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THE EFFECT OF STREPTOZOTOCIN-INDUCED DIABETES ON THE MALE WISTAR RAT HEPATIC HIGH CAPACITY, LOW AFFINITY ESTROGEN BINDING PROTEIN

by

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ABSTRACT

Several parallels have been noted between aryl hydrocarbon hydroxylase (AHH) activity (a measure of drug and steroid metabolism) and the high capacity low affinity (HCLA) estrogen binding protein. These include a sex and age dependency, as well as parallel changes in the AHH and HCLA levels due to various physiological manipulations (e.g. gonadectomy and hypophysectomy). It has been suggested from this evidence that there is a regulatory action of the HCLA estrogen binding protein on hepatic AHH activity. Since streptozotocin (STZ) induced diabetes is known to cause alterations in AHH activity we investigated the effects of this condition on the HCLA estrogen binding protein, and sought the hormonal control mechanism for the HCLA estrogen binding protein in this state.

At both four and ten days post-induction of diabetes with STZ, there was approximately a 50% decrease in the binding capacity of the HCLA estrogen binding protein, with no alterations in the Kd value. Hormonal replacement was undertaken to restore the normal physiological levels of testosterone, insulin, triiodothyronine, and growth hormone; all of which are depressed in the diabetic. None of the treatment regimens carried out were able to restore the reduced binding capacity of the HCLA binding protein. Treatment with testosterone and insulin had previously been shown to restore AHH activity in gonadectomized and diabetic

rats, respectively. Since we were unable to restore HCLA binding protein levels with these treatments in the diabetic rat, we conclude that there is no direct regulatory action of the HCLA binding protein on AHH activity in the rat.

Several other species were also examined for the presence of a hepatic HCLA estrogen binding protein. We were unable to detect any such component in any of the other species examined, indicating that the HCLA binding protein may be rat specific. What physiological role the HCLA binding protein may be playing remains unclear at this time.

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Supervisor

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LIST OF ABBREVIATIONS

AHH	aryl hydrocarbon hydroxylase
BB	bio breeding
Bmax	maximum binding capacity
BSA	bovine serum albumin
cpm	counts per minute
DCC	dextran coated charcoal
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
estradiol	$\Delta^{1,3,5(10)}$-estratrien-3,17β-diol
F	free steroid concentration
GH	growth hormone
HCLA	high capacity, low affinity
i.v.	intravenous
Kd	equilibrium dissociaton constant
NSB	nonspecific binding
pmol/mg	picomoles per milligram
PZI	protamine zinc insulin
SB	specific binding
s.c.	subcutaneous
STZ	streptozotocin
T_3	3,3,5-triiodothyronine
T.C.D.D.	2,3,7,8-tetracholorodibenzo-p-dioxin
TED	Tris base, ethylenediamine tetraacetic acid, dithiothreitol, sodium molybdate, glycrerol

TES	testosterone
testosterone	Δ^4-androsten-17β-ol-3-one
To.I	Toronto regular insulin

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INTRODUCTION

1) Diabetes mellitus

Diabetes mellitus is a disease characterized by excess glucose in the blood and urine. Its prevalence in North America has been estimated at 2% of the population. Complications of diabetes include: atherosclerosis; coronary artery disease, which is the leading cause of death amongst middle aged diabetics; peripheral vascular disease; hypertension; cerebrovascular disease; visual disorders, such as glaucoma, cataracts, retinopathy, and blindness; renal disease; proteinurea; peripheral neuropathy; complications during pregnancy, such as excessive birth weights and toxemia. Even though diabetes was identified as a disease entity as early as 1500 B.C. in Egypt, it is only very recently that the etiology has been realized to be multifactorial. There appear to be several steps in the development of diabetes: a genetic predispostion (probably two genes on chromosome 6), in combination with the influence of environmental agents (such as cell toxins or viruses) which act as amplifying factors, leading to direct β-cell destruction *via* an autoimmune mechanism and/or the lack of β-cell regeneration after injury. Thus diabetes is not actually one disease but a group of syndromes. It reflects a variety of genetic and environmental causes and amplifying factors leading to metabolic and tissue changes

as chronic complications secondary to insufficient insulin activity (Fajans et al., 1978). The prevalence of diabetes and the many manisfestations which it can take in the clinical picture make it one of the leading health concerns today.

Research into diabetes has led to many different animal models of diabetes being available (for reviews see Mordes and Rossini, 1981; Grodsky et al., 1982; Like, 1985) but since no one model is capable of precisely corresponding to any type of human diabetes the model should be used to examine specific phenotypic, pathologic, or genotypic expressions of the disease. There are a number of animal models available which spontaneously develop diabetes. These include: C57BL/6J mice, Zucker and BB rats, the Chinese hamster, New Zealand white rabbits, the Keeshound dog, and the Celebes black ape. These models all vary in the severity and pathogenesis of the diabetes they develop and not all animals in the colony will become diabetic. Due to difficulties which can arise in trying to maintain a colony of spontaneously diabetic animals (or in trying to obtain them from others) much research has been done to create chemical or biological methods which will create a diabetic state in research animals. The main methods which have been developed center on the use of cytotoxic chemicals.

The first diabetogenic agent used for research was alloxan. It was widely used as the classical diabetogen

until streptozotocin (STZ) was introduced in 1959 (Vavra et al., 1960). Studies using STZ to induce diabetes, quickly showed that it produced a lower mortality rate than alloxan (Hoftiezer and Carpenter, 1973), it had a lower general toxicity, and the characteristic lesions (i.e. β-cell toxicity) were more reproducible (Arison et al., 1967; Junod et al., 1969). It was possible to produce a series of diabetic states of graded severity by varying the dose (Junod et al., 1969). Most importantly, the changes in tissue metabolic patterns more closely resemble those seen in human diabetes mellitus (Mansford and Opie, 1968) and the lesions produced by STZ most clearly resemble those seen in the insulitis of human diabetes mellitus (Kloppel, 1985). These factors give weight to the validity of STZ-induced diabetes as a relevant model for the disease. Due to these factors, alloxan has largely been replaced by STZ as the main diabetogenic agent for experimentally induced diabetes mellitus, except in strains highly resistant to STZ, such as the rabbit.

The diabetogenic action of STZ is due to its selective destruction of the pancreatic β-cells (Mordes et al., 1981). The diabetic state induced by STZ is marked by hyperglycemia, hypoinsulinemia, hyperlipemia, hyperketonemia, and decreased growth and body weight (Montoya et al., 1974; Chen et al., 1982). There is an inverse relationship between sensitivity to, and the severity of, the diabetogenic properties of STZ and the age

of the animal (Masiello et al., 1979). There is also evidence that the male animal is more senstive than the female in both single dose (MacLeren et al., 1980) and multiple dose regimens (Rossini et al., 1978).

Besides producing a diabetic state, STZ affects hormone supply and secretion. There is a decrease in insulin due to β-cell destruction. Serum testosterone is significantly reduced in STZ-treated male animals (Baxter et al., 1981) to levels similar to those seen in spontaneously diabetic male rats (Warren et al., 1983), while STZ-treated female rats show an increase in serum testosterone to normal male levels (Leaming et al., 1982). This testosterone increase is mainly of adrenal origin and correlates with the severity of ketosis. Thyroid dysfunction is also reported to be due to a decrease in the release of hypothalamic thyrotropin releasing hormone which causes pituitary and thyroid alterations. As well, the plasma levels of thyroxin, 3,3,5-triiodothyronine, and 3,3-diiodothyronine fall, the plasma level of 3,5-diiodothyronine increases and there is a decrease in the conversion of thyroxin to 3,3,5-triiodothyronine (Mitsuma et al., 1982; Jennings, 1984; Ortiz-Caro et al., 1984). A decrease in the release of growth hormone is seen due to deficient synthesis and storage (Bluet-Pajot et al., 1984). However, Tannenbaum (1981) measured normal levels of growth hormone in the pituitary, thus pointing to growth hormone release as the deficiency. The release pattern of growth hormone in the

male diabetic rat is also changed from its normal 'peak and trough' (ultradiene) release to the female 'continuous level' release pattern (Tannenbaum and Martin, 1976; Tannenbaum, 1981). This alteration in release patterns seems to be due to increased levels of somatostatin in the STZ-diabetic animal (Tannenbaum, 1981). Decreases in the circulating levels of luitenizing hormone and prolactin are also seen (Perez-Diaz et al., 1982).

STZ also has direct effects on the liver. It produces degranulation of the rough endoplasmic reticulum and mitochondrial swelling with the loss of cristea, independent of whether or not diabetes is produced (Languens et al., 1980). Its also been reported that other hepatic changes include decreased weight, glycogen concentration, citric acid content, and increased acetyl coA content, proteins, and DNA phospholipid P concentration (Montoya and Herrera, 1974). Warren et al. (1983) reported alterations in the protein distribution in the cytochrome P-450 region. These alterations (increases in some bands and decreases in others) have also been seen by other authors (Reinke et al., 1978; Stohs et al., 1979; Past and Cook, 1980; Peng et al., 1983). Overall, the effect was to make drug metabolism less differentiated between the sexes. Insulin treatment was able to reverse the biochemical and metabolic abnormalities seen (Reinke et al., 1978; Reinke et al., 1979). These changes in drug metabolism were similar in the STZ-induced diabetic animal and the spontaneously

diabetic animals (Warren et al., 1983).

2) Hepatic estrogen binding proteins

a) Estrogen receptor

The first estrogen binding protein to be found in the rat liver was the estrogen receptor. This receptor showed physical properties and binding characteristics which were identical to the 'classical' estrogen receptor found in the uterus: sedimentation in the 8-9 S region on sucrose density gradients; specificity for steroidal and non-steroidal estrogens; high affinity (10^{-9}M) and low capacity (fmol/mg) for estrogens; and a similar isoelectric focusing profile (Chamness et al., 1975; Viladiu et al., 1975; Eisenfeld et al., 1976; Beers and Rosner, 1977; Wrange et al., 1980). The estrogen receptor has also been found in other tissues (e.g. kidney, heart) but at lower levels than in the liver or uterus (Eisenfeld et al., 1977; Singletary et al., 1982). The estrogen receptor has also been found in the liver of both sexes of other species such as the rabbit, green monkey (Eisenfeld et al., 1977), and human (Duffy and Duffy, 1978).

The hepatic estrogen receptor is found in similar levels in both male and female rats (Powell-Jones et al., 1980; Thompson et al., 1981) and reaches its highest level in the post-pubertal animal (Rumbaugh et al., 1983). The hepatic estrogen receptor in both sexes is under

multihormonal regulation. Gonadectomy causes an increase in the levels of receptor in both male and female rats (Beers and Rosner, 1977; Eagon et al., 1980; Powell-Jones et al., 1981), while hypophysectomy causes a decrease in receptor levels in both sexes (Norstedt et al., 1981 a; Eriksson, 1982), as does adrenalectomy (Norstedt et al., 1981 a). The evidence for the regulatory mechanism of the hepatic estrogen receptor points towards glucocorticoids in combination with growth hormone and prolactin as the probable agents (Chamness et al., 1975; Norstedt et al., 1981 a; Gustafsson et al., 1983).

The hepatic estrogen receptor in the cytosol has been demonstrated to undergo nuclear translocation where it complexes with chromatin after it has complexed with an estrogenic ligand. This occurs in both males and females, but in males the translocation process is slower and nuclear retention longer (Eisenfeld et al., 1976; Aten et al., 1978; Aten et al., 1980; Dickson and Eisenfeld, 1980). The levels of estrogen receptor have been correlated with increases in renin substrate (Eisenfeld et al., 1976), plasma levels of very low density lipoproteins (Thompson et al., 1983), and an increase in the levels of the specific mRNA sequence for apo very low density lipoprotein-II in avian liver (Snow et al., 1978). Recently debate has arisen as to whether the estrogen receptor is actually present in the cytosol or if it truly resides in the nucleus or on the nuclear membrane (i.e. is the accepted model of

receptor-ligand complexing in the cytosol then translocating to the nucleus to bind with chromatin based on an artifact of experimental procedure) (Gorski et al., 1984; King and Greene, 1984; Schrader, 1984). This is an important question to answer, but either way it will not change the end result that estrogens do elicit a series of responses from the liver which are mediated by estrogen receptors. Not all of the hepatic responses seen due to estrogens can be accounted for by estrogen receptor-ligand complexes interacting with chromatin (Tamulevicius et al., 1982; Lax et al., 1983). It has been found that estrogens also bind with receptors that are associated with the plasma membrane (Pietras and Szego, 1979; 1980), lysosomal membranes (Hirsch and Szego, 1974), and microsomal membranes (Blyth et al., 1971; Helton et al., 1977; Yamada and Miyaji, 1982). Binding to these membranes could cause effects by altering intracellular Ca^{2+} concentrations and distribution, or rearranging some biologically important components of the membrane or microsomal system.

b) High capacity, low affinity binding protein

Besides the estrogen receptor there is a second class of estrogen binding protein in male rat liver which shall be referred to as the high capacity, low affinity (HCLA) binding protein.

The HCLA binding protein has been reported by several groups to have binding characteristics which show lower

affinity and higher capacity than those seen with receptor systems. Values which have been reported are: $Kd=10^{-7}$-$10^{-8}M$, binding capacity 10^{-12} mol/mg protein (Eagon et al., 1980); $Kd=10^{-7}$-$10^{-8}M$, binding capacity 10^{-13} mol/mg protein (Rogerson and Eagon, 1984); $Kd=10^{-7}M$, binding capacity 10^{-15} mol/mg protein (Dickson et al., 1978); $Kd=10^{-8}M$, binding capacity 10^{-11} mol/mg protein (Smirnov et al., 1977); $Kd=10^{-7}M$, binding capacity 10^{-12} mol/mg protein (Warren, 1982; Finlayson, 1983). Differences in the values reported may be due to the various methodologies used to measure the protein (e.g. sucrose density gradients, gel filtration, single point analysis, Scatchard analysis of multi-point competitive binding curves), differences in the buffers used to prepare the cytosol, and in the analysis of the data obtained. The HCLA binding protein sediments in the 3-4 S region of sucrose density gradients (Smirnov et al., 1977; Eagon et al., 1980; Powell-Jones et al., 1980) as compared to the estrogen receptor which sediments in the 8-9 S region (Eagon et al., 1980). The HCLA binding protein also shows up as a unique peak in gel filtration which is not seen in the analysis of cytosol from adult female or prepubescent male and female rats (Dickson et al., 1978; Eagon et al., 1980; Thompson et al., 1981). The HCLA binding protein is capable of binding estrogens and some androgens with the affinity being highest for steroidal estrogens (estriol, estradiol, estrone) and approximately 10-20 times less for

the androgens which bind (2α-hydroxytestosterone, 5α-androstane-3α,17β-diol, 5α-dihydrotestosterone) (Miroshnechenko et al., 1983; Rogerson and Eagon, 1984). The important functional groups for binding (for both estrogens and androgens) are the 3-α and 17-β hydroxy groups, removal of which sharply decreases the binding affinity of the steroid (Miroshnechenko et al., 1983).

The levels of the HCLA binding protein are much higher in the mature male than in the mature female. Thompson et al. (1981) reported a ten-fold difference in levels, while Dickson et al. (1978) reported a two hundred-fold difference. Other groups have been unable to detect the HCLA binding protein in the mature female liver (Eagon et al., 1980; Finlayson, 1983). Thompson et al. (1981) reported that sex differences were not seen in the immature rat and the levels found in their livers were similar to those seen in the mature female. Powell-Jones et al. (1980) found that the HCLA binding protein was not seen in the immature male, but became evident after puberty (days 34-42) due to the testosterone surge seen in the male at this time. This testosterone surge is the cue for the expression of the HCLA binding protein which has been neonatally imprinted during days 7-12. This is demonstrated by neonatal castration preventing the development of the HCLA binding protein in the mature male, and testosterone treatment of the neonatal castrates allowing expression of normal male levels when these animals reach adulthood (Sloop

et al., 1983; Thompson and Lucier, 1983). Once expressed, androgens are neccesary for the maintanence of the HCLA binding protein levels as demonstrated by castration of the adult male resulting in a testosterone-reversible decrease in the HCLA binding protein levels (Finlayson, 1983).

In addition to the androgen dependency for expression and maintanence, the HCLA binding protein also requires an intact pituitary. Hypophysectomy of adult male and female rats resulted in the abolition of sex differences in the expression of the HCLA binding protein (i.e. HCLA binding protein levels were decreased in the male and increased in the female) (Powell-Jones et al., 1980; Lucier et al., 1981; Finlayson, 1983). Growth hormone seems to be the agent playing a role in the sex differences seen in the HCLA binding protein levels. While Lucier et al. (1981) reported that growth hormone administration to hypophysectomized animals had no effect on HCLA binding protein levels, Finlayson (1983) reported a further 50-80% decrease in binding capacity in the hypophysectomized animal (male and female). This repression of HCLA binding protein levels was also seen by Rumbaugh et al. (1983) when an ectopic pituitary was implanted in the hypophysectomized animal (male or female). The main hormone(s) secreted by an ectopic pituitary, which seemed to be responsible for the feminization of HCLA binding protein levels, has not been identified with certainty but the evidence points to growth hormone. Growth hormone and prolactin are secreted in the

largest amounts (Lam et al., 1976; Gustafsson et al., 1980) and since prolactin had no measurable feminizing effect on other hepatic parameters (e.g. steriod metabolism) (Eneroth et al., 1977) it was concluded there must be another "feminizing" factor being released which was termed feminotropin. Feminotropin was later identified as growth hormone (Rumbaugh and Colby, 1980; Mode et al., 1983). The feminizing effect on HCLA binding protein levels of growth hormone secreted from the ectopic pituitary is the same as that seen upon the administration of exogenous growth hormone (Finlayson, 1983), which strengthens the probability that growth hormone is responsible for the decreased level. That growth hormone can have a feminizing effect on a steroid binding system is not a novel concept. Its administration to hypophysectomized male rats leads to female (i.e. increased) concentrations of prolactin receptors in the liver (Norstedt et al., 1981 b; Norstedt et al., 1984).

Functions for the HCLA binding protein have been suggested to be: the uptake and concentration of estrogens and androgens (and their metabolites) thereby regulating the distribution between receptors and the enzymes of metabolism (Miroshnechenko et al., 1982), and the binding and disposition of sex steroids and their metabolites (Dickson et al., 1978; Singletary et al., 1983). There is also the possibility that the HCLA binding protein may serve as a modifying mechanism for the translocation of the estrogen

receptor (Powell-Jones et al., 1980) although it does not show translocation itself (Dickson et al., 1978). An experimental use for the HCLA binding protein may be as an indicator for the initial screening of male reproductive toxicants, as demonstrated by the decreased levels (feminization) in adult males (and masculinization in adult females) exposed neonatally to toxicants such as methyl mercury, 1,2-dibromo-3-chloropropane, and chlordecone (Lawrence et al., 1984; Lui, 1985; Lawrence-Domey and Lui, 1985).

3) Hypothesis

Previous work in our lab (Warren, 1982; Finlayson, 1983) has shown a number of correlations between the HCLA estrogen binding protein and the levels of aryl hydrocarbon hydroxylase (AHH) activity in the male rat, reflecting the observed sexual differences in drug and steroid metabolism. These include: an age dependent sex difference in the levels of the HCLA binding protein and AHH activity; gonadectomy produces a testosterone-reversible decrease in the capacity of the HCLA binding protein and in AHH activity; hypophysectomy abolishes the sex differences seen in the capacity of the HCLA binding protein and in AHH activity; growth hormone administration to the hypophysectomized male or female rat causes a reduction in AHH activity and further decreases the capacity of the HCLA binding protein. Since there is a reduction of the sex

differences in drug and steroid metabolism in the STZ-diabetic animal we hypothesized that this may be being mediated in part by the HCLA estrogen binding protein. We thus investigated the effects of STZ-induced diabetes on the HCLA estrogen binding protein at time periods where the changes in AHH activity were evident (4 and 10 days). We also sought the hormonal control mechanism of the HCLA estrogen binding protein in this pathological condition and therefore investigated the effects of hormonal replacement in physiological doses to the STZ-diabetic animal. Testosterone enanthate, protamine zinc insulin, 3,3,5-triiodothyronine were administered once a day. Toronto regular insulin was administered twice a day. With growth hormone four possibilities had to be examined: mean levels of circulating GH need to be maintained, the peaks of the ultradiene pattern need to be present, the troughs of the ultradiene pattern need to be present, or both the peaks and troughs of the ultradiene pattern need to be maintained.

MATERIALS AND METHODS

1) Chemicals and Reagents

The following chemicals and reagents were obtained from Sigma Chemical Company (St. Louis, Mo.): activated charcoal, bovine serum albumin (BSA), Coomassie brilliant blue G, DL-dithiothreitol (DTT), disodium ethylenediamine tetraacetic acid (EDTA), sodium molybdate, streptozotocin (STZ), testosterone enanthate, 3,3',5-triiodo-L-thyronine (T_3), 17β-estradiol, Trizma® base. [6,7-^{3}H(N)]-Estradiol, 40-60 Ci/mmol and Biofluor scintillation cocktail were obtained from New England Nuclear (Boston, Ma.). Dextran T-70 was obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Alzet® osmotic-minipumps, Model 2001 were obtained from Alza Corporation (Palo Alta, Ca.). Ovine growth hormone (1.5 I.U./mg) was a gift of the National Institute of Diabetes, Digestive, and Kidney Diseases. Protamine zinc and Toronto insulin (Connaught Labs) were purchased at retail outlets as was the Tes-tape® (Eli Lilly and Company, Toronto, Ont). All other chemicals and reagents were of analytical quality.

2) Animals

Male Wistar rats weighing 300-350 grams, male C57BL/6 and male DBA/2 mice weighing 20-30 grams were obtained from Canadian Breeding Farms (Montreal, P.Q.). Male guinea pigs weighing 325-375 grams were obtained from local suppliers

(U.B.C Animal Care). Animals were housed in a separate animal room on Lobund® bedding (Paxton Processing Ltd., Paxton, Il.) under controlled light (6 a.m. on, 8 p.m. off) and temperature (22°C). They were allowed free access to food (Purina Laboratory Chow, Ralston Purina of Canada Ltd, Woodstock, Ont.) and tap water ad libitum.

3) Animal Treatments

Streptozotocin was administered four or ten days prior to animal use via a tail vein injection of 60 mg/kg in citrate buffer. Controls were injected with vehicle only under ether anaesthetic. Before injection, rats were allowed a minimum of 3-4 days to equilibrate after shipment. The following hormones were injected s.c. once a day: protamine zinc insulin (PZI) (10 units/kg), testosterone enanthate (TES) (1 mg/kg in corn oil), triiodothyronine (T_3) (30 µg/kg in normal saline at pH 8.5). Toronto insulin was administered s.c. twice a day (15 units/kg). Ovine growth hormone (GH) was dissolved in normal saline (pH = 8.5) and: 1) administered s.c. in a dose of 30 µg/injection seven times per day at 6:15 a.m., 10:15 a.m., 2:15 p.m., 6:15 p.m., 9:00 p.m., 11:40 p.m., and 2:40 a.m.; 2) for continuous infusion of GH, Alzet® Model 2001 minipumps were implanted subcutaneously in the rostral half of ether aneasthetized rats. The GH solution was loaded into the pumps and the pumps were weighed before and after filling to ensure complete filling. The pumps had a life

expectancy of 7 days at a pumping rate of 1.04 µL/hr ± 0.06 µL/hr (equivalent to a dose of 0.02 units/hr). For i.v. injection GH was administered *via* the tail vein in conscious animals four times per day (9:00 a.m., 1:00 p.m., 5:00 p.m., and 9:00 p.m.) in a dose of 30 µg/injection. A related set of experiments used the same GH i.v. injection method and had TES and PZI added to the treatment regimen in the same injection pattern and dose as they were when given alone.

Rats were cannulated under halothane anaesthesia following standard surgical procedures. Rats were anaesthetized with halothane and a midabdominal incision made through the skin and muscle layers. The vena cava was located and sutures passed underneath and over the area where the cannula was to be placed. A trocar was pushed through the back muscle beside the spine and allowed to exit dorsally at the neck region to create a cavity under the skin of the back. The cannula was then placed in the trocar and taken to the exit point and flow through the cannula was checked. The other end of the cannula was inserted into the vena cava and loosely tied to nearby muscle tissue for anchoring. Topical antibiotics were sprinkled over the wound, and the muscle and skin layers were carefully sutured closed. Lidocaine solution was applied topically to alleviate pain from the surgery. The animal was removed from the halothane, treatments started, and placed in an individual cage for recovery and use.

4) High capacity, low affinity protein binding assay

Rats and mice were stunned with a blow to the head and killed by decapitation. Guinea pigs were killed *via* cervical dislocation followed by decapitation. Livers were perfused *via* the portal vein with ice cold TED buffer, containing 50mM Tris, 1.5mM EDTA, 0.5mM DTT, 20mM sodium molybdate, and 10% glycerol, pH 7.5 at 4°C. The livers were then excised into TED buffer, blotted dry and the weight recorded. Tes-tape® was used to test for glycosuria at the time of killing to confirm the presence of diabetes. Livers were then minced and homogenized (1:10 w/v) in TED buffer using a Potter-Elvehjem tissue homogenizer. After homogenization samples were centrifuged at 10,000g for 10 minutes at 4°C using an International Equipment Company (Needham Hts., Ma.) Model B-20 refrigerated centrifuge. The resulting supernatant was centrifuged at 100,000g for 30 minutes at 4°C (to obtain the cytosolic fraction) using a Beckman L5-50 ultracentrifuge with a type 65 fixed angle rotor (Beckman Instruments Corp., Palo Alto Ca.). The supernatant was removed and diluted with TED buffer to a protein concentration of 1-3 mg/ml determined *via* the Bradford protein assay method (Bradford, 1976) and placed on ice.

The binding characteristics of estradiol were determined by Scatchard (1949) analysis of competitive binding curves. The incubation mixture contained 500 µL cytosol, 10 µL [^{3}H]-estradiol (10 Ci/mmol) 10-200 nM (in

absolute ethanol) in the presence or absence of a 100 fold excess of unlabelled estradiol. Incubations were carried out for 90 minutes at 4°C. The reaction was terminated and bound steroid was separated from free by the addition of 500 µL of dextran T-70 (0.05% w/v) coated charcoal (0.5% w/v) (DCC) in TED buffer, as prepared previously minus the molybdate. Following the addition of DCC the samples were mixed and DCC sedimented by centrifugation at 1500g for 10 minutes at 4°C using a Beckman J6-B refrigerated centrifuge with a type JS 4.2 rotor. The resulting supernatants were sampled (0.5 mL) and mixed with 10 mL Biofluor liquid scintillation cocktail. Samples were counted for bound estradiol using a Searle Mark III Liquid Scintillation System, Model 6880 (Searle Analytical Inc., Des Plains, Il.). The Mark III was interfaced with an Apple Plus II computer so data could be recorded directly to disk. All points were assayed in duplicate. The tubes which contained [^{3}H]-estradiol without competitor measured total binding (TB), while the tubes with both [^{3}H]-estradiol and competitor represented the non-specific binding (NSB) present.

Analysis of binding data proceeded as follows. The counts per minute (cpm) recorded for [^{3}H]-estradiol were converted to disintegrations per minute (dpm) based on the percent efficiency of the instrument. Efficiency for tritium was 47%. Thus dpms were calculated on the basis of efficiency, which was determined for each sample from

previously prepared quench standards. The dpms were converted to Curies on the basis that 1 Ci = 2.2×10^{12} dpm. The specific activities of the ligands were employed to calculate the picomoles of binding which was normalized for protein content. Then the NSB tube values were subtracted from the TB tube values to give the specific binding (SB) at any point. Duplicates were then averaged. The total ligand (total counts) added, less the SB, represents the free concentration (F) used in the calculation of specific bound over free (B/F). In cases where displacement was observed the B/F was graphed as a function of SB to yield a curve-linear plot with a negative slope (Scatchard plot). The inverse of the slope of the linear portion of the plot represented the dissociation constant (Kd), and the x-intercept the uncorrected measure of capacity. (The numbers for the Scatchard plot were obtained by subjecting the data to computer analysis (Input/Calc programme by Sunahara and Fawzi) and by confirming the results by hand).

5) Determination of Protein Content

Protein concentration was determined by the method of Bradford (1976). Using BSA as a standard, the assay is linear for protein concentrations from 0.1 - 0.5 mg/mL. A Perkin-Elmer Model 124 double beam spectrophotometer was used to assess protein concentration. Samples were read 5 minutes after the addition of 5 mL Bradford reagent which contained: 100 mg Coomassie-brilliant blue-G; 50 mL

absolute ethanol; 100 mL 85% phosphoric acid; diluted to 1L and filtered using a Buchner suction apparatus.

6) Statistical Analysis

Differences were considered significant from control at $p<0.05$ using ANOVA and the Newman-Keuls multiple range test.

RESULTS

1) Effect of possible blood contamination

Early in our studies the suggestion that blood contamination could produce artifactual results was made (Poellinger *et al.*, 1983). To determine whether there was a blood-borne component which may interfere with our assay, various perfusion volumes were used to flush the blood out *in situ*. As well, whole rat blood (taken from the animals at time of decapitation) was assayed for the presence of any component which could bind estradiol in a specific and saturable manner. As shown in Table I and Figure 1 there were no significant differences in the apparent binding capacity or Kd in livers perfused with 10 or 100 mL of TED buffer. It can also be seen that whole blood contained no agent capable of binding estradiol.

2) Effect of STZ-induced diabetes

The effect of a single injection of 60 mg/kg of STZ upon the binding capacity of the HCLA binding protein is shown in Table II and Figure 2. The binding capacity is decreased by 50-80% at both the 4 day and 10 day time points. The apparent Kd is not affected by the diabetic state. Diabetes was confirmed by the presence of glycosuria, 1/2% or greater.

Table I

EFFECT OF LIVER PERFUSION VOLUME

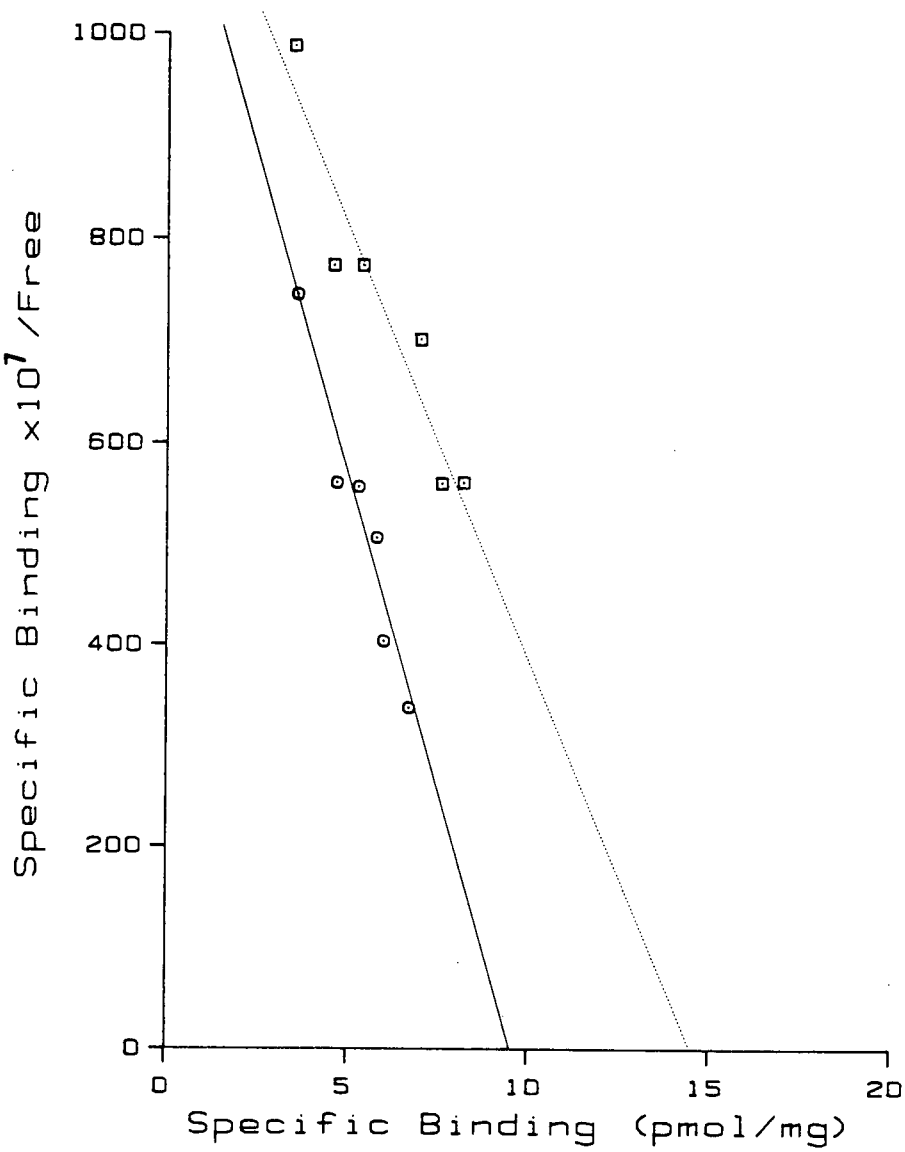
The effect of varying the liver perfusion volume was determined in citrate injected control animals (citrate buffer pH 4.5). Rat blood was assayed for the presence of any component which was capable of binding the ligand in a specific and saturable manner. Incubations were carried out as described in the methods section. Binding kinetics were determined by Scatchard analysis. Data were expressed as mean±S.E.M. Numbers in brackets denote the number of animals tested.

Perfusion Volume	Apparent Binding Kinetics		
	Kd (10^{-7}M)	Bmax (pmol/mg)	
10 mL	1.08±0.34	14.83±3.97	(11)
100 mL	1.41±0.11	17.91±4.82	(8)
Rat blood	no detectable binding*		(2)

* denotes no detectable displaceable and saturable binding

Fig. 1

Effect of varying the liver perfusion volume. Different volumes (10 mL⊙———⊙, 100 mL⊡·····⊡) of TED buffer were perfused through the vena cava before removal of the liver. Incubations were carried out according to the Methods section. Graphs shown are of a single experiment and are representative of the results found.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Specific Binding x10⁷/Free
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20
Specific Binding (pmol/mg)

Table II

EFFECT OF STREPTOZOTOCIN-INDUCED DIABETES AND INSULIN REPLACEMENT ON THE MALE RAT HEPATIC HCLA BINDING PROTEIN

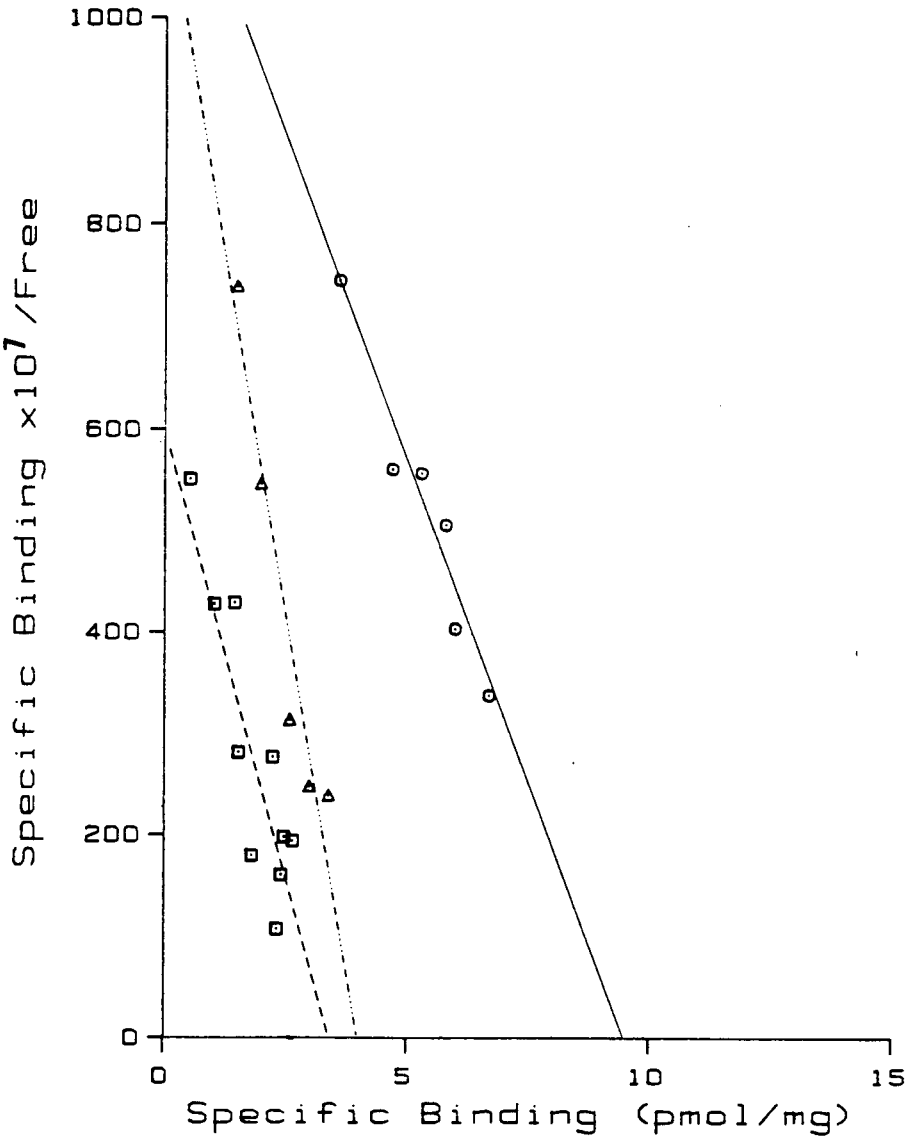
Adult male rats were rendered diabetic by a single injection of streptozotocin (STZ) (60 mg/kg) in citrate buffer (pH 4.5) under ether anaesthetic (controls were injected with vehicle only). Protamine zinc insulin (PZI) was administered in a dose of 10 units/kg s.c. once a day, Toronto insulin (To.I) was given in a dose of 15 units/kg s.c. twice a day. Incubations were carried out as described in the Methods section. Binding kinetics were determined by Scatchard analysis. Data were expressed as mean ± S.E.M. Numbers in brackets denote number of animals tested.

Treatment	Apparent Binding Kinetics		
	Kd (10^{-7}M)	Bmax (pmol/mg)	
Control	1.08±0.34	14.83±3.97	(11)
4 day STZ	0.82±0.18	4.82±0.95*	(8)
4 day STZ +PZI	0.79±0.19	3.41±0.99*	(5)
4 day STZ +To.I	0.57±0.06	1.41±0.38*	(7)
10 day STZ	0.51±0.26	4.37±1.59*	(5)
10 day STZ +PZI	1.08±0.18	5.72±1.23*	(5)

* denotes significant difference compared to control ($p<0.05$) according to ANOVA and the Newman-Keul range test

Fig. 2

Effect of streptozotocin-induced diabetes on the levels of the HCLA binding protein of rat liver. Adult male rats were rendered diabetic by a single tail vein injection of streptozotocin (60 mg/kg) in citrate buffer (pH 4.5) under ether anaesthesia; controls (o——o) were injected with buffer only. Animals were left for 4 (□-----□) or 10 (Δ------Δ) days before use. Incubations were carried out as described in the Methods section. Graphs shown are of a single experiment and are representative of the results found.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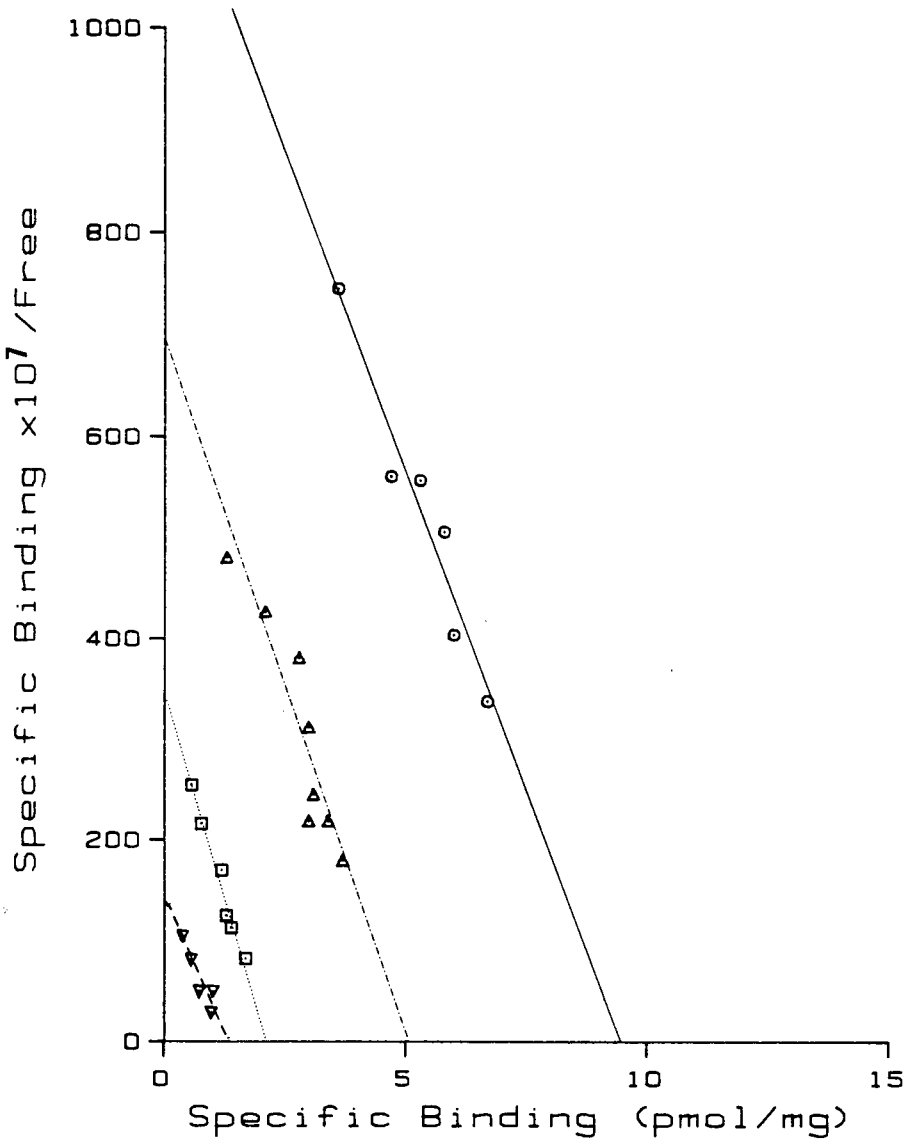
Specific Binding (pmol/mg)

3) Effect of insulin replacement in the STZ-diabetic animal

The effect of protamine zinc insulin treatment in a dose of 10 units/kg s.c. once a day can be seen in Table II and Figure 3. This treatment regimen was ineffective in restoring the levels of the HCLA binding protein in both the 4 day and 10 day diabetic animals. The apparent binding capacity remained depressed to levels 50-80% below of those seen in control animals and the Kd was unaffected. Protamine zinc insulin was effective in controlling the diabetic state as evidenced by the lack of glycosuria in the treated animals. Since there were no differences seen between the 4 day and 10 day time points the 4 day model was used for the rest of the experiments. Toronto regular insulin was tested to see if it could cause the reversal of the decrease in binding capacity of the HCLA binding protein due to a report in the literature. Ortiz-Caro et al. (1984) found that Toronto regular insulin was effective in reducing some of the thyroid dysfunction seen in the STZ-diabetic animal when given in a regimen of 15 units/kg s.c. twice a day, whereas protamine zinc insulin once a day had been ineffective. The lack of restoration of HCLA estrogen binding protein levels with this treatment can be seen in Table II and Figure 3. With respect to the HCLA binding protein it would appear that the type of insulin and its dosage did not make a difference as we were again unable to restore the reduced binding capacity. The diabetic state was being controlled as

Fig. 3

Effect of insulin on the level of the hepatic HCLA binding protein in streptozotocin-induced diabetic rats. Adult male rats were rendered diabetic by a single tail vein injection of streptozotocin (60 mg/kg) in citrate buffer (pH 4.5) under ether anaesthesia; controls (○———○) were injected with buffer only. Protamine zinc insulin was given in a dose of 10 units/kg s.c. once a day. Animals were treated for 4 (□·····□) or 10 (△-·-·-·-△) days before use. Toronto regular insulin was given in a dose of 15 units/kg s.c. twice a day for 4 days (▽----▽) before use. Incubations were carried out as described in the Methods section. Graphs shown are of a single experiment and are representative of the results found.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Specific Binding (pmol/mg)

evidenced by the lack of glycosuria.

4) Effect of subcutaneous injections

The effect of subcutaneous injection of vehicles was determined in citrate buffer (pH=4.5) injected (via a tail vein under ether aneasthesia) control animals. Normal saline was injected s.c. at a volume of 0.3 mL once a day, or corn oil was injected s.c. at a volume of 0.5 mL once a day. The results can be seen in Table III. It is apparent that there is no change in either the apparent binding capacity or Kd between injected or non-injected control animals.

5) Effect of hormone replacement

a) Testosterone enanthate

Due to the decrease in serum testosterone seen in the diabetic male and the restorative effect of testosterone on the HCLA binding protein in the gonadectomized male, we investigated the effect of testosterone replacement in the diabetic male. The effect of testosterone enanthate at a dose of 1 mg/kg can be seen in Table IV and Figure 4. It is observed that this dose, which is sufficient to restore normal plasma testosterone levels, (Sunahara, 1984) is unable to restore the decreased binding capacity seen in the STZ-induced diabetic animal. The Kd remains the same as that seen in the control animals. The diabetic animals

Table III

EFFECT OF SALINE AND CORN OIL S.C. INJECTIONS IN CONTROL ANIMALS

Adult male rats were injected with 0.3 ml citrate buffer (pH 4.5) under ether aneasthetic. Normal saline was injected s.c. at a volume of 0.3 ml once a day. Corn oil was injected at a volume of 0.5 ml once a day. Incubations were carried out as described in the Methods section. Binding kinetics were determined by Scatchard analysis. Data were expressed as mean ± S.E.M. Numbers in brackets denote number of animals tested.

Treatment	Apparent Binding Kinetics		
	Kd (10^{-7})	Bmax (pmol/mg)	
Saline s.c.	1.01±0.01	14.71±1.53	(4)
Corn oil s.c.	0.90±0.01	12.68±2.83	(4)
Control	1.08±0.34	14.83±3.97	(11)

treated with testosterone still showed signs of glycosuria at the time of killing.

b) 3,3,5-triiodothyronine

Since there are alterations in thyroid hormone levels in the diabetic animal, we investigated the replacement of triiodothyronine. The effect of administering triiodothyronine at a dose of 30 µg/kg can be seen in Table IV and Figure 4. This dosage is effective in restoring the thyroid to normal status (Tahiliana, 1983). It can be seen that the replacement of triiodothyronine in the diabetic animal is unable to restore the decreased binding capacity but the Kd was unaltered from the control values. The diabetic animals treated with triiodothyronine tested postive for glycosuria at the time of killing.

c) Growth hormone

The effect of replacement of growth hormone can be seen in Table IV and Figure 5. Calculating from known blood levels (Tannenbaum and Martin, 1976) two treatment regimens were carried out with growth hormone: i) replacement *via* continuous infusion with the use of minipumps; ii) replacement *via* a seven times per day s.c. injection schedule. Neither of these treatment regimens (done to restore absolute levels of growth hormone and to duplicate the natural male release pattern respectively) were able to restore the decreased binding capacity of the HCLA binding

Table IV

EFFECT OF HORMONE REPLACEMENT ON THE RAT HEPATIC HCLA BINDING PROTEIN IN 4-DAY DIABETIC ANIMALS

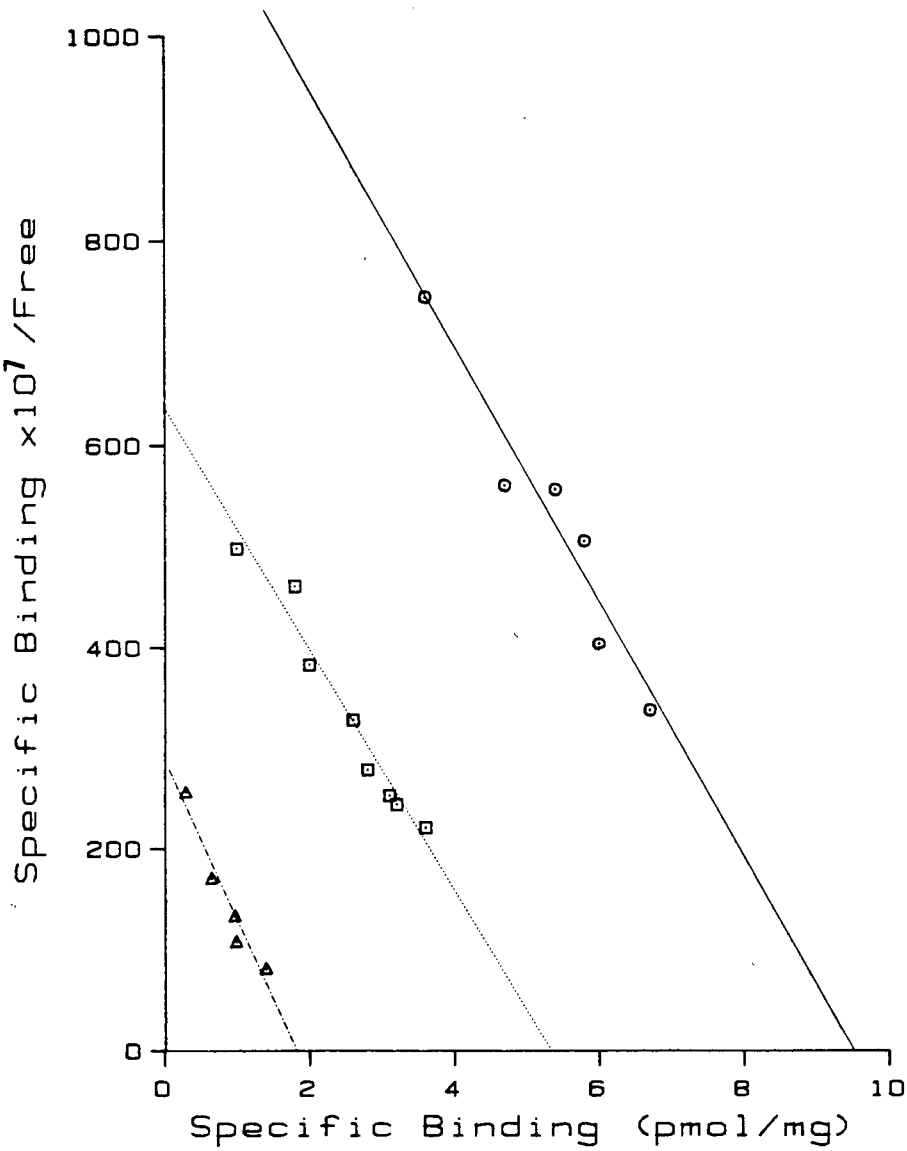
Adult male rats were rendered diabetic by a single injection of streptozotocin (STZ) (60 mg/kg) in citrate buffer (pH 4.5) under ether anaesthesia. Testosterone enanthate was given in a dose of 1 mg/kg in corn oil once a day. Triiodothyronine was given in a dose of 30 ug/day s.c. in normal saline. The animals which had osmotic minipumps implanted were given a dose of ovine growth hormone (1.5 I.U./mg) at 0.02 units/hour for four days. The dose of ovine growth hormone given s.c. was 30 ug/injection seven times a day. Incubations were carried out as described in the Methods section. Binding kinetics were determined by Scatchard analysis. Data were expressed as mean ± S.E.M. Numbers in brackets denote number of animals tested.

Treatment	Apparent Binding Kinetics		
	Kd (10^{-7}M)	Bmax (pmol/mg)	
Control	1.08±0.34	14.83±3.97	(11)
4 day STZ	0.82±0.18	4.82±0.95*	(8)
4 day STZ +testosterone enanthate	0.56±0.10	3.65±0.82*	(5)
4 day STZ +triiodothyronine	0.40±0.10	1.83±0.40*	(5)
4 day STZ +growth hormone (minipump)	0.56±0.11	1.81±0.22*	(5)
4 day STZ +growth hormone (s.c.)	0.52±0.06	1.54±0.28*	(7)

* denotes significant difference compared to control ($p<0.05$) according to ANOVA and the Newman-Keuls range test

Fig. 4

Effect of testosterone and 3,3,5-triiodothyronine replacement in 4 day streptozotocin-induced diabetic animals on the level of the HCLA binding protein of rat liver. Adult male rats were made diabetic by a single tail vein injection of streptozotocin (60 mg/kg) in citrate buffer (pH 4.5) under ether anaesthesia; controls (○———○) were injected with buffer only. Animals were treated with testosterone enanthate at a dose of 1 mg/kg in corn oil s.c. once a day (□·····□), or triiodothyronine at a dose of 30 ug/kg in normal saline s.c. once a day (△-·-·-·-△). Incubations were carried out as described in the Methods section. Graphs shown are of a single experiment and are representative of the results found.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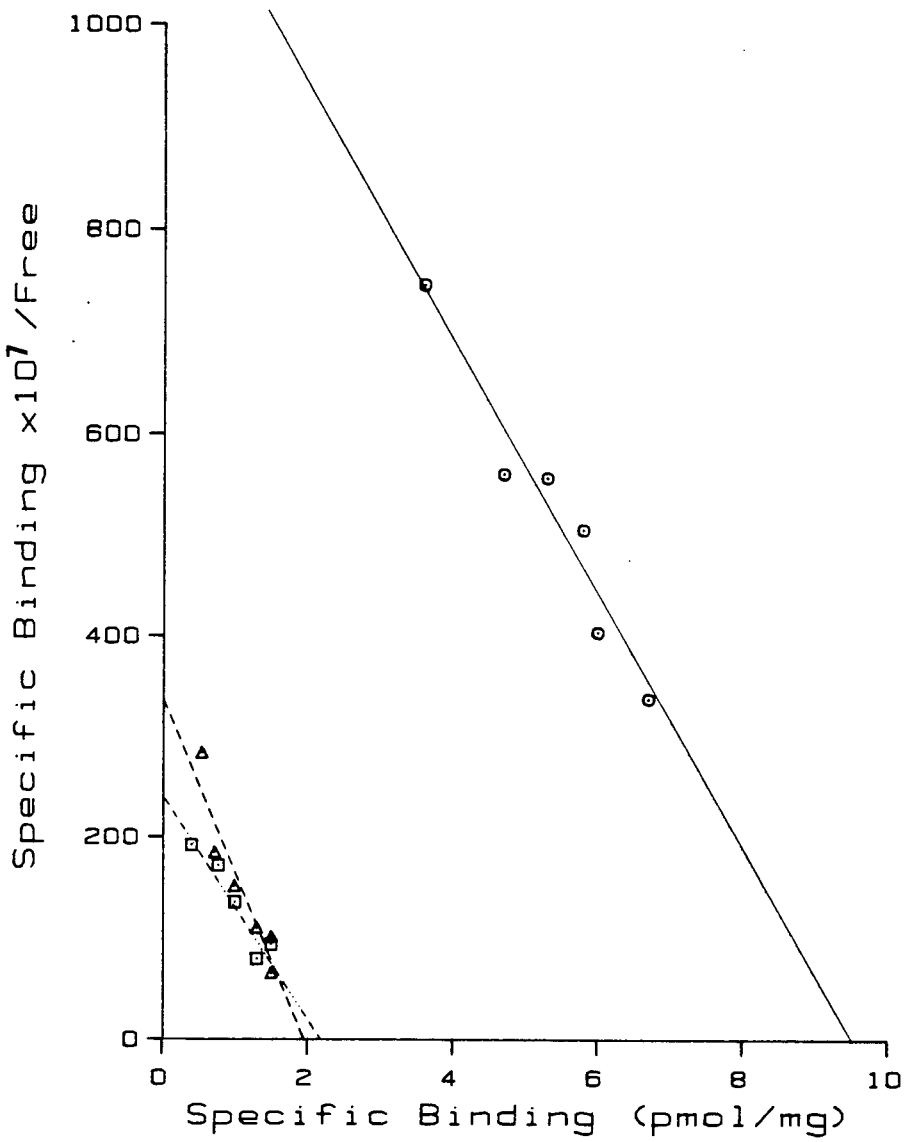
Specific Binding (pmol/mg)

Fig. 5

Effect of ovine growth hormone (1.5 I.U./mg) replacement on the hepatic HCLA binding protein in 4 day streptozotocin-induced diabetic rats. Adult male rats were rendered diabetic by a single tail vein injection of streptozotocin (60 mg/kg) in citrate buffer (pH 4.5) under ether anaesthesia; controls (o———o) were injected with buffer only. Growth hormone was dissolved in basic (pH 8.5) saline and administered by either osmotic minipump (□- - - - □) implanted in the rostral portion of the rat's back and given a dose of 0.02 units/hr, or injected s.c. (Δ- - - - Δ) at a dose of 30 ug/injection seven times a day. Animals were treated for 4 days before use. Incubations were carried out as described in the Methods section. Graphs shown are of a single experiment and are representative of the results found.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Specific Binding (pmol/mg)

protein in the diabetic rat. Again, the Kd value was unaltered from the value seen in control animals, and the animals tested positive for glycosuria.

6) Effect of intravenous growth hormone administration

a) Vena cava cannulation

The effect of vena cava cannulation and the subsequent administration of growth hormone to the STZ-diabetic animal can be seen in Tabel V. This was undertaken to more closely duplicate the normal physiologic level seen in the blood (i.e. to get marked blood peaks). There was no restoration of the decreased binding capacity and this treatment actually gave the lowest levels of the HCLA binding protein that we observed; the Kd remained unaltered from the control value. Because of this very low binding capacity concern was raised over the potential hepatotoxiciy of halothane as an anaesthetic and a control experiment was done to compare the effects of halothane versus ether as the surgical anaesthetic. The results of this can be seen in Table V. There were no apparent differences in the binding capacity values obtained with either halothane or ether anaesthesia, but these values were significantly lower than the values obtained from control animals which had not been surgically manipulated; the Kd values were the same in all three cannulated groups and were not different from the control Kd values. The low number of animals used to obtain data precluded statistical manipulation of the figures. Larger

Table V

EFFECT OF VENA CAVA CANNULATION ON THE THE RAT HEPATIC HCLA BINDING PROTEIN

Adult male rats were cannulated as described in the Methods section under either ether or halothane anaesthesia. One group was rendered diabetic by a single injection of streptozotocin (STZ) (60 mg/kg) in citrate buffer (pH 4.5) while under the operative anaesthetic (controls were injected with buffer only). Ovine growth hormone (1.5 I.U./mg) was given in a dose of 30 ug/injection s.c. seven time a day. Binding kinetics were determined by Scatchard analysis. Numbers in brackets denote the number of animals tested. Data were presented as the mean ± S.E.M. Incubations were carried out as described in the Methods section.

Conditions of Cannulation	Apparent Binding Kinetics	
	Kd (10^{-7} M)	Bmax (pmol/mg)
ether anaesthetic	0.53±0.17	2.85±1.67 (3)
halothane anaesthetic	0.59±0.13	4.25±2.44 (3)
halothane anaesthetic +STZ +growth hormone	0.46±0.20	0.51±0.19 (2)

numbers of animals were used, but because of the intolerance of the rat to the cannula, as demonstrated by its pulling the cannula out, chewing the injection end down to where it was unusable, and the problem of blood clots plugging the cannula inside the animal, the final numbers were low.

b) Tail vein administration in the conscious animal

The effect of tail vein administration of growth hormone is shown in Table VI and Figure 6. All treatment groups showed a decrease in the binding capacity of the HCLA binding protein compared to the untreated (i.e. uninjected) controls, with no differences in the Kd values. This decrease in binding capacity was also evident in the vehicle injected control animals which were only being injected with saline in the tail vein. These animals showed an approximately 50% decrease in binding capacity compared to non-injected controls, i.e. from 10-15 pmol/mg protein to 5 pmol/mg protein. The STZ inside controls (injected with saline) showed a further 50% decrease below this level, i.e. to 2 pmol/mg protein, as did the growth hormone treated diabetics. A second treatment group in which testosterone enanthate and protamine zinc insulin were also given, in doses described previously, in addition to the i.v. growth hormone showed a decrease similar to that seen with the growth hormone alone and the STZ-diabetic animal injected with saline. All the STZ injected animals except those treated with insulin showed glycosuria at the time of

Table VI

EFFECT OF HORMONE REPLACEMENT BY TAIL VEIN INJECTION IN CONSCIOUS 4-DAY DIABETIC ANIMALS ON THE RAT HEPATIC HCLA BINDING PROTEIN

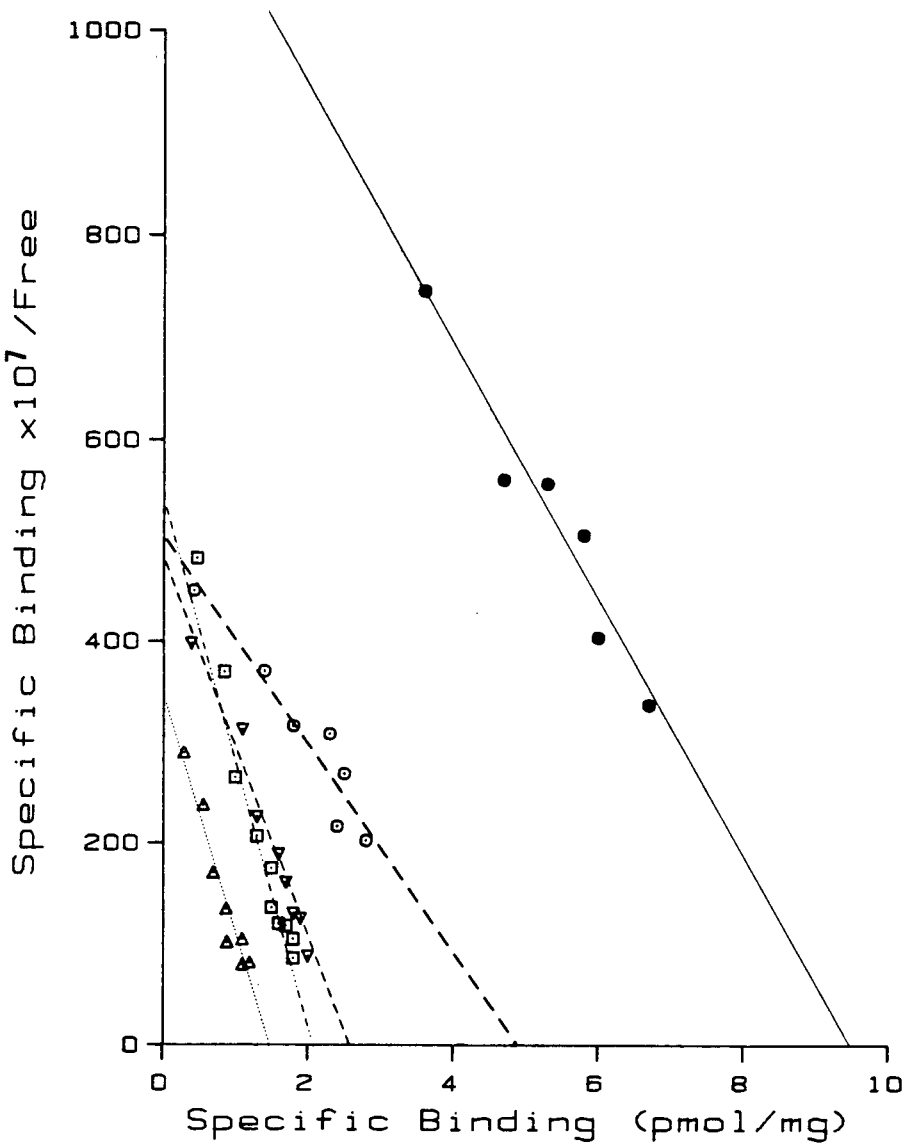
Adult male rats were rendered diabetic by a single injection of streptozotocin (STZ) (60 mg/kg) in citrate buffer (pH 4.5) under ether anaesthesia (controls were injected with buffer only). Ovine growth hormone (1.5 I.U./mg) was injected i.v. at a dose of 30 ug/injection four times a day in normal saline (vehicle injected controls and vehicle injected diabetic controls were injected with saline only). Testosterone enanthate was given at a dose of 1 mg/kg s.c. in corn oil once a day. Protamine zinc insulin (PZI) was given in a dose of 10 units/kg s.c. once a day. Incubations were carried out as described in the Methods section. Binding kinetics were determined by Scatchard analysis. Data were expressed as mean $\pm$ S.E.M. Numbers in brackets denote number of animals tested.

Treatment	Apparent Binding Kinetics	
	Kd (10^{-7}M)	Bmax (pmol/mg)
Control	1.08$\pm$0.34	14.83$\pm$3.97 (11)
Vehicle injected Control	0.66$\pm$0.11	4.05$\pm$0.47* (4)
Vehicle injected 4 day STZ	0.36$\pm$0.03	2.07$\pm$0.37** (4)
4 day STZ +growth hormone	0.43$\pm$0.04	2.25$\pm$0.34** (4)
4 day STZ +growth hormone +testosterone enanthate +PZI	0.54$\pm$0.18	2.37$\pm$0.37** (4)

* denotes a significant difference from control ($p<0.05$) according to ANOVA and the Newman-Keuls range test
** denotes a significant difference from control and vehicle injected control ($p<0.05$) according to ANOVA and the Newman-Keuls range test

Fig. 6

Effect of tail vein injections in conscious 4 day streptozotocin-induced diabetic rats on the hepatic HCLA binding protein. Adult male rats were made diabetic by a single tail vein injection of streptozotocin (60 mg/kg) in citrate buffer (pH 4.5) under ether anaesthesia; controls (●———●) were injected with buffer only. Control (○— — —○) and one group of diabetics (□- - - - - □) were injected in a tail vein with saline alone 4 times a day for 4 days before use. Another group of diabetic animals was treated with growth hormone dissolved in basic (pH 8.5) saline at a dose of 30 ug/injection 4 times a day in a tail vein for 4 days before use (△.....△). The last group of diabetics was treated with growth hormone in basic (pH 8.5) saline at a dose of 30 ug/injection 4 times a day in a tail vein , plus testosterone enanthate at a dose of 1 mg/kg in corn oil s.c. once a day, plus protamine zinc insulin at a dose of 10 units/kg s.c. once a day for 4 days before use (▽- - - -▽) . Incubations were carried out as described in the Methods section. Graphs shown are of a single experiment and are representative of the results found.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Specific Binding (pmol/mg)

killing. Thus, none of the treatments involved in this study were able to reverse the decrease in binding capacity seen in the STZ-diabetic animal. The difference between the binding capacities of the vehicle injected controls, and the vehicle injected STZ controls, growth hormone treated, and growth hormone + testosterone + insulin treated animals were significant, indicating that there is still a difference between the non-diabetic animals and the diabetic animals That is, even though this protocol is causing an effect on the animal, probably through the stress of handling and injection, the diabetic effect is also occuring in parallel.

7) Other species examined

Several other species were examined for the presence of the hepatic HCLA binding protein. The results of these experiments can be seen in Table VII. None of the other species examined showed any evidence of the HCLA binding protein in their liver cytosol.

8) Stability studies

It had been suggested that the differences in binding capacity seen between the control and STZ-diabetic animal may be due to differences in the stability of the protein during the incubation period. This was examined and the results can be seen in Table VIII. Both the control and STZ-diabetic cytosols demonstrated a stability of binding

capacity and Kd up to the longest time point measured (in this case 3 hours). The observed difference in the binding capacity was evident (i.e. controls being approximately 3 times higher than STZ-diabetics) and the Kd values between the two groups were the same.

Table VII

OTHER MODELS EXAMINED FOR THE PRESENCE OF THE HCLA BINDING PROTEIN

Mouse and guinea pig liver was assayed for the HCLA binding protein in the same manner as rat liver. Binding kinetics were determined by Scatchard analysis. Numbers in brackets denote the number of animals tested. Incubations were carried out as described in the Methods section.

Animal model	Apparent Binding Kinetics	
	Kd (10^{-7} M)	Bmax (pmol/mg)
C57BL/6 mouse liver	no detectable binding* (4 pools of 3)	
DBA/2 mouse liver	no detectable binding* (4 pools of 3)	
guinea pig liver	no detectable binding* (4)	

* denotes no detectable displacable and saturable binding

Table VIII

STABILITY STUDY OF THE HCLA BINDING PROTEIN IN CONTROL AND 4-DAY DIABETIC ANIMALS

Adult male rats were made diabetic by a single injection of streptozotocin (60 mg/kg) in citrate buffer (pH 4.5) under ether aneasthetic (controls were injected with buffer only). Cytosol was incubated immediately (0 hr.), or left sitting on ice for 1 hr before use, or left sitting on ice 3 hr. before use. Incubations were carried out as described in the Methods section. Binding kinetics were determined by Scatchard analysis. Data were expressed as mean±S.E.M. Numbers in brackets denote number of animals tested.

Treatment	Apparent Binding Kinetics		
	Kd (10^{-7}M)	Bmax (pmol/mg)	
Control			
0 hr.	0.72±0.01	12.59±0.66	(2)
1 hr.	0.70±0.01	13.29±0.68	(2)
3 hr.	0.89±0.02	12.96±0.58	(2)
4 day diabetic			
0 hr.	0.47±0.02	2.78±1.56	(2)
1 hr.	0.60±0.01	3.98±0.15	(2)
3 hr.	0.68±0.01	3.47±0.46	(2)

DISCUSSION

1) Binding kinetics

The results obtained in these experiments for the binding kinetics of the HCLA estrogen binding protein of male rat liver ($Kd=10^{-7}M$, binding capacity=14.83 pmol/mg protein) are similar to those reported by other authors (Eagon *et al.*, 1980; Warren, 1982; Finlayson, 1983). The saturation curves (see Appendix I) indicate that specific, saturable binding is taking place in the assay system used, and reliable Scatchard plots can be generated from the data. Since there was no effect of varying the liver perfusion volume, and no specific binding could be detected in rat whole blood, the HCLA binding protein is a component of liver cytosol and not the plasma transport proteins such as albumin and sex hormone binding globulin which can also bind steroids (Anderson, 1974; Siiteri *et al.*, 1982). Since there was no binding detected in whole blood there were no blood borne contaminants interfering with the assay system. The lack of non-specific binding which might lead to artifactual results had also been demonstrated by Finlayson (1983) who failed to detect binding to bovine serum albumin and rat plasma.

2) Effect of STZ-induced diabetes

STZ-induced diabetes caused a decrease of 50-80% in the

binding capacity of the HCLA binding protein at both the 4 and 10 day time points. No change in the Kd was noted. The levels of the HCLA protein, being equal at both time points, indicate that the reduction in binding capacity occurs early and is not subject to regeneration within this time period. As can be seen in Appendix I, both the control and diabetic animals demonstrated specific, saturable binding, indicating that reliable Scatchard plots could be generated for both groups. The results in Table VIII indicate that there appears to be no apparent alteration in the stability of the protein *per se* as a result of the STZ treatment, as seen by its remaining as stable as the control over the time course studied (but at its reduced capacity). It could be questioned as to whether the decrease seen in the HCLA binding protein levels could be due to a direct effect of STZ, unrelated to its diabetogenic action (i.e. is there a generalized decrease in hepatic proteins). This does not seem to be the case, as Montoya and Herrara (1974) observed an overall increase in hepatic protein concentration in the STZ-diabetic animal. Observations by other groups (Reinke *et al.*, 1978; Stohs *et al.*, 1979; Past and Cook, 1980; Peng *et al.*, 1983; Warren *et al.*, 1983) also show that there is not a generalized depression of proteins in the diabetic state; some proteins are decreased and other proteins are increased. Alterations in the levels of the affected proteins occur in parallel in the spontaneously diabetic rats and the STZ-induced diabetic rats. More

specifically, with regard to the HCLA binding protein, Warren (1982) using a similar assay system and examining spontaneously diabetic B.B. rats did not see as marked a change in the binding capacity using Scatchard analysis, but upon the application of the non-linear curve fitting computer program (NONLIN UBC) a 60% decrease was noted from control values. These experiments were done with tissue pools and on a small number of animals. Unfortunately, we were unable to obtain any spontaneously diabetic B.B. rats to examine and compare results to those obtained by Warren to see if the lack of change seen with Scatchard analysis could be due to the individual variation observed in the HCLA binding protein levels noted in the animals using this assay system (Finlayson, 1983). However, this report and that of Warren agree in that there is a general trend towards reduced capacity of the HCLA binding protein in the diabetic animal (regardless of the etiology of the disease state i.e. either spontaneous or chemically induced) indicating that the reduction is due to the diabetic state and not a nonspecific effect of the STZ-treatment. The lack of any effect on the Kd in the diabetic state was also observed by Warren. This lack of change in the Kd indicates that probably all that is being altered in the diabetics is the number of binding sites available on the protein, the absolute amount of the protein produced, or the amount available to the ligand in the cytosol in a functional form.

3) Hormone replacement

a) Insulin

Insulin replacement (either with protamine zinc insulin once per day, or Toronto regular insulin twice per day) at levels which were effective in controlling glycosuria, and which had previously been shown to reverse the effects of diabetes on hepatic drug/steroid metabolism, were ineffective in reversing the decrease in binding capacity of the HCLA binding protein. This was evident at both of the treatment time points investigated (4 and 10 days). In contrast, restoration of normal drug/steroid metabolism was evident with 4 days of insulin treatment (Warren, 1982). This demostrates that insulin levels per se are not a controlling factor in the HCLA binding protein levels, and that there does not appear to be an interplay between the two. This is in contrast to the estrogen receptor system where there is an interrelationship between insulin, insulin receptors, and estrogen receptor levels (Shafie and Hif, 1978; Lui et al., 1981).

b) Testosterone

The dose of testosterone enanthate used in this study had previously been shown to be able to restore circulating levels of testosterone and normalize AHH activity in the adult castrate male (Sunahara, 1984) as well as restoring the levels of the HCLA binding protein in the gonadectomized

adult male (Finlayson, 1983). There was, however, no evidence of restoration of the HCLA binding protein levels with this treatment in the diabetic animal. It could be that a longer treatment course is necessary as the restoration seen by Finlayson in the gonadectomized group was after 10 days; it may have occured sooner but a time course was not done. However, it should be noted that the testosterone treatment was initiated at the same time as STZ.

c) 3,3,5-triiodothyronine

The dose of triiodothyronine used in this study has previously been shown to restore normal thyroid status in the diabetic animal but not cause any other alterations in the diabetic state (Tahiliani, 1983). There was no effect of this hormone treatment on the levels of the HCLA binding protein, indicating that thyroid hormones are not playing a role in the regulation of the HCLA binding protein, though they do work to correct some of the abnormalities in pituitary growth hormone content seen in the diabetic animal (Ortiz-Caro et al., 1984).

d) Growth hormone

Growth hormone secretion patterns show a marked difference between the male and female animal. In the female there is a continuous release of growth hormone which leads to stable, constantly detectable levels in the

circulation. In the male though, the secretion follows an ultradiene rhythm which leads to high levels (peaks) which are rapidly cleared from the circulation, leaving no detectable growth hormone (troughs) until the next secretory peak. This rhythmic cycle occurs approximately every 3.5 hours in the male rat. The level of growth hormone seen during a peak in the male's cycle is about twice that which is continuously present in the female (Tannenbaum and Martin, 1976). The first two experiments carried out with growth hormone were done to restore absolute circulating levels of growth hormone (via minipumps) which are decreased in diabetics, and to mimic the normal male release pattern (via s.c. injections seven times per day) which is feminized in the diabetic male. Neither of these experiments resulted in the elevation of the HCLA binding protein to control levels. In retrospect, that there was no difference between the effects of the two treatment regimens is not suprising, since a s.c. injection probably acts in rather similar manner to a depot preparation such as the minipump. That is, there is a slow constant diffusion into the blood, and no marked peaks and valleys are seen in the circulatory system. Thus, the two treatments probably result in a similar dosing pattern. That these treatments failed to restore the decreased levels or the HCLA binding protein, does not negate the theory that growth hormone is the feminizing factor involved in the changes seen in liver function related to drug/steroid metabolism (Wilson, 1969;

1970; Mode et al., 1981; Vockentanz and Virgo, 1985). Its presence is associated with feminization and its absence causes masculinization of drug/steroid metabolism (Virgo, 1985). This would explain the ultradiene pattern seen in the normal male as some growth hormone (the peak) is necessary for normal growth and development, while the clearance of growth hormone from the circulation (the trough) allows masculinization of various physiological parameters to be maintained. The maintenance of a low level of growth hormone in the circulation is probably dependent upon circulating testosterone levels (Jansson et al., 1984) which are capable of inhibiting growth hormone release from the pituitary (Hall et al., 1984). Since testosterone levels are decreased in diabetes this inhibitory and clearing effect is not seen, leading to feminization.

Since the s.c. injection of growth hormone did not duplicate the normal secretory pattern, we decided to try intravenous injections to obtain the plasma peaks. The effectiveness of intravenous injections over either s.c. injections or continuous intavenous infusion in duplicating normal growth hormone patterns in the male rat has recently been demonstrated (Clark et al., 1985). The ineffectiveness of growth hormone to restore the HCLA binding protein levels was again seen (Tables V and VI). This lack of effect remained when testsoterone and insulin were added to the treatment regimen to restore a more normal

physiology, e.g. testosterone to obtain a more normal baseline of growth hormone after the injection. We were injecting four times per day, not the physiological seven of the ultradiene pattern. This may have had an effect on the lack of restoration of HCLA binding protein levels although normalization of hepatic drug and steroid metabolism has been seen with four injections per day (Skett and Young, 1982).

These studies were marked by a further complication. Animals which had been cannulated and those non-diabetic controls undergoing tail vein injections 4 times per day showed markedly reduced levels of the HCLA binding protein compared to non-manipulated controls (Tables V and VI, Fig. 6). The level of decrease in the non-diabetic tail vein injected controls was approximately 50% from the non-manipulated controls. The levels of the HCLA binding protein in the tail vein injected diabetic animals (Table VI) was a further 50% below the reduced control value. It thus appeared that these manipulations (i.e. surgery and conscious tail vein injection) caused a decrease which was additive to that of diabetes. It has been reported (Nakashima et al., 1975) that the stress of surgery can lower serum testosterone levels for several days. The animals undergoing tail vein injections four times per day appeared to be undergoing stress probably due to pain and handling; presumably this would be similar to the stress of surgery and subsequent intolerance of the

cannula. This lowering of testosterone could lead to the decreased levels of the HCLA binding protein in controls, and the further decrease in the already depressed diabetics. Another alteration of testosterone levels could be *via* an interference with the prolactin and corticotropin interaction which leads to adrenal androgen release (Higuchi *et al.*, 1984). Since prolactin levels are decreased in diabetes, a reduction in the ability of this pathway to produce testosterone would also be seen. Catecholamines are elevated by stress and there is evidence that an α-adrenergic mechanism is involved in the regulation of growth hormone secretion (Terry and Martin, 1981) (i.e. catecholamines act to cause release). However, rats react to stress by inhibiting the secretion of growth hormone (Terry *et al.*, 1977) which would suggest that this factor is not of major involvement in the effect seen.

4) Other species

No detectable binding was observed in the other species examined for the presence of the hepatic HCLA binding protein. These particular species (C57BL/6 mice, DBA/2 mice, guinea pigs) were selected due to the individual variation seen amongst the rats used in the experiments. Since genetics are not that well worked out in rats, we used mice where the genetics are clearly defined, to see if there might be an individual locus with which the HCLA binding protein segregated. We chose these species because of

the known differences in the Ah locus which controls the so-called T.C.D.D receptor, since there was a suggestion that there may be an relationship between this receptor and the HCLA binding protein (Finlayson, 1983). The C57BL/6 mice and guinea pigs have a high affinity T.C.D.D. receptor system, while the DBA/2 mice posess a very low T.C.D.D receptor system. The lack of evidence for the HCLA binding protein in all three species indicates that there is not an association between the T.C.D.D. receptor system and the HCLA binding protein. It also indicates that the HCLA binding protein may be a rat specific protein.

PROPOSED FUTURE EXPERIMENTS

As previously mentioned, a longer time course of hormonal treatments should be undertaken (specifically with testosterone) since it has been shown that it takes two weeks for the HCLA estrogen binding protein to appear in hypophysectomized female rats. It could be that the control mechanism in male diabetics is damaged and takes a longer amount of time (with the proper regulatory hormones) before levels of the protein can be regenerated. A time course of the depression of the HCLA binding protein level should be undertaken to see how quickly the depression occurs and if it can be correlated with any other alterations occurring within (or slighty before) the same time span.

Testosterone replacement should be attempted in conscious tail vein injected control animals to further delineate its role in the HCLA binding protein regulation. Adrenalectomy should be performed on control and diabetic rats to see what effect catecholamines may be having in the regulation in the non-stressed animal, and the same operation should be performed on the stressed animals to see if there are further alterations, and more completely examine the role of stress on the HCLA estrogen binding protein.

Ligand specificity studies should be performed on the diabetic animals to see if there are any fundamental alterations in the HCLA binding protein system other than

reduced capacity. Spontaneous diabetic B.B. rats should be studied using the same assay system to see if the effects of their disease state parallel those seen with STZ or if there are differences in these two states. If there are, these would have to be investigated to see if they are a result of the chemical used or if they are due to differences in the pathologies of the diseases. Other tissues should be examined in control and diabetic animals to see if the HCLA binding protein is exclusive to the liver or if it is more diffuse in distribution, which would have implications as to its possible function. Enucleation experiments to examine the exact intracellular distribution of the HCLA binding protein would be of interest in light of the controversy developing over the localization of estrogen receptors. Finally, isolation, further characterization, and amino acid sequencing of the HCLA binding protein should be carried out.

SUMMARY

STZ-induced diabetes causes a decrease in the capacity of the HCLA estrogen binding protein found in male rat liver without affecting the Kd. Hormonal treatments including insulin, testosterone, triiodothyronine, and growth hormone were ineffective in restoring the reduced levels when they were administered in doses and patterns which duplicated normal physiology. This inability to restore the HCLA estrogen binding protein levels indicates that there is not a direct interplay between these hormones and the HCLA levels in the diabetic male rat. The lack of effect of these hormonal treatments to restore the HCLA estrogen binding protein levels, while they do restore the alterations in drug/steroid metabolism seen in diabetes, lead us to conclude that there is no direct connection between the HCLA estrogen binding protein and the sex-dependent drug metabolism patterns observed in the rat. Also, the HCLA estrogen binding protein was not detectable in the liver cytosol of several other species examined indicating that it may be rat specific.

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APPENDIX I

1) Saturation curves

The data contained in this section were samples of saturation curves which were used to generate the Scatchard plots in the Results section. The saturation curves had to indicate that specific, saturable binding over the ligand concentration range used was taking place before a Scatchard plot could be generated with confidence.

Figure A1

Specifically bound [^{3}H]-estradiol *vs* ligand concentration ([^{3}H]-estradiol) in tail vein injected citrate control animal. Incubations carried out as described in the Methods section. (o——o) Specific bound, (□——□) non-specific bound. Graph shown is of a single experiment and representative of the results found.

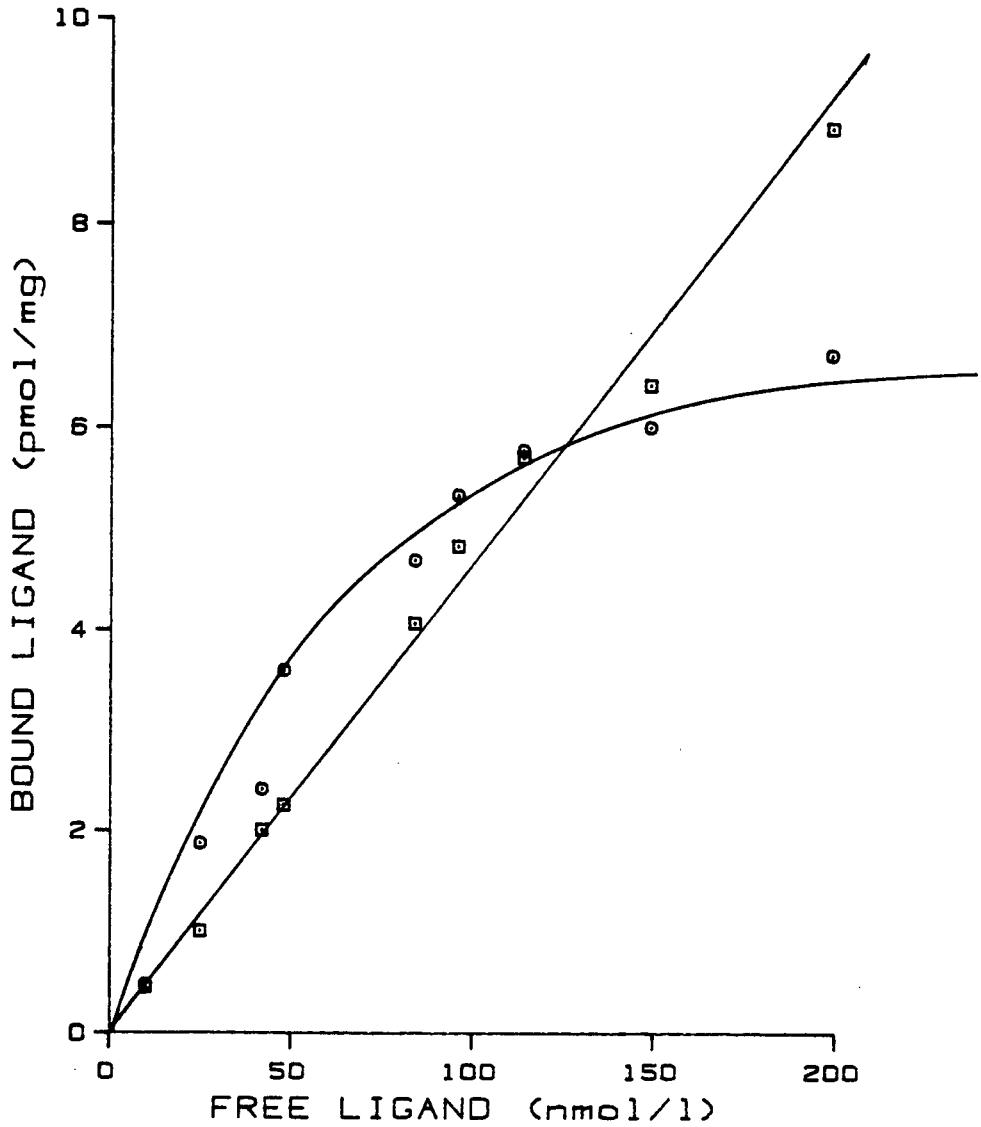
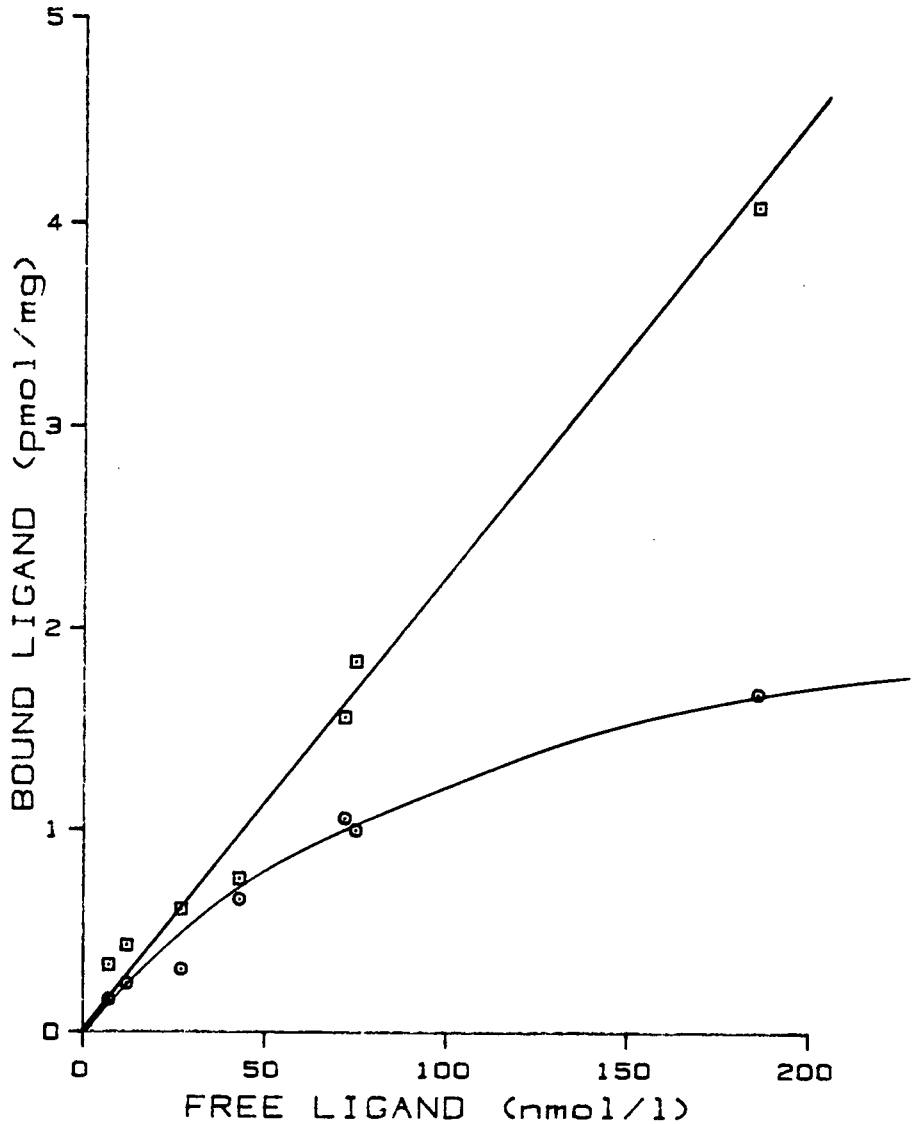

10
8
6
4
2
0
BOUND LIGAND (pmol/mg)
0
50
100
150
200
FREE LIGAND (nmol/l)

Figure A2

Specifically bound [^{3}H]-estradiol *vs* ligand concentration ([^{3}H]-estradiol) in 4 day STZ-induced diabetic animal. Incubations were carried out as described in the Methods section. (o——o) Specific bound, (□——□) non-specific bound. Graph shown is of a single experiment and representative of the results found.


BOUND LIGAND (pmol/mg)
FREE LIGAND (nmol/l)
0
1
2
3
4
5
0
50
100
150
200