HEPATIC DRUG METABOLISM STUDIES IN STREPTOZOTOCIN
AND SPONTANEOUSLY DIABETIC RATS: THE POSSIBLE INFLUENCE
OF $[^3H]$-ESTRADIOL BINDING PROTEINS

by

BETTY LYNNE WARREN

B.Sc. (Pharm.) University of British Columbia, 1974
M.Sc. University of British Columbia, 1979

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We accept this thesis as conforming
to the required standard

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July 1982
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Department of **Pharmaceutical Sciences**

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date **July 26, 1982**
We have examined the effect of recent onset diabetes on several aspects of hepatic microsomal metabolism in both chemically-induced and spontaneously BB (Bio Breeding) diabetic male and female Wistar rats. Experiments were performed either 4 days post-streptozotocin injection or 4 days after withdrawal of insulin (BB rats). Differential alterations of the diabetic state on hepatic microsomal enzyme activities were observed. Female diabetic rats exhibited no change in benzo[a]pyrene hydroxylase activity, a decrease in testosterone \( \Delta^4 \) hydrogenase, and an increase in aniline hydroxylase. On the other hand, male diabetic rats demonstrated a decrease in hepatic benzo[a]pyrene hydroxylase activity, no change in testosterone \( \Delta^4 \) hydrogenase, and an increase in aniline hydroxylase. Insulin treatment reversed these effects. Benzo[a]pyrene hydroxylase kinetic studies did not reveal marked differences between control and diabetic rats. There were no marked differences between the chemically-induced and genetic models of diabetes with respect to the metabolism studies.

Serum testosterone levels were significantly lower than control in BB diabetic males, whereas no change was apparent in female diabetics. Serum insulin determinations suggested that the BB diabetic animals we examined were not severely diabetic although they did exhibit hyperglycemia. Electrophoresis of hepatic microsomal proteins indicated that spontaneous diabetes of short duration altered the protein distribution in the cytochrome P450 region.
Two $[^3H]$-estradiol binding sites were detected in rat liver cytosol by Scatchard analysis with a ligand concentration range of 0.05 to 200 nM. The high affinity site, which was specific for estrogens, exhibited a $K_d$ of $\sim 10^{-10}$ M and a capacity of $\sim 100$ fmol/mg protein in the 50% ammonium sulfate fraction. Unexpectedly, the data suggested that the capacity of this site was greater in males than in females. The moderate affinity binding site exhibited a $K_d$ of $\sim 10^{-7}$ M and a capacity of $\sim 10$ pmol/mg protein in the whole cytosol fraction. Binding at this site was markedly pH dependent. Both estradiol and dihydrotestosterone competed for binding to this site. A sex difference existed for moderate affinity binding because it was present only in males. We obtained unexpected results in binding studies conducted on a relatively small number of BB diabetic rats. In diabetic males, the capacity of the high affinity site was reduced to 50% of control, whereas the reduction in moderate affinity binding was not nearly so marked. Additional studies using a larger sample size and more sophisticated data analysis are required to verify these results.

We concluded that alterations in sex dependent drug metabolism evident in streptozotocin-induced diabetic rats were also seen in the spontaneously diabetic rat model, and were accompanied by changes in the relative disposition of electrophoretically separable microsomal proteins. Changes in circulating androgen levels were also found in BB diabetic males, along with changes in the capacities of certain hepatic steroid binding sites. It is not yet possible to establish mechanistic relationships between these $[^3H]$-estradiol binding sites and modulation of hepatic drug metabolism.
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LIST OF ABBREVIATIONS AND TRIVIAL CHEMICAL NAMES

ALX  alloxan
androstenedione  Δ<sup>4</sup> - androsten-3,17-dione
AS  ammonium sulfate
BB  Bio Breeding
BP  benzo[a]pyrene
dexamethasone  9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione
DHT  dihydrotestosterone
EDTA  ethylenediamine tetraacetic acid
EH  epoxide hydrase
ER  endoplasmic reticulum
estradiol  Δ<sup>1,3,5,(10)</sup>-estratrien-3,17β-diol
IC  nondiabetic inside control
3-MC  3-methylcholanthrene
MFO  mixed function oxidases
OC  outside Wistar control
progesterone  Δ<sup>4</sup>-pregnene-3,20-dione
PZ  protamine zinc
SDS  sodium dodecyl sulfate
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
STZ  streptozotocin
testosterone  Δ<sup>4</sup>-androsten-17β-ol-3-one
triamcinolone  triamcinolone acetonide
  9α-fluoro-11β, 16α, 17,21-tetra-ol-pregna-1,4-diene-3,20-dione cyclic 16,17 acetal with acetone
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INTRODUCTION

A. The role of androgens and estrogens in modulating levels of hepatic microsomal enzyme activities in rats

Precisely how the levels of hepatic microsomal drug and steroid-metabolizing monooxygenase activities are regulated is unknown. This is an important area since hepatic metabolism is pivotal in controlling the biological activity of many endogenous hormones and xenobiotics. It is well recognized that a wide variety of physiological (e.g., sex, age, species), pathological (e.g., starvation, stress, disease), and pharmacological (e.g., phenobarbital, ethanol, SKF 525A) factors influence cytochrome P-450-associated metabolizing activities. In the rat, among the more important physiological regulators are endocrine hormones, in particular estrogens and androgens. It is generally believed that these two gonadal steroids are responsible for producing sex differences in hepatic microsomal metabolism. However, the underlying mechanisms by which the steroids accomplish this are not yet known. Moreover, it is still controversial whether sex steroid effects on the liver are mediated directly or indirectly. Clearly, if we could understand how sex dependency of hepatic cytochrome P-450 activities is established we would learn much about the endocrine control of hepatic drug and steroid metabolism.

Sex dependent drug metabolizing activities in the rat are usually higher in the male than in the female. As such, these enzymes have been viewed as being dependent on some kind of stimulatory action mediated by androgens. Sex dependency varies with substrate. Hydroxylation of aniline, zoxazolamine, biphenyl, and androstenedione (7α), and O-deethylation of 7-ethoxycoumarin have been observed to be
sex independent (El Defrawy El Masry et al., 1974; Litterst et al., 1976); whereas, ethylmorphine and aminopyrine N-demethylases, and benzo[a]-pyrene (BP), hexobarbital, and androstenedione - 16α hydroxylations have been observed to be sex dependent. For instance, Quinn et al. (1958) found that the basal rate of metabolism of hexobarbital in adult male rats was approximately 5 times that of female rats. Moreover, administration of testosterone to female rats (treatment for up to 7 weeks) enhanced hexobarbital, ethylmorphine, and BP metabolism several fold. (Quinn et al., 1958; Booth and Gillette, 1962; Hamrick et al., 1973; Gurtoo and Parker, 1977). On the other hand, administration of estradiol to male rats lowered hexobarbital metabolism, by about 4 fold (Quinn et al., 1958). The stimulatory role of androgens was also observed by these and other workers in that hexobarbital, aminopyrine, ethylmorphine and codeine metabolism were similar in male and female rats up to about 4 to 5 weeks of age. Beyond that, metabolic activity became markedly increased in male animals, a response that coincided with enhanced gonadal production of androgens (Quinn et al., 1958; El Defrawy El Masry and Mannering, 1974). In the female, the metabolism of these substrates did not change markedly at puberty, although there may have been a trend to decreased metabolism after maturity in females (El Defrawy El Masry and Mannering, 1974). Other evidence which implicates androgens as stimulators of drug metabolism has been demonstrated in castration experiments (Booth and Gillette, 1962; Kato and Gillette, 1965; El Defrawy El Masry and Mannering, 1974). Castration of adult male rats resulted in substantially lower rates of metabolism of ethylmorphine (El Defrawy El Masry et al., 1974), hexobarbital (Booth and Gillette, 1962), and aminopyrine (Kato and Gillette, 1965), almost to control female levels. When androgen
was replaced exogenously in male rats, the drug metabolizing activities returned to control levels. Similarly, in ovariectomized female rats, androgen therapy enhanced drug metabolizing activity above the usual female control values, closer to basal male levels. The sex independent metabolizing activities represented by aniline and zoxazolamine hydroxylases were not different in female and male rats, and were not altered by castration (Kato and Gillette, 1965).

While androgens have been established as inducers of sex dependent metabolism, the role of estrogenic influences is less clear. Recently, however, several laboratories have confirmed that estrogen treatment decreases sex dependent activities in normal male rats (Sweeney and Cole, 1980), with further decreases to the female level observed in castrated male rats treated with estrogen (Berg and Gustafsson, 1973; Kramer et al., 1978; Kramer and Colby, 1976). In female rats, neither ovariectomy nor subsequent estrogen treatment altered sex dependent ethylmorphine or BP metabolism (El Defrawy El Masry and Mannering, 1974; Al Turk et al., 1980a). On the other hand, estradiol or mestranol treatment of intact adult females led to increased activities of BP hydroxylase, 7-ethoxycoumarin O-deethylase (Al Turk et al., 1980b) and p-nitroanisole O-demethylase (Briatico et al., 1976). By 28 days of age female rats attained peak levels of aminopyrine and ethylmorphine N-demethylase activities which progressively decreased by 56 days of age to levels that were 3 to 6 fold less than in males (El Defrawy El Masry et al., 1974). These authors suggested that sex dependency of hepatic enzymes in mature rats results not only from temporal increases in males, but also from temporal decreases in females.
B. Altered sex dependent drug metabolism in chemically-induced diabetic rats

Various pathophysiological situations will alter the ability of an animal to metabolize endogenous and exogenous substrates. For instance, hormone imbalances caused by thyroxine treatment, adrenalectomy or hypophysectomy alter microsomal enzyme activities. Likewise, liver microsomal preparations from male and female chemically-induced diabetic rats have decreased ability to metabolize certain drug substrates, whereas other substrates may be more readily metabolized. The accompanying table summarizes previous workers' findings with respect to the effect of diabetes mellitus on drug metabolizing activities in rats (Table I). It is apparent that chemically induced diabetes lowers sex dependent microsomal activities in the male rat, but raises them in the diabetic female. On the other hand, the sex independent enzymes are unchanged or increased in both male and female diabetics. This situation suggests that in the diabetic state androgen sensitive activities are impaired, while androgen insensitive activities are not impaired or may even be enhanced. This impression is further supported by the work of Kato and Gillette (1965). They observed that castrated adult male rats did not exhibit lowered activities towards hexobarbital or aminopyrine when they were rendered diabetic. That is, castrated male rats already exhibited lower-than-control androgen dependent activities, and superimposed diabetes mellitus did not reduce this further. It is interesting to note that alloxan (ALX) diabetes in male castrates prevented methyltestosterone induction (10 mg/kg on alternate days for 18 days) of hexobarbital and aminopyrine metabolism. In the case of the females, hexobarbital and aminopyrine metabolism was similar to control levels.
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<td>hexobarbital</td>
<td>+</td>
<td>induced by methyltestosterone</td>
<td>Kato and Gillette (1965)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aminopyrine</td>
<td>+</td>
<td>induced by methyltestosterone</td>
<td>Kato and Gillette (1965)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aniline</td>
<td>+</td>
<td>induced by methyltestosterone</td>
<td>Kato and Gillette (1965)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>zoxazolamine</td>
<td>+</td>
<td>induced by methyltestosterone</td>
<td>Kato and Gillette (1965)</td>
</tr>
<tr>
<td>Sprague-Dawley F castrated</td>
<td>none</td>
<td>hexobarbital</td>
<td>no change</td>
<td>no further +</td>
<td>Kato et al. (1970)</td>
</tr>
<tr>
<td></td>
<td>ALX 130 mg/kg</td>
<td>hexobarbital</td>
<td>no change</td>
<td>induced by methyltestosterone</td>
<td>Kato et al. (1970)</td>
</tr>
<tr>
<td></td>
<td>aminopyrine</td>
<td>no change</td>
<td>induced by methyltestosterone</td>
<td>SL. + by methyltestosterone</td>
<td>Kato et al. (1970)</td>
</tr>
<tr>
<td></td>
<td>aniline</td>
<td>no change</td>
<td>induced by methyltestosterone</td>
<td>SL. + by methyltestosterone</td>
<td>Kato et al. (1970)</td>
</tr>
<tr>
<td>Wistar M intact 180 g</td>
<td>ALX 130 mg/kg</td>
<td>hexobarbital</td>
<td>+</td>
<td>reversed by insulin</td>
<td>Reinke et al. (1978a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aminopyrine</td>
<td>+</td>
<td>reversed by insulin</td>
<td>Reinke et al. (1978a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aniline</td>
<td>+</td>
<td>reversed by insulin</td>
<td>Reinke et al. (1978a)</td>
</tr>
<tr>
<td>Wistar F intact 160 g</td>
<td>ALX 130 mg/kg</td>
<td>hexobarbital</td>
<td>+</td>
<td>reversed by insulin</td>
<td>Reinke et al. (1978a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aminopyrine</td>
<td>+</td>
<td>reversed by insulin</td>
<td>Reinke et al. (1978a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aniline</td>
<td>+</td>
<td>reversed by insulin</td>
<td>Reinke et al. (1978a)</td>
</tr>
<tr>
<td>Sprague-Dawley M intact 180 g</td>
<td>STZ 60 mg/kg</td>
<td>aminopyrine</td>
<td>SL. +</td>
<td>reversed by insulin</td>
<td>Reinke et al. (1978a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aniline</td>
<td>+</td>
<td>reversed by insulin</td>
<td>Reinke et al. (1978a)</td>
</tr>
<tr>
<td>Sprague-Dawley F intact 160 g</td>
<td>STZ 60 mg/kg</td>
<td>aminopyrine</td>
<td>+</td>
<td>reversed by insulin</td>
<td>Reinke et al. (1978a)</td>
</tr>
</tbody>
</table>

....cont'd
<table>
<thead>
<tr>
<th>Strain, sex and status of animals</th>
<th>Diabetogenic agent and dose</th>
<th>Drug substrate tested</th>
<th>Effect of diabetic state compared to control</th>
<th>Effect of other treatments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprague-Dawley M intact 180 g</td>
<td>STZ 60 mg/kg</td>
<td>benzo[a]pyrene</td>
<td>+</td>
<td>reversed by insulin</td>
<td>Reinke et al. (1979)</td>
</tr>
<tr>
<td>Sprague-Dawley F intact 160 g</td>
<td>STZ 60 mg/kg</td>
<td>benzo[a]pyrene</td>
<td>+</td>
<td>induced by 3MC, but to a lesser extent than with 3 MC induction in controls</td>
<td>Reinke et al. (1979)</td>
</tr>
<tr>
<td>Sprague-Dawley M intact 180 g</td>
<td>STZ 60 mg/kg</td>
<td>androstenedione</td>
<td>16α-hydroxylation + 7α-hydroxylation +</td>
<td>reversed by insulin</td>
<td>Reinke et al. (1978b)</td>
</tr>
<tr>
<td>Sprague-Dawley F intact 180 g</td>
<td>STZ 60 mg/kg</td>
<td>benzo[a]pyrene</td>
<td>intestine + lung + kidney no change adrenal no change</td>
<td>reversed by insulin</td>
<td>Stohs et al. (1979)</td>
</tr>
<tr>
<td>Sprague-Dawley M intact 140 g</td>
<td>STZ 60 mg/kg</td>
<td>7-ethoxycoumarin</td>
<td>liver + intestine +</td>
<td>reversed by insulin</td>
<td>Al-Turk et al. (1980c)</td>
</tr>
<tr>
<td>Sprague-Dawley F intact 140 g</td>
<td>STZ 60 mg/kg</td>
<td>7-ethoxycoumarin</td>
<td>liver + intestine +</td>
<td>reversed by insulin</td>
<td>Al-Turk et al. (1980c)</td>
</tr>
</tbody>
</table>

* 3MC = 3-methylcholanthrene
<table>
<thead>
<tr>
<th>Strain, sex and status of animals</th>
<th>Diabetogenic agent and dose</th>
<th>Drug substrate tested</th>
<th>Effect of diabetic state compared to control</th>
<th>Effect of other treatments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fischer M intact 160-210 g</td>
<td>ALX 120 mg/kg</td>
<td>hexobarbital</td>
<td>all +</td>
<td>all +</td>
<td>Chewalit et al. (1982)</td>
</tr>
<tr>
<td></td>
<td>6-aminonicotinamide 35 mg/kg</td>
<td>aminopyrine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N-methyl-acetamide 6.5 ml/kg</td>
<td>aniline</td>
<td>all +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sprague-Dawley M intact 160-200 g</td>
<td>ALX 50 mg/kg</td>
<td>aminopyrine</td>
<td></td>
<td></td>
<td>Cook and Past (1979)</td>
</tr>
<tr>
<td></td>
<td>STZ 80 mg/kg</td>
<td>p-chloro-N-methyl-aniline (in isolated perfused liver)</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Sprague-Dawley M intact 175-250 g</td>
<td>STZ 65 mg/kg</td>
<td>p-nitroanisole</td>
<td>reversed by insulin</td>
<td></td>
<td>Eacho et al. (1981)</td>
</tr>
<tr>
<td></td>
<td>(in isolated hepatocytes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sprague-Dawley F intact 175-250 g</td>
<td>STZ 65 mg/kg</td>
<td>p-nitroanisole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(in isolated hepatocytes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

....cont'd
Table I/cont'd

<table>
<thead>
<tr>
<th>strain, sex and status of animals</th>
<th>diabetogenic agent and dose</th>
<th>drug substrate tested</th>
<th>effect of diabetic state compared to control</th>
<th>effect of other treatments</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long Evans M intact 75-125 g</td>
<td>STZ 120 mg/kg</td>
<td>$[^3]H$-acetaminophen</td>
<td>+ glucuronide conjugation</td>
<td>no change in mercapturate formation (cyt. P450 dependent)*</td>
<td>Eacho et al. (1981)</td>
</tr>
</tbody>
</table>

* cytochrome P450 dependent
whether the animal was ALX diabetic, ovariectomized, or both. Alloxan diabetes did cause reduction in sex dependent activities in the females if, after they had been castrated, they were administered exogenous methyltestosterone.

The effects of chemically-induced diabetes on drug metabolizing activities were apparently a result of insulin lack (Table I). When a diabetic rat received insulin replacement, measured effects of the diabetic state were reversed, returning enzyme activities to control levels in a matter of a few days (Dixon et al., 1961; Reinke et al., 1978a). Reinke et al., (1978a) observed a slight influence of insulin treatment alone upon the enzyme activities they measured. However these data are flawed since non-diabetic animals were tested, producing hyperinsulinemic animals. These same workers as well as Ackerman and Leibman (1977) showed that in vivo infusion of glucose or addition of glucose to microsomes in vitro did not affect drug metabolism. These experiments showed that hyperglycemia was not responsible directly for the changes in drug metabolizing activities in diabetic rats. More evidence in this regard was provided by Rouer and Leroux (1980). They monitored various mono-oxygenase activities in 3 strains of mice, obese (ob/ob), streptozotocin (STZ) injected lean, and diabetic (db/db), which were all hyperglycemic. Despite the similarities of their elevated blood glucose levels, the different strains did not exhibit similar alterations in their mixed function oxidase (MFO) systems.

Effects of chemical diabetes upon drug metabolizing enzyme activities in extrahepatic tissues have been observed. Stohs et al., (1979) and Al-Turk et al., (1980c) noted that the metabolism of BP
and 7-ethoxycoumarin in STZ diabetic female rats was increased in liver and intestine, but was decreased in lung tissue. Values returned to control with administration of insulin. In diabetic male rats, the sex independent activity of 7-ethoxycooumarin O-deethylase followed a similar pattern to that in the diabetic female (Al-Turk et al., 1980c).

Not only are cytochrome P-450 dependent reactions altered by the diabetic state, but so are phase II conjugation reactions. Interestingly, the male diabetic rat appears to have increased glucuronidation and sulfation capabilities reversible by insulin treatment (Eacho et al., 1981; Price and Jollow, 1982) and control levels of mercapturate formation. Conjugation reactions in the female diabetic are not different from control (Eacho et al., 1981). Hassing et al., (1979) reported that STZ diabetic female rats were more susceptible to the glutathione depleting effects of acetaminophen; while Price and Jollow (1982) observed that STZ diabetic male rats were more resistant to acetaminophen-induced hepatotoxicity. Again, there is a dissociation between the male and female rat in their metabolic response to diabetes.

Since androgenic stimulation of hepatic mixed function oxidases is apparently abnormal in diabetic rats it is possible that other induction processes are also affected. Dixon et al. (1963) examined the effect of enzyme induction on hexobarbital hydroxylation in ALX diabetic male rats. Phenobarbital treatment of diabetic animals enhanced metabolizing activity to the same level as that observed in control rats receiving phenobarbital treatment. Hence, the impaired metabolizing ability of male diabetic rats toward hexobarbital was not only restored to control levels but was restored to the elevated levels attained with phenobarbital
induction evident in control male rats. Similarly, Reinke et al., (1979) pretreated diabetic female rats with the inducing agent 3-methylcholanthrene (3-MC) and observed enhanced BP hydroxylase activity, that in this case was just slightly less than that attained in control females receiving 3-MC. These data suggest that at least some hepatic induction pathways are preserved in diabetic rats.
C. Serum testosterone levels in diabetic rats

It is possible that the expression of androgen sensitive drug metabolism is impaired in diabetic rats due to a lack of circulating testosterone. Tesone et al. (1980) and Murray et al. (1981) found that prostate and other sexual tissue weights were reduced in STZ diabetic male rats in association with a 50% and 75% decrease in serum and testicular testosterone levels respectively. Baxter's group has also demonstrated a decrease in serum testosterone levels in STZ male diabetic rats which returned to control with insulin treatment (Baxter et al., 1981). Administration of testosterone to diabetic male rats did not restore lowered sex dependent enzyme activities to control (Chawalit et al., 1982), but in this case duration of treatment was probably insufficient (testosterone acetate 12mg/kg/day for 2 days).

Litterest (1980) observed that genetically obese male Zucker (fafa) rats were substantially deficient in various in vitro hepatic monooxygenase activities, including sex dependent aminopyrine N-demethylation and BP hydroxylase. $K_m$ and $V_{max}$ values for aminopyrine N-demethylation were significantly less in obese rats than in lean rats. Although serum testosterone levels were found to be 44% of the value in normal Sprague-Dawley males, testosterone treatment (testosterone propionate 2.5 mg/kg twice weekly for 3 weeks) failed to restore drug metabolizing activity in the obese rats. It is possible that these animals also may have a defective testosterone-mediated mechanism for increasing enzyme activity. These findings are of interest because obese rodents generally have an abnormal insulin physiology which leads to insulin resistance, hyperglycemia, and decreased insulin receptor binding (Kahn and Roth, 1976).
Therefore a parallel can be drawn between diabetic and obese rats. That is, decreased testosterone levels along with abnormal insulin control may together disrupt the regulation of sex dependent drug metabolism.
D. Possible influence of hepatic androgen binding proteins on sex dependency of hepatic monooxygenase

Although it is still speculative, there is indirect evidence to suggest that androgenic stimulation of drug metabolism in the liver may be mediated by hepatic steroid binding protein(s) (Gustafsson et al., 1975; Roy et al., 1974; Sato et al., 1980a). This is assumed to occur in a parallel fashion to that in "classical" target tissues, where the interaction between the steroid and cytoplasmic receptor determines the hormone responsiveness of the cell/tissue. Fang and Liao (1971) combined radioactive dihydrotestosterone (DHT) in vitro with either whole cytosol or cytoplasmic DHT binding protein from rat ventral prostate. These fractions were then incubated with purified hepatic nuclei. When the labelled steroid-protein complex was isolated from the nuclei, it was found that the liver nuclei retained more radioactive DHT than did non-target tissue nuclei in blood, spleen, lung, thymus, and diaphragm. The work of Gustafsson et al., (1975) also supports the hypothesis that an androgen "receptor" is present in liver tissue. Radiolabelled testosterone was injected intraperitoneally to castrated male rats. (Castration causes androgen receptors to be localized in the cytoplasm, since the binding of endogenous androgen ligand and subsequent transport to the nucleus no longer occurs). The liver cytosol obtained from these rats contained a protein-labelleandrostenedione complex. This complex was found to have a sedimentation coefficient of 10S, was destroyed by incubation with pronase, and was stable at 37°C for at least 60 minutes. Similar adrostenedione binding was observed in in vitro studies where unlabelled cytosol from castrated rats was incubated with labelled testosterone. The data indicated that under these experimental conditions
testosterone had been metabolized to androstenedione. The authors interpreted these findings to suggest the existence of specific mechanisms in liver cytosol which produce androstenedione from testosterone, protect it from further metabolism, and provide for its uptake into liver nuclei. However, there were no data presented to indicate the specificity of the androstenedione binding protein. This would have been of interest since Roy et al. (1974) had previously reported the existence of a DHT binding protein in liver cytosol which demonstrated a sedimentation coefficient of 3.5S. It bound DHT, testosterone, and estradiol with moderate affinity. Specific binding of DHT to the protein had a dissociation constant ($K_d$) of $4.5 \times 10^{-8}$ M; and the $K_d$ for estradiol binding to the same protein was $3.5 \times 10^{-7}$ M. No data were presented regarding nuclear binding of the DHT-receptor complex. These workers correlated the existence of the androgen binder in hepatic cytosol with the urinary output of $\alpha_{2u}$-globulin, a protein synthesized under the influence of androgen in the liver which appears in the urine of male rats. Immature and senescent male and female rats did not produce $\alpha_{2u}$-globulin and were found to lack liver androgen binding activity as well. Castration or estradiol treatment of male rats led not only to the loss of cytoplasmic androgen binding activity but also to the loss of $\alpha_{2u}$-globulin synthesis. These data indicated that cytoplasmic androgen binding protein was induced by androgens and was suppressed by estradiol. Another significant finding was that pseudohermaphroditic male rats which show androgen insensitivity at the end organ level, did not synthesize $\alpha_{2u}$-globulin (Milin and Roy, 1973). In turn, these animals were found not to show any hepatic androgen binding activity, indicating further the correlation between the liver cytosol binding protein and hepatic synthesis of $\alpha_{2u}$-globulin.

In addition, other studies carried out on pseudohermaphroditic rats indirectly support the hypothesis that androgen binders in the liver
may be associated with androgenic stimulation of sex dependent drug metabolism. Since the pseudohermaphroditic male rats have tissue insensitivity to androgens, it would be predicted that androgen dependent drug metabolising enzyme activities would be reduced in these animals. In fact, their metabolizing enzyme levels were similar to female levels, and they were significantly different from normal male activities with respect to ethylmorphine N-demethylase, \( \Delta^4 \)-steroid reductase (Bullock et al., 1971), aminopyrine N-demethylase, and cytochrome P-450 content (Sonawane et al., 1979). Administration of testosterone and DHT did not increase these enzyme activities in pseudohermaphrodities although it did in normal female rats or castrated male rats (Bullock et al., 1971).

These observations may be explained on the basis of a defect in pseudohermaphroditic rats which renders them unable to concentrate androgen in target cell nuclei (Bullock and Bardin, 1973). This could result from reduced or absent androgen binding proteins, or from altered affinity of protein for androgens.

Recently, another group has detected a testosterone - and androstenedione binding protein in male rat liver (Sato et al., 1980a; Ota et al., 1980). This partially purified cytosolic component exhibited a sedimentation coefficient of 10S, a \( K_d \) and capacity for testosterone binding of \( 5.8 \times 10^{-7} \) M and 170 fmol/mg protein, a \( K_d \) and capacity for androstenedione binding of \( 1.1 \times 10^{-6} \) M and 158 fmol/mg protein. Both cytosolic and nuclear binding sites were decreased following castration or estradiol treatment, and were increased by testosterone (Sato et al., 1980b, Sato et al., 1980c). Estradiol did not appear to compete with testosterone in the in vitro binding experiments (Sato et al., 1980).
These data, summarized in Table II, provide a basis on which to hypothesize the existence of androgenic binding protein(s) in rat liver which correlate with sex-hormone status, and with certain physiological endpoints, eg. synthesis of $\alpha_{2u}$-globulin. However, as yet, mechanistic studies demonstrating the existence of these binding proteins and their correlated regulation of cytochrome P-450 drug metabolism have not been carried out.
### Table II. Some properties of hepatic androgen binding protein

<table>
<thead>
<tr>
<th>Source</th>
<th>male rat liver</th>
<th>female rat liver</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K_d</strong></td>
<td>4.5 \times 10^{-8} M (dihydrotestosterone) &lt;br&gt; 3.5 \times 10^{-7} M (estradiol binding) &lt;br&gt; 5.8 \times 10^{-7} M (testosterone binding) &lt;br&gt; 1.1 \times 10^{-6} M (androstenedione binding)</td>
<td>no dihydrotestosterone &lt;br&gt; or estradiol binding</td>
</tr>
<tr>
<td><strong>Capacity</strong></td>
<td>170 fmol/mg protein (testosterone) &lt;br&gt; 158 fmol/mg protein (androstenedione)</td>
<td>no \alpha_2\gamma-globulin synthesis</td>
</tr>
<tr>
<td>pH optimum for binding</td>
<td>pH 8.5</td>
<td></td>
</tr>
<tr>
<td>Compounds which compete for binding sites</td>
<td>dihydrotestosterone, testosterone &lt;br&gt; (estradiol), androstenedione</td>
<td></td>
</tr>
<tr>
<td>Compounds which do not compete for binding sites</td>
<td>androstandiol, 17α-methyltestosterone, corticosterone, cortisol</td>
<td></td>
</tr>
<tr>
<td>Sedimentation coefficient</td>
<td>3.5S, 10S</td>
<td></td>
</tr>
<tr>
<td>Evidence for nuclear translocation</td>
<td>yes (cytosolic levels replenished within 60 minutes)</td>
<td></td>
</tr>
<tr>
<td>Effect of androgens</td>
<td>induced; absent in pseudohermaphrodites</td>
<td></td>
</tr>
<tr>
<td>Effect of estrogens</td>
<td>suppressed</td>
<td></td>
</tr>
<tr>
<td>Effect of age</td>
<td>absent in immature and senescent rats</td>
<td></td>
</tr>
<tr>
<td>Effect of castration (prepubertal) (adult)</td>
<td>absent in adult &lt;br&gt; trace only by 18 days</td>
<td></td>
</tr>
<tr>
<td>Physiological correlates</td>
<td>\alpha_2\gamma-globulin synthesis</td>
<td></td>
</tr>
</tbody>
</table>

References: Roy et al., 1974; Sato et al., 1980a; Ota et al., 1980 <br> Sato et al., 1980b; Sato et al., 1980c; Ota et al., 1981.
E. Possible influence of hepatic estrogen binding proteins on sex dependency of hepatic monooxygenases

The influence of estrogens on the liver must also be considered, although their role in affecting the levels of hepatic sex dependent monooxygenase activities is less clear than it is for androgens, as discussed above. The existence of a hepatic estrogen "receptor" in rats was recently demonstrated by 4 groups of workers (Beers and Rosner, 1977; Viladiu et al., 1975; Chamness et al., 1975; and Eisenfeld et al., 1976). Beers and Rosner detected a saturable estrogen binding 4S protein in liver cytosol with an association constant of 5.98 ± .95 x 10^9 1/mol. (Kd ~ 1.7 x 10^{-10} M). Eisenfeld et al. (1977) found that estrogen binding in rat liver was lower than that found in uterus, but was higher than plasma, spleen, and heart. The "receptor" showed specificity in that estrone, estriol and mestranol bound to it, whereas DHT, testosterone, progesterone, cortisol and dexamethasone did not. It was shown that the hormone-"receptor" complex was also able to translocate to the nucleus. Therefore, the liver appeared to contain a protein which bound estradiol with high affinity and specificity. These properties and sedimentation characteristics were essentially similar to those of the estrogen receptor identified in the rat uterus. In addition, Eisenfeld's group has been able to correlate estrogen receptor levels in rat liver with the hepatic synthesis of a plasma protein. "Plasma renin substrate" levels were elevated along with estrogen receptor levels in both adult and estrogen treated female rats compared to prepubescent female rats (Eisenfeld et al., 1976, 1977a, 1977b).

Not only were estrogen receptors present in female rat liver, but they were also found in male rat liver (Aten et al., 1978). In male
rat liver there appeared to be at least two types of estrogen binding proteins: a moderate affinity and capacity one, and a high affinity low capacity receptor protein. The high affinity, low capacity receptor had similar properties in male and female rats. However, the less specific receptor additionally present in male rat liver was present in much smaller amounts in immature male and female rats and was barely detectable in mature females (Powell-Jones et al., 1980) as measured in sedimentation studies. This group also noted that castration decreased the levels of this binding protein in the male by only 20%, although Dickson et al. (1978) found approximately a 50% decrease. From the work of the previously mentioned groups along with several other reports (Mulder et al. 1974; Aten et al., 1978a and 1978b; Dickson and Eisenfeld, 1980; Dickson et al., 1980, Powell-Jones et al., 1976, 1978, and 1981) various properties of the two types of hepatic estrogen binding proteins may be described (Tables III, IV). It should be noted that there are certain striking similarities between the moderate affinity estrogen binding protein (Table IV) and the androgen binder defined by Roy's group (Table II). It is also apparent that various parameters which affect the moderate affinity estrogen binding protein alter sex dependent enzyme activities in a similar fashion. For instance, levels of the binding protein in the male rat are increased at puberty and by exogenous androgen treatment, and are higher in the adult male than in the female. Castration, hypophysectomy, and estradiol treatment reduce male levels towards those of the female. Because parallel shifts in activities are observed in sex dependent monooxygenases under these same influences, it is postulated that hepatic steroid binding proteins influence the control of sex dependent metabolism.
Table III. Some properties of hepatic high affinity estrogen binding protein

<table>
<thead>
<tr>
<th>Source</th>
<th>Female Rat Liver</th>
<th>Male Rat Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$</td>
<td>$0.7-1.7 \times 10^{-10}$ M</td>
<td>$1.4 \times 10^{-10}$ M</td>
</tr>
<tr>
<td>$K_d$ (ammonium sulfate ppt)</td>
<td>$2.2 \times 10^{-10}$ M</td>
<td>$0.8-3.5 \times 10^{-10}$ M</td>
</tr>
<tr>
<td>Capacity</td>
<td>$\sim 14-76$ fmol/mg protein</td>
<td>$\sim 38$ fmol/mg protein</td>
</tr>
<tr>
<td>Capacity (ammonium sulfate ppt)</td>
<td>$\sim 25$ fmol/mg protein</td>
<td>$9 \sim 25$ fmol/mg protein</td>
</tr>
<tr>
<td>Typical compounds which compete for binding sites</td>
<td>estradiol, diethylstilbestrol, estrone, estriol, ethinyl estradiol, nafoxidine</td>
<td>estradiol, diethylstilbestrol, estrone, ethinylestradiol</td>
</tr>
<tr>
<td>Typical compounds which do not compete for binding</td>
<td>dihydrotestosterone, testosterone, progesterone, cortisol, corticosterone, dexamethasone</td>
<td>dihydrotestosterone, progesterone, corticosterone</td>
</tr>
<tr>
<td>Sedimentation coefficient</td>
<td>8-9 S</td>
<td>8 S</td>
</tr>
<tr>
<td>Evidence for nuclear translocation and binding</td>
<td>yes (mainly unchanged estradiol bound)</td>
<td>yes - but slower onset of translocation, longer nuclear retention (mainly a polar metabolite bound)</td>
</tr>
<tr>
<td>Effect of age</td>
<td>low levels in immature animals, at puberty, high levels develop</td>
<td>low levels in immature animal; at puberty, high levels develop</td>
</tr>
<tr>
<td>Effect of castration (adult) (immature)</td>
<td>1 day - slight decrease 2 weeks - 2½ fold increase</td>
<td>3 weeks - 30% increase increased binding</td>
</tr>
<tr>
<td>Effect of hypophysectomy</td>
<td>decreased binding</td>
<td>decreased binding</td>
</tr>
<tr>
<td>Physiological correlate</td>
<td>stimulation of plasma renin substrate</td>
<td>stimulation of plasma renin substrate</td>
</tr>
</tbody>
</table>

References: Mulder et al., 1974; Viladiu et al., 1975; Chamness et al., 1975; Eisenfeld et al., 1976; 1977a, 1977b; Powell-Jones et al., 1976; 1978; Beers and Rosner, 1977; Aten et al., 1978a; Dickson et al., 1978, 1980; Dickson and Eisenfeld, 1980.
Table IV. Some properties of hepatic moderate affinity estrogen binding protein

<table>
<thead>
<tr>
<th>source</th>
<th>female rat liver</th>
<th>male rat liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ capacity</td>
<td>(10-200 fold less than present in adult male)</td>
<td>0.6-1.3 x 10^{-7} M</td>
</tr>
<tr>
<td>typical compounds which compete for binding sites</td>
<td>estradiol, estriol, ethynyl estradiol, dihydrotestosterone, 5α-androstan-3α,17β-diol, testosterone</td>
<td></td>
</tr>
<tr>
<td>typical compounds which do not compete for binding sites</td>
<td>diethylstilbestrol</td>
<td>diethylstilbestrol, corticosterone, cortisol, progesterone</td>
</tr>
<tr>
<td>sedimentation coefficient</td>
<td>4-5-S</td>
<td>4 S</td>
</tr>
<tr>
<td>evidence for nuclear translocation</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>(remains in cytoplasm)</td>
<td>(minimum translocation of estradiol at short time periods; increased after longer time periods)</td>
</tr>
<tr>
<td>effect of age</td>
<td>little</td>
<td>adult has 4-10 fold greater amount than immature male (main binding component of adult male liver)</td>
</tr>
<tr>
<td>effect of castration (adult)</td>
<td>no change</td>
<td>20-50% decrease or no change decreased binding to female level</td>
</tr>
<tr>
<td></td>
<td>(neonatal)</td>
<td></td>
</tr>
<tr>
<td>effect of hypophysectomy</td>
<td>increased</td>
<td>decreased to female level; no response to estradiol treatment</td>
</tr>
</tbody>
</table>

References: Dickson et al., 1980; Powell-Jones et al., 1980, 1981.
Recently, various inducers of the hepatic mixed function oxidases have been shown to decrease high affinity cytosolic estrogen binding in ovariectomized rats (Duvivier et al., 1981). For example, one 40 mg/kg dose of 3-MC decreased binding by 44% after 24 hours and 80% after 72 hours. The authors found an increase of 17% 24 hours after a 30 mg/kg dose of phenobarbital. However, the discrepancy between the low dose and short time period in relation to maximal enzyme induction by phenobarbital, in contrast to the supramaximal dose of 3-MC calls into question any conclusion about the phenobarbital results. Further studies with higher doses over a longer time period would clarify if a difference in pattern does exist. However, the findings with the other inducers are of considerable importance. In particular, these authors found an excellent negative correlation between the effects of 3-MC or BP on cytosolic estrogen receptor and BP hydroxylase activity. Since the estrogen binding changes were not observed in other tissues, such as uterus, and were not accompanied by a decrease in lactic dehydrogenase (another cytosolic protein), the effect did not appear to be a general non-specific one. The authors postulated that due to structural similarities between the polycyclic hydrocarbons and estrogens, these inducers or their electrophilic metabolites could interact specifically with the estrogen receptor and produce a modification of the estrogen binding sites. Alternatively, it was suggested that one of the consequences of the nuclear effects of polycyclic hydrocarbons, besides that of induction of MFO's, could be a decrease in synthesis of cytosolic estrogen receptors. These studies enhance the notion that regulation of hepatic monooxygenase activities is modulated by cytosolic steroid binding proteins.
F. Interrelation of insulin with steroid hormones and hepatic steroid binding proteins

Insulin is known to affect protein synthesis in various cell types, and there is evidence to suggest that this hormone may influence steroid binding proteins in the liver. Using an isolated liver perfusion system, Murakami et al. (1980) showed that insulin promoted the synthesis of plasma renin substrate (angiotensin I) in female rats. This is of interest because Eisenfeld et al. (1976) correlated hepatic plasma renin substrate synthesis with hepatic estrogen receptor levels. These studies indicated that an estrogen receptor mediated function can be influenced by insulin levels. Further support for this was provided by Lui et al. (1981) who found that insulin (5 μg/ml) partially protected against the loss of estrogen receptors during perfusion of isolated hepatocytes.

Insulin receptors in humans have been observed to fluctuate in the presence or absence of estrogen and progesterone. Serum monocytes from pregnant women were shown to exhibit less insulin binding (as a result of lowered capacity) compared to nonpregnant controls, even though fasting plasma insulin was significantly higher in pregnant women. Under the influence of progesterone and estrogen, insulin binding to monocytes has been observed to be reduced in the luteal phase of the menstrual cycle (Bertoli et al., 1980). These data indicate that not only do steroids appear to affect insulin receptors, but also that insulin appears to affect steroid receptors, as discussed above. Recently, estrogen receptors have been detected in rat pancreas (Sandberg and Rosenthal, 1979; Tesone et al., 1979).

Additional evidence for hypothesizing an interplay between
steroids and insulin has been put forth by Shafie and Hilf (1978) through their work with 7,12-dimethylbenz(a)anthracene induced mammary tumors in rats. They observed that in STZ diabetic animals, growing mammary tumors exhibited decreased insulin binding and increased estradiol binding. In contrast, regressing tumors in diabetic animals exhibited increased insulin binding and decreased estrogen binding. When female rats were castrated, growing mammary tumors showed increased insulin binding, while regressing tumors showed decreased insulin binding. In this tissue, tumor growth was modulated by insulin and female sex hormones.

Insulin has been observed to exert a synergistic influence on the androgen dependent hepatic synthesis of $\alpha_{2u}$-globulin (Roy and Leonard, 1973). ALX diabetic male rats had a significantly reduced urinary output of $\alpha_{2u}$-globulin which was reversed by insulin administration. Although these workers did not determine hepatic androgen binding protein levels, it was apparent that insulin lack somehow interfered with androgenic stimulation of hepatic $\alpha_{2u}$-globulin synthesis. It is possible that hepatic androgen and/or estrogen binding proteins were altered in the diabetic male rats to cause the observed effects. This suggestion is plausible since recently Tesone et al. (1980) have reported that STZ diabetic rats exhibited a decrease in androgen receptor content and binding in prostate cytosol.
G. Hypothesis

In summary, it is evident that rat liver contains various steroid binding proteins. It would seem likely that androgenic and estrogenic binding proteins, stimulated by the appropriate hormonal signal, interact to modulate the general metabolic response of the animal. One aspect of this regulation could be to control the type and amount of specific hepatic proteins, e.g. sex dependent enzymes. Since insulin influences protein synthesis, we hypothesize that alterations of sex dependent drug metabolism in diabetic rats may reflect disturbances in hepatic steroid binding proteins.

H. Objectives

As indicated above, observations of the effect of diabetes mellitus on drug metabolism parameters have, to date, been made in chemically-induced diabetic animals. The majority of work has been carried out on ALX or STZ diabetic animals. This procedure has been accepted since there was no other rat model in which to study diabetes mellitus. But direct hepatic effects of the β-cell toxins cannot be definitively ruled out. There is evidence that STZ, for example, may cause liver damage (Laguens et al., 1980). Female C3H-S mice were administered a single injection of STZ and 21 days later ultrastructural changes in hepatocytes were observed. These included degranulation of
rough ER, swelling of mitochondria, and some dilatation of smooth ER. Furthermore, it was observed that liver damage was apparent whether diabetic symptoms were present or not. In future studies, therefore, it would be advantageous to eliminate direct toxic effects of STZ on the liver. In order to accomplish this we have utilized the Wistar-derived BB genetically diabetic rat (Bio Breeding Laboratories). The diabetic syndrome in these animals develops spontaneously before 120 days of age with a variable frequency and spectrum of severity. The affected animals are nonobese, hyperglycemic, hypoinsulinemic, and glycosuric. Plasma ketone and glucagon levels are elevated in relation to the severity of the diabetic state. Progressive Beta cell pancreatic destruction with insulin deficiency appears to be a result of marked inflammatory changes (insulitis) in the pancreatic islets (Nakhooda et al., 1977). Diabetes occurs with equal frequency in both sexes and the frequency is increased with selective inbreeding. The etiology of the genetic syndrome is not known, however, recent studies have suggested a cell-mediated autoimmune mechanism (Like et al., 1979). In addition, it is likely that the diabetic syndrome of the BB rat is not dependent on recognized infectious agents (Rossinni et al., 1979).

Using the spontaneously diabetic BB rat we have undertaken studies to determine the nature of various drug metabolism parameters in comparison to those in chemically induced diabetics. Furthermore our objective was to observe whether there was an association between alterations in drug metabolism and changes in hepatic steroid binding proteins in diabetic and control rats. The specific parameters we have monitored are described below.
1) We have measured the sex dependent activities BP hydroxylase and testosterone $\Delta^4$ hydrogenase (Appendix II) in hepatic microsomes of control and diabetic male and female rats. Diabetes was induced with ALX or STZ, or developed spontaneously in the BB rat. We carried out these studies at early time points after the onset of diabetes. We were interested in observing initial changes in control mechanisms, as opposed to those manifestations which would not be causally related to the disease process. We chose BP hydroxylase to monitor for the following reasons: sensitivity of the assay, it reflects aryl hydrocarbon hydroxylase activity which is responsible for the metabolism of numerous foreign lipophilic compounds, and its activity is approximately 5 to 6 fold higher in the normal male than in the female. Hepatic testosterone $\Delta^4$ hydrogenase was monitored because its sex dependency is manifested in the reverse direction to that of BP hydroxylase (that is, higher in females than males). We reasoned that if the diabetic state was responsible for specific drug metabolizing alterations (vs those due to general metabolic decline), then the pattern of changes, if any, should not be the same for BP hydroxylase and testosterone $\Delta^4$ hydrogenase activities. Also in contrast to BP hydroxylase, the hydrogenase metabolizes various endogenous compounds, e.g. steroids. We also chose to measure kidney BP hydroxylase since this tissue may be affected by the diabetic state, and because it may be sex dependent (Chabra and Fouts, 1974; Gurtoo and Parker, 1977).

2) We have monitored aniline hydroxylase (Appendix I) because it is sex independent, and it was of interest to observe whether this activity was altered similarly in male and female diabetics.
3) We have examined the enzyme kinetic parameters associated with hepatic BP hydroxylase activity in male and female chemically induced and spontaneously diabetic rats. It would not be unexpected for the altered pathophysiological diabetic state to affect apparent $V_{\text{max}}$ and/or $K_m$ of an enzyme.

4) We have measured hepatic epoxide hydrase activity in control and diabetic rats. This enzyme is located within the endoplasmic reticulum membrane, and although it catalyzes the hydration of various monooxygenase system metabolites, epoxide hydrase is not dependent on cytochrome P-450. It was of interest to observe whether the diabetic state altered non-cytochrome P-450 mediated metabolism.

5) We have carried out sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on microsomal preparations. Because this technique gives an indication of changes in the distribution of specific cytochromes P-450, it was of interest to determine if changes in cytochrome P-450 dependent metabolism were accompanied by alterations in electrophoretic mobility of microsomal hemeproteins.

6) We have determined if insulin therapy restored control drug metabolizing activities in spontaneously diabetic rats.

7) We have compared the spontaneously and chemically induced diabetic syndromes in terms of liver and pancreatic histology and insulin deficiency.

8) We have quantitated serum testosterone levels in control and diabetic rats because androgen sensitive drug metabolism appears to
be decreased in male diabetics.

9) We have established hepatic steroid binding assays which could be used to assess the androgen and/or estrogen sensitivity of the control and diabetic liver.
MATERIALS AND METHODS

A. Chemicals and reagents

STZ, ALX, BP, NADP, NADPH, bovine serum albumin, activated charcoal (untreated), sodium dodecyl sulfate (SDS), acrylamide, Coomassie brilliant blue G, Trizma base, dithiothreitol, ethylenediamine tetraacetic acid (EDTA) and molybdic acid (sodium salt), were obtained from Sigma Chemical Co. (St. Louis, Mo.), as were nonlabelled steroids used in the binding experiments (estradiol, diethylstilbestrol, testosterone, DHT, androstenedione, progesterone, triamcinolone, and dexamethasone). Tritium labelled estradiol ([6,7-\(^3\)H(N)]40-60 Ci/mmol and [2,4,6,7,16,17-\(^3\)H(N)]130-170 Ci/mmol) and Biofluor\(^R\) scintillation cocktail were obtained from New England Nuclear, Boston, Mass. Tritium labelled (70-87 Ci/mmol) synthetic steroids moxestrol, [\(^11\beta\)-methoxy-\(^3\)H]- (R2858), methyltrienolone [\(^17\alpha\)-methyl-\(^3\)H] (R1881), and promegestone [\(^17\alpha\)-methyl-\(^3\)H]-(R5020) were supplied by New England Nuclear under licensed agreement of Roussel-Uclaf (Romainville, France), along with nonlabelled steroids. BP was further purified by dissolving it in benzene, then filtering and recrystallizing from cold methanol. \([^3\)H]-styrene oxide was prepared in our laboratory by the method of Oesch et al. (1971) and after dilution with styrene oxide had a specific activity of approximately 36 \(\mu\)Ci/mmol. Standard proteins for calibration and N,N,N',N'-tetramethylenediamine were obtained from Pharmacia Chemical Co. (Piscataway, N.J.); 2-mercaptoethanol and N,N'methylenebisacrylamide from Eastman Kodak Co. (Rochester, N.Y.); ammonium persulfate from
E-C Apparatus Corp. (St. Petersberg, Fla.), and ammonium sulfate (AS) from British Drug House (Vancouver, B.C.). Protamine zinc (PZ) insulin 100 units/ml (Connaught Laboratories, Willowdale Ont.) or AlzetR mini-osmotic pumps (Alza Corp., Palo Alto, CA) filled with IletinR II (purified pork insulin 500 units/ml) (Eli Lilly and Co., Indianapolis, Ind.) was used to maintain diabetic animals where indicated. All other chemicals and reagents were of analytical grade purity.

B. Animals

1. Benzo[a]pyrene (BP) hydroxylase kinetics experiments

Male and female Wistar rats were obtained from Canadian Breeding Farms (Montreal, Quebec). ALX (50 mg/kg) or STZ (60 mg/kg) diabetes was induced in female rats when their body weight was approximately 250 g. Injection of the freshly prepared diabetogen (50 mg/ml or 60 mg/ml, respectively) was given intravenously (i.v.) via tail vein. Control animals received a similar injection with vehicle only (0.1 M citrate buffer pH 4.5). These animals were housed in wire cages under controlled light and temperature conditions (0800-2000 hr, 22°C) and were allowed ad lib. access to water and food (laboratory chow, Ralston Purina of Canada Ltd., Woodstock, Ont.). Animals were tested
for glycosuria with Tes-TapeR (Eli Lilly and Co. Ltd., Toronto, Ont.).
The observed incidence of chemical diabetes was approximately 90% in
the STZ treated animals and 40% in the ALX treated animals. These female
rats were part of a collaborative study and were not sacrificed until
3 months after the date of injection. All animals were fasted 16 to 18 hours
prior to sacrifice to ensure consistency of serum insulin levels.
Diabetes was induced in male rats with ALX (50 mg/kg i.v.) and STZ
(60 mg/kg i.v.) when the body weight was approximately 350 g. These
animals were housed in polycarbonate cages on corncob bedding (Lobund
grade, Paxton Processing Ltd., Paxton, Ill.), and were allowed free
access to food (Purina laboratory chow) and water. These male animals
were sacrificed 5-10 days after detection of glycosuria. The incidence
of diabetes in the STZ treated animals was greater than 95% but less
than 10% in the ALX treated group. A second group of ALX treated male
animals was injected, but in this instance the animals were starved 24 hours
prior to injection to enhance the diabetogenic effect of ALX. In this
case, the incidence of diabetes was closer to 50%.

BB spontaneously diabetic male and female rats were obtained
in the pre-diabetic state from Bio Breeding Laboratories (Ottawa, Ont.).
The animals were received when the females were 70-110 g and the males
110-135 g. Animals were housed in groups of littermates so that the
nondiabetic littermates served as the controls for the diabetics. Onset
of diabetes was monitored by testing for glycosuria. Although the
expected incidence of diabetes in these animals was approximately 70%
by 80 days of age, we observed an incidence of only 32% by 154 days of
age. For this reason, in future experiments we obtained recently detected
diabetic animals directly from the supplier. In the first set of experiments animals were sacrificed as soon as possible after detection of glycosuria (2 to 6 days post detection).

2. Other microsomal metabolism experiments

In all experiments involving the BB Wistar derived spontaneously diabetic rats, two control groups obtained from the same supplier (Bio Breeding Laboratories) were followed. The directly comparable group for these BB diabetics were nondiabetic littermates, designated "inside" nondiabetic controls (IC). In addition, we obtained normal "outside" Wistar controls (OC) which were from the same supplier but were not from the diabetic colony. Other Wistar rats were obtained from Canadian Breeding Farms (Montreal, Quebec) for experiments utilizing STZ diabetic rats and the corresponding citrate buffer injected controls. All animals were allowed to equilibrate for at least 7 days in controlled light and temperature conditions (0600-1800 hr, 22°C). Experimental and control animals were matched for age (120-160 days). Spontaneously diabetic animals were administered 0.8 units/100 g body weight PZ insulin 12 hours after arrival, then on a daily basis. In experiments involving insulin-treated diabetics, insulin therapy was maintained until 18 hours before sacrifice. As before chemically-induced diabetic animals received 60 mg/kg STZ i.v. and control animals 0.1 M citrate buffer i.v. Experiments were carried out either 4 days post-STZ injection in chemically-induced animals or 4 days after withdrawal of insulin in BB diabetic animals. This time period was chosen since we were interested in early detectable changes and
BP hydroxylase time course studies showed that at approximately 96 hours this could be achieved (Figure 1). Animals were fasted 16 hours prior to sacrifice unless they were being maintained on insulin. At sacrifice, blood samples were taken for insulin, glucose, and testosterone determinations, and extent of glycosuria was estimated using Tes-Tape.

3. Hepatic steroid binding experiments

Preliminary studies were conducted using control Wistar rats (Canadian Breeding Farms, Montreal, Quebec) maintained in the usual controlled light and temperature conditions. Pseudohermaphroditic rats and appropriate controls were obtained from the International Foundation for Study of Rat Genetics and Rodent Control (Oklahoma City, Oklahoma). Upon arrival these animals were held in quarantine and received anthelmintic therapy for 3 weeks on the following schedule: 1 week on/1 week off/1 week on. They were utilized in the binding studies when they were approximately 150 days of age. Hypophysectomized and sham operated rats were obtained from Canadian Breeding Farms (Montreal, Quebec). Surgical procedures were carried out by Charles River Breeding Laboratories (Stone Ridge, N.Y.) when the animals were 50-60 days of age. To increase calorie intake after the stress of surgery and airflight these animals were given 5% sucrose in their drinking water for 1 week after their arrival, then given free access to tap water and Purina laboratory chow. They were sacrificed at approximately 120 days of age. Wistar rats from Canadian Breeding Farms (Montreal, Quebec)
Figure 1.

Time course of streptozotocin (STZ)-induced effects and subsequent insulin replacement on hepatic benzo[a]pyrene (BP) hydroxylase activity in male Wistar rats. n = 4 - 16.
BP HYDROXYLASE ACTIVITY (% of control)

STZ
60 mg/kg

insulin replacement

TIME (hours)
were rendered diabetic with 60 mg/kg STZ via tail vein injection. Control animals received a similar injection with vehicle only (0.1 M citrate buffer pH 4.5). Female STZ diabetics were part of a collaborative study and were sacrificed 6 weeks post injection. Male STZ diabetics were sacrificed 4 days post injection. Insulin treated females and males were maintained for 6 weeks post STZ injection using Alzet® miniosmotic pumps filled with 500 units/ml purified pork insulin. The pumps were replaced approximately every 2 weeks. Supplemental PZ insulin was injected subcutaneously if necessary. BB spontaneously diabetic rats and control nondiabetic littermates for these studies were received either from the Animal Facilities, Hospital for Sick Children (Toronto, Ont.) or from Bio Breeding Laboratories. One group of animals was 120-160 days of age and the other group was 30-40 days. Both groups were approximately 2 weeks post detection. Diabetics were maintained on PZ insulin 0.8 units/100 g body weight until 4 days prior to sacrifice.

C. Tissue preparation

1. Microsomal enzyme assays

Animals were either stunned, decapitated and bled or were killed by cervical dislocation if serum samples were being collected. Liver and kidney tissues were perfused with ice cold 1.15% KCl and then placed in this same solution. All following procedures were performed at
4°C. Tissues were blotted, minced, and then homogenized in 1.15% KCl 1:4 (w/v) in a Potter-Elvejhem tissue homogenizer (liver) or Polytron tissue homogenizer (Brinkmann Instruments, Rexdale, Ont.) (kidney). Homogenates were centrifuged at 10,000 g for 10 minutes. To obtain the microsomal fraction, the post mitochondrial fractions were centrifuged at 100,000 g for 60 minutes. Both liver and kidney microsomal pellets were washed, resuspended in 0.1 M phosphate buffer pH 7.2, and diluted 1:3.75 (liver) or 1:2 (kidney) to yield appropriate protein concentrations.

2. Histopathology

Tissue sections were taken from the main lobe of the liver and from the pancreas of some control, STZ and BB diabetic animals at time of sacrifice. Liver specimens were fixed in 10% buffered formalin with calcium acetate 2%. Pancreatic sections were fixed in Bouin's solution. Hematoxylin and eosin staining was performed in the usual manner (Dept. of Zoology, U.B.C.).

Some nondiabetic and BB diabetic pancreatic samples were prepared for islet cell immunocytochemistry in the Department of Physiology, U.B.C. (See Appendix II).

3. Serum hormone and steroid measurements

Blood samples were collected by cardiac puncture through the left ventricle at time of sacrifice. Whole blood was allowed to sit
at room temperature to clot and was then centrifuged at room temperature for 30 minutes. Serum was gently aspirated. Insulin and glucose levels were measured in sera that had been stored frozen at -20°C. Testosterone levels were measured in fresh serum.

4. Hepatic steroid binding experiments

Animals were killed by cervical dislocation, livers were perfused with ice cold TED buffer (50 mM Trizma base, 1.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 20 mM molybdate either pH 7.5 or 8.8, 4°C), and then were placed in fresh buffer. All procedures were carried out at 4°C. Livers were homogenized 1:10 (w/v) in TED buffer using a Potter-Elvehjem homogenizer. Uteri were homogenized similarly, with a Polytron homogenizer. Homogenates were centrifuged at 800 g for 10 minutes. The resulting supernatant was centrifuged at 100,000 g for 30 minutes to yield the cytosolic fraction which was diluted 1:3 for use in the incubation. Where indicated AS precipitation was carried out. To produce a 50% AS fraction an equal volume of 100% AS solution (at room temperature) was added dropwise to undiluted whole cytosol at 4°C and stirred for 30 minutes at 4°C. The precipitate was separated by centrifugation at 12,000 g for 30 minutes and resuspended in one-half the original cytosol volume. When tissue requirements were such, the precipitate was resuspended in the original cytosol volume.
D. Assay protocols

1. Microsomal enzyme assays

   a) Benzo[a]pyrene (BP) hydroxylase activity

BP hydroxylase activities in liver and kidney were measured fluorometrically by the method of Nebert and Gelboin (1968) except that albumin was added to the incubation mixture (Alvares et al., 1970). The requirements for saturating substrate concentration and linearity of enzyme concentration and incubation time in control and diabetic rats were established in preliminary studies and are discussed below (see Results section). The final volume of the incubation mixture was 1.0 ml at pH 7.4. It contained 0.36 μmol NADH, 0.36 μmol NADPH, 3 μmol MgCl₂·6H₂O, 8.7 nmol bovine serum albumin, 150 μl enzyme preparation, 80 nmol BP in 40 μl acetone, and 25 μmol Tris buffer pH 7.5. Protein concentration in the incubation mixture was 0.2-0.5 mg/ml, unless otherwise indicated. The incubation was carried out at 37°C for 2.5 minutes in liver, and for 5 minutes in kidney. Enzyme activities were determined in duplicate and were compared to 2 different blanks, one in which enzyme was added to the incubation mixture after termination of the reaction, and the other in which BP was added after termination. The reaction was terminated with 1.0 ml cold acetone. Hexane 3.3 ml was added to allow extraction of BP compounds. A 2.0 ml aliquot of the organic phase was added to 4.0 ml 1N NaOH. Polar metabolites in the NaOH phase were obtained after freezing and discarding the hexane phase. Fluorescence was determined spectrophotofluorometrically with activation at 396 nm and fluorescence at 522 nm. Quinine sulfate standard was used to calibrate the fluorometer. Activity as derived from a standard curve.
is expressed as pmol 3-hydroxybenzo[a]pyrene per minute per mg protein. Protein determinations were carried out according to the method of Sutherland et al. (1949) as modified by Robson et al. (1968) for the kinetics experiments, and by the method of Bradford (1976) for subsequent enzyme and binding studies.

To determine BP hydroxylase kinetic parameters, incubations were carried out in the presence of increasing substrate concentration (BP 2.5-80 μM). Enzyme activity was plotted graphically as a function of [BP]. Specific activity was determined at saturated conditions and is expressed as pmol 3-hydroxybenzo[a]pyrene produced per minute per mg protein. Apparent $K_m$ was determined at half maximum activity and is expressed as μM.

b) Epoxide hydrase (EH) activity

Hepatic EH activity was measured radiometrically by the method of Oesch et al. (1971) except that the incubation period was 10 minutes. The final volume of incubation mix was 0.4 ml. It contained 12.5 μmol Tris HCl buffer pH 9.0 with 0.025% Tween 80, 0.08 ml distilled water, 0.81 μmol $[^3H]$-styrene oxide in 0.02 ml acetonitrile and 0.2 ml enzyme preparation which contained 1.5-2.5 mg protein. The incubation was carried out at 37°C and was terminated with petroleum ether. Enzyme activities were determined in duplicate and were compared to a blank in which enzyme was added to the incubation mixture after termination of the reaction. Extraction of polar metabolites was carried out by two freeze separations, the products being present in the final ethyl acetate phase. A 0.4 ml aliquot of the ethyl acetate phase was counted in 10 ml
Biofluor\textsuperscript{R} scintillation cocktail (efficiency for $[^3]H$ 46 to 48\%) in a Mark III Searle liquid scintillation counter. The radioactivity detected represented the amount of product formed which was converted from cpm to dpm and nmol. Assay results are expressed as nmol styrene glycol formed per minute per mg protein (See Appendix I for additional enzyme assays carried out.)

2. Insulin and glucose determinations

Insulin levels were measured by the radioimmunoassay method of Pederson and Brown (1979) with modification for application to rat serum (see Results section). The method employed a guinea pig antibody prepared against human insulin. A rat insulin standard curve was prepared in the presence of charcoal extracted rat plasma with each assay. Each incubation tube initially contained 0.7 ml 0.04 M phosphate buffer pH 7.5 with 5\% charcoal extracted human plasma, 0.1 ml antiserum, and 0.1 ml appropriate insulin standard or serum sample.

All tubes were preincubated at 4°C for 24 hours after which 0.1 ml $[^{125}]$-insulin was added to each tube and an additional 24 hour incubation at 4°C was carried out. Bound radioactivity was separated from free using the dextran coated charcoal method. Dextran coated charcoal (0.2 ml) was added to each tube, samples were equilibrated at 4°C for approximately 10 minutes; and then centrifuged at 1500 g for 30 minutes. The supernatant fraction (containing the antigen-antibody complex) was discarded and the pellet (containing the free label) was counted in a gamma counter. Percent $[^{125}]$-insulin bound (B) was calculated by:
Nonspecific binding of labelled insulin was accounted for in each sample. Sample values were obtained directly from a standard curve of percent $[^{125}\text{-I}]-\text{insulin bound vs } \mu\text{Units insulin/ml}$. Serum glucose levels were measured with a Beckman Glucose Analyzer (Beckman Instruments Inc., Fullerton, CA) which was calibrated with a standard glucose solution (150 mg/100 ml). Glucose was determined by means of an oxygen rate method employing a Beckman Oxygen Sensor. Ten $\mu$l of sample was pipetted into a vessel containing glucose oxidase reagent. A sensor in the vessel responded to oxygen concentration and solid-state electronic circuitry determined the rate of oxygen consumption, which was directly proportional to the concentration of glucose in the sample. Results are expressed as mg glucose/100 ml serum.

3. Serum testosterone determinations

A direct procedure for radioimmunoassay of serum testosterone was employed using an $[^{125}\text{-I}]-\text{testosterone}$ kit (Radioassay Systems Laboratories, Inc., Carson, California). Fresh sera were used for this assay because freezing produced unreliable assay results. The method employed rabbit antisera generated against testosterone-19-carboxy-methylether-BSA. Duplicate serum samples and testosterone standards (0.1 ng/ml - 10 ng/ml) were incubated in the presence of testosterone binding globulin inhibitor (0.1 ml) and testosterone antiserum (0.1 ml) at $37^\circ\text{C}$ for 60 minutes. $[^{125}\text{-I}]-\text{testosterone}$ (0.1 ml) was added and another incubation at $37^\circ\text{C}$ for 60 minutes was carried out. Bound radioactivity was separated from free during incubation ($37^\circ\text{C}$, 60 minutes) with goat antirabbit globulins. The precipitate obtained after centri-
fugation (1000 g for 15 minutes) was counted in a gamma counter. Non-specific binding was taken into account for each sample. Percent total $[^{125}\text{I}]-\text{testosterone bound}$ ($\frac{B}{B_0}$) was calculated according to the formula:

$$\% \frac{B}{B_0} = \frac{\text{sample (cpm)} - \text{nonspecific binding (cpm)}}{\text{0 standard (cpm)} - \text{nonspecific binding (cpm)}} \times 100$$

where the "0 standard" represents 100% binding (no added cold testosterone standard). Sample values were obtained directly from a standard curve of percent $[^{125}\text{I}]-\text{testosterone bound}$ vs ng/ml testosterone. Total unconjugated testosterone was measured using this method.

4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis of microsomal preparations was carried out according to Weber and Osborn (1969) with modifications. Glass gel tubes approximately 10 cm long with an inner diameter of 6 mm were used for preparation of the gels. The gels consisted of gel buffer 0.1 M sodium phosphate pH 7.0 containing 0.1% SDS (w/v), 7.5% (w/v) acrylamide, .07% (w/v) methylene bis-acrylamide, .06% (w/v) ammonium persulfate, and .15% (v/v) N,N,N',N'-tetramethylenediamine. Microsomal preparations (8-12 mg/ml) were diluted 1:10 in 0.1 M phosphate buffer. Forty µl of the protein suspension was solubilized in 40 µl 0.5 M sodium phosphate buffer pH 7.0 containing 3.3% (w/v) SDS, 30% (v/v) glycerol, and 16% (v/v) 2-mercaptoethanol. Bromphenol blue .048% was added and the mixture was boiled for 5 minutes. Approximately 25 µg solubilized protein which was contained in a volume of 50 µl was applied to each gel. Electrophoresis
was carried out at 33-35°C with constant current of about 12 mAmperes/gel (Power Supply EPS 500/400, Pharmacia, Montreal) for about 4 to 5 hours, or until the bromphenol blue tracking dye was within 1 cm of the bottom of the tube. The gels were stained in Coomassie brilliant blue G 0.025% (w/v), with 10% (v/v) glacial acetic acid and 25% (v/v) isopropyl alcohol in distilled water. Gels were destained in 10% acetic acid. The gels were scanned at 550 nm using a Gilford Model 250 spectrophotometer.

5. Hepatic steroid binding assays

Both 50% AS and whole cytosol fractions were analyzed for estrogen binding. To detect high affinity binding, aliquots (0.5 ml) of 50% AS fraction were incubated with 10 μl [3H]-estradiol both in the presence and absence of 10 μl competitor (100-fold excess estradiol). The label which was displaced by the excess unlabelled competitor was considered specifically bound (total binding - nonspecific binding = specific binding). Ligands and competitors were dissolved in 95% ethanol. For Scatchard analysis the ligand concentration ranged from 0.05 to 2.5 nM with a specific activity of 130-140 Ci/mmol. To measure moderate affinity binding aliquots (0.5 ml) of whole cytosol were incubated with 10 μl [3H]-estradiol both in the presence and absence of 10 μl competitor (100-fold excess estradiol or 1000-fold excess DHT). For Scatchard analysis the ligand concentration ranged from 10 to 200 nM with a specific activity of 7-10 Ci/mmol. Other ligands were used where indicated. Incubations were carried out for 90 minutes at 0-4°C except when otherwise stated, and were terminated by addition of 0.5 ml dextran-coated charcoal (0.5% w/v activated charcoal and
0.05% w/v dextran-T 70 in TED buffer with no molybdate). The mixtures were Vortex\textsuperscript{R} mixed once and held at 0-4\textdegree{} for 10 minutes. The charcoal was pelleted by centrifugation (1500 g for 10 min) and 0.5 ml aliquots of the supernatant in 10 ml Biofluor\textsuperscript{R} scintillation cocktail were counted for bound radioactivity in a Mark III Searle liquid scintillation counter (% efficiency for \[^{3}\text{H} = 48\%\]). Protein content was determined using the method of Bradford (1976).

In some studies uterine whole cytosol was assayed for high affinity \[^{3}\text{H}\]-estradiol binding. This was accomplished in a similar fashion to that described above.

Steroid binding capacity (fmol/mg protein or pmol/mg protein) and \(K_d\) (M) were determined by Scatchard plot analysis. Graphs were drawn by hand. Data points which deviated from linearity either due to excess protein concentration or involvement of another binding site were not used to obtain the reported slope and capacity parameters.

E. Statistics

Students t-test for unpaired sample means was carried out where indicated. Differences were considered statistically significant if \(p < 0.05\). In some instances, non-linear curve fitting (UBC NONLIN) was applied to binding data.
RESULTS

A. Preliminary experiments to establish assay conditions

1. Hepatic-microsomal benzo[a]pyrene (BP) hydroxylase assay

Although the BP hydroxylase assay has been used widely in the field of drug metabolism, certain problems have been identified and have to be taken into account when establishing assay conditions with hepatic microsomes (Hansen and Fouts, 1972; Robie et al., 1976; and Cumps et al., 1977). These problems include the following: 1) reaction velocity is linear only over relatively narrow ranges of protein concentration and incubation time; 2) BP associates with microsomal proteins and the availability of the substrate to the enzyme is dependent upon the protein concentration in the incubation mixture; and 3) increases in enzyme activity are often accompanied by apparent changes in enzyme kinetics. Therefore, we carried out experiments in order to determine optimum assay conditions for hepatic BP hydroxylase activity both in control and diabetic rats. Data shown in each of the Figures 2 to 7 are from an experiment using a single animal. However, similar results were obtained in up to 6 repeat experiments.

It was observed that BP hydroxylase activity varied with substrate concentration and that at 80 μM BP concentration, activity was saturated. This was apparent both in females and males (Figure 2 and 3). Although chemically induced and spontaneous diabetes altered the specific activity of BP hydroxylase compared to control, saturation was achieved at 80 μM BP in microsomal preparations from diabetic rats also.
Figure 2.

Hepatic benzo[a]pyrene (BP) hydroxylase activity vs substrate concentration in control and diabetic female rats. Data represent the results of one typical experiment. Microsomal protein concentration in the incubation mixture was ~0.5 mg/ml; incubation time was 2.5 minutes.

C: citrate control
ALX: alloxan diabetic
BB: BB spontaneously diabetic

STZ: streptozotocin diabetic
IC: nondiabetic inside control
HEPATIC BP HYDROXYLASE ACTIVITY (pmol/min/mg protein)

chemically-induced

STZ

ALX

spontaneous

BB

IC

BENZO[a]PYRENE (μM)
Figure 3.

Hepatic benzo[a]pyrene (BP) hydroxylase activity vs substrate concentration in control and diabetic male rats. Data represent the results of one typical experiment. Microsomal protein concentration in the incubation mixture was ~0.5 mg/ml; incubation time was 2.5 minutes.

Symbols are as in Figure 2.
HEPATIC BP HYDROXYLASE ACTIVITY (pmol/min/mg protein)

BENZO[a]PYRENE (μM)

chemically induced

spontaneous

STZ

ALX

IC

BB
As expected, higher amounts of microsomal protein present in the incubation mixture apparently reduced the production of 3-hydroxybenzo-[a]pyrene as indicated by the curvilinear plot (Figures 4 and 5). Therefore it was desirable to utilize as little protein as possible without going below limits of detectability. This was achieved at the level where the resuspended microsomal pellet was diluted to approximately 1/4 of the original content. This corresponded to a concentration in the incubation mixture of 0.3 to 0.5 mg protein per ml. As before, the same trends were observed in females, males, controls and diabetics.

The effect of incubation time on hepatic BP hydroxylase activity is shown in Figures 6 (females) and 7 (males). Based on these and additional studies we observed that the enzyme activity was linear for up to 5 minutes in the incubation conditions indicated. This was a consistent finding among the control and diabetic animals.

Based on these studies we selected the following assay conditions to be employed to monitor hepatic microsomal BP hydroxylase activity: 80 µM substrate concentration, approximately 0.50 mg/ml protein concentration in the incubation mixture, and 2.5 minutes incubation time. Furthermore, we concluded that these conditions were applicable to female, male, control and diabetic microsomes.

It should be noted that optimum pH (7.4-7.8) and cofactor conditions were selected on the basis of other workers' results (Nebert and Gelboin, 1968; Cumps et al., 1977) and previous determinations in our laboratory.
Hepatic benzo[a]pyrene (BP) hydroxylase activity vs protein content in control and diabetic female rats. Data represent the results of one typical experiment. BP concentration was 80 μM; incubation time was 2.5 minutes.

Symbols are as in Figure 2.
HEPATIC BP HYDROXYLASE ACTIVITY (fluorescence units)

PROTEIN CONCENTRATION (mg/ml incubation mixture)
Figure 5.

Hepatic benzo[a]pyrene (BP) hydroxylase activity vs protein content in control and diabetic male rats. Data represent the results of one typical experiment. BP concentration was 80 μM; incubation time was 2.5 minutes.

Symbols are as in Figure 2.
Hepatic benzo[a]pyrene (BP) hydroxylase activity vs incubation time in control and diabetic female rats. Data represent the results from one typical experiment. BP concentration was 80 μM; microsomal protein concentration in the incubation mixture was ~0.5 mg/ml.

C: citrate-injected control
IC: nondiabetic inside control
BB: BB spontaneously diabetic
Figure 7.

Hepatic benzo[a]pyrene (BP) hydroxylase activity vs incubation time in control and diabetic male rats. Data represent the results from one typical experiment. BP concentration was 80 µM; microsomal protein concentration in the incubation mixture was ~2 mg/ml.

STZ: streptozotocin diabetic
ALX: alloxan diabetic
IC: inside nondiabetic control
BB: BB spontaneously diabetic
HEPATIC BP HYDROXYLASE ACTIVITY
(fluorescence units)

TIME (min)

0.40  0.80  1.20  1.60  2.00

STZ  BB  K  ALX
2. Kidney microsomal benzo[a]pyrene (BP) hydroxylase assay

Previously in our laboratory the kidney BP hydroxylase assay was carried out on the 10,000 g supernatant fraction. However, we observed that the enzyme in this tissue behaved similarly to that in liver. That is, beyond a certain level additional protein added to the incubation mixture reduced reaction rate even to the point of inhibiting it (Figure 8), and there was a marked limit to the time over which activity was linear (Figure 9). For these reasons we developed the kidney assay using the microsomal fraction since it would contain a greater proportion of enzyme activity compared to the amount of total protein present. Kidney microsomes were prepared from control male and female Wistar rats (tissue pool from 2 to 4 rats) and BP hydroxylase activity was determined at different protein concentrations (Figure 10) and incubation times (Figure 11). In these animals kidney microsomal enzyme activity was linear up to a protein concentration of approximately 0.3 mg/ml and an incubation time of slightly more than 5 minutes. In nondiabetic inside control rats similar results with respect to linearity of protein concentration and incubation time were obtained (Figure 12), although microsomal protein content was slightly less in these animals than it was in the Wistar rats i.e., ~60% that of regular controls as in Figure 10 (0.45 mg/ml vs 0.60 mg/ml incubation mixture).

Based on these studies we selected the following assay conditions to monitor kidney BP hydroxylase activity in a range which produced reliable fluorometric readings: 80 μM substrate concentration, approximately 0.2 to 0.4 mg per ml protein concentration in the incubation mixture, and 5 minutes incubation time. Before assays were carried out in the different
Figure 8.

Kidney benzo[a]pyrene (BP) hydroxylase activity vs protein content in 10,000 g supernatant fraction in control male rats (tissue pool from 2 animals). BP concentration was 80 μM; incubation time was 5 minutes.
PROTEIN CONCENTRATION (mg/ml incubation mixture)

KIDNEY BP HYDROXYLASE ACTIVITY (fluorescence units)
Figure 9.

Kidney benzo[a]pyrene (BP) hydroxylase activity vs incubation time in 10,000 g supernatant fraction in control male rats (tissue pool from 3 animals) at 2 different protein concentrations. BP concentration was 80 µM.
Figure 10.

Kidney benzo[a]pyrene hydroxylase (BP) activity vs protein content in 100,000 g microsomal fraction in control female and male Wistar rats. BP concentration was 80 μM; incubation time was 5 minutes.
Figure 11.

Kidney benzo[a]pyrene (BP) hydroxylase activity vs incubation time in 100,000 g microsomal fraction from control female and male Wistar rats. BP concentration was 80 μM; protein concentration in the incubation mixture was ~ 0.2 mg/ml.
Figure 12.

Kidney benzo[a]pyrene (BP) hydroxylase activity \textit{vs} protein content or \textit{vs} incubation time (o—o) in 100,000 g fraction for nondiabetic inside control male and female rats. BP concentration was 80 \(\mu\text{M}\).
KIDNEY BP HYDROXYLASE ACTIVITY (fluorescence units)

INCUBATION TIME (min)

5 10 15 20

PROTEIN CONTENT (mg/ml)

1.15 2.3 3.45 4.5

Male

Female

protein concentration

0.0 0.2 0.4 0.6 0.8

incubation time

5 min = 5 min

0.45 mg/ml
experimental groups, protein content curves were obtained in order that we could verify the linearity of each particular system.

We did not investigate substrate and cofactor concentrations in this instance, since kidney BP hydroxylase was generally 10-fold less than in liver and therefore would have been saturated at this protein concentration, assuming the enzymes are similar in liver and kidney.

3. Effect of rat plasma on insulin radioimmunoassay standard curve

In order to determine the effect of rat plasma on the binding of insulin in the radioimmunoassay conditions, standard curves were prepared in the absence and presence of rat plasma (100 μl plasma/1.0 ml incubation volume). The plasma was previously subjected to a charcoal extraction procedure to remove endogenous insulin. The results of this experiment are shown in Figure 13. Nondiabetic control (IC) and Wistar control (OC) charcoal extracted plasma altered the insulin standard curve. This was likely due to increased nonspecific binding. For instance, with no additions, nonspecific binding was 3% bound, with human plasma added it was 8.4% bound; and for IC plasma and OC plasma added it was 24% bound and 22% bound respectively. It was concluded that future insulin radioimmunoassays would be carried out in the presence of charcoal extracted rat plasma.
Figure 13.

Effect of added charcoal extracted plasma (CEP) on insulin standard curve. (100 μl plasma/1 ml incubation volume).

control: no additions to standard curve incubation
human: human CEP added to incubation
IC: nondiabetic inside control rat CEP added to incubation
OC: outside Wistar control rat CEP added to incubation
EFFECT OF ADDED CHARCOAL EXTRACTED PLASMA (CEP) ON INSULIN STANDARD CURVE
4. Hepatic steroid binding assays: high and moderate affinity $[^3H]$-estradiol binding

a. Preliminary studies to measure high affinity $[^3H]$-estradiol binding

Initially we followed the method of Powell-Jones et al. (1980 and 1981) to detect high affinity $[^3H]$-estradiol binding in rat liver cytosol. Details of this assay are as follows: 10 mM Tris buffer pH 7.4 at 4°C containing 1.0 mM dithiothreitol and 1 mM EDTA; 30% AS fraction (we used 50% AS fraction); incubation mixture contained 100 μl AS fraction (0.7 mg/ml protein), 100 μl $[^3H]$-estradiol (we used 0.2 to 10 nM) with and without 100 μl 100-fold excess diethylstilbestrol; ligand and competitor delivered in buffer; incubation performed at 4°C for 2 hours; unbound $[^3H]$-estradiol was removed by treatment with 200 μl dextran coated charcoal solution (0.5% charcoal and 0.05% dextran in buffer) for 30 minutes at 4°C; after sedimentation (800 g for 10 minutes) 100 μl aliquots of supernatant were counted for bound radioactivity. Results of this experiment carried out on male rat liver are shown in Figure 14. The binding curve showed that specific $[^3H]$-estradiol binding was saturated below 4 nM. Nonspecific binding was linear with ligand concentration. Using Scatchard analysis we obtained a $K_d$ and capacity for $[^3H]$-estradiol binding in male rat liver cytosol of $1.8 \times 10^{-9}$ M and 44 fmol/mg protein, respectively. Powell-Jones reported a $K_d$ of $\approx 10^{-10}$ M.

It was of interest to compare the Powell-Jones assay technique to that used by the Hormone Receptor Laboratory (Portland, Oregon). This method was originally established in order to measure uterine and mammary gland high affinity $[^3H]$-estradiol binding. Details of this assay are as follows: TED buffer with 20 mM sodium molybdate (see Methods
Figure 14.

Scatchard analysis of high affinity $[^3H]$-estradiol binding in 50% ammonium sulfate fraction of male rat liver (pool of 2). The method employed was that of Powell-Jones et al., (1980, 1981). Incubation conditions are described in Results section.

Inset: Binding curve of $[^3H]$-estradiol bound vs ligand concentration ($[^3H]$-estradiol 0.2 - 10 nM) in control male rat liver.

- Total binding (T)
- Nonspecific binding (NS)
- Specific binding (S)
$K_d = 1.8 \times 10^{-9} M$
whole cytosol fraction; incubation mixture contained 0.5 ml whole cytosol fraction (~2 mg/ml protein), 10 μl [3H]-estradiol (0.2 to 10 nM) with and without 10 μl 100-fold excess diethylstilbestrol; ligand and competitor delivered in 95% ethanol; incubation performed at 4°C for 2 hours; unbound [3H]-estradiol was removed by treatment with 0.5 ml dextran coated charcoal solution (0.5% charcoal and 0.05% dextran in TED buffer) for 10 minutes at 4°C; after sedimentation (1500 g for 10 minutes) 0.50 ml aliquots of supernatant were counted for bound radioactivity.

Results of this experiment carried out on male rat liver whole cytosol are shown in Figure 15. By Scatchard analysis we found that [3H]-estradiol binding in male rat liver whole cytosol was of higher (~10^-7 M vs 10^-9 M) and higher capacity (pmol/mg vs fmol/mg) than that in the 50% AS fraction (Figure 14). The specific binding curve (inset Figure 15) suggested that the ligand concentration range (0.2 to 10 nM) detected more than one binding site in the whole cytosol fraction. One site appeared to be saturated below the 2 nM range and the other became evident above the 5 nM range. This latter site was not apparent in the 30% AS fraction (inset Figure 14) at 0.2 to 10 nM [3H]-estradiol and presumably altered our ability to measure high affinity [3H]-binding in the whole cytosol fraction. Therefore, in subsequent experiments we employed a ligand concentration range of 0.05 to 1.0 nM [3H]-estradiol, and the 30% AS fraction to measure high affinity binding (K_d ~ 10^-9 to 10^-10 M). In addition we modified and combined various features of the two previously mentioned protocols to optimize our assay conditions. For instance we employed the TED buffer with 20 mM molybdate because molybdate has been reported to stabilize various binding proteins (Noma et al., 1980; Krozowski and Murphy, 1981) including estrogen
Figure 15.

Scatchard analysis of $[^3H]$-estradiol binding in whole cytosol fraction of male rat liver (pool of 2). Incubation conditions are described in Results section.

Inset: Specifically bound ($[^3H]$-estradiol 0.2 to 10nM) in control male rat liver.
Specific Binding (dpm x 10^-3)

B/F x 10^7

[3H] Estradiol Added (nm)

K_d = 1.43 x 10^-7 M

Specific Bound (pmol/mg protein)
receptors. We prepared and stored the ligands and competitors in 95% ethanol rather than in buffer because of enhanced solubility and stability of steroids in the alcohol. In addition, we used estradiol as the competitor whereas the other 2 protocols used diethylstilbestrol. The Scatchard plot shown in Figure 16 indicated that estradiol and diethylstilbestrol competitors gave similar high affinity binding characteristics with $[^3\text{H}]-\text{estradiol}$ as the ligand under these assay conditions ($K_d \sim 10^{-10}$ M).

b. Studies to measure moderate affinity $[^3\text{H}]-\text{estradiol}$ binding in the 50% ammonium sulfate (AS) fraction

The results of our preliminary experiments indicated that the assay as described to this point in the AS fraction detected high affinity $[^3\text{H}]-\text{estradiol}$ binding sites with the expected $K_d$ and capacity. In addition we detected moderate affinity $[^3\text{H}]-\text{estradiol}$ binding sites ($K_d \sim 10^{-7}$ M) in the whole cytosol fraction of male rat liver (Figure 15). When the ligand concentration range was extended to 1000 nM $[^3\text{H}]-\text{estradiol}$ we more clearly detected moderate affinity binding sites in the 50% AS fraction. The results of an experiment where the 50% AS fraction of male rat liver cytosol was incubated with increasing ligand concentrations from 0.025 to 1000 nM $[^3\text{H}]-\text{estradiol}$, are presented in the following figures, with either estradiol (Figure 17) or moxestrol (Figure 18) as competitor. With estradiol as the competitor, the high affinity binding site exhibited a $K_d$ of $1.8 \times 10^{-9}$ M and a capacity of 140 fmol/mg protein, while the moderate affinity site had a $K_d$ of $1.2 \times 10^{-7}$ M and capacity of 4.8 pmol/mg protein. With moxestrol (R2858) as the competitor, the high affinity binding site exhibited a $K_d$ of $2.4 \times 10^{-9}$ M and a capacity of 184 fmol/mg protein, while the low affinity site had a $K_d$ of $4.3 \times 10^{-7}$ M and capacity of 6.6 pmol/mg.
Figure 16.

Scatchard analysis of high affinity $[^3H]$-estradiol binding in male rat liver (pool of 2) using 2 different competitors.

Inset: Specifically bound $[^3H]$-estradiol vs ligand concentration ($[^3H]$-estradiol 0.25 to 1 nM) in control male rat liver.

$E_2$: estradiol

DES: diethylstibestrol
Specific bound (fmol/mg protein)

$B/F \times 10^7$

$E_2$: $K_d = 4.1 \times 10^{-10} \text{ M}$

$DES$: $K_d = 2.7 \times 10^{-10} \text{ M}$

$[^3H] - \text{ESTRADIOL ADDED (nM)}$
Figure 17.

Scatchard analysis of high and moderate affinity $[^3H]$-estradiol binding in male rat liver cytosol 50% ammonium sulfate fraction. Conditions were as follows: 0.025 to 1000 nM $[^3H]$-estradiol concentration; ~ 4 mg/ml protein; incubation carried out in absence and presence of 100-fold excess nonlabelled estradiol for 2 hours at 4°C. Data were obtained from a tissue pool from 2 rats.
estradiol competitor

$K_d: 1.8 \times 10^{-9} \text{ M}$

$K_d: 1.2 \times 10^{-7} \text{ M}$
Figure 18.

Scatchard analysis of high and moderate affinity [\(^{3}\text{H}\)]-estradiol binding in male rat liver cytosol 50% ammonium sulfate fraction. Conditions were as follows: 0.025 to 1000 nM [\(^{3}\text{H}\)]-estradiol concentration; ~4 mg/ml protein; incubation carried out in absence and presence of 100-fold excess nonlabelled moxestrol (R2858) for 2 hours at 4°C. Data were obtained from a tissue pool from 2 rats.
moxestrol competitor

\[ K_d: 2.4 \times 10^{-9} \text{ M} \]

\[ K_d: 4.3 \times 10^{-7} \text{ M} \]

SPECIFIC BOUND (fmol/mg protein)
c. Binding studies in the 50% ammonium sulfate (AS) and whole cytosol fractions

Because earlier indications were that we could measure moderate affinity binding in the male whole cytosol fraction (Figure 15), we carried out further experiments to compare the 2 binding sites in both tissue fractions using 2 different competitors (estradiol and moxestrol (R2858)). Both males and females were studied. Results are shown in Table V. We observed the following: both male and female 50% AS fractions exhibited high affinity \([^3H]\)-estradiol binding of similar \(K_d\) and capacity characteristics with either competitor; while male high affinity binding did not produce a Scatchard plot in the whole cytosol fraction, in the female it did. In females, the high affinity binding site had a slightly greater capacity in the 50% AS fraction than in whole cytosol. Male moderate affinity binding (\(K_d \sim 10^{-7}\) M) was evident in both 50% AS and whole cytosol fractions. There was a trend for this binding site to have a slightly greater capacity in the whole cytosol fraction than in the 50% AS fraction. Females did not exhibit moderate affinity \([^3H]\)-estradiol binding.

d. Incubation time course studies of high and moderate affinity \([^3H]\)-estradiol binding

Before further refinements in the assay were explored we carried out time course studies in order to determine the optimum incubation time
TABLE V. High and moderate affinity $[^3]$H-estradiol binding characteristics in male and female rat liver cytosol fractions. $K_d$ and capacities were determined by Scatchard analysis. Incubation conditions are as described in the Methods section, except incubation time was 2 hours.

$E_2$ = estradiol  
$R2858$ = moxestrol

<table>
<thead>
<tr>
<th>Competitor concentration</th>
<th>Male 50% AS$^1$</th>
<th>Male Whole Cytosol</th>
<th>Female 50% AS</th>
<th>Female Whole Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$: $1.8 \times 10^{-9}$ M</td>
<td>displaceable binding but no Scatchard</td>
<td>$K_d$: $2.2 \times 10^{-9}$ M</td>
<td>$K_d$: $1.5 \times 10^{-9}$ M</td>
</tr>
<tr>
<td>0.025 to 15 nM capacity:</td>
<td>140 fmol/mg</td>
<td>no Scatchard</td>
<td>capacity: 115 fmol/mg</td>
<td>capacity: 50 fmol/mg</td>
</tr>
<tr>
<td>25 to 1000 nM capacity:</td>
<td>$4.8 \times 10^{-7}$ M</td>
<td>no displaceable binding</td>
<td>$3.4 \times 10^{-7}$ M</td>
<td>no displaceable binding</td>
</tr>
<tr>
<td></td>
<td>4.8 pmol/mg</td>
<td>capacity: 7.5 pmol/mg</td>
<td>no displaceable binding</td>
<td>no displaceable binding</td>
</tr>
</tbody>
</table>

$R2858$

| 0.025 to 15 nM capacity: | $2.4 \times 10^{-9}$ M | no displaceable binding | $1.4 \times 10^{-9}$ M | $1.8 \times 10^{-9}$ M |
| 25 to 1000 nM capacity:  | $4.3 \times 10^{-7}$ M | no displaceable binding | $5.5 \times 10^{-7}$ M | no displaceable binding |
|                          | 6.6 pmol/mg | capacity: 10 pmol/mg | capacity: 84 fmol/mg |

$^1$ The ammonium sulfate (AS) and whole cytosol fraction originated from the same tissue pool (n = 4 rats) for each competitor concentration range of 0.025 - 1000 nM.
for high and moderate affinity $[^3\text{H}]$-estradiol binding. We prepared large tissue pools and measured specific binding in them at 4°C for various incubation periods ranging from 15 minutes to 24 hours. High affinity binding was maximal by 1.5 hours in both males and females (Figure 19) remained stable for 5 hours, but was decreased at 24 hours. Male moderate affinity binding was maximal by approximately 1 hour (Figure 20) and had decreased by 24 hours. Therefore, for convenience, in future experiments we performed all incubations at 4°C for 1.5 hours, unless otherwise stated.

e. Studies comparing $[^3\text{H}]$-estradiol binding in various tissue fractions

As mentioned previously, we had observed some differences in binding characteristics between the AS and whole cytosol fractions (Table V). Therefore, we measured high and moderate affinity binding in a series of cytosol fractions in order to determine which one could be used for optimal conditions. We tested the 50% AS fraction we had been using up to this point, the 30% and 50% AS fractions using the Powell-Jones (1980) method and the whole cytosol fraction (i.e., unfractionated). Furthermore, it was of interest to attempt to separate the high and moderate affinity binding components by AS fractionation. We prepared a large pool of adult male liver cytosol (pool of 4 rats) which was the tissue from which all the fractions were subsequently
Figure 19.

Time course of specific high affinity $[^3H]$-estradiol binding at 4°C in male and female rat liver, 50% ammonium sulfate fraction. The binding in each sex was determined on a single tissue pool at 2 nM $[^3H]$-estradiol ligand concentration and 100-fold excess estradiol competitor. Points joined by a dashed line were determined in more than 1 tissue pool.
Figure 20.

Time course of specific moderate affinity $[^3\text{H}]$-estradiol binding at 4°C in male rat liver, 50% ammonium sulfate fraction. The binding was determined on a single tissue pool at 200 nM $[^3\text{H}]$-estradiol ligand concentration and 100-fold excess estradiol competitor. Points joined by a dashed line were determined in more than 1 tissue pool.
derived. High affinity $[^3H]$-estradiol binding (assays carried out by M.J. Finlayson, thesis in preparation) was observed to be similar in our 50% AS fraction compared to the other two AS fractions. In the male whole cytosol fraction, as before, no Scatchard plot could be derived. Therefore, in future experiments we continued to use the 50% AS fraction to detect high affinity binding in males and in females because it produced a larger tissue fraction. Results of the moderate affinity $[^3H]$-estradiol binding experiments are given in Figure 21. The capacity in the whole cytosol fraction was approximately 6 times greater than in the various AS fractions, and the calculated $K_d$ was in the expected range ($\sim 10^{-7}$ M). Therefore we monitored moderate affinity binding in male whole cytosol in subsequent experiments, unless otherwise stated. We also concluded from these experiments that our AS fractionation procedure did not separate the high and moderate affinity binding sites since moderate affinity binding could be detected in the AS fractions (Figure 21). Moreover, we reasoned that high affinity binding in the male whole cytosol fraction (Table V) did not produce a Scatchard plot because the presence of the moderate affinity binder interfered with the assay.

f. Specificity studies

Specificity of high and moderate affinity $[^3H]$-estradiol binding in male and female rat liver was studied by incubating appropriate tissue fractions with ligand alone or together with a range of concentrations of
Figure 21.

Scatchard analysis of moderate affinity $[^3H]$-estradiol binding in various fractions of male rat liver cytosol. Conditions were as follows: 25 to 500 nM $[^3H]$-estradiol concentration; ~2 mg/ml protein; incubation carried out in absence and presence of 100-fold excess nonlabelled estradiol for 1.5 hours at 4°C. Data were obtained from one tissue pool (n = 4 rats).

WC: whole cytosol
AS: ammonium sulfate
P-J: AS prepared by the method of Powell-Jones et al. (1980)
$WC \ K_d: \ 1.8 \times 10^{-7} \ M$

50% AS fraction

50% AS fraction (P-J)

30% AS fraction (P-J)

SPECIFIC BOUND (fmol/mg protein)
various potential competitors. Binding specificity of high affinity sites (determined at 0.5 nM $[^3\text{H}]$-estradiol) in male and female 50% AS fraction is given in Tables VI and VII. The most effective competitor for high affinity binding was estradiol in both males and females, although diethylstilbestrol and R2858 were nearly as effective. None of the other steroids competed for high affinity binding sites.

The specificity of moderate affinity $[^3\text{H}]$-estradiol binding sites in the whole cytosol fraction from male rat liver is given in Table VIII. Again, estradiol was the most effective competitor (34% displacement at 100-fold excess). R2858 competed nearly as well as estradiol, but diethylstilbestrol did not compete at all for these moderate affinity binding sites. Both DHT and testosterone competed for $[^3\text{H}]$-estradiol binding, especially at the 1000-fold excess competitor level. The other androgenic steroids exhibited slight competition while the progestins and corticosteroids did not compete for moderate affinity binding sites. In female liver whole cytosol fraction (Table IX) none of the potential competitors inhibited $[^3\text{H}]$-estradiol binding to an appreciable extent.

g. Moderate affinity $[^3\text{H}]$-estradiol binding with dihydrotestosterone (DHT) as competitor

As a result of the specificity studies described above, and because other workers had previously reported moderate affinity DHT-
Table VI. Ligand specificity of high affinity $[^3H]$-estradiol binding sites in the 50% ammonium sulfate (AS) fraction of liver from male rats. Samples of 50% AS fraction were incubated for 1.5 hours at 4°C with $[^3H]$-estradiol (0.5 nM) alone or in the presence of 10, 100, or 1000-fold excess of competing ligand.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>% of control binding(^1) in the presence of excess competitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-fold</td>
</tr>
<tr>
<td>estradiol</td>
<td>59</td>
</tr>
<tr>
<td>diethylstilbestrol</td>
<td>59</td>
</tr>
<tr>
<td>moxestrol (R2858)</td>
<td>64</td>
</tr>
<tr>
<td>testosterone</td>
<td>-</td>
</tr>
<tr>
<td>dihydrotestosterone</td>
<td>91</td>
</tr>
<tr>
<td>androstenedione</td>
<td>92</td>
</tr>
<tr>
<td>methyltrienelone (R1881)</td>
<td>-</td>
</tr>
<tr>
<td>progesterone</td>
<td>91</td>
</tr>
<tr>
<td>promegestone (R5020)</td>
<td>-</td>
</tr>
<tr>
<td>triamcinolone</td>
<td>97</td>
</tr>
<tr>
<td>dexamethasone</td>
<td>88</td>
</tr>
</tbody>
</table>

\(^1\) The binding of $[^3H]$-estradiol in the presence of competitor is given as % of control values (control = binding in the absence of competitor).
Table VII. Ligand specificity of high affinity [\(^3\)H]-estradiol binding sites in the 50% ammonium sulfate (AS) fraction of liver from female rats. Samples of 50% AS fraction were incubated for 1.5 hours at 4°C with [\(^3\)H]-estradiol (0.5 nM) alone or in the presence of 10, 100, or 1000-fold excess of competing ligand.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>% of control binding in the presence of excess competitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-fold</td>
</tr>
<tr>
<td>estradiol</td>
<td>63</td>
</tr>
<tr>
<td>diethylstilbestrol</td>
<td>61</td>
</tr>
<tr>
<td>moxestrol (R2858)</td>
<td>79</td>
</tr>
<tr>
<td>testosterone</td>
<td>100</td>
</tr>
<tr>
<td>dihydrotestosterone</td>
<td>100</td>
</tr>
<tr>
<td>androsterone</td>
<td>100</td>
</tr>
<tr>
<td>methyltrienelone (R1881)</td>
<td>100</td>
</tr>
<tr>
<td>progesterone</td>
<td>100</td>
</tr>
<tr>
<td>promegestone (R5020)</td>
<td>98</td>
</tr>
<tr>
<td>triamcinolone</td>
<td>100</td>
</tr>
<tr>
<td>dexamethasone</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^1\) The binding of [\(^3\)H]-estradiol in the presence of competitor is given as % of control values (control = binding in the absence of competitor).
Table VIII. Ligand specificity of moderate affinity $[^3]H$-estradiol binding sites in the whole cytosol fraction of liver from male rats. Samples of whole cytosol were incubated for 1.5 hours at 4°C with $[^3]H$-estradiol (50 nM) alone or in the presence of 10, 100, or 1000-fold excess of competing ligand.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>% control binding$^1$ in the presence of excess competitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-fold</td>
</tr>
<tr>
<td>estradiol</td>
<td>92</td>
</tr>
<tr>
<td>diethylstilbestrol</td>
<td>100</td>
</tr>
<tr>
<td>moxestrol (R2858)</td>
<td>100</td>
</tr>
<tr>
<td>testosterone</td>
<td>100</td>
</tr>
<tr>
<td>dihydrotestosterone</td>
<td>100</td>
</tr>
<tr>
<td>androstenedione</td>
<td>86</td>
</tr>
<tr>
<td>methyltrienelone (R1881)</td>
<td>85</td>
</tr>
<tr>
<td>progesterone</td>
<td>100</td>
</tr>
<tr>
<td>promegestone (R5020)</td>
<td>100</td>
</tr>
<tr>
<td>triamcinolone</td>
<td>100</td>
</tr>
<tr>
<td>dexamethasone</td>
<td>100</td>
</tr>
</tbody>
</table>

$^1$ The binding of $[^3]H$-estradiol in the presence of competitor is given as % of control values (control = binding in the absence of competitor).
Table IX. Ligand specificity of moderate affinity $[^3\text{H}]$-estradiol binding sites in the whole cytosol fraction of liver from female rats. Samples of whole cytosol were incubated for 1.5 hours at 4°C with $[^3\text{H}]$-estradiol (50 nM) alone or in the presence of 10, 100, or 1000-fold excess of competing ligand.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>10-fold</th>
<th>100-fold</th>
<th>1000-fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>estradiol</td>
<td>79</td>
<td>76</td>
<td>100</td>
</tr>
<tr>
<td>diethylstilbestrol</td>
<td>80</td>
<td>81</td>
<td>100</td>
</tr>
<tr>
<td>moxestrol (R2858)</td>
<td>90</td>
<td>89</td>
<td>92</td>
</tr>
<tr>
<td>testosterone</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>dihydrotestosterone</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>androstenedione</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>methyltrienelone (R1881)</td>
<td>99</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>progesterone</td>
<td>95</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>promegestone (R5020)</td>
<td>91</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>triamcinolone</td>
<td>99</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>dexamethasone</td>
<td>98</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

1 The binding of $[^3\text{H}]$-estradiol in the presence of competitor is given as % of control values (control = binding in the absence of competitor).
displaceable binding in male rat liver (Roy et al., 1974; Dickson et al., 1978), we compared estradiol and DHT-displaceable $[^3H]$-estradiol binding in male rat liver. Samples of whole cytosol were incubated with $[^3H]$-estradiol (10 to 200 nM) alone or in the presence of 100-fold excess of either estradiol or DHT. Scatchard analysis of these data was plotted (Figure 22). Moderate affinity $[^3H]$-estradiol binding ($K_d \approx 1 \times 10^{-7}$ M) was detected using either ligand. The capacity for estradiol-displaceable binding was approximately double that for DHT-displaceable binding (9.2 pmol/mg protein vs 5.2 pmol/mg protein). We observed in additional experiments carried out using this methodology that the DHT-displaceable binding was somewhat more variable than that for estradiol. Therefore in future determinations we used 1000-fold excess DHT competitor because the specificity studies indicated that competition for $[^3H]$-estradiol binding sites by DHT was more marked at this concentration.

In summary, we chose the following assay conditions to measure high and moderate affinity $[^3H]$-estradiol binding in rat liver cytosol:

<table>
<thead>
<tr>
<th></th>
<th>Hi affinity binding</th>
<th>Moderate affinity binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>tissue fraction</td>
<td>50% AS fraction</td>
<td>whole cytosol</td>
</tr>
<tr>
<td>ligand concentration</td>
<td>$[^3H]$-estradiol 0.05-2.5 nM</td>
<td>$[^3H]$-estradiol 10-200 nM</td>
</tr>
<tr>
<td>competitor(s)</td>
<td>estradiol 100-fold excess</td>
<td>estradiol 100-fold excess or DHT 1000-fold excess</td>
</tr>
<tr>
<td>incubation time/T°</td>
<td>1.5 hours at 4°C</td>
<td>1.5 hours at 4°C</td>
</tr>
</tbody>
</table>

Other details of the binding assays are included in the Methods section.
Scatchard analysis of moderate affinity $[^3H]$-estradiol binding in male rat liver cytosol using two different competitors. Conditions were as follows: 10 to 200 nM $[^3H]$-estradiol concentration; ~2 mg/ml protein; incubation carried out in absence and presence of 100-fold excess estradiol or dihydrotestosterone (DHT) for 1.5 hours at 4°C. Data were obtained from one tissue pool (n = 2 rats).
B/F × 10^7 vs. SPECIFIC BOUND (fmol/mg protein)

- Estradiol competitor: K_d = 0.8 × 10^{-7} M
- DHT competitor: K_d = 0.5 × 10^{-7} M
B. Studies in chemically-induced and spontaneously diabetic rats

1. Hepatic benzo[a]pyrene (BP) hydroxylase kinetics

Previous workers have demonstrated that microsomal BP metabolism exhibits curvilinear Lineweaver-Burk plots under "usual" assay conditions (Nebert and Gelboin, 1968; Robie et al., 1976; Cumps et al., 1977). This is thought to be due mainly to poor availability of the substrate (BP) to the enzyme. Cumps et al. (1977) observed that at higher microsomal membrane concentrations (greater than 10-20 μg/ml) there was a nonspecific interaction between BP and noncatalytic membrane sites which significantly reduced the free BP concentration. The nonspecific binding to microsomal proteins was reversible and was determined to be similar to an adsorption process. The authors reasoned that the nonspecific sites on the membrane acted as a competitive inhibitor for BP hydroxylation by reversibly combining with BP at sites which were structurally distinct from the catalytic site of cytochrome P-450. In this situation, in contrast to the classical one, the inhibitor (nonspecific microsomal sites) was competing with the enzyme (BP hydroxylase) for the substrate (BP). Another factor which limited the availability of BP to the enzyme is its poor water solubility. Robie et al. (1976) detected that BP formed molecular aggregates during incubation which occurred to a greater extent at higher levels of added BP (greater than 40 μM). Another problem contributing to non-linearity of BP kinetics with time was that 3-hydroxybenzo[a]pyrene was metabolized by the enzyme system to nonfluorescent products and may have competed with BP for the same enzyme site (Robie et al., 1976).
All these factors predispose toward artificially high $K_m$ values. Higher microsomal protein levels cause high $K_m$ values due to rapid substrate depletion and disappearance of fluorescent products. Molecular aggregation of BP at high substrate concentration leads to obtaining high $K_m$ values possibly as a reflection of the differences in the affinity of the BP aggregates for the enzyme. High $K_m$ values may also reflect a composite value for different routes of BP metabolism.

For these reasons, the conditions which allow measurement of initial reaction velocity must avoid changing substrate levels since these determinations are substrate dependent. The conditions used to determine microsomal BP hydroxylase kinetics were the following: low microsomal protein content (less than 20 μg/ml), short assay times (less than 3 minutes), and low substrate concentration (0.2-7 μM BP) (Robie et al., 1976; Cumps et al., 1977). These two groups calculated the $K_m$ of BP hydroxylase activity under these conditions to be 1.0 μM and 2.5 μM respectively.

The sensitivity of our assay system would not permit the "low level" determinations necessary for Michaelis-Menten kinetics as mentioned above. The conditions in our system were ~0.5 mg/ml protein concentration, 2.5 minutes incubation time, and 2.5 to 80 μM BP concentration. However, we accepted the limitations of our assay to carry out kinetic determinations because we were interested only in relative similarities or differences between control and diabetic rats. The apparent $K_m$ values which we obtained were approximately 10-fold higher than what Robie et al. (1976) and Cumps et al. (1977) obtained, but were similar to those reported by Hansen and Fouts (1972) and Alvares et al. (1970). The 10-fold difference probably relates to our higher protein and
substrate concentration, which as discussed above may lead to artificially high $K_m$ values. However, we reasoned that estimating the apparent $K_m$ of overall BP metabolism in nonpurified microsomal preparations would be a valid qualitative basis on which to compare hepatic drug metabolism in control and diabetic rats.

Table X summarizes the results of hepatic BP hydroxylase kinetic experiments in control and diabetic female and male rats. The values in the table were obtained from the graphs of enzyme activity vs [BP], examples of which are shown in Figure 23. In both females and males, nondiabetic IC rats demonstrated a decrease in specific BP hydroxylase activity and in apparent $K_m$ compared to regular Wistar OC rats. Kinetic parameters in female BB, ALX, or STZ diabetics were not significantly different from those in the corresponding control groups. Whereas there was no significant difference in specific activity in BB male diabetics compared to the IC group, there was a statistically significant decrease in this parameter in ALX and STZ diabetic males. However, the BB diabetics exhibited an increased apparent $K_m$ value for BP hydroxylase compared to the IC group and the STZ diabetics demonstrated a decreased $K_m$ compared to control. The graphical representation of some of these data (Figure 23) indicates the variability of results obtained within the experimental groups. In light of this large variability among individual animals, the small differences noted in specific activity and apparent $K_m$ of BP hydroxylase were unimpressive.
Table X. Hepatic benzo[a]pyrene hydroxylase kinetics in control and diabetic rats. Assay conditions as described in "Methods"; incubation time 2.5 min; 0.5 mg/ml protein concentration in incubation mixture.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Number of animals</th>
<th>Specific Activity (pmol/min/mg protein) (± S.E.M.)</th>
<th>Apparent $K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FEMALES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outside Control (OC)</td>
<td>4</td>
<td>451 ± 60</td>
<td>20.4 ± 0.6</td>
</tr>
<tr>
<td>Inside Nondiabetic control (IC)</td>
<td>7</td>
<td>223 ± 7†</td>
<td>12.6 ± 0.5†</td>
</tr>
<tr>
<td>RB Diabetic (BB)$^a$</td>
<td>8</td>
<td>255 ± 30</td>
<td>15.9 ± 1.4</td>
</tr>
<tr>
<td>Citrate Control (C)$^b$</td>
<td>4</td>
<td>325 ± 93</td>
<td>16.6 ± 2.0</td>
</tr>
<tr>
<td>Alloxan Diabetic (ALX)$^b$</td>
<td>4</td>
<td>446 ± 96</td>
<td>16.4 ± 1.7</td>
</tr>
<tr>
<td>Streptozotocin Diabetic (STZ)$^b$</td>
<td>6</td>
<td>379 ± 59</td>
<td>17.7 ± 2.0</td>
</tr>
<tr>
<td><strong>MALES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outside Control (OC)</td>
<td>4</td>
<td>1986 ± 316</td>
<td>20.4 ± 0.4</td>
</tr>
<tr>
<td>Inside Nondiabetic control (IC)$^b$</td>
<td>10</td>
<td>1251 ± 132†</td>
<td>13.1 ± 0.7†</td>
</tr>
<tr>
<td>BB Diabetic (BB)$^a$</td>
<td>9</td>
<td>1138 ± 196</td>
<td>19.0 ± 1.8*</td>
</tr>
<tr>
<td>Citrate Control (C)$^c$</td>
<td>5</td>
<td>2397 ± 171</td>
<td>22.8 ± 0.8</td>
</tr>
<tr>
<td>Alloxan Diabetic (ALX)$^c$</td>
<td>4</td>
<td>1425 ± 246*</td>
<td>19.5 ± 1.7</td>
</tr>
<tr>
<td>Streptozotocin Diabetic (STZ)$^d$</td>
<td>6</td>
<td>707 ± 126*</td>
<td>12.1 ± 0.5*</td>
</tr>
</tbody>
</table>

† = Significantly different from "outside control" at $p < 0.05$
* = Significantly different from "inside control" or "citrate control" at $p < 0.05$

$^a$Animals were 2 to 6 days post detection or 4 days post insulin withdrawal.
$^b$Animals were 3 months post injection.
$^c$Animals were 6 to 7 days post injection.
$^d$Animals were 18 to 24 days post injection.
Figure 23.

The effect of increasing substrate concentration on hepatic benzo-[a]pyrene (BP) hydroxylase activity in female and male spontaneously diabetic rats. Each curve represents data obtained from 1 animal. Protein concentration was ~0.5 mg/ml; incubation time 2.5 minutes.
HEPATIC BP HYDROXYLASE ACTIVITY (pmol/min/mg protein)

females

males

BENZO[a]PYRENE (uM)
2. Additional studies of hepatic benzo[a]pyrene (BP) hydroxylase activity

Because of the variable results we had previously obtained, we carried out further measurements of BP hydroxylase activity in large numbers of control and diabetic rats at saturating substrate concentration. Diabetic animals in these experiments were either discontinued from insulin for 4 days (BB) or were 4 days post-STZ injection. As expected, control female levels were approximately 5 to 7 fold less than male control levels (394 ± 35 pmol/min/mg protein vs 2185 ± 178 pmol/min/mg protein for the OC group; 227 ± 20 vs 1485 ± 118 for the IC group and 614 ± 52 vs 2950 ± 208 for the C group) (Figure 24 vs Figure 25). Our data indicated that this activity in diabetic females was not different from the appropriate control levels, whether the animal was genetically (BB) (213 ± 21 pmol/min/mg protein vs 227 ± 20) or chemically (STZ) diabetic (551 ± 39 pmol/min/mg protein vs 614 ± 52) (Figure 24). On the other hand, in males hepatic BP hydroxylase activity was reduced to approximately 50% of control in both BB (903 ± 91 pmol/min/mg protein vs 1485 ± 118) and STZ diabetics (1652 ± 149 pmol/min/mg protein vs 2950 ± 208) (Figure 25). The nondiabetic littermate controls of the BB rats (IC) of both sexes exhibited significantly lower hepatic BP hydroxylase activity than the normal Wistar "outside" controls (OC) (227 ± 20 pmol/min/mg protein vs 394 ± 35 for females; 1485 ± 118 vs 2185 ± 178 for males). Although the citrate injected (C) control levels of BP hydroxylase were higher than the OC Wistar controls in both sexes, values of 400 to 600 and 2200-3000 pmol/min/mg protein for females and males respectively are normal ranges of activity in our laboratory.
Figure 24.

Hepatic benzo[a]pyrene (BP) hydroxylase activity in control and 4 day diabetic female rats. Results are expressed as mean ± SEM. Numbers in the bars refer to the number of animals.

OC: outside Wistar control
IC: inside nondiabetic control
BB: spontaneously diabetic
C: Wistar citrate control
STZ: streptozotocin-induced diabetic

* Significantly different from outside Wistar control at p<0.05.
HEPATIC BP HYDROXYLASE ACTIVITY
(pmol/min/mg protein)
Figure 25.

Hepatic benzo[a]pyrene (BP) hydroxylase activity in control and 4 day diabetic male rats. Results are expressed as mean ± SEM. Abbreviations are as for Figure 24.

* Significantly different from outside Wistar control at p<0.05.

* Significantly different from inside nondiabetic control at p<0.05 or significantly different from citrate control at p<0.05.
These results are remarkably similar to those obtained in the kinetics experiments (Table X), despite the following differences in experimental protocol and time course of diabetes. Some of the BB diabetic animals in the kinetic experiments were obtained from the supplier in the prediabetic state and the diabetic state was detected in our laboratory. These animals never received insulin. Other BB rats in the kinetic experiments were obtained from the supplier in the diabetic state and were maintained on insulin until 4 days before sacrifice. The female chemically induced animals in the kinetic studies were obtained from another laboratory in our department and were 3 months post injection. The male chemically induced diabetics were 6 days (ALX) or 18 to 24 days (STZ) post injection. In contrast, diabetic animals in the later experiments were uniformly either discontinued from insulin for 4 days (BB) or were 4 days post STZ injection. The only dissimilarity in the 2 sets of experiments was apparent in the kinetic experiments (Table X) where hepatic BP hydroxylase in the BB diabetic males was not decreased compared to the nondiabetic control (IC) values. The most likely explanation for this difference is that these particular BB male diabetics tended to be stable diabetics and therefore were more similar to their nondiabetic littermates. The spectrum of severity of spontaneous diabetes became apparent when we studied a larger number of animals (n = 26 to 32). From this enlarged pool of data, the overall trend in male BB diabetics was to exhibit approximately 50% of control hepatic BP hydroxylase activity (Figure 25).
To determine the effects of insulin replacement on hepatic BP hydroxylase activity, BB diabetic rats were maintained on insulin therapy until 18 hours before they were sacrificed. It was observed that insulin maintenance reversed the effects of spontaneous diabetes on hepatic BP hydroxylase activity in male BB diabetics towards the level of the inside controls (IC) (1787 ± 188 pmol/min/mg protein vs 1113 ± 167) (Figure 26). Insulin treated BB females exhibited about 30% higher levels of activity than BB diabetic and control groups (471 ± 41 pmol/min/mg protein vs 308 ± 40 and 350 ± 34). It should be noted that the levels of BP hydroxylase in the insulin treated females were still within the physiologically normal range. A specific stimulation effect would be expected to raise BP hydroxylase by more than 30% in order to be physiologically relevant.

3. Kidney benzo[a]pyrene (BP) hydroxylase activity

We observed no difference in kidney BP hydroxylase between control and STZ diabetic females as has been reported previously (71.4 ± 5.7 pmol/min/mg protein vs 73.2 ± 5.9) (Stohs et al., 1979), or between control and STZ diabetic male rats (54.4 ± 11 pmol/min/mg protein vs 52.0 ± 5.5) (Figures 27 and 28). However, in both sexes, BP hydroxylase activity progressively decreased from OC to IC controls to BB diabetics (41.1 ± 4.3 pmol/min/mg protein vs 22.2 ± 2.6 vs 14.2 ± 1.5 in
Effect of insulin (INS) replacement on hepatic benzo[a]pyrene (BP) hydroxylase activity in spontaneously diabetic (BB) female and male rats. BB diabetics were 4 days post insulin withdrawal; insulin treated diabetics received insulin for those 4 days.

IC: inside nondiabetic control

* Significantly different from inside nondiabetic control at \( p<0.05 \).

\* Significantly different from untreated BB diabetic at \( p<0.05 \).
Kidney benzo[a]pyrene (BP) hydroxylase activity in control and 4 day diabetic female rats. Results are expressed as mean ± SEM. Abbreviations are as for Figure 24.

* Significantly different from outside Wistar control at p<0.05.

** Significantly different from inside non-diabetic control at p<0.05 or significantly different from citrate control at p<0.05.
Figure 28.

Kidney benzo[a]pyrene (BP) hydroxylase activity in control and 4 day diabetic male rats. Results are expressed as mean ± SEM. Abbreviations are as for Figure 24.
MALES

KIDNEY BP HYDROXYLASE ACTIVITY (pmol/min/mg protein)

<table>
<thead>
<tr>
<th>OC</th>
<th>IC</th>
<th>BB</th>
<th>C</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>19</td>
<td>25</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>
females; 37.4 ± 4.2 pmol/min/mg protein vs 29.1 ± 4.4 vs 21.7 ± 3.2 in males). The differences were statistically significant only in the females. These results along with those in liver (Figures 24 and 25) would suggest that there is a strain difference in tissue BP hydroxylase activity between the nondiabetic control and regular Wistar rats. This is not unexpected considering the presumed genetic predisposition of the nondiabetic littermates (IC).

It was apparent that kidney microsomal BP hydroxylase was not a sex dependent activity in our hands, in contrast to the results of Gurtoo and Parker (1977). However, those workers used the 15,000 g supernatant fraction in their studies.

4. Hepatic epoxide hydrase (EH) activity

The non-cytochrome P450 dependent activity EH was measured in regular Wistar controls (OC), nondiabetic littermates (IC) and spontaneously diabetic (BB) rats (Figure 29). Diabetes of 4 days duration (i.e., post insulin withdrawal) had no effect on EH activity in females (OC = 3.12 ± 0.19 mmol/min/mg protein vs IC = 3.31 ± 0.20 vs BB = 3.40 ± 0.64) or in males (OC = 6.01 ± 0.58 vs IC = 5.29 ± 0.33 vs BB = 4.56 ± 0.24). These data along with that from the BP hydroxylase, testosterone Δ⁴ hydrogenase, and aniline hydroxylase (Appendix I) studies supported the concept that diabetes may alter various microsomal enzyme
Figure 29.

Hepatic microsomal epoxide hydrase activity in Wistar control (OC), non-diabetic inside control (IC) and spontaneously diabetic (BB) female and male rats.
activities due to effects on cytochrome P450.

5. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

The effect of diabetes on the appearance of microsomal protein bands separated by electrophoresis is shown in Figures 30 and 31. We did not observe major changes in the overall pattern of protein bands (28,000-130,000 molecular weight) in diabetic animals. However, some bands in the P450 region (46,000-61,000) did change in relation to one another. For instance, the BB diabetic females (Figure 30) in comparison to the nondiabetics showed an increase in the 52,000 molecular weight band without a decrease in the 46,000 and 61,000 molecular weight regions. On the other hand, in male BB diabetics (Figure 31), the prominent series of 4 peaks (46,000-61,000) evident in nondiabetic controls (IC) was altered. The 52,000 molecular weight band increased, the 56,000 decreased, and the 46,000 and 61,000 regions nearly disappeared. Insulin treatment reversed these changes in both male and female BB diabetics. Citrate-injected controls (C) and BB nondiabetic controls (IC) demonstrated some differences in the protein bands compared to one another. For example in males, the 46,000 and 61,000 molecular weight regions were not prominent in citrate controls. Generally, within each strain of animal there were no major differences observed between male and female densitometric scans.
Figure 30.

Densitometric scans of Coomassie blue stained microsomal protein bands separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis from control and diabetic female rats. Molecular weight ranges are shown \( \times 10^{-3} \), with the range 46-61 designating 46,000, 52,000, 56,000, and 61,000 molecular weight regions.

IC: inside nondiabetic control
BB: spontaneously diabetic
BB + ins: BB diabetic treated with insulin
C: Wistar citrate control
STZ: streptozotocin induced diabetic
Figure 31.

Densitometric scans of Coomassie blue stained microsomal protein bands separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis from control and diabetic male rats. Molecular weight ranges are shown x 10^-3, with the range 46-61 designating 46,000, 52,000, 56,000, and 61,000 molecular weight regions. Abbreviations are as for Figure 30.
6. Effects of diabetes on body weight, serum glucose, and serum insulin levels

It was of interest to further characterize the diabetic rats on the basis of body weight and serum glucose and insulin levels. Whether rats were chemically induced or spontaneously diabetic, short term diabetes (4 days) produced a significant decrease in body weight (Table XI). Control rats had fasted serum glucose levels of approximately 100 mg/100 ml, whereas diabetic rats exhibited significant hyperglycemia (~3 to 4 fold above control). It was observed that BB diabetic animals exhibited only slightly lower serum insulin levels than the nondiabetic controls (IC), even though the diabetics were definitely hyperglycemic. On the other hand, the STZ diabetic animals did show a statistically significant reduction in serum insulin levels. We would have predicted that the serum levels in the BB diabetics would be significantly lower than control because the diabetic syndrome in these animals is characterized by marked insulitis in the pancreatic islets (Nakhooda et al., 1976). For this reason we examined diabetic and control pancreatic tissue under light microscopy. In addition, results from immunocytochemical techniques were employed as a more precise means of studying the nature of the pancreatic islets of diabetic and control rats.
Table XI. Effects of spontaneous and streptozotocin-induced diabetes on body weight, serum glucose, and serum insulin levels in male and female rats. Data are expressed as mean \( \pm \) S.E.M.

<table>
<thead>
<tr>
<th>Animal Status</th>
<th>n</th>
<th>Body Weight (g)</th>
<th>n</th>
<th>Serum Glucose(^a) (mg/100 ml)</th>
<th>n</th>
<th>Serum Insulin(^a) ((\mu)U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female OC</td>
<td>32</td>
<td>265 (\pm) 9</td>
<td>7</td>
<td>115.1 (\pm) 5.2</td>
<td>19</td>
<td>28.3 (\pm) 4.7</td>
</tr>
<tr>
<td>Female IC</td>
<td>27</td>
<td>247 (\pm) 4</td>
<td>8</td>
<td>92.8 (\pm) 6.6</td>
<td>23</td>
<td>23.3 (\pm) 4.5</td>
</tr>
<tr>
<td>Female BB</td>
<td>28</td>
<td>215 (\pm) 5*</td>
<td>7</td>
<td>354.9 (\pm) 93.1*</td>
<td>25</td>
<td>19.0 (\pm) 3.1</td>
</tr>
<tr>
<td>Female C</td>
<td>8</td>
<td>251 (\pm) 2</td>
<td>8</td>
<td>91.3 (\pm) 9.7</td>
<td>7</td>
<td>17.3 (\pm) 5.6</td>
</tr>
<tr>
<td>Female STZ</td>
<td>12</td>
<td>233 (\pm) 5*</td>
<td>12</td>
<td>285.8 (\pm) 38.7*</td>
<td>9</td>
<td>6.5 (\pm) 1.3*</td>
</tr>
<tr>
<td>Male OC</td>
<td>32</td>
<td>414 (\pm) 16</td>
<td>7</td>
<td>102.6 (\pm) 10.3</td>
<td>17</td>
<td>25.9 (\pm) 3.7</td>
</tr>
<tr>
<td>Male IC</td>
<td>29</td>
<td>415 (\pm) 7</td>
<td>8</td>
<td>119.8 (\pm) 10.7</td>
<td>27</td>
<td>20.2 (\pm) 2.1</td>
</tr>
<tr>
<td>Male BB</td>
<td>28</td>
<td>352 (\pm) 11*</td>
<td>4</td>
<td>375.8 (\pm) 104.2*</td>
<td>19</td>
<td>19.1 (\pm) 1.5</td>
</tr>
<tr>
<td>Male C</td>
<td>8</td>
<td>419 (\pm) 7</td>
<td>8</td>
<td>118.9 (\pm) 7.9</td>
<td>12</td>
<td>27.3 (\pm) 3.2</td>
</tr>
<tr>
<td>Male STZ</td>
<td>12</td>
<td>377 (\pm) 3*</td>
<td>12</td>
<td>403.8 (\pm) 53.1*</td>
<td>16</td>
<td>14.1 (\pm) 1.6*</td>
</tr>
</tbody>
</table>

OC = outside Wistar control  
IC = inside nondiabetic control  
BB = spontaneously diabetic  
C = Wistar citrate control  
STZ = streptozocin-induced diabetic  

* Significantly different from appropriate control at \( p < 0.05 \)

\( ^a \) Animals were fasted 16 hr prior sacrifice
7. Hepatic and pancreatic histology

Light microscopy studies of hemotoxylin and eosin-stained sections of pancreas and liver were carried out. Under these conditions minimal histologic change was apparent in tissues of STZ or spontaneously diabetic animals. Pancreatic islets were present in the BB diabetic rats although there was evidence of lymphocytic infiltration. The results of immunocytochemical studies carried out by Dr. Alison Buchan are present in Appendix II.

8. Serum testosterone levels

BB diabetic male rats were found to have significantly reduced serum testosterone levels compared to nondiabetic littermate controls (IC) (Table XII). Furthermore, the nondiabetic littermates had lower testosterone levels than the nonlittermate controls (OC). The steroid levels in females were much lower than in males but no difference was observed between controls and diabetics.
Table XII. Effect of spontaneous diabetes on serum testosterone levels in male and female rats. Data are expressed as a mean ± S.E.M. in ng/ml.

<table>
<thead>
<tr>
<th>Animal Status</th>
<th>n</th>
<th>Serum Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female OC</td>
<td>4</td>
<td>0.18 ± .04</td>
</tr>
<tr>
<td>Female IC</td>
<td>4</td>
<td>0.12 ± .04</td>
</tr>
<tr>
<td>Female BB</td>
<td>2</td>
<td>0.15 ± .02</td>
</tr>
<tr>
<td>Male OC</td>
<td>4</td>
<td>6.40 ± 1.25</td>
</tr>
<tr>
<td>Male IC</td>
<td>7</td>
<td>2.99 ± 0.42*</td>
</tr>
<tr>
<td>Male BB</td>
<td>4</td>
<td>1.16 ± 0.44†</td>
</tr>
</tbody>
</table>

OC = outside Wistar control
IC = inside nondiabetic control
BB = spontaneously diabetic

* Significantly different from OC at p < 0.05.
† Significantly different from IC at p < 0.05.
9. Hepatic steroid binding assays

High and moderate affinity $[^{3}\text{H}]-\text{estradiol}$ binding was measured in nondiabetic littermate control (IC) and BB diabetic rats. High affinity binding in control females and males was similar and was within the expected range ($K_d$: $7.2$ and $7.7 \times 10^{-10}$ M and capacity: $52$ and $116$ fmol/mg protein) (Figures 32 and 33). High affinity binding in BB diabetic females was not different from control (Figure 32), however in male diabetics (Figure 33) there was a 50% decrease compared to control in the capacity of high affinity binding sites ($116$ fmol/mg protein vs $48$ fmol/mg protein). The decrease in binding was evident in the binding curve data as well (inset Figure 33).

Moderate affinity $[^{3}\text{H}]-\text{estradiol}$ binding was detected in both nondiabetic and diabetic males within the expected range ($K_d$: $0.73$ to $1.3 \times 10^{-7}$ M and capacity of $1.2$ to $2.1$ pmol/mg protein), whether estradiol or DHT was the competitor (Figure 34 and Figure 35). There was a trend for moderate affinity binding sites to be somewhat reduced in male BB diabetics, but the reduction was not as marked as it was in the high affinity range (Figure 33). Moderate affinity $[^{3}\text{H}]-\text{estradiol}$ binding was not detected in female rats, as expected (Figure 36).

During a series of routine experiments in our laboratory it was observed that pH changes affected the hepatic binding assays in an unexpected fashion (see Ph.D. thesis, Malcolm J. Finlayson). Therefore further studies were carried out at assay conditions of pH 8.8 in control and diabetic rats. Each binding curve is from a single liver preparation. The examples given are typical experiments from a series of four. The
Scatchard analysis of high affinity $[^{3}\text{H}]-\text{estradiol}$ binding in control and diabetic female rat liver. Conditions were as follows: pH 7.4; 50% ammonium sulfate fraction; ~ 3 mg/ml protein; incubation carried out in absence and presence of 100-fold excess nonlabelled estradiol for 1.5 hours at 4°C.

Inset: Specifically bound $[^{3}\text{H}]-\text{estradiol}$ vs ligand concentration ($[^{3}\text{H}]-\text{estradiol}$) in control and diabetic female rat liver.

- Nondiabetic inside control (IC) (pool of 2)
- BB diabetic (BB) (pool of 4)
females
pH 7.4

SPECIFIC BOUND (fmol/mg)

[\textsuperscript{3}H] - ESTRADIOL ADDED (nM)

SPECIFIC BOUND (fmol/mg protein)

BB: $K_d = 6.8 \times 10^{-10}$ M

IC: $K_d = 7.2 \times 10^{-10}$ M
Scatchard analysis of high affinity $[^3H]-estradiol$ binding in control and diabetic male rat liver. Conditions were as follows: pH 7.4; 50% ammonium sulfate fraction; ~3 mg/ml protein; incubation carried out in absence and presence of 100-fold excess nonlabelled estradiol for 1.5 hours at 4°C.

Inset: Specifically bound $[^3H]-estradiol$ vs ligand concentration ($[^3H]-estradiol$) in control and diabetic male rat liver.

Non-diabetic inside control (IC) (pool of 3)
BB diabetic (BB) (pool of 2)
males
pH 7.4

SPECIFIC BOUND (fmol/mg)

150
120
90
60
30
0.5 1.0 1.5 2.0 2.5
[3H] - ESTRADIOL ADDED (nM)

SPECIFIC BOUND (fmol/mg protein)

IC: $K_d = 7.7 \times 10^{-10}$ M

BB: $K_d = 6.4 \times 10^{-10}$ M
Scatchard analysis of moderate affinity $[^3H]$-estradiol binding in control and diabetic male rat liver. Conditions were as follows: pH 7.4; whole cytosol fraction; ~ 2 mg/ml protein; incubation carried out in absence and presence of 100-fold excess nonlabelled estradiol for 1.5 hours at 4°C.

Inset: Specifically bound $[^3H]$-estradiol vs ligand concentration ($[^3H]$-estradiol) in control and diabetic male rat liver.

Nondiabetic inside control (IC) (pool of 3)
BB diabetic (BB) (pool of 2)
males
estradiol competitor
pH 7.4

\[ \text{SPECIFIC BOUND (fmol/mg)} \]

\[ \text{SPECIFIC BOUND (fmol/mg protein)} \]

IC: \( K_d = 1.05 \times 10^{-7} \text{M} \)
BB: \( K_d = 1.3 \times 10^{-7} \text{M} \)
Figure 35.

Scatchard analysis of moderate affinity $[^3H]$-estradiol binding in control and diabetic male rat liver. Conditions were as follows: pH 7.4; whole cytosol fraction; ~2 mg/ml protein; incubation carried out in absence and presence of 1000-fold excess nonlabelled dihydrotestosterone (DHT) for 1.5 hours at 4°C.

Inset: Specifically bound $[^3H]$-estradiol vs ligand concentration ($[^3H]$-estradiol) in control and diabetic male rat liver.

- Nondiabetic inside control (IC) (pool of 3)
- BB diabetic (BB) (pool of 2)
males
DHT competitor
pH 7.4

\[ [^3H] - ESTRADIOL ADDED (nM) \]

\[
\begin{align*}
\text{SPECIFIC BOUND (fmol/mg)} & \quad 2000 \\
& \quad 1600 \\
& \quad 1200 \\
& \quad 800 \\
& \quad 400 \\
& \quad 0 \\
\text{SPECIFIC BOUND (fmol/mg protein)} & \quad 50 \\
& \quad 100 \\
& \quad 150 \\
& \quad 200 \\
& \quad 250 \\
& \quad 300 \\
\end{align*}
\]

B/F \times 10^7

IC: \[ K_d = 0.97 \times 10^{-7} \text{M} \]

BB: \[ K_d = 0.73 \times 10^{-7} \text{M} \]
Figure 36.

Scatchard plot of moderate affinity $[^3H]$-estradiol binding in control and diabetic female rat liver. Conditions were as follows: pH 7.4; whole cytosol fraction; ~ 3 mg/ml protein; incubation carried out in absence and presence of 100-fold excess nonlabelled estradiol for 1.5 hours at 4°C.

Inset: Specifically bound $[^3H]$-estradiol vs ligand concentration ($[^3H]$-estradiol) in control and diabetic female rat liver.

Nondiabetic inside control (IC) (pool of 2)

BB diabetic (BB) (pool of 4)
females
estradiol competitor
pH 7.4

SPECIFIC BOUND (fmol/mg protein)

SPECIFIC BOUND (fmol/mg)

B/F x 10^7.
Scatchard plots obtained from high affinity binding data in females and males is shown in Figures 37 and 38 respectively. At pH 8.8, high affinity binding sites exhibited a higher $K_d$ ($10^{-9}$ vs $10^{-10}$ M) and capacity ($400$ fmol/mg protein vs $100$ fmol/mg protein) than at pH 7.4 in control females and males. However, the trends in the diabetic animals were similar to those observed at pH 7.4, i.e. no changes were observed in the $K_d$ values, or in the capacity of binding in diabetic females, while there was a marked decrease in binding in the diabetic males (450 decreased to 120 fmol/mg protein).

Moderate affinity $[\text{H}]-\text{estradiol}$ binding was remarkably different at pH 8.8 than at pH 7.4. At pH 8.8 control females exhibited moderate affinity binding with a $K_d$ of $2.5$ and $2.9 \times 10^{-8}$ M and capacity of $2.1$ to $3$ pmol/mg protein, with estradiol and DHT as competitors (Figures 39 and 40). At pH 8.8 diabetic females exhibited marked decreases in moderate affinity binding compared to control (e.g. to $0.28$ pmol/mg protein or less). On the other hand, moderate affinity $[\text{H}]-\text{estradiol}$ binding in control males was detected at pH 8.8 but linear Scatchard plots could not be derived from the data with either DHT or estradiol competitors (Figure 41 and Figure 42). However, it was observed that BB diabetic males exhibited considerably less specific binding than the controls (insets, Figures 41 and 42).
Figure 37.

Scatchard analysis of high affinity [\(^3\)H]-estradiol binding in control and diabetic female rat liver. Conditions were as follows: pH 8.8; 50% ammonium sulfate fraction; ~ 2 mg/ml protein; incubation carried out in absence and presence of 100-fold excess nonlabelled estradiol for 1.5 hours at 4°C.

Inset: Specifically bound [\(^3\)H-estradiol] vs ligand concentration ([\(^3\)H-estradiol]) in control and diabetic female rat liver.

Nondiabetic inside control (IC) (typical experiment)
BB diabetic (BB) (typical experiment)
SPECIFC BOUND (fmol/mg protein)

B/F x 10^7

females pH 8.8

BB: $K_d = 1.04 \times 10^{-9}$ M

IC: $K_d = 1.65 \times 10^{-9}$ M

$[^3H]$ - ESTRADIOL ADDED (nM)
Figure 38.

Scatchard analysis of high affinity [³H]-estradiol binding in control and diabetic male rat liver. Conditions were as follows: pH 8.8; 50% ammonium sulfate fraction; ~ 3.3 mg/ml protein; incubation carried out in absence and presence of 100-fold excess nonlabelled estradiol for 1.5 hours at 4°C.

Inset: Specifically bound [³H]-estradiol vs ligand concentration ([³H]-estradiol) in control and diabetic male rat liver.

Nondiabetic inside control (IC) (typical experiment)
BB diabetic (BB) (typical experiment)
males
pH 8.8

B/F \times 10^7

SPECIFIC BOUND (fmol/mg)

SPECIFIC BOUND (fmol/mg protein)

IC: $K_d$: $1.79 \times 10^{-9}$ M

BB: $K_d$: $1.29 \times 10^{-9}$ M

[3H]-ESTRADIOL ADDED (nM)
Figure 39.

Scatchard analysis of moderate affinity $[^3\text{H}]$-estradiol binding in control and diabetic female rat liver. Conditions were as follows: pH 8.8; whole cytosol fraction; ~2 mg/ml protein; incubation carried out in absence and presence of 100-fold excess nonlabelled estradiol for 1.5 hours at 4°C.

Inset: Specifically bound $[^3\text{H}]$-estradiol vs ligand concentration ($[^3\text{H}]$-estradiol) in control and diabetic female rat liver.

Nondiabetic inside control (IC) (typical experiment)

BB diabetic (BB) (typical experiment)
females
estradiol competitor
pH 8.8

IC: $K_d = 2.94 \times 10^{-8}$ M

BB: $K_d = 0.98 \times 10^{-8}$ M

[3H] Estradiol Added (nM)
Figure 40.

Scatchard analysis of moderate affinity $[^3\text{H}]$-estradiol binding in control and diabetic female rat liver. Conditions were as follows: pH 8.8; whole cytosol fraction; $\sim 2 \text{ mg/ml}$ protein; incubation carried out in absence and presence of 1000-fold excess nonlabelled dihydrotestosterone (DHT) for 1.5 hours at 4°C.

Inset: Specifically bound $[^3\text{H}]$-estradiol vs ligand concentration ($[^3\text{H}]$-estradiol) in control and diabetic female rat liver.

- Nondiabetic inside control (IC) (typical experiment)
- BB diabetic (BB) (typical experiment)
females
DHT competitor
pH 8.8

IC: $K_d = 2.5 \times 10^{-8}$ M

SPECIFIC BOUND (fmol/mg protein)

SPECIFIC BOUND (fmol/mg)

$[\text{H}^3] - \text{ESTRADIOL ADDED (nM)}$
Figure 41.

Scatchard plot of moderate affinity $[^3\text{H}]-\text{estradiol}$ binding in control and diabetic male rat liver. Conditions were as follows: pH 8.8; whole cytosol fraction; ~3 mg/ml protein; incubation carried out in absence and presence of 100-fold excess nonlabelled estradiol for 1.5 hours at 4°C.

Inset: Specifically bound $[^3\text{H}]-\text{estradiol}$ vs ligand concentration ($[^3\text{H}]-\text{estradiol}$) in control and diabetic male rat liver.

Nondiabetic inside control (IC) (typical experiment)

BB diabetic (BB) (typical experiment)
males
estradiol competitor
pH 8.8

SPECIFIC BOUND (fmol/mg protein)

[3H] - ESTRADIOL ADDED (nM)

B/F x 10^7

SPECIFIC BOUND (fmol/mg protein)
Figure 42.

Scatchard plot of moderate affinity $[^3H]$-estradiol binding in control and diabetic male rat liver. Conditions were as follows: pH 8.8; whole cytosol fraction; ~ 3 mg/ml protein; incubation carried out in absence and presence of 1000-fold excess nonlabelled dihydrotestosterone (DHT) for 1.5 hours at 4°C.

Inset: Specifically bound $[^3H]$-estradiol vs ligand concentration ($[^3H]$-estradiol) in control and diabetic male rat liver.

Nondiabetic inside control (IC) (typical experiment)
BB diabetic (BB) (typical experiment)
males
DHT competitor
pH 8.8

\[^{3}H\] -ESTRADIOL ADDED (nM)

SPECIFIC BOUND (fmol/mg protein)

B/F x 10^7

1000 2000 3000 4000
DISCUSSION

A. Alterations in hepatic drug metabolism in diabetic rats

We observed that spontaneous and chemical diabetes tended to exert similar influences on the hepatic drug metabolism system, although the mechanisms responsible may not be identical. For instance, BP hydroxylation, a sex dependent enzyme activity, was reduced in BB spontaneously diabetic and STZ diabetic male rats. Enzyme kinetic studies revealed that changes in enzyme activity were not accompanied by physiologically relevant alterations of apparent $K_m$ values in diabetic males. These data indicated that in diabetic rats BP hydroxylase activity was altered quantitatively rather than qualitatively. Previous workers have suggested that diabetes interferes with androgenic stimulation of sex dependent enzyme activities. For instance, Kato and Gillette (1965) reported that ALX diabetes in castrated male rats did not cause a reduction of sex dependent metabolism, although it did in methyltestosterone treated castrates. Other workers have reported that STZ diabetic male rats exhibit lower than control serum testosterone levels (Tesone et al., 1980; Murray et al., 1981; Baxter et al., 1981) and we observed that BB diabetics followed the same trend (Table XII). It is likely that one of the factors contributing to altered sex dependent drug metabolism in male diabetics is lack of circulating testosterone.

Baxter et al. (1981) reported an increase in serum testosterone levels in female STZ diabetic rats compared to control. This could be related to previous reports where sex dependent metabolism has been observed to increase in STZ diabetic female rats (Kato and Gillette,
1965; Kato et al., 1970; Reinke et al., 1978a; Reinke et al., 1979). However, in our studies using either model of diabetes, female rats did not demonstrate an increase in hepatic BP hydroxylase activity compared to control, even when we examined 10 day rather than 4 day diabetic animals. (Previous studies utilized 10 day post injection animals.) In addition, we did not observe any increase in serum testosterone levels in these female diabetics. At present, we are unable to account for this difference between our studies and those of others.

The other sex dependent enzyme we measured was testosterone $\Delta^4$ hydrogenase activity, an index of endogenous substrate metabolism. Generally, androgens exert opposite effects on steroid $\Delta^4$ hydrogenases compared to mixed function oxidases (Colby, 1980). In control rats testosterone $\Delta^4$ hydrogenase was approximately four fold greater in females than in males (Figures 43 and 44). We observed a significant decrease in activity in female diabetics, and no change in male diabetics. Again, the trends were the same in STZ-induced and spontaneously diabetic animals. The data are not what we had predicted in relation to the lowered serum testosterone levels observed in diabetic male rats. In this situation one would expect an increase in hydrogenase levels in diabetic males in association with decreased serum testosterone levels, but this was not observed. These data indicate that there are more factors than serum testosterone levels involved in the control of sex dependent drug metabolism. Baxter et al. (1981) reported that serum estrogen levels in female diabetics were not different from control, while male diabetics exhibited lower than control estradiol values. Steroid levels were not restored to normal with 1 week of insulin
therapy. This is the opposite of what would be predicted in relation to the testosterone $\Delta^4$ hydrogenase results because, in theory, the decrease in $\Delta^4$ hydrogenase activity in diabetic females would have been associated with a decrease in serum estradiol levels. (Estradiol stimulates testosterone $\Delta^4$ hydrogenase activity). In addition, male diabetics exhibited no change in enzyme activity although serum estradiol levels were reduced, according to Baxter et al. (1981). It would have been expected that the enzyme activity would have followed the same trend. It appears from these observations that there is not a simple direct relationship between androgen and estrogen levels and activity of these enzymes.

We found that the sex independent activity aniline hydroxylase was increased in both male and female diabetic rats. The increase was evident in both STZ and spontaneously diabetic rats. Since in this case, the diabetic state produced a similar effect in males and females, it might be predicted that a relatively nonspecific mechanism is responsible for this alteration. There is evidence to suggest that ketonic compounds alter MFO activities, including aniline hydroxylase (Mehendale et al., 1977). Metabolic ketosis could be the mechanism responsible for elevated aniline hydroxylase, even in stable nonketonuric diabetic animals, because local tissue concentrations of ketone bodies could accumulate and produce the observed stimulation.

Other workers have found that total hepatic microsomal cytochrome P-450 was increased in diabetic female rats (Kato et al., 1971; Reinke et al., 1978; Stohs et al., 1979). Reinke et al. (1979) observed that female cytochrome P-450 levels remained unchanged until 48 hours post STZ injection and were maximally increased at 96 hours.
In male diabetics, increases in total cytochrome P-450 have been reported to be statistically significant (Reinke et al., 1978) or not statistically significant (Kato et al., 1971). In the present study, we obtained densitometric scans of diabetic microsomal proteins which had been separated electrophoretically. The banding patterns in the tracings suggested that the composition of proteins in the cytochrome P-450 region (~50,000 molecular weight) was altered in STZ and BB diabetic rats compared to their corresponding controls. Some bands were increased in relation to one another, while others were decreased. That is, there was not a uniform increase or decrease in the overall electrophoretic profiles, but rather, more subtle changes in individual protein bands. Insulin treatment reversed the observed alterations. Past and Cook (1980) reported that ALX diabetes altered the electrophoretic profile of rat liver heme microsomal proteins in the cytochrome P-450 region. It is possible that relatively subtle changes in individual cytochrome P450 proteins could effect marked alterations in substrate specific microsomal metabolism. It may be that the regulatory processes involved in the control of hepatic metabolism are disrupted in STZ and BB diabetic animals, thereby altering specific cytochromes P-450 and producing differential changes in enzyme activities. Further experiments utilizing purified forms of the cytochromes would be necessary to clarify this suggestion.
B. The diabetic syndrome in BB spontaneously diabetic rats

We observed that significant changes in various hepatic microsomal enzyme activities occurred in both STZ and spontaneously diabetic rats at a very early time point in the course of the disease (4 days). It is of interest that these alterations occurred even though animal weights and serum insulin levels revealed relatively minor effects due to diabetes. Therefore, it would appear from these parameters that the spontaneously diabetic animals employed in our study tended toward the stable diabetic syndrome, as opposed to the unstable ketotic syndrome of diabetes (Nakhooda et al., 1976).

It was unexpected that serum insulin levels in the BB diabetics, although reduced, were not significantly different from control (Table X). It should be pointed out that the insulin standard curve was becoming rather flat in the region where we were making determinations (between 20 and 35% bound) and therefore would have minimized differences within this range (Figure 13). Recently Dr. Ray Pederson's laboratory has measured insulin levels in serum samples from fasted and non-fasted BB diabetic rats which were part of ongoing studies in our laboratory. Fasted males had serum insulin values of 10.3 ± 0.8 μU/ml (n = 4) and females 13.5 ± 1.9 μU/ml (n = 3). Both groups were markedly hyperglycemic (634 ± 71 mg/100 ml and 668 ± 75, respectively). Nondiabetic inside control insulin values in this group ranged widely (8.6 to 47 μU/ml) with a mean value of 23.2 ± 8.4, as did the serum glucose values (117 to 400 mg/100 ml; mean, 205 ± 66). Non-fasted male BB diabetics had significantly lower serum insulin levels (p < 0.05) than nondiabetic inside controls (14.3 ± 2.2 μU/ml vs 33.3 ± 3.1) as did female BB
diabetics (17.9 ± 3.8 µU/ml vs 33.7 ± 4.7). These data suggest that the fasted serum insulin values which we obtained (Table XI) were slightly high but were still within the range observed for BB diabetic rats. Moreover, the insulin response of these animals was abnormal in view of the marked hyperglycemia which they exhibited. It was of interest that the fasted insulin levels in the nondiabetic controls were lower than those for the outside Wistar controls.

The pancreatic histology studies (hemotoxylin and eosin stain) showed the presence of islets in the BB animals. However, as a result of immunocytochemical studies in the BB diabetics, it was revealed that the islets that were present contained markedly reduced numbers of immunoreactive insulin cells, a reported characteristic of BB diabetic rats.

Tannenbaum et al. (1981) have monitored BB rats over the entire spectrum of the diabetic syndrome. In contrast to our findings, the stable diabetics in their study demonstrated significant hypoinsulinemia 1 to 5 days post detection, and exhibited pancreatic insulitis well in advance of detection. The difference between our observations and theirs is not unexpected in view of certain differences in experimental protocol. For instance, the BB diabetic animals we employed were those that had survived the stress of a long airflight. Such a preselection of "healthier" animals in our case was evident in the observed body weight ranges. The diabetic males in our study were approximately 100 g heavier than the animals monitored by Tannenbaum et al. (1981). In addition, the diabetic animals which we received had been maintained on insulin prior to shipment and then received insulin for at least one
more week in our laboratory. The diabetic rats in Tannenbaum's study never received insulin. It is apparent that there is a marked spectrum of severity of genetic diabetes, and it is important to monitor several parameters of the disease process when studying this model of diabetes.
C. Alterations in hepatic steroid binding proteins in diabetic rats

Using Scatchard analysis, high affinity $[^3H]$-estradiol binding in nondiabetic and diabetic male and female rat liver cytosol (50% AS fraction) exhibited $K_d$ values of between 6.4 and $7.4 \times 10^{-10}$ M. The capacity of the binding site in the nondiabetic male was 116 fmol/mg protein compared to 53 fmol/mg protein in the nondiabetic female, and 47 fmol/mg protein in the diabetic male (Figures 32 and 33). The phenomenon of reduced high affinity $[^3H]$-estradiol binding in male diabetic rat liver has not been previously reported. Furthermore, it was unexpected that high affinity binding capacity was higher in nondiabetic control males than in nondiabetic females. We also examined moderate affinity $[^3H]$-estradiol binding in male rat liver whole cytosol and found only a slight reduction in the capacity of this binding site in diabetics (Figures 34 and 35).

In addition, we analyzed this data using a nonlinear curve fitting program NONLIN (University of British Columbia Computing Centre). Our objectives in doing this were two-fold: 1) to verify the Scatchard plot-derived binding parameters we had previously obtained, and 2) to see if the data would fit a two site mathematical model of binding. However, we had assayed for high affinity $[^3H]$-estradiol binding in the 50% AS fraction and for moderate affinity binding in the whole cytosol fraction in order to obtain optimum conditions. The NONLIN program required that the data be obtained under identical experimental conditions. Future experiments therefore would have to establish the appropriate tissue fraction for analysis over the range of 0.5 to 200 nM $[^3H]$-estradiol concentration before nonlinear curve fitting could be performed adequately.
The 50% AS fraction would be the logical choice based on our preliminary experiments.

For the reasons just mentioned we did not obtain binding parameters using a 2 site model. However we did obtain $K_d$ and capacity values by analyzing single binding sites with the NONLIN program. The comparison of the Scatchard and NONLIN calculations is given in Table XIII. The NONLIN program used in this fashion, predictably overestimated high affinity binding capacity and $K_d$ in all the animal models because it could not account for the influence of the moderate affinity site. Moderate affinity binding in males was also overestimated most likely because at 200 nM $[^3H]$-estradiol ligand concentration, saturation was not achieved or was influenced by nonspecific binding sites. With the assay protocol we used, we concluded that Scatchard analysis provided the more accurate $K_d$ and capacity values. It was of interest that the NONLIN derivation showed BB diabetic males to exhibit $\sim50\%$ of control high affinity $[^3H]$-estradiol binding and also reduced moderate affinity binding; i.e. our conclusions remained the same.
Table XIII. Comparison of hepatic \([^{3}\text{H}]-\text{estradiol}\) binding data parameters derived by Scatchard analysis or by nonlinear curve fitting (NONLIN UBC). Experiments were performed on tissue pools of 2-4 rats. Capacity is expressed as fmol/mg protein or pmol/mg protein.

<table>
<thead>
<tr>
<th></th>
<th>MALE RAT LIVER</th>
<th>FEMALE RAT LIVER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC(^1)</td>
<td>BB</td>
</tr>
<tr>
<td>High affinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>binding(^2):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scatchard</td>
<td>(K_d: 7.7 \times 10^{-10} \text{ M})</td>
<td>(K_d: 6.4 \times 10^{-10} \text{ M})</td>
</tr>
<tr>
<td></td>
<td>capacity: 116 fmol/mg</td>
<td>capacity: 47 fmol/mg</td>
</tr>
<tr>
<td>NONLIN UBC</td>
<td>(K_d: 1.7 \times 10^{-9} \text{ M})</td>
<td>(K_d: 1.5 \times 10^{-9} \text{ M})</td>
</tr>
<tr>
<td></td>
<td>capacity: 176 fmol/mg</td>
<td>capacity: 77 fmol/mg</td>
</tr>
<tr>
<td>Moderate affinity binding(^3):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scatchard</td>
<td>(K_d: 1.1 \times 10^{-7} \text{ M})</td>
<td>(K_d: 1.3 \times 10^{-7} \text{ M})</td>
</tr>
<tr>
<td></td>
<td>capacity: 2.1 pmol/mg</td>
<td>capacity: 1.8 pmol/mg</td>
</tr>
<tr>
<td>NONLIN UBC</td>
<td>(K_d: 9.4 \times 10^{-7} \text{ M})</td>
<td>(K_d: 3.6 \times 10^{-7} \text{ M})</td>
</tr>
<tr>
<td></td>
<td>capacity: 12 pmol/mg</td>
<td>capacity: 4.1 pmol/mg</td>
</tr>
</tbody>
</table>

\(^1\) Abbreviations used: IC = Nondiabetic Inside Control
BB = Spontaneously BB Diabetic
ND = not detectable

\(^2\) Determined on the 50% ammonium sulfate fraction with \([^{3}\text{H}]-\text{estradiol}\) ligand concentrations ranging from 0.5 to 2.5 nM.

\(^3\) Determined on the whole cytosol fraction with \([^{3}\text{H}]-\text{estradiol}\) ligand concentrations ranging from 10-200 nM; estradiol was the competitor.
D. Speculation of what $[^3$H]-estradiol binding could be and how it could relate to modulation of hepatic drug metabolism

We observed two types of $[^3$H]-estradiol binding sites in rat liver cytosol, high affinity and moderate affinity sites. The high affinity sites appear to correspond to an estrogen receptor, having similar characteristics to that described by Eisenfeld et al. (1976, 1977a), Aten et al. (1978), Powell-Jones et al. (1976, 1980), and others (Chamness et al., 1975; Viladiu et al., 1975; Beers and Rosner, 1977). These sites exhibited a $K_d$ of $\approx 10^{-10}$ M and capacity of $\approx 100$ fmol/mg protein, were not markedly pH dependent, were stable to storage at -80°C for periods of time up to at least 2 months (Appendix III), bound only estrogenic steroids (estradiol, diethylstilbestrol, and moxestrol) and had similar characteristics in males and females, although the respective capacities were not always similar. As yet, we have not correlated the presence of this high affinity receptor to a physiological function, although Eisenfeld observed that presence of the receptor was associated with stimulation of hepatic synthesis of plasma renin substrate (Eisenfeld et al., 1976 and 1977b).

Moderate affinity $[^3$H]-estradiol binding sites have been referred to as "unusual sex-specific binding protein" (Dickson et al., 1978), male steroid binding protein (Powell-Jones et al., 1980), and "nonreceptor" binding protein (Powell-Jones et al., 1981b). The moderate affinity sites we quantitated possessed similar characteristics to those measured by these previous workers. We observed these sites, present only in male rat liver cytosol, to exhibit the following characteristics: a $K_d$ of $\approx 10^{-7}$ M and a capacity of $\approx 2$ to 5 pmol/mg protein, reduced steroid specificity (DHT and testosterone competed for binding.
sites as well as the estrogenic steroids, but not diethylstilbestrol; poor stability on storage at -80°C (Appendix III); and marked pH dependency. These data suggest that the moderate affinity binding sites may be an enzyme. We have investigated the possibility that various compounds in serum could account for moderate affinity sites, but when male and female rat serum was analyzed for these sites (10-200 nM ligand concentration) no binding was observed (Appendix III). As yet, no physiological function has been attributed to the presence of the moderate affinity binding protein in the liver.

It is of interest that recently two cytosolic \(^{3}H\)-estradiol binding sites have been reported to exist in rat uterus (Eriksson et al., 1978; Clark et al., 1978) and mouse mammary tumors (Watson and Clark, 1980). Type I sites represented the classical receptor sites \((K_d \approx 10^{-10} \text{ M and capacity } \approx 0.5 \text{ pmol/100 } \mu\text{g DNA})\). The cytoplasmic type II sites had an affinity for estradiol about forty times lower than that of the type I sites \((K_d \approx 3 \times 10^{-8} \text{ M})\) and a four-fold increased capacity. These sites were present in higher concentrations in target tissue cytosols (uterus and vagina) but were found at low levels in some other tissue cytosols (kidney and spleen) but not in hypothalamus or pituitary (Kelner and Peck, 1981). As opposed to the classical cytoplasmic receptor (type I) sites, which are translocated to the nucleus by the hormone, these type II sites remained in the cytoplasm. Their function is not known at present. It has been suggested that they may serve as a mechanism for concentrating estrogen in target cells, or may be intermediates in the formation of type I sites (Clark et al., 1978). Kelner and Peck (1981) concluded that the absence of type II
sites in pituitary and hypothalamus was consistent with the idea that type II sights may be involved with the trophic response of cells to estrogen (hypertrophy and hyperplasia).

The physiological significance of the different forms of $[^3H]$-estradiol binding sites in rat liver cytosol is at present not clear. We have been interested in the possible role they may have in determining levels of activity of various sex dependent monooxygenase enzymes. However, more data has to be compiled before we can define the relationship, if any, between hepatic steroid binding proteins and modulation of microsomal enzyme activity. The necessity of additional experiments is particularly relevant because some of the results we obtained were not what had been predicted. For instance, there was a trend toward a sex difference in the capacity of the high affinity $[^3H]$-estradiol binding sites, which was not anticipated. In several experiments, the capacity of binding was greater in males than in females (Table V; Figure 33 vs Figure 32; Figure 38 vs Figure 37). In addition, diabetic males exhibited a more marked alteration of high affinity than moderate affinity binding sites (Figure 33 vs Figure 34). We had predicted that changes in sex dependent drug metabolism would be more closely related to changes in the male moderate affinity binding site, but this was not observed. However, it must be pointed out that these experiments in nondiabetic and diabetic animals have to be repeated before we can verify these observations. We were not able to perform these essential experiments due to lack of availability of the BB rats.
E. Proposed future experiments

We observed that sex dependent drug metabolism was altered in diabetic rats, as was the apparent disposition of cytochrome P450 in these animals. We were interested in examining whether changes in hepatic steroid binding proteins could account for the diabetes-associated alterations in drug metabolism. We obtained preliminary evidence to suggest that high, and possibly moderate, affinity $[^3H]$-estradiol binding sites were altered in the diabetic state. However, further experiments must be performed before it would be possible to assess the precise effects of the binding proteins on the levels of cytochrome P450 and substrate specific drug metabolism in control and diabetic rats.

As discussed above, repeat experiments must be performed in order to verify the trends in the disposition of the binding proteins which we observed. The protocol in such experiments would have to include utilization of identical tissue fractions in the incubation conditions (0.05 to 200 nM $[^3H]$-estradiol). This would enable the data to be analyzed on a more sophisticated basis, e.g. using computerized nonlinear curve fitting programs. Data analyzed in this fashion could provide a mathematical basis for understanding the physiology of the various binding proteins in rat hepatic cytosol. If indeed there turned out to be a sex difference in the high affinity binding site, and if it were decreased in the diabetic state, then one could consider a role for this binding site in modulation of sex dependent drug metabolism. On the other hand, if it were verified that
the moderate affinity binding site was also altered in male diabetics, there could be a basis on which to hypothesize an interplay between the high and moderate affinity binding sites.

It would also be important to test if insulin replacement reversed the effects of diabetes on the binding proteins. Furthermore, it would be necessary to determine if insulin replacement corrected alterations in circulating testosterone and/or estrogen levels. It could be established during time course studies whether changes in the steroid levels either preceded or followed the insulin-associated changes in the binding proteins. Depending on what was observed, cause-and-effect relationships between hepatic steroid binding proteins, insulin levels, and steroid hormone levels could be established. Not until this point would it be possible to delineate the precise role of these factors in the modulation of sex dependent enzymes.
F. Summary and Conclusions

We observed that early onset spontaneous and chemical (STZ-induced) diabetes tended to exert similar influences on the hepatic drug metabolism system. The sex dependent metabolizing activity BP hydroxylase, was reduced in 4 day diabetic male rats to approximately 50% of control values. Serum testosterone levels were reduced in the BB diabetic males compared to nondiabetic controls levels (1.16 ± 0.44 ng/ml vs 2.99 ± 0.42). Microsomal testosterone Δ⁴ hydrogenase, also a sex dependent activity, was not different from control in diabetic males, but was reduced in diabetic females by approximately 30%. The sex independent enzyme activity aniline hydroxylase was increased in both male and female diabetics. Electrophoretic studies of male and female diabetic microsomal proteins showed that the disposition of cytochromes P-450 was altered in the diabetic state. Changes in drug metabolism parameters were reversed with insulin replacement.

Preliminary experiments showed that high affinity (K_d ∼ 10⁻¹⁰) [³H]-estradiol binding capacity was reduced by approximately 50% in spontaneously diabetic male rats. Moderate affinity binding sites (K_d ∼ 10⁻⁷ M) were only slightly reduced in male diabetics. Whereas, there was a sex difference noted with respect to the presence of moderate affinity binding (it was not detectable in females), the data were not conclusive as to a possible sex difference in the binding capacity of the high affinity sites between male and female rats.
We conclude the following:

1. Spontaneous and chemically-induced (STZ) diabetes produced similar alterations in hepatic drug metabolism.

2. Diabetes-associated changes in hepatic drug metabolism occurred at early time points (4 days) and were reversible by insulin replacement therapy.

3. Alterations in hepatic drug metabolism were accompanied by changes in the relative disposition of electrophoretically separated microsomal (cytochrome P-450) proteins in diabetic rats.

4. The diabetes-associated changes in hepatic drug metabolism were not solely due to changes in circulating androgen levels.

5. Two types of $[^3\text{H}]$-estradiol binding sites were detected in rat liver cytosol, high affinity and moderate affinity sites. The high affinity sites probably corresponded to an estrogen receptor, ($K_d \approx 10^{-10} \text{ M}$), while the moderate affinity sites ($K_d \approx 10^{-7} \text{ M}$) appeared to have characteristics of an enzyme.

6. Preliminary evidence indicated that high affinity $[^3\text{H}]$-estradiol binding sites were reduced by 50% in male diabetics, and moderate affinity sites were only slightly reduced. However, additional determinations are necessary before this trend can be verified.

7. It was not possible to establish exact relationships between high and moderate affinity $[^3\text{H}]$-estradiol binding sites and modulation of hepatic drug metabolism.
REFERENCES


Al-Turk, W., Stohs, S., Roche, E.B., Drug Metab. Dispos. 8: 44-45, 1980c.


APPENDIX I - Hepatic testosterone $\Delta^4$ hydrogenase and aniline hydroxylase activities in spontaneous and streptozotocin diabetic rats. Assays were carried out by Dr. R. Pak, L.S. Gontovnick, M.J. Finlayson, and B. Warren

A. METHODS

1. Testosterone $\Delta^4$ hydrogenase assay

Hepatic testosterone $\Delta^4$ hydrogenase activity was measured colorimetrically by the method of McGuire and Tomkins (1959). The final volume of the incubation mixture was 2.0 ml. It contained 12.5 $\mu$mol MgCl$_2$·6H$_2$O, 1.0 $\mu$mol NADP, 20.0 $\mu$mol glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 0.5 $\mu$mol testosterone, 200 $\mu$l of enzyme suspension, and 1.72 ml phosphate buffer pH 7.2. The incubation was carried out for 10 minutes at 37°C and was terminated with dichloromethane. The aqueous phase was aspirated and the absorption at 240 nm was measured. The decrease in absorption compared to testosterone blank was related to the amount of testosterone metabolized. Approximately 0.5-1.5 mg of microsomal protein was added to the incubation mixture. Activity is expressed as nmol testosterone metabolized per minute per mg protein.

2. Aniline hydroxylase assay

Hepatic aniline hydroxylase activity was measured colorimetrically by the method of Imai, Ito, and Sato (1966). The final volume

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of the incubation mixture was 2.0 ml. It contained 2.5 μmol aniline, 0.25 ml phosphate buffer 0.1 M pH 7.2, 0.25 ml enzyme preparation, 2.5 μmol NADP, 5 μmol glucose-6-phosphate, 2.5 units glucose-6-phosphate dehydrogenase, 12.5 μmol MgCl₂·6H₂O, and 0.94 ml phosphate buffer pH 7.5. The incubation was carried out for 15 minutes at 37°C and was stopped with 1.0 ml 20% trichloroacetic acid. The samples were centrifuged to separate the precipitate. To a 1.0 ml aliquot of the supernatant 0.5 ml of 10% Na₂ CO₃ was added, followed by 1.0 ml 2% phenol in 0.2 N NaOH. Color development was measured at 640 nm after incubation for 30 minutes at 37°C. Approximately 1.5-3.0 mg of microsomal protein was added to the incubation mixture. Activity is expressed as ng p-aminophenol produced per minute per mg protein.

B. RESULTS

1. Testosterone Δ⁴ hydrogenase assay

We observed the expected sex difference in testosterone Δ⁴ hydrogenase activity between control females and male rats, where female levels were approximately 4 times greater than male levels(Figure 43 vs Figure 44). In female rats, both spontaneous (BB) and STZ diabetes led to a significant decrease in testosterone Δ⁴ hydrogenase activity; whereas in males, diabetes had no effect. Interestingly, the pattern
Figure 43.

Hepatic testosterone Δ⁴ hydrogenase activity in control and 4 day diabetic female rats. Results are expressed as mean ± SEM.

OC: outside Wistar control
IC: inside nondiabetic control
BB: spontaneously diabetic
C: Wistar citrate control
STZ: streptozotocin-induced diabetic

* Significantly different from inside nondiabetic control at p<0.05 or significantly different from citrate control at p<0.05.
Figure 44.

Hepatic testosterone $\Delta^4$ hydrogenase activity in control and 4 day diabetic male rats. Results are expressed as mean ± SEM. Abbreviations are as for Figure 43.
TESTOSTERONE $\Delta^4$ HYDROGENASE ACTIVITY
(nmol/min/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>MALES</th>
</tr>
</thead>
<tbody>
<tr>
<td>OC IC</td>
<td>24</td>
</tr>
<tr>
<td>BB</td>
<td>18</td>
</tr>
<tr>
<td>STZ</td>
<td>12</td>
</tr>
</tbody>
</table>

0  5  10  15
was the reverse to that observed with BP hydroxylase activity, which was decreased in male diabetics and unaltered in female diabetics. Also in contrast to the pattern observed with BP hydroxylase, there was no difference between regular Wistar controls (OC) and nondiabetic littermate controls (IC).

2. Aniline hydroxylase assay

As has been reported previously (Table I) we observed the sex independent activity, aniline hydroxylase, to be significantly increased over control in chemically induced female and male diabetic rats (Figures 45 and 46). In addition, we found that the same effect was apparent in BB spontaneously diabetic rats. In this instance there was no difference between the non-littermate (OC) and littermate controls (IC).

3. Effect of insulin replacement on enzyme activities

BB diabetic rats were maintained on insulin therapy until 18 hours before they were sacrificed. It was observed that insulin
Figure 45.

Hepatic aniline hydroxylase activity in control and 4 day diabetic female rats. Results are expressed as mean ± SEM. Abbreviations are as for Figure 43.

*Significantly different from inside nondiabetic control at p<0.05 or significantly different from citrate control at p<0.05.
Figure 46.

Hepatic aniline hydroxylase activity in control and 4 day diabetic male rats. Results are expressed as mean ± SEM. Abbreviations are as for Figure 43.

* Significantly different from inside nondiabetic control at p<0.05 or significantly different from citrate control at p<0.05.
maintenance reversed the effects of spontaneous diabetes on testosterone \( \Delta^4 \) hydrogenase and aniline hydroxylase activities (Figures 47 and 48). That in insulin treated BB females (Figure 47) testosterone \( \Delta^4 \) hydrogenase activity appeared slightly higher than control levels (IC) was probably a reflection of the different sample sizes \( n = 6 \) vs \( 22 \) rather than a stimulatory effect of insulin.
Figure 47.

Effect of insulin (INS) replacement on hepatic testosterone $\Delta^4$ hydrogenase activity in spontaneously diabetic (BB) female and male rats. BB diabetics were 4 days post insulin withdrawal; insulin-treated diabetics received insulin for those 4 days.

IC: Inside non-diabetic control

* Significantly different from inside nondiabetic control at $p<0.05$.

** Significantly different from untreated BB diabetic at $p<0.05$. 
TESTOSTERONE Δ^4 HYDROGENASE ACTIVITY

(nmol/mg protein)

TESTOSTERONE Δ^4 HYDROGENASE ACTIVITY

(nmol/min/mg protein)

Males

<table>
<thead>
<tr>
<th>Group</th>
<th>Activity (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>24</td>
</tr>
<tr>
<td>BB</td>
<td>18</td>
</tr>
<tr>
<td>BB+NS</td>
<td>8</td>
</tr>
</tbody>
</table>

Females

<table>
<thead>
<tr>
<th>Group</th>
<th>Activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC</td>
<td>22</td>
</tr>
<tr>
<td>BB</td>
<td>18 (*</td>
</tr>
<tr>
<td>BB+NS</td>
<td>6    (*</td>
</tr>
</tbody>
</table>
Figure 48.

Effect of insulin (INS) replacement on hepatic aniline hydroxylase activity in spontaneously diabetic (BB) female and male rats. BB diabetics were 4 days post insulin withdrawal; insulin-treated diabetics received insulin for those 4 days.

IC: inside nondiabetic control

* Significantly different from inside nondiabetic control at p<0.05.

* Significantly different from untreated BB diabetic at p<0.05.
APPENDIX II - Immunocytochemistry study of spontaneously diabetic rat pancreatic islets

*Work carried out by Dr. Alison Buchan, Department of Physiology, UBC.

Samples of pancreatic tissue were collected and fixed in Bouin's solution for 2 hours at room temperature, dehydrated and embedded in paraffin wax. Serial 5 micron sections were cut and immunostained by the peroxidase antiperoxidase method\(^1\) with antisera to insulin (1:2000). Some sections were exposed to somatostatin (1:1000) or pancreatic glucagon (1:1000) antisera. After development of the peroxidase reaction with diaminobenzidine, the sections were counterstained with conventional hemotoxylin and eosin. Insulin-containing cells stained black and were easily visible under light microscopy. Using this technique, it was possible to compare the hemotoxylin and eosin and anti-insulin stains on the same slide. In pancreatic sections obtained from nondiabetic control animals and stained with hemotoxylin and eosin, islets were visible (although they were not quantitated). The anti-insulin stain showed the usual central location of insulin containing cells (Figure 49). Somatostatin cells and glucagon cells ringed the islet (not shown). BB diabetic rats that had been withdrawn from insulin 4 days prior to sacrifice exhibited islets on hemotoxylin and eosin stain but there was evidence of lymphocytic infiltrate in them and there were fewer islets than there were in nondiabetic controls. A few insulin containing cells could be visualized with

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Figure 49.

the anti-insulin stain, but there were fewer immunoreactive insulin cells than in the nondiabetics (Figure 50). There was also a reduction in glucagon and somatostain containing cells in the BB diabetic islets (not shown).
Figure 50.

Photomicrograph of an immunocytochemically stained pancreatic islet from a spontaneously diabetic rat. Anti-insulin stain. Hemotoxylin and eosin counterstain. The animal had been withdrawn from insulin maintenance 4 days previously. Magnification: X16.
APPENDIX III - Additional studies of high and moderate affinity $[^3H]$-estradiol binding in rat liver cytosol

Assays were performed by B. Warren.

The data contained in this appendix were collected in ongoing studies in our laboratory, and are included here for the sake of completeness.

1. The effect of freezing on the stability of $[^3H]$-estradiol binding sites

Identical pools of male and female rat liver were prepared and stored for various time periods at -80°C. Assays were carried out as described in the Methods section. High affinity $[^3H]$-estradiol binding sites were stable to freezing for at least up to 4 weeks. Moderate affinity sites were stable for 1 week (Table XIV).

2. $[^3H]$-Estradiol binding in male and female rat serum

We performed Scatchard analysis over the entire concentration range (0.10-200 nM) on pools of male and female rat serum to see if any binding components could be detected. The assays were performed in the usual manner except rat serum (~6 mg/ml) was included in the incubation mixture instead of liver cytosol. We did not detect any appreciable specific (total - nonspecific) $[^3H]$-estradiol binding in rat serum in the concentration ranges tested (Figure 51).
Table XIV. The effect of freezing (-80°C) on [3H]-estradiol binding sites in male and female rat liver cytosol 30% ammonium sulfate fraction. Assays were performed according to the protocols described in the Methods section. Determinations were made on similar tissue pools.

<table>
<thead>
<tr>
<th>Time period (weeks)</th>
<th>High affinity sites</th>
<th>Moderate affinity sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>0</td>
<td>$K_d: 4.2 \times 10^{-10}$ M&lt;sup&gt;1&lt;/sup&gt;</td>
<td>$K_d: 7.0 \times 10^{-10}$ M</td>
</tr>
<tr>
<td></td>
<td>capacity: 47 fmol/mg protein</td>
<td>capacity: 203 fmol/mg protein</td>
</tr>
<tr>
<td>1 week</td>
<td>$K_d: 9.4 \times 10^{-10}$ M</td>
<td>$K_d: 17 \times 10^{-10}$ M</td>
</tr>
<tr>
<td></td>
<td>capacity: 68 fmol/mg protein</td>
<td>capacity: 200 fmol/mg protein</td>
</tr>
<tr>
<td>2 weeks</td>
<td>$K_d: 6.9 \times 10^{-10}$ M</td>
<td>$K_d: 12.5 \times 10^{-10}$ M</td>
</tr>
<tr>
<td></td>
<td>capacity: 96 fmol/mg protein</td>
<td>capacity: 141 fmol/mg protein</td>
</tr>
<tr>
<td>4 weeks</td>
<td>$K_d: 5.8 \times 10^{-10}$ M</td>
<td>$K_d: 8.6 \times 10^{-10}$ M</td>
</tr>
<tr>
<td></td>
<td>capacity: 102 fmol/mg protein</td>
<td>capacity: 89 fmol/mg protein</td>
</tr>
</tbody>
</table>

<sup>1</sup> Binding parameters were obtained by Scatchard analysis.
Figure 51.

High (A) and moderate (B) affinity $[^{3}\text{H}]-\text{estradiol}$ binding in male and female rat serum. Assays were run as described in Methods for liver cytosol, except 0.50 ml serum (6 mg/ml) was added to the incubation mixture at pH 8.8.
3. Effect of incubation time on moderate affinity $[^3H]$-estradiol binding in male and female rat liver cytosol (pH 8.8)

Tissue pools were prepared from 3 rats of each sex. $[^3H]$-estradiol binding over the concentration range 10-100 nM was determined at 1.5 and 24 hours incubation time (4°C) according to the protocols described in the Methods section except the buffer was pH 8.8. The binding curves are shown in Figure 52. Whereas at 24 hours of incubation, moderate affinity binding was markedly increased in females, it was decreased in males.

4. High affinity $[^3H]$-estradiol binding in control and streptozotocin (STZ) diabetic male rats

One month after injection (citrate buffer or STZ) control and diabetic male rat liver cytosol was analyzed for high affinity binding sites. The assays were performed as described in the Methods section, except the buffer was pH 8.8. High affinity $[^3H]$-estradiol binding was reduced in the diabetic animals (50% of that observed in citrate-injected controls). (Table XV).

5. The effect of spontaneous diabetes on high affinity $[^3H]$-estradiol binding in rat uterine cytosol

Uteri from nondiabetic controls and 4 day (post insulin withdrawal) diabetic rats were frozen in liquid nitrogen and stored
Figure 52.

The effect of incubation time (1.5 vs 24 hours) on moderate affinity \(^{3}\text{H}\)-estradiol binding at pH 8.8 in male and female rat liver cytosol. Conditions were as follows: 10 to 100 nM \(^{3}\text{H}\)-estradiol concentration; ~3 mg/ml protein; incubation carried out in absence and presence of 100-fold excess estradiol for 1.5 hours at 4°C. Specific binding (cpm) was corrected for protein content. Data was obtained on tissue pools (n = 3).
Table XV. Effect of streptozotocin-induced (STZ) diabetes on high affinity $[^3H]$-estradiol binding in male rats. Experiments were carried out 1 month post injection with either buffer (0.1 M citrate pH 4.5) or STZ (60 mg/kg intravenously). Assay protocol was as described in the Methods section, except pH 8.8 was used. Data are from single animals.

<table>
<thead>
<tr>
<th>K_d (M)</th>
<th>Capacity (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$8.2 \times 10^{-10}$</td>
<td>120</td>
</tr>
<tr>
<td>$13.4 \times 10^{-10}$</td>
<td>96</td>
</tr>
<tr>
<td>$3.8 \times 10^{-10}$</td>
<td>48</td>
</tr>
<tr>
<td>$6.2 \times 10^{-10}$</td>
<td>64</td>
</tr>
</tbody>
</table>

$^1$ Parameters were obtained by Scatchard analysis.
at -80°C for 6 to 10 days. Tissues were pooled (4 uteri per pool) and assayed as for liver cytosol (Methods section) except pH was 8.8. Diabetes of short duration had no effect on high affinity binding sites in rat uteri (nondiabetic control $K_d$: $2.9 \times 10^{-10}$ M and capacity 849 fmol/mg protein; and BB diabetic $K_d$: $3.4 \times 10^{-10}$ M and capacity 1037 fmol/mg protein).

6. High and moderate affinity [$^3$H]-estradiol binding in hypophysectomized (HYPO-X) and pseudohermaphroditic (Pseudo) rats

We examined [$^3$H]-estradiol binding in hepatic cytosol from HYPO-X and Pseudo rats (Tables XVI and XVII). Assays were performed as described in the Methods section, except pH was 8.8. High affinity sites were reduced in HYPO-X females and males compared to control. Binding data in the Pseudo experiment was highly variable.
Table XVI. $[\text{H}]-\text{Estradiol}$ binding in sham-operated and hypophysectomized rats. Binding parameters were obtained by Scatchard analysis. Assays were performed as described in the Methods section, except pH was 8.8. Results are for a single experiment.

<table>
<thead>
<tr>
<th></th>
<th>High affinity binding</th>
<th>Moderate affinity binding</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control female</strong></td>
<td>$K_d$: $4 \times 10^{-10}$ M</td>
<td>$K_d$: $0.2 \times 10^{-7}$ M</td>
</tr>
<tr>
<td>(sham-operated)</td>
<td>capacity: 72 fmol/mg protein</td>
<td>capacity: 0.55 pmol/mg protein</td>
</tr>
<tr>
<td><strong>HYPO-X female</strong></td>
<td>$K_d$: $2.2 \times 10^{-10}$ M</td>
<td>not detectable</td>
</tr>
<tr>
<td></td>
<td>capacity: 7.4 fmol/mg protein</td>
<td></td>
</tr>
<tr>
<td><strong>Control male</strong></td>
<td>$K_d$: $3.2 \times 10^{-9}$ M</td>
<td>displacement but no Scatchard</td>
</tr>
<tr>
<td>(sham-operated)</td>
<td>capacity: 270 fmol/mg</td>
<td></td>
</tr>
<tr>
<td><strong>HYPO-X male</strong></td>
<td>$K_d$: $0.51 \times 10^{-9}$ M</td>
<td>displacement but no Scatchard</td>
</tr>
<tr>
<td></td>
<td>capacity: 24 fmol/mg</td>
<td></td>
</tr>
</tbody>
</table>

1 HYPO-X = hypophysectomized
Table XVII. [3H]-Estradiol binding in control and pseudohermaphroditic (Pseudo) rats. Binding parameters were obtained by Scatchard analysis. Assays were performed as described in the Methods section, except pH was 8.8. Results are for a single experiment.

<table>
<thead>
<tr>
<th></th>
<th>High affinity binding</th>
<th>Moderate affinity binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control male</td>
<td>$K_d: 1.1 \times 10^{-9}$ M</td>
<td>displacement but no Scatchard</td>
</tr>
<tr>
<td></td>
<td>capacity: 234 fmol/mg protein</td>
<td></td>
</tr>
<tr>
<td>Control female</td>
<td>$K_d: 1.3 \times 10^{-9}$ M</td>
<td>displacement but no Scatchard</td>
</tr>
<tr>
<td></td>
<td>capacity: 381 fmol/mg protein</td>
<td></td>
</tr>
<tr>
<td>Pseudo</td>
<td>$K_d: 4.0 \times 10^{-9}$ M</td>
<td>$K_d: 0.12 \times 10^{-7}$ M</td>
</tr>
<tr>
<td></td>
<td>capacity: 1182 fmol/mg protein</td>
<td>capacity: 0.12 pmol/mg protein</td>
</tr>
<tr>
<td>Pseudo</td>
<td>$K_d: 0.67 \times 10^{-9}$ M</td>
<td>$K_d: 1.6 \times 10^{-7}$ M</td>
</tr>
<tr>
<td></td>
<td>capacity: 208 fmol/mg protein</td>
<td>capacity: 1.2 pmol/mg protein</td>
</tr>
</tbody>
</table>
PUBLICATIONS:


ABSTRACTS

Pharmacologist 21: 262, 1979. Abstract #615