Zeist) weighing 150-200 g were used.

Chemicals.

d- and l-hexobarbital, $(\alpha)_D^{20} = +12.0^\circ$; m.p.: 155°C and $(\alpha)_D^{20} = -12.5^\circ$;

m.p.: 154-155°C, were kindly donated by Prof. Dr. J. Knabe, University of Saarland, Saarbrücken, Germany. Racemic hexobarbital was obtained from the O.P.G., Utrecht. All other chemicals and solvents used were of analytical grade purity.

Hexobarbital metabolism.

Hexobarbital metabolism was assayed in 9000 g supernatant, which was prepared by centrifuging (20 min) a 1:2 (w/v) liver homogenate in 0.1 M Na-K phosphate buffer (pH 7.4) containing 0.1 mM EDTA. Enzyme incubations were done in the same buffer, containing 0.75 µmol NADP, 12.5 µmol glucose-6-P, 5 µmol nicotinamide, 12.5 µmol $MgCl_2$, hexobarbital as indicated and 1 ml (mouse) or 0.5 ml (rat) supernatant in a total volume of 3 ml. The incubates were shaken in 25 ml bottles at 37°C in air. The reaction was started by adding the supernatant to the prewarmed incubation medium and stopped after 5 min (rats) or 10 min (mice) by addition of 12 ml of heptane containing 1.5% n-amyl alcohol with shaking.

Hexobarbital was determined by means of GLC, as UV absorbing metabolites may interfere with spectrophotometric determination (21). Hexobarbital was extracted from the incubates with 12 ml heptane-amylalcohol containing 0.2 µmol methohexital as an internal standard, by shaking for 15 sec on a Vortex mixer as described by Breimer and Van Rossum (22). After centrifugation 10 ml of the organic layer were vigorously shaken for 5 minutes with 5 ml phosphate buffer, pH 11 (23), 4 ml of which were acidified with 0.4 ml 12.5 N HCl and shaken for 5 minutes with 20 ml $CHCl_3$. The chloroform layer was removed and evaporated to dryness. The residue was dissolved in 100 µl chloroform and 1 µl portions were injected into a Varian 2100 gaschromatograph, using a 1.8 m x 4 mm column containing 3% OV-17 on 80-100 mesh Chromosorb W (HP). The following temperatures were used: injection port: 260°C, oven: 210°C and FID detector 300°C. In each series of determinations a standard curve was included, which proved to be linear and showed small day-to-day variations.

Difference spectra and cytochrome P-450 reduction.

Difference spectra and cytochrome P-450 reduction were determined in microsomes. 9000 g Supernatant was prepared from a 1:4 (w/v) liver homogenate and subsequently centrifuged at 75,000 g for 90 min. The microsomal pellet was suspended in 0.1 M phosphate buffer (pH 7.4).

Spectra were recorded in an Aminco-Chance spectrophotometer in the split-beam mode. Microsomal suspensions contained 3 mg prot./ml. The difference

between maximum and minimum absorbance was taken as the magnitude of spectral interaction.

NADPH-cytochrome P-450 reductase activity was determined at 30°C under anaerobic conditions (protein concentration 1 mg/ml), essentially as described by Gigon et al. (19). An Aminco DW-2 UV-VIS spectrophotometer was used in the dual wavelength mode. Reductase stimulation represents the difference between the initial rates of reduction with and without added substrate. Further details will be provided elsewhere (24).

Microsomal protein was estimated according to the method of Lowry (25), using bovine serum albumin as a standard.

Calculations.

Kinetic constants for metabolism (K_m, V_{max}) and type I binding (K_s, ΔA_{max}) were calculated using the method of Wilkinson (26). In some cases more than 10% of substrate was metabolized. Therefore, the substrate concentrations used in the calculations were corrected for substrate disappearance as described by Lee and Wilson (27).

Statistical significance of differences was tested by analysis of variance and Student's t-test.

RESULTS.

Metabolism in male and female mice.

9000 g Supernatant of livers from both male and female CPB-SE mice metabolizes l-hexobarbital faster than its optical antipode, as can be seen in Table VIII,1. Female mice metabolize both enantiomers faster than males, as

Table VIII,1. Metabolism of hexobarbital enantiomers by 9000 g liver supernatant (nmoles/min/incubate) of male and female mice (0.167 mM) and rats (0.333 mM) (means ± SE).

	Mice		Rats	
	male	female	male	female
d	7.8 ± 1.2 (7)	14.6 ± 0.8 (8)	73.7 ± 3.9 (3)	16.9 ± 0.8 (4)
l	13.4 ± 1.1 (7)	22.9 ± 0.8 (8)	25.8 ± 1.9 (4)	0.91± 0.40 (3)
	p<0.001	p<0.001	p<0.005	p<0.001

was previously observed for racemic hexobarbital and other type I compounds (5,6). This result is in contrast to the work of McCarthy and Stitzel (15), who found not the l- but rather the d-enantiomer to be metabolized faster in their mice.

Kinetics of metabolism and type I binding in female mice.

Because the d-enantiomer of hexobarbital is metabolized very slowly in male mice (Table VIII,1), our method of measuring hexobarbital metabolism by dissappearance of substrate does not enable us to determine kinetic constants of this reaction in these animals. Similarly, the kinetics of type I binding could not be determined in male mice, due to the extremely small difference spectra produced by d-hexobarbital. Therefore, only values in females are given. Table VIII,2 summarizes the results. The substrate concentrations used in metabolic studies were 3, 4.5, 6, 7.5 and 9 mM^{-1}. In binding studies 2, 4, 6, 8 and 10 mM^{-1} hexobarbital were used. Linear Lineweaver-Burk plots were obtained for metabolism as well as type I binding of the enantiomers. A significant difference ($p<0.02$) was found for the K_m values of the enantiomers, calculated with or without corrected substrate concentrations. No difference was found in V_{max}. For type I binding an analogous result was obtained. K_s values were different ($p<0.005$) whereas ΔA_{max} values were not. The values for K_m, calculated using uncorrected substrate concentrations exceeded the analogous K_s, but calculation from corrected concentrations gave K_m values which did not exceed K_s.

Table VIII,2. Kinetic constants of metabolism and type I binding of hexobarbital enantiomers in female mice and male rats (means ± SE).

	Mice		Rats	
	d	l	d	l
K_m (mM)	0.180 ± 0.029	0.092 ± 0.009	0.114 ± 0.014	0.225 ± 0.33
K_m (mM)*	0.112 ± 0.022	0.053 ± 0.005	0.071 ± 0.009	0.193 ± 0.026
K_s (mM)	0.114 ± 0.011	0.073 ± 0.004	0.014 ± 0.0004	0.036 ± 0.005
V_{max}	38.9 ± 2.3	37.3 ± 2.7	95.6 ± 5.4	44.3 ± 4.0
V_{max}*	35.4 ± 2.1	35.2 ± 2.2	90.6 ± 4.8	44.0 ± 3.3
ΔA_{max}	18.0 ± 0.7	19.8 ± 0.6	24.5 ± 0.6	24.6 ± 2.0

*Corrected for substrate disappearance.
n = 12 (K_m, V_{max}) or 5 (K_s, ΔA_{max}) in rats and 8 in mice.
V_{max} is expressed as nmol/min/incubate and ΔA_{max} as ΔA/mg protein.

Metabolism in male and female rats.

Table VIII,1 gives the rate of metabolism of hexobarbital enantiomers in male and female rats. In this species the opposite result was obtained from that in mice. The d-enantiomer was metabolized faster than its antipode in males as well as in females, which is in contrast to the results of Furner et al. (13). Male animals metabolized each enantiomer faster than females.

Kinetics of metabolism and type I binding in rats.

As can be seen from Table VIII,1, female rats metabolized l-hexobarbital very slowly. Therefore, no kinetic studies on hexobarbital metabolism were carried out in these animals. Type I binding spectra, however, were of sufficient magnitude to study binding kinetics. Table VIII,2 summarizes kinetic constants for metabolism and type I binding obtained in male rats. The concentrations used in metabolic studies were 1.5, 3, 4.5, 6, 7.5, 9 and 20.5 mM^{-1}, whereas 10, 50, 100, 150 and 200 mM^{-1} hexobarbital were used in binding studies. Linear Lineweaver-Burk plots were obtained both for metabolism and type I binding. The values of K_m obtained in male rats using our method agree with those for racemic hexobarbital reported by Sitar and Mannering (28) and Kupfer and Rosenfeld (29), who used product formation instead of substrate disappearance for the determination of reaction rates. For the more slowly metabolized l-hexobarbital a higher K_m ($p<0.001$) and a lower V_{max} ($p<0.001$) were observed than for the d-enantiomer, as was also reported by McCarthy and Stitzel (15). For type I binding a significant parallel difference in K_s ($p<0.01$) was found, whereas ΔA_{max} values were similar. The K_m values were in this case higher than the K_s for the same enantiomer, even after correction for substrate disappearance. In female rats not only K_s ($p<0.01$), but also ΔA_{max} ($p<0.01$) was different for the enantiomers, indicating that the nature of the type I binding sites are different for d- and l-hexobarbital. Hexobarbital concentrations of 2, 4, 6, 8 and 10 mM^{-1} were used in these determinations.

Enhancement of cytochrome P-450 reductase activity.

Due to the rather low enhancements of cytochrome P-450 reduction produced by hexobarbital it was not possible to obtain reliable values in some cases in concentration regions where differences in the hydroxylation rates of the enantiomers are the most prominent. This relates in particular to the male mouse, of which no data are presented. In female mice at 1.0 mM no difference

Table VIII,3. Enhancement of NADPH-cytochrome P-450 reductase activity, rate of metabolism, and magnitude of spectral interaction of d- and l-hexobarbital in female mice and male and female rats (means ± SE).

		reductase stimulation	rate of metabolism	type I spectral change
female mice	d	2.45 ± 0.11 (14)	31.6 ± 1.9 (8)	16.2 ± 0.7 (8)
1.0 mM	l	2.33 ± 0.11 (14)	33.4 ± 2.1 (9)	18.5 ± 0.6 (8)*
male rats	d	1.81 ± 0.14 (5)	53.5 ± 2.2 (12)	23.5 ± 0.9 (5)
0.1 mM	l	1.80 ± 0.13 (4)	15.5 ± 1.0 (12)	17.4 ± 0.6 (5)**
female rats	d	2.29 ± 0.16 (10)		8.0 ± 0.3 (8)
0.5 mM	l	1.83 ± 0.11 (10)*		5.5 ± 0.3 (8)**

Spectral changes and metabolic rates were derived from the kinetic studies presented in Table VIII,2 and are expressed in the units indicated there. Reductase stimulation is expressed as nmol cytochrome P-450 reduced/min/mg prot.
* Significant difference between d and l, $p<0.05$; ** id., $p<0.001$.

in reductase stimulations produced by the enantiomers could be detected (Table VIII,3). However, at this concentration also no significant difference in hydroxylation rates was apparent. These findings cannot prove the involvement of cytochrome P-450 complex reduction as a rate-limiting step, but the combined data on binding and hydroxylation kinetics and cytochrome P-450 reduction leaves this possibility open.

In male rats d- and l-hexobarbital (0.1 mM) enhanced cytochrome P-450 reduction to the same extent, although at this concentration the metabolic rates differed by a factor of 3.5. In female rats at 0.5 mM a small, but significant difference was found in reductase stimulation. In Table VIII,1 it was shown that at a concentration of 0.33 mM d-hexobarbital was metabolized about 18 times faster than the l-enantiomer. These large discrepancies make it very unlikely that in rats the reduction of cytochrome P-450-substrate complex is rate-limiting. Furthermore, Table VIII,3 demonstrates statistically significant differences between the magnitudes of type I spectra of the enantiomers in all cases. This could mean either that reductase stimulation is not as dependent on type I binding as is currently believed, or that the initial rate of reduction of cytochrome P-450-substrate complex is not only dependent on the concentration of this complex.

DISCUSSION.

The present results indicate that, in the Swiss mouse strain we use, stereospecific metabolism of hexobarbital takes place. In contrast to the results of McCarthy and Stitzel (15) the l-enantiomer was metabolized faster. Apparently strain differences in stereoselectivity exist. The linear Lineweaver-Burk plots obtained for hexobarbital metabolism in female mice suggest a completely ordered system, as discussed by Gillette et al. (30). The difference found in K_m correlates with a similar difference in K_s for type I binding. The similarity of both V_{max} and ΔA_{max} of the enantiomers indicates that they interact with the same cytochrome(s) P-450. The K_m values of the enantiomers, if corrected for substrate disappearance, did not exceed K_s, as was also observed for dl-hexobarbital (data not given). Furthermore, the rates of metabolism and the enhancements of NADPH-cytochrome P-450 reductase activity at the same concentration did not differ for the enantiomers. This suggests that in female mice the reduction of the cytochrome P-450-substrate complex is rate-limiting and, moreover, because K_m and K_s values are similar, that the type I binding site is the active site of the enzyme.

In rats, the situation with regard to stereoselectivity and sex difference is the opposite of that in mice. In this species the d-enantiomer is metabolized faster and males metabolize both enantiomers faster than females. The stereoselectivity is seen in both males and females, whereas Furner et al. (13) found it only in males. Their in vivo results, however, clearly demonstrate that in females the d-enantiomer is eliminated faster. In male rats we found a difference in both K_m and V_{max} for the enantiomers. A similar difference was found for the K_s of type I binding, although, even after correction for substrate disappearance, K_m exceeded K_s. This difference may be due to a fast product dissociation, which influences K_m, but not K_s. Moreover, K_s may be concentration range dependent (31) and the difference between K_m and K_s might be caused by the different concentration ranges used for the determination of these constants. The large difference in rates of metabolism of the enantiomers was not paralleled by a similar difference in the enhancements of NADPH-cytochrome P-450 reductase activity, measured at the same substrate concentration. This indicates that the reduction of the cytochrome P-450-substrate complex is not rate-limiting for the total reaction, which would also explain why the kinetic constants of type I binding and metabolism are different.

In female rats only type I binding could be investigated. Here again, the faster metabolized enantiomer had a lower K_s, but, in contrast to the results

in males, also a higher ΔA_{max}. The latter observation suggests that the enantiomers bind to different populations of type I binding sites in female rats. Recently Grasdalen et al. (32) demonstrated a heterogeneity of type I binding sites in male rats, which may occur in females as well. Moreover, Degkwitz et al. (14) and Feller and Lubaway (16) reported ΔA_{max} differences for d- and l-hexobarbital in phenobarbital-induced male rats, which may also be explained by binding to different type I site subspecies.

Comparing the kinetics of type I binding in female and male rats, a sex difference in the composition of cytochrome P-450 is apparent. This is concluded from the sex differences in the respective ΔA_{max} values, which are much larger than the sex difference in cytochrome P-450 (data not given), as also observed for racemic hexobarbital by Schenkman et al. (1) and by the presence of a ΔA_{max} difference for the enantiomers in females, which is absent in males. On the basis of other evidence El Defrawy et al. (33) came to a similar conclusion.

In all experiments significant differences were found between the magnitudes of the spectral changes induced by the hexobarbital enantiomers at the concentrations used for the reductase stimulation assays. However, only in female rats a concomitant, but relatively small difference in reductase stimulation was observed. This apparent discrepancy between type I binding and reductase stimulation seems to be at variance with the concept that by type I binding an additional amount of cytochrome P-450 is involved in the first phase of reduction, thereby causing an increase in initial reduction rate (19,20). In support with this we have observed simultaneous variations of type I binding and reductase stimulation in mice, using ethylmorphine as a substrate (24). The present results, however, are not necessarily in conflict with these findings. For we have also observed, that different substrates, which elicit type I spectra of comparable size, can produce diverging reductase stimulations:

	reductase stim. (nmol/min/mg prot.)	spectral change ($\Delta A \times 10^3$/mg prot.)
Hexobarbital (dl) 1.0 mM	2.46 ± 0.15	16.9 ± 0.4
Ethylmorphine 1.0 mM	6.52 ± 0.05	17.4 ± 0.2

(means ± SE of 4 determinations)

This suggests that the reductase stimulation is not simply the expression of an additional amount of cytochrome P-450 being involved in the first fast

phase of reduction. It could very well be that substrates activate similar amounts of cytochrome P-450, but that the rate constants of cytochrome P-450-substrate complex reduction differ. For the hexobarbital enantiomers such differences in rate constants could be opposed to differences in amounts of complex formed, which occasionally might lead to reductase stimulations of similar size. Alternatively, or perhaps additionally, the binding sites for reductase stimulation and type I binding might be distinct (although tightly coupled) and have different substrate affinities, as suggested by Holtzman and Rumack (3). A significant difference in reductase stimulation was found only in a case where ΔA_{max} values were different (female rats). This might indicate that type I and activation sites are present in equal amounts (situated on the same cytochrome P-450 species), but that the binding to the activation sites is less or not stereoselective.

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CHAPTER IX. THE RELATION BETWEEN THE SEX-DEPENDENCY OF TYPE I BINDING OF ETHYLMORPHINE AND THE 1-BUTANOL-INDUCED SPECTRAL CHANGE IN MOUSE LIVER MICROSOMES.

SUMMARY.

1. A sex difference in the spectral interaction of 1-butanol with liver microsomes from adult mice was observed. In males a profound reverse type I spectrum was elicited, whereas only a small spectral change of irregular shape was apparent in females. This sex difference is the opposite of that observed in the type I binding of ethylmorphine. In immature animals no sex difference was found. Testosterone pretreatment of female mice increased the size of the 1-butanol spectrum concomitantly with a decrease in ethylmorphine binding.

2. Microsomes from males and females did not contain different levels of endogenous substrates. Thus, the presence or displacement of such substrates does not explain the sex differences in type I and reverse type I binding respectively.

3. 1-Butanol was found to interfere with both type II and type I binding. It is concluded that the 1-butanol-induced spectral change consists of at least two components and that the sex difference is due to a sex-dependent type I component.

INTRODUCTION.

Many compounds give rise to characteristic spectral changes, when added to a liver microsomal suspension. This is thought to reflect binding to cytochrome P-450 (1). Two main types of difference spectra have been defined: type I (peak at 385-390 nm, trough at 420 nm), caused by binding to a hydrophobic part of the cytochrome, and type II (peak at 425-430 nm, trough at 390-400 nm), caused by direct binding to the heme (2). The difference spectra elicited by 1-butanol and several other compounds did not fit this classification and were called modified type II or reverse type I (2,3).

In this paper we describe a sex difference in the 1-butanol-induced spectral change in mouse liver microsomes. Thus far, this spectral change has been investigated mostly in male rats. The reverse type I spectral change has

been observed, which may reflect the reconversion of the (type I) substrate-bound form of cytochrome P-450 into the substrate-free form. Several authors have tried to confirm this concept, though with varying results (3-6). Lately, some evidence has been obtained that the 1-butanol spectrum might represent a distinct type of spectral change rather than the displacement of endogenous substrates (7-9).

Previously, we have demonstrated a sex difference in type I binding in mice (10), which is opposite to that observed in rats (11). Female mice have a much larger capacity for binding type I substrates (ethylmorphine, hexobarbital) than males as the result of an inhibitory action of androgen (10,12, 13). The sex difference in the magnitude of the 1-butanol spectrum appeared to be the opposite of that found with the type I spectrum, suggesting the presence of different levels of endogenous substrates. On the other hand, we have obtained evidence that the sex difference in type I binding is caused by the existence of different amounts of cytochrome P-450 capable of type I binding (13). It was, therefore, of interest to further investigate the nature of the 1-butanol-induced spectral change in mouse liver microsomes.

MATERIALS AND METHODS.

Animals.

Male and female mice of the CPB-SE strain were obtained from the Central Animal Breeding Station TNO, Zeist, The Netherlands. They were kept in Makrolon cages with pinewood shavings, received standard food pellets (Hope Farms) and tap water *ad lib.*, and were used after they had reached the age of 11 weeks, unless stated otherwise.

Chemicals.

1-Butanol (p.a.) was obtained from Merck, ethylmorphine from Brocacef, and crystalline bovine serum albumin from Poviet. Testosterone propionate was a generous gift from Organon. All other chemicals used were at least reagent grade.

Treatments.

In one experiment female mice were pretreated with testosterone. This was carried out by subcutaneous injection of testosterone propionate (2.5 mg in

0.1 ml arachis oil/animal) 14, 10, 7, and 3 days prior to the experiments. Control animals received an equivalent volume of oil.

Preparation of microsomes.

After starving overnight, the animals were killed by a blow on the head. Livers were homogenized in 3 volumes of 0.1 M phosphate buffer (pH 7.4), using glass Potter tubes with a Teflon pestle. The homogenate was centrifuged for 20 minutes at 9000 g and the microsomal fraction was sedimented from the resulting supernatant by centrifuging at 75,000 g for 90 minutes. The pellet was suspended in 0.1 M phosphate buffer (pH 7.4). For each determination the livers of several animals were pooled.

Organic solvent extraction.

Extraction of liver microsomes was carried out essentially as described by Vore et al. (6). Microsomal suspensions (7-8 mg protein/ml) were quickly frozen in liquid nitrogen, and subsequently freeze-dried. A portion of freeze-dried microsomes, containing about 36 mg protein, was homogenized in 9 ml acetone and centrifuged for 5 min at 35,000 g (-20°C). The pellet was resuspended in 9 ml of acetone and again centrifuged. A final homogenate in 9 ml of acetone was filtered on a Büchner funnel. The filter contents were kept in a desiccator *in vacuo* for 30 min at 4°C. The dried powder was homogenized in 18 ml Na/K-phosphate buffer (0.1 M, pH 7.4) and sonicated for three 5-sec intervals, in order to obtain a stable suspension. This sonication did not result in any loss of cytochrome P-450. Extraction with 1-butanol was performed by first treating the freeze-dried microsomes with 9 ml of 1-butanol, followed by two subsequent extractions with acetone, according to the above procedure. As a control, freeze-dried microsomes were directly suspended in buffer.

Spectral measurements.

Difference spectra were recorded using either an Aminco-Chance or an Aminco DW-2 UV-VIS spectrophotometer in the split-beam mode. Microsomal suspensions, containing 1-2 mg protein/ml, were equally divided between sample and reference cuvettes, and a baseline of zero absorbance was established. Substrates were added as indicated in the text.

The concentration of cytochrome P-450 was estimated by gassing the microsomes for 1 minute with carbon monoxide, dividing them between sample

and reference cuvettes, and then reducing the contents of the sample cuvette by the addition of a few crystals of sodium dithionite. The absorbance difference between 450 and 490 nm was taken as a measure of the cytochrome P-450 concentration, using an absorption coefficient of 91 $cm^{-1}mM^{-1}$ (14).

Protein determination.

Microsomal protein was determined according to the method of Lowry et al. (15), using crystalline bovine serum albumin as a standard.

RESULTS.

1-Butanol-induced spectral changes.

Fig. IX,1 shows typical recordings of the spectral changes induced by 1-butanol in male and female mice. The male spectrum is characterized by a

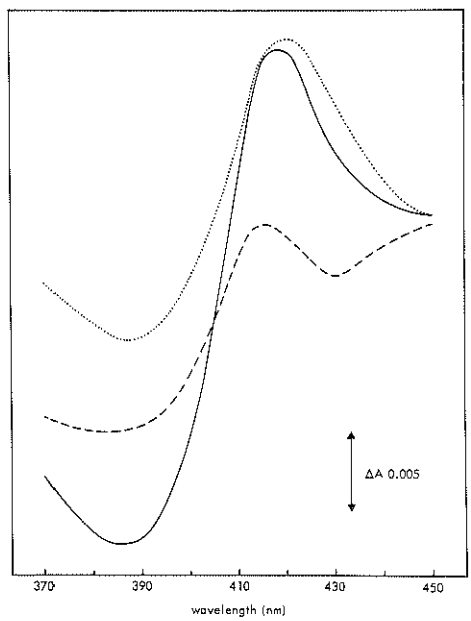

Fig. IX,1. 1-Butanol-induced difference spectra in liver microsomes from male and female mice. ——— Males; - - - - females; ····· difference between ——— and - - -. Cytochrome P-450 contents: males 1.46 nmol/ml; females 1.44 nmol/ml. 1-Butanol concentration: 66 mM.

broad trough around 385-390 nm and a peak at wavelengths varying between 416 and 420 nm. The female spectrum has a rather anomalous appearance: a slight trough around 385 nm and a peak between 410 and 420 nm. The general appearance of the spectra suggests that a different type of absorbance change interferes with that due to cytochrome P-450-substrate interaction, causing the absorbance to rise with increasing wavelength. This might represent a solvent effect of butanol, causing a change in turbidity of the microsomal suspension (it was not reversed by the addition of an equivalent volume of buffer to the reference cuvette). When the female spectrum is subtracted from the male spectrum, a spectrum is obtained which is the reverse of the type I spectral change (Fig. IX,1).

Effects of testosterone pretreatment.

Both testosterone pretreatment and castration of male and female mice have previously been shown to abolish the sex difference in type I binding of

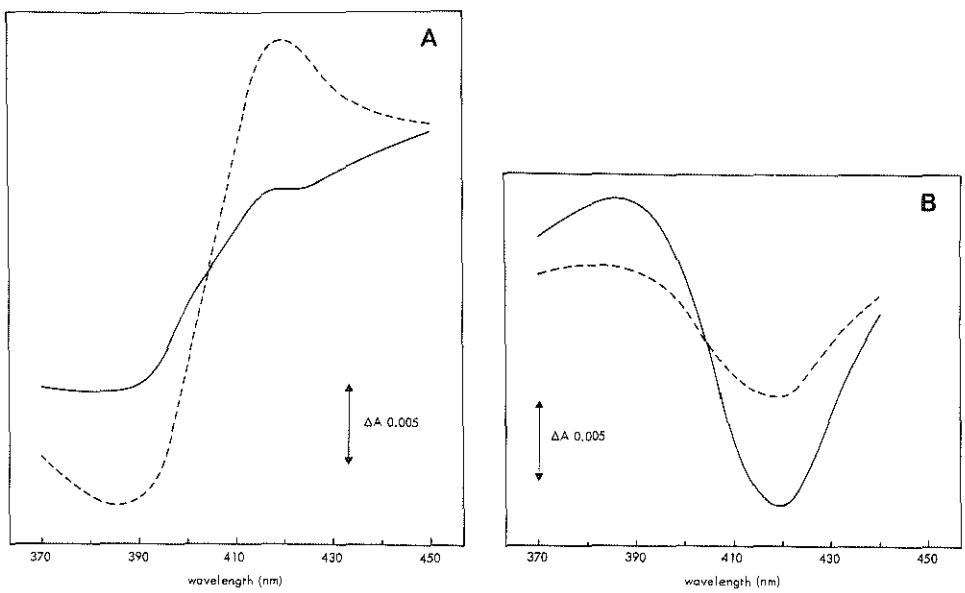

Fig. IX,2. Effects of testosterone pretreatment on the 1-butanol (A) and ethylmorphine (B) induced difference spectra in liver microsomes of female mice. ———— Control (oil-treated); - - - - testosterone-treated. Cytochrome P-450 contents: 1.19 (contr.) and 1.23 (test.) nmol/ml. Substrate concentrations: 1-butanol, 88 mM; ethylmorphine, 2 mM.

ethylmorphine (12). Testosterone pretreatment, in the present study, appeared to enlarge the 1-butanol-induced spectral change in females (Fig. IX,2A) together with a reduction in the size of the ethylmorphine-induced spectral change (Fig. IX,2B). This suggests that these effects are interrelated.

Effects of age.

There was no sex difference in the type I binding capacity of ethylmorphine when 3 week old mice were used (13). Table IX,1 shows that at 3 weeks the 1-butanol-induced spectral change in females is slightly larger than that observed in males, when the values are calculated on the basis of microsomal protein content. When the magnitude of the spectral change is related to the cytochrome P-450 content, however, a sex difference is not observed. The magnitudes of the spectral changes in immature males and females are equal to those observed in adult males. This indicates that the development of the sex difference in the 1-butanol-induced spectral change results solely from a decrease in the spectral change in females.

Extraction of microsomes.

Simply washing the microsomes with 1-butanol, in a concentration used for the spectral measurements (i.e. 25 ml microsomal suspension containing approx. 2 nmol P-450/ml, mixed with 200 µl 1-butanol, gently stirred for 15 min, sedimented and resuspended), did not yield significant effects. This procedure even tended to slightly decrease the type I spectrum and increase the 1-butanol spectrum (data not shown). Extraction of freeze-dried microsomes with 1-butanol and/or acetone has been shown to increase the type I spectral change in male rats (6). Table IX,2 shows that this treatment did not significantly increase the ethylmorphine binding spectrum, but decreased the 1-butanol spectrum.

Difference between the absolute absorption spectra of males and females.

The absolute absorption spectra of cytochrome P-450 in microsomes from male and female mice were investigated by a direct spectrophotometric comparison (Fig. IX,3), in order to detect a possible sex difference in the amount of the substrate-bound form of cytochrome P-450. The amounts of cytochrome b_5 were balanced by diluting the microsomes from females by 25%. Fortunately, this eliminated the difference in the cytochrome P-450 contents almost completely, so that the resulting difference spectrum essentially

Table IX,1. The magnitude of the 1-butanol-induced spectral change in liver microsomes from immature and mature male and female mice.

	$\Delta A_{420-385}$/mg protein		$\Delta A_{420-385}$/nmol P-450	
	3 weeks	adult	3 weeks	adult
Males	14.4 ± 0.7 (3)	21.18 ± 1.28 (14)[b]	19.5 ± 0.7 (3)	21.38 ± 1.01 (12)
Females	17.2 ± 0.7 (3)[d]	8.24 ± 0.94 (16)[a,c]	20.0 ± 0.9 (3)	6.93 ± 0.98 (14)[a,c]

Values represent means ± SE of the number of experiments indicated in parentheses.
a Significant effect of age, $p<0.001$; b id., $p<0.05$ (Student's t-test).
c Significant sex difference, $p<0.001$; d id., $p<0.05$.

Table IX,2. The effects of organic solvent extraction of freeze-dried liver microsomes from male mice on the ethylmorphine- and 1-butanol-induced spectral changes.

	spectral changes after three acetone extractions (n=3)		spectral changes after one 1-butanol and two acetone extractions (n=2)	
	control	control minus extracted	control	control minus extracted
3 mM ethylmorphine	5.2 ± 0.5	1.5 ± 1.1	5.0 ± 0.8	2.4 ± 0.7
88 mM 1-butanol	18.3 ± 0.9	-2.3 ± 0.4 *	17.5 ± 0.5	-4.3 ± 0.0 *

Values are expressed as $\Delta A_{385-420}$ (ethylmorphine) or $\Delta A_{420-385}$ (1-butanol)/nmol P-450, and represent means ± SE. * Significant effect of extraction, $p<0.05$ (paired t-test).

reflects only differences in the composition of the male and female cytochrome P-450 populations. Fig. IX,3 shows a decrease in absorbance with increasing wavelength, which is due to a difference in the turbidity of the two preparations, caused by the dilution of one of them. In addition to this only a small shoulder seems to be present around 410 nm.

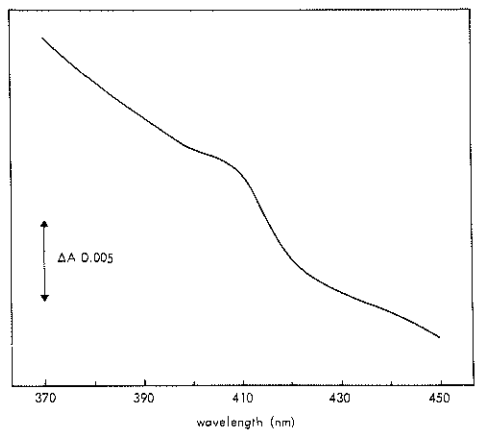

Fig. IX,3. Difference between the absolute absorption spectra of liver microsomes from male and female mice (males minus females).
The spectrum was obtained essentially according to the method used by Kinoshita and Horie (16) for the determination of the effect of induction. The cytochrome b_5 contents of the male and female microsomal preparations (approx. 1 mg prot/ml) were estimated by reduction with NADH. The cytochrome b_5 concentrations were balanced by diluting the female preparation. The final cytochrome P-450 concentrations after this balancing were 1.05 (males) and 1,06 nmol/ml (females). A baseline of zero absorbance was established with female microsomes in both sample and reference cuvettes. The contents of the reference cuvette were then replaced by the male microsomal preparation, and the difference spectrum was recorded.

Effects of type I and type II compounds on the 1-butanol-induced spectral change.

Fig. IX,4A shows that the presence of aniline in sample and reference cuvettes diminishes the magnitude of the 1-butanol-induced difference spectrum in males, and alters the position of the absorbance maximum,

indicating the disappearance of a type II component. An analogous effect was observed in females, although the resulting spectrum is complex (Fig. IX,4B). This indicates either that aniline and 1-butanol compete for type II binding sites, or that 1-butanol inhibits aniline binding non-competitively.

When, in addition, ethylmorphine was added, the final spectra were the same in males and females. The female spectrum lost a large type I component, whereas the male spectrum was only slightly changed. This indicates that 1-butanol also interferes with type I binding, which is particularly evident in females. In Fig. IX,5 this interference is further demonstrated by the effect of hexobarbital on the female 1-butanol spectrum. The resulting spectrum resembles the 1-butanol spectrum observed in males. These results suggest that the sex difference in the 1-butanol-induced spectral change may be due to sex-dependent type I binding.

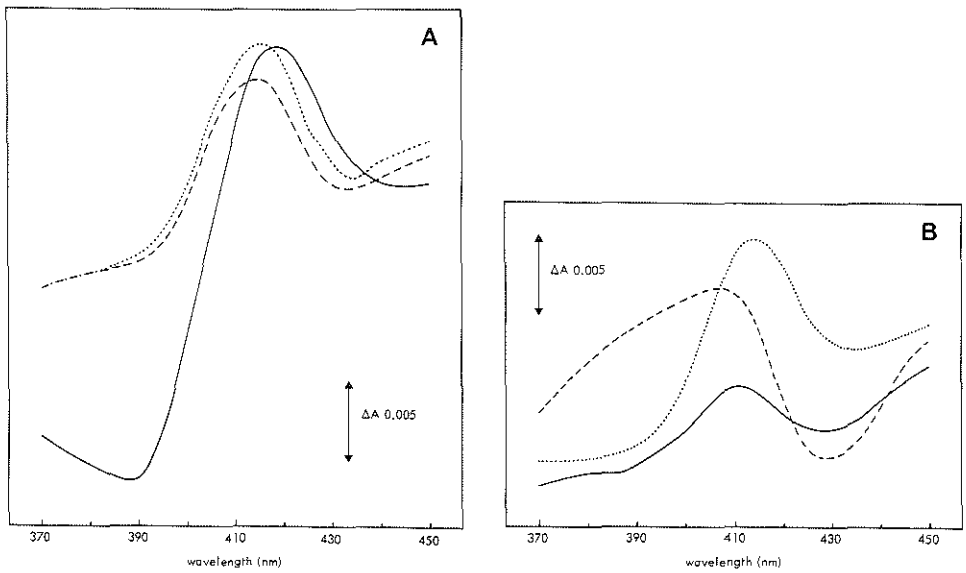

Fig. IX,4. The effects of aniline and ethylmorphine on the 1-butanol-induced spectral changes in liver microsomes from male (A) and female (B) mice. ———— 88 mM 1-butanol in the sample cuvette; - - - - 88 mM 1-butanol + 3 mM aniline in the sample cuvette and 3 mM aniline in the reference cuvette; ····· 88 mM 1-butanol + 3 mM aniline + 3 mM ethylmorphine in the sample cuvette and 3 mM aniline + 3 mM ethylmorphine in the reference cuvette. Cytochrome P-450 contents: males 1.28 nmol/ml; females 1.22 nmol/ml.

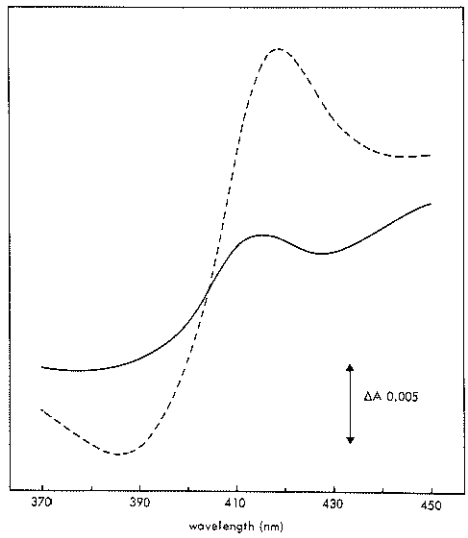

Fig. IX,5. The effect of hexobarbital on the 1-butanol-induced spectral change in liver microsomes from female mice. ———— 88 mM 1-butanol in the sample cuvette; - - - - 88 mM 1-butanol in the sample cuvette and 3 mM hexobarbital in both sample and reference cuvettes. Cytochrome P-450 concentration: 1.10 nmol/ml.

DISCUSSION.

If the 1-butanol-induced spectral change represents the displacement of endogenous substrates (4), the sex difference in this spectral change, observed in this study (Fig. IX,1), indicates the existence of a high level of endogenous substrates in microsomes from male mice as compared with females. This may explain the sex difference in the type I binding of ethylmorphine and hexobarbital (the binding capacity in males is approximately threefold lower than in females), which is due to the unveiling of an inhibitory action of androgens during sexual maturation (10,12,13). Fig. IX,2 shows that testosterone pretreatment, in fact, caused an increase in the 1-butanol spectrum and a concomitant decrease in the ethylmorphine spectrum.

Several observations, however, make the role of endogenous substrates less obvious. (1) The sex difference in the 1-butanol spectrum is due to a developmental decrease occurring in females (Table IX,1). (2) The sex-depend-

ency of type I binding is due to ΔA_{max} and not to K_s (13) (but the endogenous substrates might be bound very tightly). (3) Washing the microsomes from male mice with 1-butanol had no appreciable effect (the presumably very lipophilic compounds, however, might remain with the microsomal membrane rather than be removed with the supernatant). (4) Organic solvent extraction of freeze-dried microsomes from male mice only slightly decreased the 1-butanol spectrum, and had no effect on type I binding.

Convincing evidence that male microsomes do not contain more endogenous substrates than females was presented in Fig. IX,3. The binding of endogenous type I substrates should result in an increase in the amount of the substrate-bound form of cytochrome P-450 at the expense of the substrate-free form. The recording of a difference spectrum with male and female microsomes (balanced with respect to the cytochrome b_5 and cytochrome P-450 contents) in sample and reference cuvettes respectively, should, therefore, simply yield a type I spectrum. No such spectrum was found, thus indicating that male microsomes do not contain more substrate-bound cytochrome P-450 than females, if it is present at all.

The reverse type I spectral change may represent a conformational change in cytochrome P-450, due to a loading of the membrane with substrate (17,18). Although 1-butanol affects the membrane structure (19,20) and the microsomal membrane might have a sex-dependent lipid composition (21) this is not likely to explain the sex-dependent 1-butanol spectra. Organic solvent extraction, which removes a great deal of the lipid material (6), only slightly decreased the spectral change (Table IX,2).

It has been suggested that 1-butanol interacts directly with the heme-iron (7-9), due to the nucleophilic OH-group, and thus may interfere with the binding of the type II compound aniline. Our results demonstrate that 1-butanol interferes with type II as well as type I binding. Multiple interaction between a substrate and cytochrome P-450 has been often reported (22-26). Type I binding of 1-butanol, however, has not been observed before. Only some 1-alkanols with hydrocarbon chains longer than 5 C-atoms were shown to have a type I component in their spectra in male rats (7) and male mice (8).

The 1-butanol spectra obtained in the presence of aniline together with ethylmorphine show the same size in males and females (Fig. IX,4). Type II binding is not sex-dependent, at least when related to the cytochrome P-450 content, as demonstrated with aniline (10). These observations thus indicate that the sex difference in the 1-butanol-induced spectral change in the mice we investigated is due to a sex-dependent type I binding. This elegantly

explains all observed phenomena, and in particular the results presented in Table IX,1. Based on the total amount of cytochrome P-450, the size of the 1-butanol-induced spectrum is the same in male and female immature animals and mature males. The low spectral size observed in mature females is due to type I binding, which increases selectively in females during sexual maturation (13).

In the accompanying paper it is shown that 1-butanol competitively inhibits the type I binding of ethylmorphine (Chapter X).

ACKNOWLEDGEMENTS.

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CHAPTER X. THE USE OF COMPETITIVE INHIBITION OF SUBSTRATE-BINDING TO CYTOCHROME P-450 IN THE DETERMINATION OF SPECTRAL DISSOCIATION CONSTANTS FOR SUBSTRATES WITH MULTIPLE TYPES OF BINDING, AS ILLUSTRATED WITH 1-BUTANOL.

SUMMARY.

A method is described, which allows (a) the detection of competitive inhibition of binding to cytochrome P-450 between two substrates which elicit the same type of spectral change, and (b) the estimation of dissociation constants for one type of spectral binding of a substrate which exhibits multiple interaction with cytochrome P-450.

This method was used to investigate the interaction of 1-butanol with the type I binding site of cytochrome P-450 in liver microsomes from female mice. 1-Butanol was found to competitively inhibit the binding of ethylmorphine, and has an apparent dissociation constant for type I binding of 30 mM.

INTRODUCTION.

The spectral interaction of substrates of the microsomal mixed-function oxidase with cytochrome P-450 (1) has been defined in terms of the spectral dissociation constant (K_s) and maximum spectral interaction (ΔA_{max}) (2). The spectral changes are determined by means of difference spectroscopy (3). Several compounds have been shown to generate different types of spectral interaction simultaneously, which leads to composite difference spectra (4-7). The presence of a particular component can be demonstrated by the addition of a 'modifier' compound, which is known to elicit only one type of spectral change, to both sample and reference cuvettes, thus inhibiting the corresponding component of the compound in the sample cuvette. It is shown in this paper that, if the inhibition is competitive, the classical equations for competitive inhibition do not apply. Suitable equations are formed to deal with this situation. In addition, these equations permit the calculation of the dissociation constant for one type of spectral interaction of a compound which shows multiple interaction with cytochrome P-450.

The method described is applied to the type I binding component of 1-butanol. In the preceding paper, we demonstrated a sex difference in the spectral change produced by 1-butanol in mouse liver microsomes. It was shown that this spectral change consisted of at least two components and that the sex difference was due to a type I component (characterized by a difference spectrum with a peak at 385 nm and a trough at 420 nm). Previously, we have demonstrated a similar sex difference in the binding of the type I substrates ethylmorphine and hexobarbital (8-11). In this paper, we demonstrate that 1-butanol interacts with type I binding sites, as evidenced by a competitive inhibition of ethylmorphine binding.

MATERIALS AND METHODS.

Animals.

Male and female mice of the CPB-SE strain were obtained from the Central Animal Breeding Station TNO, Zeist, The Netherlands. They were kept in Makrolon cages with pinewood shavings, received standard food pellets (Hope Farms) and tap water *ad lib.*, and were used after they had reached the age of 11 weeks.

Chemicals.

1-Butanol (p.a.) was obtained from Merck, and ethylmorphine from Brocacef. All other chemicals used were at least reagent grade.

Biochemical methods.

Preparation of liver microsomes and performance of spectral measurements are described in the preceding communication (Chapter IX).

RESULTS.

Validity of difference spectroscopy as a method for studying competitive inhibition of binding to cytochrome P-450.

The simultaneous binding of two compounds to the same site on cytochrome P-450 may be examined, in principle, using the classical method for determining

enzyme inhibition patterns, by measuring the spectral changes elicited by several concentrations of one substrate in the presence of varying concentrations of the other. The first compound (S) is added to the sample cuvette, whereas the second (I) is present in both sample and reference cuvettes. It is not usually recognized, however, that double reciprocal plots of spectral change vs. substrate concentration obtained in this fashion cannot be interpreted in the classical way. The difference spectrum which is actually measured is not the spectral change elicited by compound S. It represents the sum of the spectral changes produced by both S and I in the sample cuvette minus the spectrum of I alone in the reference cuvette. This subtraction does not simply cancel the spectral contribution of I in the sample cuvette, because the magnitude of this contribution is diminished by the presence of S.

The magnitude of the spectral change (ΔA_s) elicited by compound S is given by

$$\Delta A_s = \frac{\Delta A_{max}.[S]}{[S] + K_s} \qquad (1)$$

If a second compound (I) is added (to the sample cuvette only) the observed spectral change is determined by two equilibria:

$$K_s = \frac{[E].[S]}{[ES]} \qquad \text{and} \qquad K_i = \frac{[E].[I]}{[EI]} \qquad (2,3)$$

This spectral change (ΔA_{si}) is proportional to the total amount of complex formed:

$$\Delta A_{si} = k([ES] + [EI]) \qquad (4)$$

$$\Delta A_{max} = k[E_t] \qquad (5)$$

E_t represents the total number of binding sites:

$$[E_t] = [E] + [ES] + [EI] \qquad (6)$$

From equations 4, 5 and 6 it can be shown that

$$\frac{\Delta A_{max}}{\Delta A_{si}} = \frac{[E_t]}{[ES] + [EI]} = \frac{[E]}{[ES] + [EI]} + 1 = \frac{1}{\frac{[ES] + [EI]}{[E]}} + 1 \qquad (7)$$

After insertion of equations 2 and 3, equation 7 is transformed to

$$\frac{\Delta A_{max}}{\Delta A_{si}} = \frac{1}{\frac{[S]}{K_s} + \frac{[I]}{K_i}} + 1 \qquad (8)$$

and

$$\Delta A_{si} = \frac{\Delta A_{max}\left(\frac{[S]}{K_s} + \frac{[I]}{K_i}\right)}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i}} \qquad (9)$$

This equation is analogous to the equation describing the simultaneous catalytic actions of an enzyme on two different substrates (12a), and does not represent a hyperbole.

When the compound I is also added to the reference cuvette, the spectral shift elicited in this cuvette is given by equation 10.

$$\Delta A_i = \frac{\Delta A_{max}\,[I]}{[I] + K_i} = \frac{\Delta A_{max} \cdot \frac{[I]}{K_i}}{1 + \frac{[I]}{K_i}} \qquad (10)$$

According to equations 9 and 10 the observed difference spectrum is now defined by

$$\Delta A_{si} - \Delta A_i = \Delta A_{max}\left(\frac{\frac{[S]}{K_s} + \frac{[I]}{K_i}}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i}} - \frac{\frac{[I]}{K_i}}{1 + \frac{[I]}{K_i}}\right) \qquad (11)$$

After simple algebraic manipulation this is simplified to

$$\Delta A_{si} - \Delta A_i = \frac{\Delta A_{max} \cdot [S]}{[S]\left(1 + \frac{[I]}{K_i}\right) + K_s\left(1 + \frac{[I]}{K_i}\right)^2} \qquad (12)$$

This evidently represents a hyperbole. A double reciprocal plot of spectral change in relation to substrate (S) concentration will therefore be linear:

$$\frac{1}{\Delta A_{si} - \Delta A_i} = \frac{K_s}{\Delta A_{max}}\left(1 + \frac{[I]}{K_i}\right)^2 \cdot \frac{1}{[S]} + \frac{1}{\Delta A_{max}}\left(1 + \frac{[I]}{K_i}\right) \qquad (13)$$

A series of plots of $1/(\Delta A_{si} - \Delta A_i)$ against $1/\{S\}$ at varying inhibitor concentrations is presented in Fig. X,1. The apparent K_s as well as the apparent ΔA_{max} depend on the inhibitor concentration, which is different from normal inhibition kinetics.

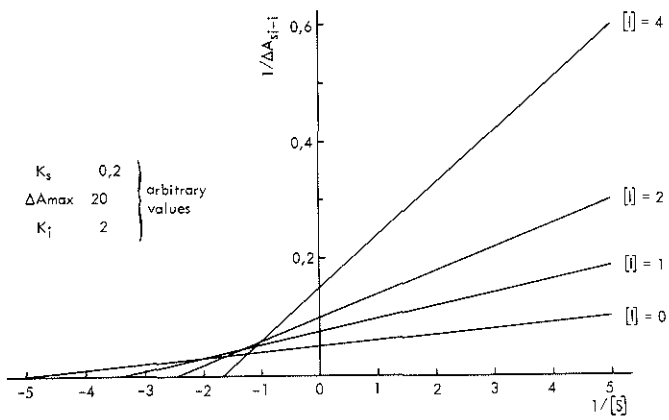

Fig. X,1. Competitive inhibition of spectral interaction with cytochrome P-450.
This figure shows the theoretical pattern of double reciprocal plots of the magnitude of the spectral change vs. substrate concentration, in the case of two substrates which each competitively inhibit the binding of the other to cytochrome P-450, and which both elicit the same type of spectral change. Microsomal suspensions are titrated with one substrate (S) in the presence of different concentrations of the other substrate (I). ΔA_{si-i} represents the size of the spectrum observed, when S is added to the sample cuvette only, and I to both sample and reference cuvettes.

The apparent ΔA_{max} is simply given by

$$\Delta A_{max_{si-i}} = \Delta A_{max}\left(\frac{1}{1 + \frac{[I]}{K_i}}\right) \qquad (14)$$

whereas the apparent K_s is obtained from the condition $1/(\Delta A_{si} - \Delta A_i) = 0$ (Eq.13).

This yields $$K_{si-i} = K_s\left(1 + \frac{[I]}{K_i}\right) \qquad (15)$$

K_i can now be calculated by replotting either $1/\Delta A_{max_{si-i}}$ or K_{si-i} against the inhibitor concentration. Such plots are straight lines which intercept the abscissa at $I = -K_i$. This procedure is the same as that appropriate for the calculation of K_i from the observed K_s in normal competitive inhibition kinetics (12b), but which is not often used because of the availability of the more convenient Dixon-plot (13). The latter method cannot be used in this case.

In the above calculation it is assumed that both compounds bind to the same number of binding sites. In the case of type I binding this might not always be true, as different classes of type I binding sites may exist (14).

Interference of 1-butanol with the type I binding of ethylmorphine and estimation of the dissociation constant for the interaction of 1-butanol with the type I site in mouse liver microsomes.

In the preceding paper (Chapter IX) it was indicated that the sex difference in the composite 1-butanol-induced spectral change is due to a sex-dependent type I component only, when calculated on the basis of the total cytochrome P-450 contents. When the 1-butanol spectrum, obtained with male liver microsomes, is subtracted from that obtained with female microsomes, a true type I component is, in fact, obtained. Sex-dependent binding has been observed, for example, with the type I substrate ethylmorphine (9,10). The sex difference was due to a difference in ΔA_{max} (female values are higher than the male ones) and not to a difference in K_s. This may also be true for 1-butanol.

When the spectral change elicited in males is subtracted from that in

females, the relationship between the difference obtained and the substrate concentration is still represented by a hyperbole:

$$\Delta A_{♀} - \Delta A_{♂} = \frac{\Delta A_{max_{♀}} \cdot [S]}{[S] + K_s} - \frac{\Delta A_{max_{♂}} \cdot [S]}{[S] + K_s} = \frac{(\Delta A_{max_{♀}} - \Delta A_{max_{♂}}) \cdot [S]}{[S] + K_s} \qquad (16)$$

A double reciprocal plot is, therefore, linear:

$$\frac{1}{\Delta A_{♀-♂}} = \frac{K_s}{\Delta A_{max_{♀-♂}}} \cdot \frac{1}{[S]} + \frac{1}{\Delta A_{max_{♀-♂}}} \qquad (17)$$

If this is applied to the spectral changes elicited by several concentrations of 1-butanol, Fig. X,2 is obtained. From the intercept on the ordinate the value of $\Delta A_{max_{♀-♂}}$ was calculated to be 12.8×10^{-3}/nmol P-450. This is in good agreement with the value of $12.1 \pm 1.0 \times 10^{-3}$/nmol P-450 obtained previously for the difference between $\Delta A_{max_{♀}}$ and $\Delta A_{max_{♂}}$ for ethylmorphine

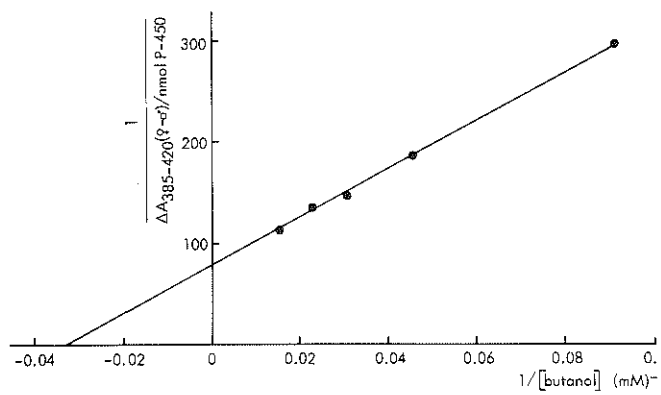

Fig. X,2. Concentration-dependent difference between the 1-butanol-induced spectral changes in microsomes from male and female mice. The size of each spectral change was measured at 385 nm, relative to 420 nm, these being the wavelengths of the peak and trough of the type I difference spectrum, and expressed per nmol P-450. Microsomal preparations contained approx. 1.3 nmol P-450/ml. For further explanation see the text.

binding (9). This is a strong indication that 1-butanol occupies the same binding sites as ethylmorphine. A dissociation constant of 30 mM is obtained from the intercept on the abscissa.

The influence of 1-butanol on ethylmorphine binding was investigated using the method described above. The sample cuvette was titrated with ethylmorphine in the presence of different concentrations of 1-butanol in sample and reference cuvettes. The data are shown in Fig. X,3. The pattern of the double reciprocal plots closely resembles that shown in Fig. X,1, thus indicating competitive inhibition between 1-butanol and ethylmorphine.

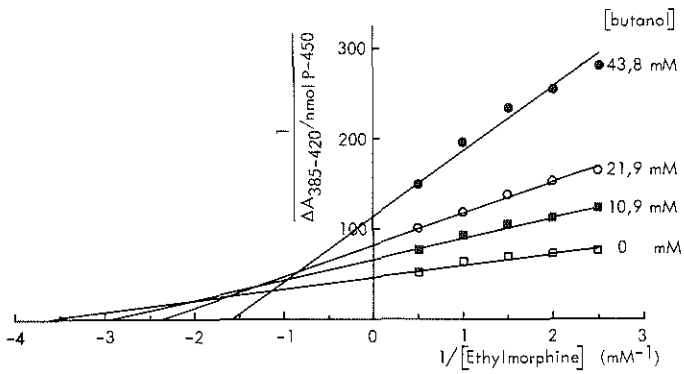

Fig. X,3. Effect of 1-butanol on type I binding of ethylmorphine in liver microsomes from female mice.
Microsomes were titrated with ethylmorphine in the presence of different concentrations of 1-butanol according to the procedure given in Fig. X,1. The data points are the means of 4 experiments. The apparent K_s and ΔA_{max} of ethylmorphine binding at each concentration of 1-butanol were calculated according to the method of Wilkinson (15). The microsomal preparations contained approx. 2.4 nmol P-450/ml.

A replot of the reciprocal of the apparent ΔA_{max} against the 1-butanol concentration, according to Eq. 14, yielded a K_i of 32 mM (Fig. X,4). A replot of the apparent K_s against the 1-butanol concentration, according to Eq. 15, yielded the same value (not shown). This value is similar to that obtained from Fig. X,2.

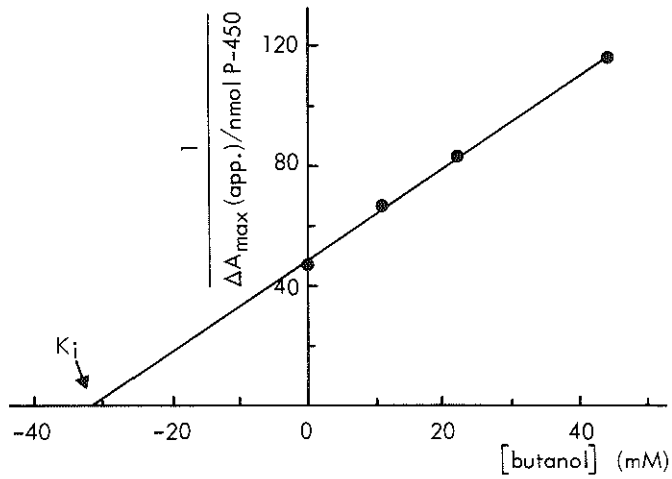

Fig. X,4. Determination of the (type I) spectral dissociation constant of 1-butanol.
The apparent ΔA_{max} values obtained from Fig. X,3 were graphically related to the 1-butanol concentration according to Eq. 14 (see the text). The value indicated by K_i represents the dissociation constant of 1-butanol.

DISCUSSION.

It is common practice to examine the mutual effects of different compounds on their binding to cytochrome P-450, using spectrophotometric methods. The results of such determinations, however, should be interpreted with care. If two substrates bind to the same site, thereby eliciting the same type of spectral change, the contributions of each substrate to the spectral change observed, on simultaneous addition to a microsomal suspension, cannot be separated. Any attempt to compensate for the spectral change elicited by the 'modifier' substrate is bound to fail, i.e. subtraction of this spectral change, either by calculation or by means of difference spectroscopy, results in spectra which are too small. Similarly, the use of a dual wavelength titration yields erroneous results. If a classical pattern of competitive inhibition is observed using these methods (e.g. 16-18), this may indicate competition, but one of the compounds will apparently not itself elicit the type of spectral change studied, which is difficult to explain. Only a

decrease in ΔA_{max}, in addition to an increase in K_s, may indicate the competitive binding of two compounds exhibiting the same type of spectral interaction (as observed by Schenkman et al. (19) with phenacetin and hexobarbital).

The quantitative description of this type of competitive inhibition, presented in this paper, allows for the determination of the dissociation constant for one type of spectral interaction if a substrate shows multiple interaction with cytochrome P-450. Using this method, we have provided evidence that 1-butanol binds to the type I binding site of cytochrome P-450, thus substantiating the conclusion of the preceding paper (Chapter IX).

The type I spectral change is usually considered to be inherent in the metabolism of a substrate (20), although there might be a few exceptions to this rule (e.g. 21). The demonstration of a type I component in the 1-butanol spectrum might, therefore, imply that 1-butanol is metabolized by cytochrome P-450. Teschke et al. (22) reported that, in rats, 1-butanol is partly metabolized via the alcohol-oxidizing system, which may involve cytochrome P-450, with an apparent K_m of 4.9 mM. No binding studies were performed, however. As the occurrence of a type I component might be strain-dependent (in addition to sex- and species-dependency), it would be interesting to know whether a type I component was present in the rat strain used.

An important complication in the determination of K_s-values (as well as K_m-values) might arise when the substrates studied are very lipophilic, since the microsomal membrane might act as a second compartment, a lipid phase, in addition to the aqueous phase. Recently Parry et al. (23) have discussed the implications of the lipophilicity of substrates which interact with membrane-embedded enzymes. The occurrence of a biphasic membrane system should be evident, in our study, from a non-linear relation between the spectral change (which is proportional to the amount of enzyme-substrate complex) and the total enzyme concentration. Our experiments, however, were performed with microsomal suspensions containing approx. 1-2 mg protein/ml, in which region the 1-butanol-induced spectral change is linear with protein concentration (unpublished observations). This means that the enzyme concentration is relatively low, so that single-compartment kinetics apply. It was demonstrated by Parry and coworkers (23) that in such a case dissociation constants do not require correction when the binding site faces the aqueous phase. For a lipid-faced binding site the observed dissociation constant depends on the membrane-water partition coefficient. It has been suggested that the binding site is concealed inside a hydrophobic pocket (24,25). In that case the true dissociation constant for the type I interaction of 1-butanol with cytochrome

P-450 might be higher than measured in the present study, as the octanol-water partition coefficient for 1-butanol is 7.6 (26).

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CHAPTER XI. FURTHER EVIDENCE FOR A HETEROGENEITY OF CYTOCHROME P-450 AS THE CAUSE OF SEX-DEPENDENT TYPE I BINDING.

INTRODUCTION.

Several compounds which elicit a type I spectral change in rat liver microsomes have been shown to have spectral dissociation constants in the micromolar range. A well-documented compound is the β-adrenoceptor blocking drug alprenolol (1,2). Initially, we interpreted the existence of this high-affinity binding as a support of the concept that tight binding of endogenous substrates might occur in microsomes, which could explain the sex difference in type I binding of ethylmorphine. As a part of the investigations presented in Chapter IX the spectral interaction of testosterone and alprenolol with mouse liver microsomes was examined. Both compounds were found to elicit a type I spectral change. Although further investigation appeared to be irrelevant in the light of the results presented, one finding prompted us to extend the study of the binding of alprenolol. The binding of this compound did not seem to be sex-dependent. This would provide an indication of a heterogeneity of type I binding sites.

MATERIALS AND METHODS.

Adult male and female mice of the CPB-SE strain were used.
Alprenolol was obtained from Astra Chemicals and Pharmaceuticals NV.
All methods were the same as those described in Chapter IX.

RESULTS AND DISCUSSION.

Alprenolol elicited a type I spectral change with mouse liver microsomes, but the binding was not sex-dependent. The maximum spectral change was the same in both sexes, when related to the cytochrome P-450 content, which is in contrast to the results obtained previously with ethylmorphine (Chapters V-VI). In Fig. XI,1 the double reciprocal plots for alprenolol binding are shown. An average line was calculated rather than the average of individual lines. ΔA_{max} was 11.9×10^{-3}/nmol P-450 in males and 11.3×10^{-3}/nmol P-450

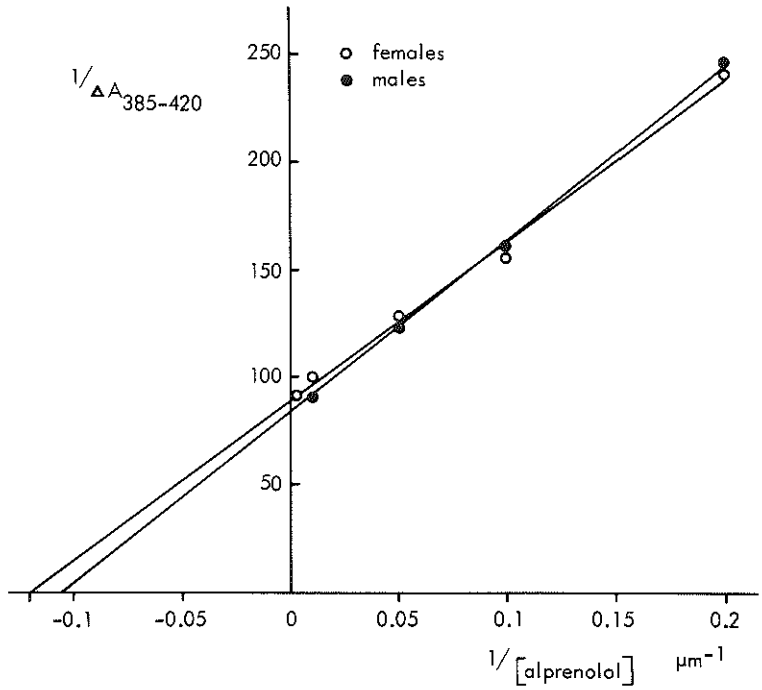

Fig. XI,1. Double reciprocal plots of the magnitude of the type I spectral change elicited by several concentrations of alprenolol in liver microsomes from male and female mice (3-4 mg protein/ml). Data points represent means of 6 (females) or 5 (males) experiments.

in females. This is not a significant sex difference, since the standard errors of the data points amounted to 5-8%. In four of these experiments ΔA_{max} for ethylmorphine binding was determined in a similar way, in order to confirm the sex-dependent binding of ethylmorphine in these microsomes. A value of 6.0×10^{-3}/nmol P-450 was found in males, whereas this value was 15.5×10^{-3}/nmol P-450 in females.

In Fig. XI,2 it is shown that the binding of ethylmorphine and alprenolol is not fully additive. In females, the spectral change elicited by 100 μM alprenolol is increased by 2 mM ethylmorphine, but not further than to the level observed with 2 mM ethylmoprhine alone. Conversely, addition of alprenolol did not add to the spectral change elicited by 2 mM ethylmorphine.

In males, ethylmorphine did not enhance the type I binding spectrum of alprenolol (a slight decrease was, in fact, observed; see below), but alprenolol enlarged the spectrum obtained with ethylmorphine to the magnitude observed when measured in the absence of ethylmorphine.

This indicates that, in females, alprenolol is able to bind only to a part of the ethylmorphine binding sites, whereas, in males, alprenolol binds to all ethylmorphine binding sites plus an additional number of other sites. For both substrates the binding affinity is not sex-dependent. The results

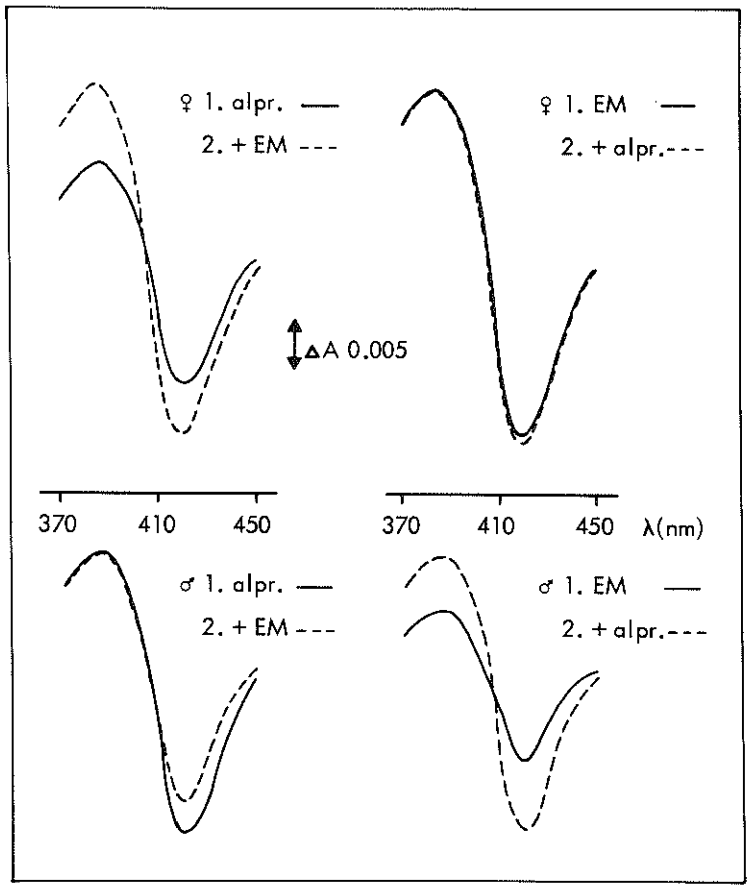

Fig. XI,2. Difference spectra observed after cumulative addition of 100 μM alprenolol (alpr.) and 2 mM ethylmorphine (EM) in liver microsomes from male and female mice (2-3 mg protein/ml).

thus present an intriguing pattern of substrate specificity, indicating the occurrence of at least three classes of type I binding sites. One class binds only ethylmorphine, the second only alprenolol, and the third class binds both of them. Grasdalen et al. (3) have observed several classes of type I binding sites in male rats, using different substrates.

In the experiments presented in Fig. XI,2 2 mM ethylmorphine was added after the final addition of 300 μM alprenolol. The absorbance change was 14.7×10^{-3}/nmol P-450 in females, which is in good agreement with the value of 14.4×10^{-3}/ nmol P-450 calculated from the ethylmorphine binding study. In males, curiously, the final addition of 300 μM alprenolol caused the spectral change to decrease slightly (which was consistently observed in each experiment), and addition of 2 mM ethylmorphine caused a further decrease to about 70% of the value measured using 100 μM alprenolol. As indicated in Fig. XI,2 the addition of ethylmorphine in the presence of 100 μM alprenolol had less effect on the spectral change. In females, concentrations up to 3 mM alprenolol still caused minor increases. This might indicate that high concentrations of alprenolol are deleterious to the type I site in males, or that a different type of spectral change arises (type II). The significance of this remains to be established.

The value of 10 μM for the K_s of alprenolol is not as low as that observed in rats. We have found a similar, but smaller difference between mice and rats with ethylmorphine and hexobarbital (not shown). Von Bahr et al. (2) indicated the presence of two phases of alprenolol binding: a high affinity (K_s = 0.17 μM) and a low affinity phase (K_s not given), both with the same binding capacity. There might be a low affinity phase in mice (operative at concentrations below 5 μM), but then its capacity will be much smaller than that of the phase presently measured.

One interesting aspect of high-affinity binding, which is not recognized in the literature, may be discussed here. When the apparent dissociation constant is determined using substrate concentrations in the micromolar range, these concentrations approach the cytochrome P-450 concentrations usually employed for these assays, or may be even lower. For example, the value of 0.17 μM determined by Von Bahr et al. (2) was obtained with a microsomal preparation containing approx. 2 nmol cytochrome P-450/ml, i.e. 2 μM (not actually stated, but this may be assumed on the basis of other data). Although it was calculated that only 13-14% of cytochrome P-450 is involved in this binding, the substrate range chosen and the apparent K_s measured are lower than the enzyme concentration. In such a case the Michaelis-Menten

equation cannot be applied, since the concentration of bound substrate is no longer negligible with respect to that of the free substrate. Any linear transformation of this equation will yield curves. Nevertheless, a straight line was obtained. Similar observations were made with other type I substrates, e.g the tricyclic antidepressant drug imipramine (K_s = 0.22 μM) (4) and the vitamin D_3 metabolite 25-hydroxycholecalciferol (K_s = 0.84 nM) (5).

Two explanations may be given for this. (1) Only very small fractions of the total cytochrome P-450 population are involved in the binding of these substrates (in particular 25-hydroxycholecalciferol), which is interesting in view of the heterogeneity of cytochrome P-450, and which would, furthermore, imply that the fraction of 13-14% calculated by Von Bahr et al. (2) is overestimated. (2) As discussed in Chapter X, Parry et al. (6) have indicated that the apparent K_s may be severely underestimated if the substrate is very lipophilic and the binding site is hidden in the lipid phase formed by the microsomal membrane. In that case the free substrate concentration represents the concentration in the lipid phase, which may be much higher than expected on the basis of the total volume of the microsomal suspension, thus exceeding the enzyme concentration.

The fact that ΔA_{max} values for many substrates, including the so-called high-affinity substrates, do not differ greatly, and that often a full or at least partial overlap of binding specificity is observed, might indicate that all type I binding is associated with an extremely small fraction of the cytochrome P-450 content. Since there is some evidence for a binding site hidden in the membrane (7,8), however, the fact that the high-affinity substrates are lipophilic leaves the second possibility as the more attractive one.

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CHAPTER XII. GENERAL DISCUSSION.

In this chapter the main aspects of this thesis are summarized and further discussed. These aspects include the hormonal basis of the sex-dependency of the cytochrome P-450 system, the sex dependency of cytochrome P-450-substrate interaction, the heterogeneity of cytochrome P-450, the mechanism of substrate-induced stimulation of NADPH-cytochrome P-450 reductase activity and the role of the reduction of the cytochrome P-450-substrate complex in the hydroxylation reaction. Finally, possible future developments and clinical aspects are discussed.

1. Sex-dependency of the cytochrome P-450 system.

It is difficult to interpret the nature of the sex differences in the cytochrome P-450-system in terms of hormonal mechanisms. The present study allows only very general conclusions in this respect. In mice of the CPB-SE strain testosterone pretreatment produces effects in females only, whereas castration produces effects in males only. This indicates that the sex differences arise through androgenic action. In rats, estrogens do not have effects of their own, but rather impair the effects of androgens. We have not investigated this in mice. It has been observed in rats that a sex difference exists in the pattern of steroid metabolism. The development of this pattern is imprinted by neonatal androgen (Chapter III,4.6). This has also been observed for the metabolism of type I substrates: the responsiveness to androgens, which results in a stimulation of metabolic activity during sexual maturation, is neonatally imprinted (1). In mice the sex differences appeared to arise as a result of developmental changes occurring in females (Chapter VII). Taking into account that, in this species, the effects of androgen were found to be inhibitory, it might be postulated that the testicular androgen produced during sexual maturation impairs a process which would occur in its absence. The smaller sex difference in ΔA_{max} as well as the lack of a sex difference in K_m of ethylmorphine demethylation observed in the CPB-V strain, may be assigned to a smaller responsiveness to androgens as compared with the SE-strain (Chapter VI).

2. Endogenous substrates and the sex difference in type I binding.

The most prominent sex-dependent process in the development of the cyto-

chrome P-450 system is evidently a change in the type I binding properties of cytochrome P-450. The low binding capacity of cytochrome P-450 in males, as compared with females, might be the result of tight binding of endogenous substrates. These substrates may be steroids, since they appear to have a high affinity for cytochrome P-450 (2). It was demonstrated, however, that, if endogenous substrate binding is important at all, this does not account for the sex-dependent type I binding (Chapter IX). In addition to this, it may be mentioned that, in a single experiment, testosterone was found to be a type I substrate, which exhibited a similar sex-dependent binding as ethylmorphine (apparent K_s approx. 5 μM). Testosterone could be accumulated (in vitro) in the microsomal membrane, but a concentration of 200 μM was easily washed out (by 2 consecutive cycles of centrifuging and resuspending) according to what might be expected on the basis of simple partitioning between membrane and supernatant. Ethylmorphine binding in controle male microsomes could not be increased by repeated washings. Thus, the mere binding of high affinity substrates is not a likely cause of the low level of type I (ethylmorphine) binding. Very recently it has been reported that treatment of microsomes with charcoal may result in the removal of endogenous substrates (3). Preliminary experiments, however, indicated that charcoal treatment does not abolish the sex difference in ethylmorphine binding. This sex difference is apparently the result of a difference in the very nature of the interaction of the substrate with cytochrome P-450, which points to a heterogeneity of cytochrome P-450.

3. Heterogeneity of cytochrome P-450 with respect to type I binding.

The developmental patterns of cytochrome P-450 in male and female mice of the CPB-SE strain indicate a heterogeneity of the cytochrome P-450 population. In Chapter VII it was shown that a large portion of cytochrome P-450 may not be involved in ethylmorphine binding. It can be calculated (using Fig. VII,4) that, in male mice, maximally 10% of the total amount of cytochrome P-450 binds ethylmorphine. This is in agreement with the finding that high concentrations of type I substrates cause only a slight attenuation of the absorbance peak of the absolute spectrum of cytochrome P-450, in contrast to the observation that the peak is fully shifted by the binding of type II substrates (which bind to the heme) (8). Females have a larger capacity for binding ethylmorphine than males, because they possess a larger amount of that particular (ethylmorphine-binding) cytochrome P-450.

Further evidence for a heterogeneity of cytochrome P-450 was presented in Chapter XI. The finding that alprenolol only partly occupies ethylmorphine binding sites, indicated a heterogeneity of type I binding sites. The fact that alprenolol binds to a fair amount of the ethylmorphine binding sites in females, and does not exhibit a sex difference in type I binding in adult animals, makes it difficult to understand that the increase in ethylmorphine binding capacity in females during sexual maturation would be the result of a selective synthesis of ethylmorphine-binding cytochrome P-450 (which was suggested by the results in Chapter VII). It would seem more likely that the increase in capacity of ethylmorphine binding is due to a modification of already existing type I sites. Since the development of alprenolol binding was not studied, however, nothing conclusive can be said about this.

4. Substrate-induced stimulation of NADPH-cytochrome P-450 reductase activity and the rate of reduction of the cytochrome P-450-substrate complex.

The results presented in Chapters V-VIII demonstrated that the stimulation of NADPH-cytochrome P-450 reductase activity by type I substrates is closely related to the magnitude of the spectral interaction with cytochrome P-450. This strongly suggests that type I binding represents the formation of an enzyme-substrate complex. The reduction of this complex did not seem to be a rate-determining factor in the demethylation of ethylmorphine. It was proposed (Chapter V), however, that a true measure of the reduction of the cytochrome P-450-substrate complex might not be obtained, since the nature of the reductase stimulation is not yet well established. In this regard four possibilities can be distinguished.

I. According to the original hypothesis by Gigon et al. (5) the substrate-induced reductase stimulation (Δ_{red}) represents the reduction of a cytochrome P-450 in the fast phase of reduction, which was reduced either in the slow phase or not at all in the absence of substrate. In the following the contribution of the slow phase to the initial rate of reduction is neglected, which is justified on the basis of the rate constants given by several authors (6-8). The (initial) basal rate of reduction is represented by kB, in which k is the pseudo first order rate constant and B is the amount of cytochrome P-450 reduced in the fast phase. This parameter may reflect the reduction of cytochrome P-450 bound to endogenous substrates (6). After the addition of substrate an extra amount of cytochrome P-450-substrate complex (B_x) is involved in the fast phase. The rate constant of the reduction of this complex (k_x) may or may not be different from that associated with the basal rate.

Thus, the total reduction rate is given by $kB + k_xB_x$. Δ_{red} then becomes $kB + k_xB_x - kB = k_xB_x$. This represents the rate of reduction of cytochrome P-450-substrate complex and is directly related to the magnitude of the type I spectrum (ΔA), which is a measure of the number of complexes.

II. The existence of a fast and a slow phase of reduction may bear no relationship to the presence or absence of substrates, but may reflect a structural difference in the assembly of cytochrome P-450 and the reductase, as proposed by Peterson et al. (8). The substrate binds to all of the cytochrome P-450 involved in the fast phase only and alters the rate constant of this phase. The basal reduction rate is kB; the rate in the presence of substrate becomes k_xB (total reduction rate, neglecting the slow phase, vide supra), which directly represents the rate of complex reduction. In that case $\Delta_{red} = (k_x - k)B$, the stimulation thus being related to the amount of complex.

III. This possibility is a variation on case II. Only a portion of the fast phase cytochrome P-450 may be involved in the binding of substrate. The basal reduction rate equals $k(B_1 + B_2)$, B_1 and B_2 representing subfractions of fast phase cytochrome P-450 with different substrate specificities. After substrate binding the rate becomes $kB_1 + k_xB_2$, and Δ_{red} is given by $kB_1 + k_xB_2 - k(B_1 + B_2) = (k_x - k)B_2$. Again Δ_{red} is related to the amount of complex. But neither Δ_{red} nor the total reduction rate represent the rate of reduction of this complex, which is given by $k_xB_2 = k_1B_2 + \Delta_{red}$, i.e. Δ_{red} plus a fraction of the basal reduction rate.

IV. An extension of cases II and III is obtained by the supposition that the substrate may also bind to slow phase cytochrome P-450. This might not have an appreciable effect on the initial rate of reduction. In that case Δ_{red} will be related to ΔA only if the relative extents of substrate-binding linked to each phase do not vary with sex, strain, or treatment, and if the reduction rate of the slow phase remains negligible. The rate of complex reduction is not well-defined: the rate constant may depend on whether cytochrome P-450 was originally involved in the fast or in the slow phase. This case may approach cases II or III, or represent a mixture of cases I and II or I and III.

Summarizing, the rate of cytochrome P-450-substrate complex reduction may be Δ_{red} (case I), the total reduction rate (case II), or lie between these values (cases II and IV). The very consistent finding that Δ_{red} is stoichiometrically related to the rate of ethylmorphine demethylation in rats (9-11), is compatible with the first mechanism. This cannot be reconciled, however, with the data obtained by Matsubara(7) and Peterson et al. (8), which require

mechanisms II or III, and possibly IV. We found Δ_{red} to be directly related to ΔA, but, as indicated above, this does not preclude any of the possible mechanisms. It only indicates that type I binding and the formation of a reducible complex are not separate phenomena. The finding that ethylmorphine and hexobarbital form the same number of type I complexes (i.e. they produce the same ΔA), but that their reductase stimulations are different (Chapter VIII), indicates that the rate constant of complex reduction depends on the substrate. This makes the role of the type I complex in metabolism (which occurs at varying rates depending on the substrate) even more probable.

The interpretation of cytochrome P-450 reduction data will remain difficult as long as the actual mechanism of substrate-induced stimulation is not known. Nevertheless, the above justifies the conclusion drawn in Chapter VII, that the rate of reduction of the cytochrome P-450-substrate complex formed by type I binding is not related to the metabolic rate whichever mechanism of stimulation is taken into consideration. In the following it is discussed, however, that yet another factor may obscure a relationship between cytochrome P-450-substrate complex reduction and metabolic rate.

5. Heterogeneity of cytochrome P-450 with respect to catalytic action; a hypothesis.

The discrepancy between the magnitude of the type I spectral change, ΔA_{max} (which reflects the formation of cytochrome P-450-substrate complex), and the rate of ethylmorphine demethylation, V_{max} (Chapters VI and VII), is difficult to explain, if it is accepted that the type I complex is the enzyme-substrate complex. The fact that a developmental increase in ΔA_{max} is observed without a concomitant increase in V_{max}, might be explained by a parallel decrease of the turnover number, for example due to less optimal conditions, which could be indicated by the decreasing K_m. This is very unlikely, however, since (1) the assay was optimalized on the basis of adult animals, (2) in the CPB-V strain a similar ΔA_{max} difference is not accompanied by a sex difference in K_m, and (3) similar K_m-changes have been observed in maturing rats (12-14).

Some interesting observations may form the basis of an attractive hypothesis. It is known that a few substrates are not metabolized, although they elicit the type I spectral change and activate the reductase accordingly (15-18). Moreover, oxygen is introduced into the complex. This inability to be metabolized may be due to chemical or sterical hindrance. Thus, it was

postulated that the substrate activates a part of the cytochrome P-450 population for the metabolic reaction. Under certain circumstances, however, this reaction does not occur: complex formation leads only to the reduction of O_2 to H_2O or H_2O_2 instead of metabolic conversion of the substrate. It is conceivable that the inability to incorporate an oxygen atom into the substrate molecule does not only depend on the nature of the substrate, but also on that of the active site of the cytochrome. It might, therefore, be speculated that some types of cytochrome P-450 bind ethylmorphine, but do not catalyze its metabolism. This is supported by the finding that after induction of rats or guinea-pigs with pregnenolone-16α-carbonitrile, the increase in type I binding of ethylmorphine is accompanied by an increase in the H_2O_2 production (19). This would explain the enormous discrepancy between V_{max} and ΔA_{max} (Chapter VII). A fraction of the cytochrome P-450 involved in establishing the value of ΔA_{max} does not contribute to V_{max}. This may be the ethylmorphine binding cytochrome, which develops during sexual maturation in females of the CPB-SE strain, since a parallel increase in V_{max} is not observed. This hypothesis might be tested by the measurement of H_2O_2 production and O_2 consumption together with type I binding and ethylmorphine demethylation under various conditions, for example in the course of sexual maturation.

6. Reappraisal of the role of NADPH-cytochrome P-450 reductase in ethylmorphine demethylation.

In Chapter VII it was concluded that the reduction of cytochrome P-450-substrate complex is not rate-limiting in the demethylation of ethylmorphine, since no parameter of reductase activity was related to the demethylation rate. However, if a certain amount of type I complexes is reduced without being further metabolized, the role of the reductase in demethylation should be reconsidered.

Upon examination of the developmental patterns of V_{max} and cytochrome P-450 reduction, V_{max} appeared to show the best correlation with the basal reduction rate, although the decrease in reduction rate between 3 and 7 weeks was much larger than in V_{max}. This is strange, since basal reductase activity does not require exogenous substrate. When it is proposed that the same amount of cytochrome P-450 is active in demethylation in males and females, according to the above hypothesis, the substrate-induced reductase stimulation associated with metabolism should be the same in both sexes. In females, this Δ_{red} (which forms a fraction of the observed Δ_{red}) may be considered equal to

the Δ_{red} obtained in males. When this value is added to the observed basal reduction rate, the descent of the sum of these reductase activities between 3 and 7 weeks is not as steep as that of the basal rate alone, thus making the resulting reductase pattern very similar to that of V_{max}. If it is assumed that reductase stimulation follows mechanism III (see section 4), it is, therefore, conceivable that ethylmorphine binds to a portion of the fast phase cytochrome P-450, and that the reduction of the type I complex actually is related to V_{max}.

Recent investigations have indicated that, in rats, the turnover numbers of cytochrome P-450 reduction well exceed those of metabolic reactions, which would imply that the reduction is not rate-limiting (7,8). The data presented, however, were based on the participation of the complete fast phase of reduction in metabolism, which was calculated to relate to approx. 50-75% of the total amount of cytochrome P-450. Since there is evidence that type I substrates bind to much smaller portions of cytochrome P-450 (see section 3 and Chapter XI), the actual turnover number of the metabolic reaction may be much higher than judged from V_{max} on the basis of this large amount of cytochrome P-450. The participation of less cytochrome P-450 would, of course, imply that the actual value of the turnover number of cytochrome P-450 reduction is proportionally higher. But the experimental values of the latter parameter may not be correct. The computer program used for the kinetic analysis of the reduction progress curve (7,8) was based on the superimposition of only two phases, which were resolved into one fast and one slow phase. One might wonder how this model would deal with a substrate-induced activation associated with only a small portion of the fast phase cytochrome P-450.

Conclusive evidence concerning the role of NADPH-cytochrome P-450 reductase in ethylmorphine demethylation and other reactions awaits more information about the amounts of cytochrome P-450 subspecies involved.

7. Future developments.

One of the crucial problems for future research is the heterogeneity of cytochrome P-450. Although it is known that factors such as lipophilicity and nucleophilicity, or possibly the presence of a certain structural entity of the substrate molecule (2), are determinants of the degree of cytochrome P-450-substrate interaction, suitable parameters which clearly relate molecular properties of the substrate to its binding to cytochrome P-450 (sub-

species) have not yet been developed. Extensive separation and purification of different forms of cytochrome P-450 may lead to a better understanding of substrate specificity. The use of physical methods (ESR, NMR) may further elucidate the nature of the binding sites and the modes of cytochrome P-450-substrate interaction, including the role of the microsomal membrane in this interaction.

Furthermore it is necessary to establish the requirements for the actual metabolism of the substrate after the oxygenated cytochrome P-450-substrate complex has been formed. Recently, it has been shown possible to study the oxygenation of cytochrome P-450 and two subsequent reactions (formation of a second complex and regeneration of the oxidized cytochrome P-450) spectrophotometrically with a purified preparation of one form of cytochrome P-450 from phenobarbital-treated rabbit liver microsomes (20). In addition, the method introduced by Hrycay and colleagues (21-24)(substrate metabolism by means of direct formation of the activated oxygen complex with oxidizing agents such as sodium periodate, thus bypassing the reductive steps) may prove useful in this respect.

The results presented in this thesis might give the impression that the further study of the sex-dependency of components of the monooxygenase chain is rather academic, since there appears to be little or no consequence for the metabolism of substrates. This is not true, however, for several reasons.

(1) Sex-dependent type I binding forms one of the many tools to obtain information about the heterogeneity of cytochrome P-450, which may help to explain functional differences displayed in a different situation. For example, the fact that a heterogeneity of cytochrome P-450 with respect to both binding and metabolism of several compounds is observed, could suggest that the development of a sex-dependent ethylmorphine-binding capacity actually reflects the development of a sex-dependent metabolic capacity for an endogenous substrate, which fits the active site better than ethylmorphine does.

(2) The fact that ethylmorphine appears to bind to a portion of cytochrome P-450 without being metabolized, may not be entirely without consequence. If ethylmorphine indeed acts as a partial uncoupler, thus forcing cytochrome P-450 to reduce O_2 to H_2O_2, this leads to an elevated cellular level of H_2O_2, which may influence other metabolic processes (as discussed by Hildebrandt et al. (19)). Moreover, if the uncoupler is not removed, it may cause the excessive consumption of cofactors or oxygen. In this respect it is important

to note that gluconeogenesis and lipogenesis were shown to be suppressed after stimulation of mixed-function oxidase activity (25,26)

8. Clinical aspects.

In Chapters I and II it was suggested that the study of the cytochrome P-450 system might be of clinical importance. A general rate-limiting step in drug metabolism might, for example, be used as a test of drug metabolizing capacity in liver biopsy samples. It has become clear, however, that the cytochrome P-450 system may be too complicated to provide such a simple measure of this capacity.

A more useful approach seems to be the further study of the factors which govern the binding of a particular substrate or class of substrates to a particular cytochrome P-450 subspecies (vide supra). The knowledge of the relationship between molecular properties and interaction with cytochrome P-450 may offer the possibility to predict whether a certain drug will interact with cytochrome P-450, and whether it will compete with other drugs for the same cytochrome P-450 or bind to different subspecies.

Furthermore, it may be of value to investigate the induction of cytochrome P-450, and to establish which subspecies is (are) induced by a particular compound. The results of such studies may offer the possibility of inducing or avoiding the induction of a specific type of metabolic activity. Human cytochrome P-450 should be investigated to establish to which extent the results obtained with animal studies can be extrapolated to man.

It may be expected that these fundamental studies will increase the understanding of drug-drug interactions and the relationships between molecular structure and biotransformation. They may further rationalize the development of new drugs by providing more detailed criteria for the manipulation of molecular structure.

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SUMMARY

In this thesis an investigation into the cytochrome P-450-dependent mixed-function oxidase in the endoplasmic reticulum of mouse liver is described. This enzyme system possesses various functions, one of which is the conversion of lipid-soluble compounds (e.g. many drugs) into products which are more readily excreted from the body than the parent compounds.

From earlier studies it was known that certain strains of mice show a sex-dependency in the metabolism of the barbiturate hexobarbital. In male mice hexobarbital-anaesthesia lasted longer than in females, and the in vitro rate of hexobarbital hydroxylation in males was slower than that observed in females. This sex-dependency was used as a tool to investigate the relationships between substrate binding to cytochrome P-450 (type I binding, measured as a change in the absorption spectrum of cytochrome P-450), NADPH-cytochrome P-450 reductase activity and the metabolism of the substrate, in view of the existing hypothesis that the reduction of the cytochrome P-450-substrate complex might be a rate-limiting step in drug metabolism. The type I compounds ethylmorphine and hexobarbital were used as model substrates.

Four introductory chapters, including a general introduction (I), a presentation of the object and framework of the investigations (II), a survey of the existing body of knowledge about cytochrome P-450-linked enzyme systems, with particular reference to the liver (III), and a description of some methodological aspects (IV), precede the experimental section.

In Chapter V it is shown that, in liver microsomes of mice of the CPB-SE strain, ethylmorphine demethylation, cytochrome P-450 content, type I binding of ethylmorphine, and substrate-induced enhancement of cytochrome P-450 reduction show sex differences, which are abolished by testosterone pretreatment and castration. A clear relationship between type I binding and reductase stimulation suggested that type I binding reflects the formation of an enzyme-substrate complex, which is subsequently reduced by NADPH-cytochrome P-450 reductase. These parameters, however, were not clearly related to the demethylation rate, which was particularly evident in the CPB-V strain.

In Chapter VI the kinetic constants of ethylmorphine demethylation and type I binding are examined in the CPB-SE and CPB-V strains. Both V_{max} and K_m appeared to be sex-dependent in the CPB-SE strain. In the CPB-V strain only a very small sex difference in V_{max} was found. The maximum spectral interaction (ΔA_{max}) observed in females appeared to be higher than in males

in both strains, but in the V-strain the sex difference was smaller than in the SE-strain. The effects of testosterone pretreatment and castration demonstrated that androgens are responsible for the observed sex differences by exerting an inhibitory action in males. Mice of the V-strain appeared to be less responsive to androgens than those of the SE-strain.

In the SE-strain, the sex differences in ΔA_{max}, reductase stimulation, cytochrome P-450 content and K_m appeared to arise during sexual maturation, mainly as the result of developmental changes occurring in females (Chapter VII). This suggested that androgens inhibit such a development in males. The sex differences in V_{max} and basal reductase activity had a more permanent character. A discrepancy between the total cytochrome P-450 content and ΔA_{max} indicated a heterogeneity of cytochrome P-450: only a small portion of cytochrome P-450 is involved in ethylmorphine binding. The discrepancy between ΔA_{max} and V_{max} in females suggested that the formation of a number of cytochrome P-450-ethylmorphine complexes does not lead to demethylation. The reduction of the cytochrome P-450-substrate complex did not seem to be rate-limiting in ethylmorphine demethylation.

In Chapter VIII the relationship between the kinetic constants of type I binding and metabolism is studied using the optical antipodes of hexobarbital. It was found that in mice (in contrast to rats) the K_s was similar to K_m, suggesting that the type I site is the active site of the enzyme, and that the reduction of cytochrome P-450-substrate complex is rate-limiting. This was supported by the measurements of reductase activities. Furthermore, it was demonstrated that the rate of complex reduction depends on the substrate, which leaves the possibility that the rate-limiting character of the reductase step depends on the substrate.

In the remaining chapters the nature of the sex difference in type I binding of ethylmorphine is further examined. In Chapter IX the possibility is excluded that the sex difference in type I binding of ethylmorphine is due to a sex difference in the level of tightly binding endogenous substrates. The 1-butanol-induced spectral change, which has been attributed to the displacement of endogenous substrates, appeared to be the resultant of different superimposed spectral components. The sex-dependency of this spectral change is due to sex-dependent type I binding of 1-butanol.

In Chapter X it is demonstrated that 1-butanol competitively inhibits ethylmorphine binding. A theoretical evaluation of competitive inhibition between two substrates eliciting the same type of spectral change is presented. It is shown how the dissociation constant of a substrate which elicits more

than one type of spectral interaction with cytochrome P-450, can be calculated. This is illustrated with 1-butanol.

Direct evidence for a heterogeneity of cytochrome P-450 as the cause of sex-dependent type I binding is presented in Chapter XI. The β - adrenoceptor blocking drug alprenolol was shown to be a type I compound, which produced the same magnitude of spectral interaction in males and females. It occupies all ethylmorphine binding sites in males, but only part of those sites in females.

A general discussion is presented in Chapter XII, in which the roles of type I binding and NADPH-cytochrome P-450 reductase in ethylmorphine demethylation are further evaluated. The measurement of NADPH-cytochrome P-450 reductase activities is discussed with regard to the question to what extent these activities might be presumed to represent the reduction of the cytochrome P-450-substrate complex. Although the data presented indicate that the reduction of the cytochrome P-450-substrate complex is not rate-limiting in ethylmorphine demethylation, a rate-limiting role of the reductase cannot be excluded, if a number of type I complexes are not involved in the metabolism of the substrate (which was indicated in Chapter VII).

The sex-dependency of the cytochrome P-450 system primarily relates to cytochrome P-450 itself, and finds expression in a sex difference in the relative amounts of cytochrome P-450 subspecies. Further research should provide insight into the factors which determine the binding of a particular (type of) substrate to a particular cytochrome P-450, and the factors which determine whether the formation of a cytochrome P-450-substrate complex leads to metabolism of the substrate or not. This information may improve the knowledge of drug-drug interactions and the relationship between chemical structure and the biotransformation of drugs.

In dit proefschrift wordt een onderzoek beschreven naar het cytochroom P-450-afhankelijke mixed-function oxidase in het endoplasmatisch reticulum van de muizelever. Dit enzym-systeem bezit een veelheid van funkties, en is onder meer betrokken bij de omzetting van vet-oplosbare verbindingen (zoals bijvoorbeeld vele farmaka) in produkten die gemakkelijker het lichaam kunnen verlaten dan de oorspronkelijke verbindingen.

Uit vroegere studies was reeds bekend, dat bepaalde muizenstammen een sekse-afhankelijk metabolisme van het barbituraat hexobarbital vertonen. Mannelijke muizen vertoonden een langduriger narkose na toediening van hexobarbital dan vrouwelijke, en de in vitro gemeten hydroxyleringssnelheid van hexobarbital was in mannetjes lager dan in vrouwtjes. Van deze sekse-afhankelijkheid is gebruik gemaakt om de relaties tussen substraat-binding aan cytochroom P-450 (type I binding, gemeten als verandering van het absorptiespektrum van cytochroom P-450), NADPH-cytochroom P-450 reduktase aktiviteit en het metabolisme van het substraat te onderzoeken, dit in het licht van de bestaande hypothese, dat de reduktie van het cytochroom P-450-substraat kompleks een snelheidsbepalende stap zou kunnen zijn in het metabolisme van farmaka. De type I verbindingen ethylmorfine en hexobarbital werden gebruikt als modelsubstraten.

Vier inleidende hoofdstukken, waaronder een algemene inleiding (I), een formulering van doel en opzet van het onderzoek (II), een overzicht van de bestaande kennis omtrent cytochroom P-450-afhankelijke enzym-systemen, toegespitst op de lever (III), en een beschrijving van enkele aspekten van de gebruikte methoden (IV), gaan aan het eksperimentele gedeelte vooraf.

Hoofdstuk V laat zien, dat in levermikrosomen van muizen van de CPB-SE stam de demethylering van ethylmorfine, de hoeveelheid cytochroom P-450, de type I binding van ethylmorfine en de substraat-geïnduceerde versnelling van cytochroom P-450 reduktie sekseverschillen vertonen, die worden te niet gedaan door testosteron behandeling en kastratie. Een duidelijk verband tussen type I binding en reduktase stimulering suggereerde, dat type I binding een weergave is van de vorming van een enzym-substraat kompleks, dat vervolgens wordt gereduceerd door NADPH-cytochroom P-450 reduktase. Deze parameters waren echter niet duidelijk gerelateerd aan de demethyleringssnelheid, hetgeen vooral duidelijk was in de CPB-V stam.

In Hoofdstuk VI worden de kinetische konstanten van ethylmorfine demethy-

lering en type I binding in de CPB-SE en CPB-V stam bestudeerd. Zowel V_{max} als K_m bleken sekse-afhankelijk in de CPB-SE stam. In de V-stam werd alleen een zeer klein sekseverschil in V_{max} gevonden. De maksimale spektrale interaktie (ΔA_{max}) die in vrouwtjes werd waargenomen, bleek groter te zijn dan die in mannetjes in beide stammen, maar in de V-stam was het sekseverschil kleiner dan in de SE-stam. De effekten van testosteron behandeling en kastratie toonden aan, dat androgenen verantwoordelijk zijn voor de waargenomen sekseverschillen door het uitoefenen van het remmende invloed in mannetjes. Muizen van de V-stam bleken in mindere mate op androgenen te reageren dan die van de SE-stam.

In de SE-stam bleken de sekseverschillen in ΔA_{max}, reduktase stimulering, hoeveelheid cytochroom P-450 en K_m te ontstaan tijdens de seksuele ontwikkeling, voornamelijk als gevolg van in vrouwtjes optredende veranderingen (Hoofdstuk VII). Dit suggereerde, dat in mannetjes dergelijke veranderingen door androgenen worden tegengegaan. De sekseverschillen in V_{max} en basale reduktase aktiviteit hadden een meer permanent karakter. Het afwezig zijn van een verband tussen de totale hoeveelheid cytochroom P-450 en ΔA_{max} duidde op een heterogeniteit van het cytochroom P-450: slechts een klein gedeelte van het cytochroom P-450 is betrokken bij de binding van ethylmorfine. Het ontbreken van een relatie tussen ΔA_{max} en V_{max} in vrouwtjes suggereerde, dat de vorming van een aantal cytochroom P-450-ethylmorfine kompleksen niet tot demethylering leidt. De reduktie van het cytochroom P-450-substraat kompleks scheen niet snelheidsbepalend te zijn in de demethylering van ethylmorfine.

In Hoofdstuk VIII wordt het verband tussen de kinetische konstanten van type I binding en metabolisme bestudeerd aan de hand van de optische antipoden van hexobarbital. In tegenstelling tot ratten bleken muizen een gelijke K_s en K_m te vertonen, wat er op wijst, dat de type I plaats het aktief centrum is voor de hydroxyleringsreaktie, en dat de reduktie van het cytochroom P-450-substraat kompleks snelheidsbepalend is. Deze opvatting werd gesteund door de meting van reduktase aktiviteiten. Voorts werd aangetoond, dat de reduktiesnelheid van het kompleks afhankelijk is van het substraat, wat de mogelijkheid openlaat, dat het snelheidsbepalend karakter van de reduktie stap substraat-afhankelijk is.

In de resterende hoofdstukken wordt de aard van het sekseverschil in type I binding van ethylmorfine verder onderzocht. In Hoofdstuk IX wordt de mogelijkheid uitgesloten, dat het sekseverschil in type I binding van ethylmorfine te wijten is aan een sekseverschil in de hoeveelheid sterk gebonden endogene substraten. De spektrale verandering die door 1-butanol wordt teweeggebracht,

en die is toegeschreven aan een verdringing van endogene substraten, bleek de resultante te zijn van verschillende gesuperponeerde spektrale komponenten. De sekse-afhankelijkheid van deze spektrale verandering is het resultaat van sekse-afhankelijke type I binding van 1-butanol.

In Hoofdstuk X wordt aangetoond, dat 1-butanol de binding van ethylmorfine kompetitief remt. Een theoretische beschrijving van de kompetitieve inhibitie tussen twee substraten die hetzelfde type spektrale verandering te zien geven, wordt gegeven. Een berekeningsmethode wordt ontwikkeld voor de dissociatiekonstante van een substraat dat meer dan één type spektrale interaktie vertoont. Dit wordt toegelicht aan de hand van 1-butanol.

Een direkt bewijs voor een heterogeniteit van cytochroom P-450 als oorzaak van sekse-afhankelijke type I binding wordt geleverd in Hoofdstuk XI. Alprenolol, een β-sympathicolyticum, bleek een type I verbinding te zijn, die in mannetjes en vrouwtjes dezelfde mate van spektrale verandering teweegbrengt. In mannetjes bezet het alle ethylmorfine bindings plaatsen, maar in vrouwtjes wordt slechts een gedeelte hiervan bezet.

Een algemene diskussie wordt geboden in Hoofdstuk XII, waarin de rol van type I binding en NADPH-cytochroom P-450-reduktase in de demethylering van ethylmorfine verder wordt getoetst. De bepaling van NADPH-cytochroom P-450 reduktase aktiviteiten wordt besproken in het licht van de vraag, in hoeverre deze verondersteld kunnen worden de reduktie van het cytochroom P-450-substraat kompleks te vertegenwoordigen. Hoewel de reduktie van dit kompleks niet snelheidsbepalend kan zijn, als men afgaat op de konkrete resultaten, kan dit niet worden uitgesloten als een aantal type I kompleksen niet betrokken is bij het metabolisme van het substraat. Voor dit laatste werd een aanwijzing gevonden in Hoofdstuk VII.

De sekse-afhankelijkheid van het cytochroom P-450 systeem betreft in de eerste plaats het cytochroom P-450 zelf, en komt tot uitdrukking in een sekseverschil in de verhouding van de hoeveelheden van verschillende soorten cytochroom P-450. Verder onderzoek moet inzicht bieden in de faktoren die de binding van een bepaald (soort) substraat aan een bepaald cytochroom P-450 bepalen, en de faktoren die bepalen of de vorming van een cytochroom P-450-substraat kompleks al of niet leidt tot het metabolisme van het substraat. Deze informatie kan leiden tot een beter begrip van de interakties tussen verschillende farmaka en de relatie tussen chemische struktuur en biotransformatie.

CURRICULUM VITAE.

Ad van den Berg werd op 10 februari 1947 te Boskoop geboren. In 1965 behaalde hij het diploma gymnasium β aan het Chr. Lyceum te Gouda. Vervolgens studeerde hij scheikunde aan de Rijks Universiteit te Utrecht. Na het kandidaatseksamen in 1969 koos hij als specialisaties biochemie (hoofdvak, bij prof. dr. L.L.M. van Deenen en dr. F.C. Reman) en analytische chemie (bijvak, bij prof. dr. G. Dijkstra en dr. J.J. de Ridder). Het doktoraal-diploma werd behaald in 1972. Van 1972 tot 1976 werd op de afdeling Farmakologie van de Erasmus Universiteit te Rotterdam in het kader van een door ZWO/FUNGO gesubsidieerd projekt het onderzoek verricht, waarop dit proefschrift is gebaseerd.