THE MODULATION OF THE HEPATIC CYTOCHROME P450-
DEPENDENT MIXED-FUNCTION OXIDASE SYSTEM BY
INSULIN-DEPENDENT DIABETES MELLITUS.

A Thesis presented for the degree of Doctor of Philosophy

by

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To my parents and family
ABSTRACT

The effects of type I (insulin-dependent) diabetes mellitus on the hepatic cytochrome P450-dependent mixed-function oxidase system was investigated in both chemically-induced and spontaneously diabetic rats. Sex-specific influences on these changes were evaluated in male and female streptozotocin-induced diabetic rats. Further studies examined the ability of hepatic fractions from both chemically-induced and spontaneously diabetic rats to bioactivate known chemical carcinogens in the Ames mutagenicity assay. The role of ketone bodies and hyperglycaemia in the diabetes-induced alterations were investigated in rats rendered hyperketonaemic by dietary manipulation. Finally, the effects on hepatic drug metabolism of hypertension, using spontaneously hypertensive rats, type II (non insulin-dependent) diabetes using the spontaneously hyperglycaemic mouse and hyperinsulinaemia, by insulinoma transplantation in the rat were also determined.

Streptozotocin-induced diabetes was associated with significant elevations of the enzyme activities catalysed by the cytochrome P450 IA, IIB, IIE, IIIA and IVA sub-families, a finding confirmed by immunological analysis. These alterations were a consequence of the diabetic state and not the diabetogen per se, as shown by the inclusion of a group of rats which received nicotinamide concurrently with streptozotocin to counter the effect of the diabetogen. Long-term streptozotocin-diabetic rats continued to display elevations of the cytochrome P450 IA and IIB families. However, after four weeks, the severity of ketosis declined and the elevation in activities of the cytochrome P450 IIE, IIIA and IVA families became less pronounced. Investigation of sex-related alterations in the diabetes-induced changes revealed that induction was always more pronounced in the male than the female.

The spontaneously diabetic BB rat exhibited similar alterations of hepatic cytochrome P450 families as those observed in the streptozotocin-induced diabetic rats.
However, in BB rats, all of the changes apparent at one month following onset of the
disease were similarly observed in six month diabetic animals. Hepatic fractions from both
chemically-induced and spontaneously-diabetic rats were markedly more efficient at
activating selected chemical carcinogens in the Ames mutagenicity assay. However, these
fractions were less efficient at activating the aromatic amine, 2-aminofluorene, suggesting
that diabetes modulates other enzyme systems capable of drug metabolism.

The mechanism responsible for the diabetes-induced changes in hepatic drug
metabolism has been proposed to involve ketone bodies. In our experiments using rats
rendered hyperketonaemic by dietary manipulation, we found that similar alterations of
hepatic drug metabolism occurred as previously observed in diabetic animals. As the
hyperketonaemic animals were normoglycaemic, it can be concluded that it is the ketone
bodies that have a major role in the diabetes-induced changes in hepatic drug metabolism
and not hyperglycaemia. However, hyperketonaemia failed to modulate the levels of
cytochrome P450 IIIA as seen in the diabetic animals, indicating that other mechanisms are
involved. Further work examining the effect of specific ketone bodies revealed that acetone
was a potent inducer of cytochrome P450 IA, IIB and IIE proteins. This study also
demonstrated that ketone bodies could not modulate the levels of cytochrome P450IV A.

These studies provide compelling evidence for a direct link between ketone bodies
and the induction of the cytochrome P450 IA, IIB and IIE subfamilies. Clearly, the
mechanism of cytochrome P450 IIIA and IVA induction are dependent on other factors.
However, in the case of cytochrome P450 IVA subfamily our studies suggest that the
increased levels of circulating free fatty acids, accompanying type I diabetes, are
responsible for the induction.

Finally, our investigations into the effects of type II diabetes, hypertension and
hyperinsulinaemia on hepatic drug metabolism revealed that type II diabetes and
hypertension have little effect on the cytochrome P450 families investigated. However, hyperinsulinaemia was associated with a selective induction of the cytochrome P450 IA subfamily, indicating that perturbations of endogenous insulin levels may have marked influences on hepatic drug metabolism and consequently the toxicity / carcinogenicity of chemicals.
ACKNOWLEDGMENTS

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<tr>
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<tr>
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Chapter 1

Introduction
1.1 Diabetes Mellitus

Diabetes mellitus has been identified in many animal species but there appears to be a higher incidence of the disease in humans where it is a leading cause of morbidity and mortality amongst the adult population (Peiris and Gustafson, 1986). Major complications associated with the disease include; retinopathy, nephropathy, peripheral vascular disease and neuropathy (Perring et al. 1985; Clements and Bell, 1986).

Diabetes can be characterised into three distinct types, in each of which subtypes have been identified (National Diabetes Data Group, 1979).

Type I, Insulin-dependent diabetes mellitus

Usually characterised by abrupt onset of symptoms, insulinopenia and dependence on injected insulin to sustain life, and proneness to ketosis. Classically, this type of diabetes occurs in juveniles and it was formerly termed juvenile diabetes. However, it can be recognised and become symptomatic for the first time at any age. In addition to the ketosis-prone stage, this type of diabetes can also be recognised in a preketosis-prone stage. Type I diabetes mellitus appears to be heterogeneous in terms of genetics and environmental factors that precipitate the disease (Rotter and Rimoin, 1978). Genetic factors are thought to be important in most patients, as expressed by the associated increased or decreased frequency of certain histocompatibility antigens (Cudworth and Woodrow, 1976). Abnormal immune response and autoimmunity are also thought to play an etiologic role, and islet cell antibodies are frequently present at diagnosis in this type of diabetes.
Type II, non insulin-dependent diabetes mellitus

This type of diabetes frequently occurs with minimal, or no symptoms of the metabolic alterations associated with type I diabetes. Patients are not dependent on insulin for prevention of ketonuria and are not prone to ketosis. However, they may become ketotic in certain instances such as severe stress caused by infection or trauma. There may be normal levels of insulin, mild insulinopenia or above normal levels of insulin associated with insulin resistance. Most patients who develop type II diabetes usually do so after the age of 40, although type II diabetes can occur in younger people. Type II diabetes has a genetic basis which appears to be more evident than for type I diabetes. Environmental factors superimposed on genetic susceptibility are undoubtedly involved in the onset of this type of diabetes. Excessive weight gain leading to obesity is probably an important factor in its pathogenesis. Type II diabetes has been subdivided according to the presence or absence of obesity, revealing that in Western societies 60 - 90% of all type II diabetics are obese. Hyperglycaemia and glucose intolerance are usually improved by weight loss. In this type of diabetes islet cell antibodies have not been found although it is possible for type II diabetics to progress to type I diabetes mellitus (National Diabetes Data Group, 1979).

Other types of diabetes

In this subclass diabetes forms part of certain other conditions and syndromes. For example, diabetes may be secondary to pancreatic disease or removal of pancreatic tissue, endocrine diseases such as acromegaly, Cushing's syndrome, glucagonoma or somatostatinoma, or the administration of certain drugs, hormones or chemicals that cause hyperglycaemia. Finally, diabetes may also be associated with defective insulin receptors present on the cells.
1.2 Animal models of diabetes mellitus

Syndromes resembling human diabetes occur spontaneously in many animal species and can also be induced by treating animals with drugs or viruses, excising the pancreas or manipulating the diet (Salans and Graham, 1982). None of the known animal models can, without reservation, be taken to reproduce all the characteristics of human diabetes, but they are believed to illustrate various types of etiology and pathogenetic mechanisms that in principle could operate in man.

1.2.1 Animal models of type II diabetes mellitus

Most animal models of type II diabetes show the same characteristics of obesity and elevated plasma insulin as human type II diabetics. One of the most common models is the genetically hyperglycaemic obese mouse. This strain originated in the early 1940's at the Jackson Laboratory, Maine. In these animals the obesity gene \((ob)\), is recessive, with the homozygous recessive animal becoming obese and weighing three to four times as much as their lean littermates. They also develop severe insulin resistance and diabetes. Other animal models are also due to the inheritance of a single gene. For example the diabetic \((db/db)\) mouse or the Zucker or fatty \((fa/fa)\) rat. The spontaneously obese rhesus monkey may be a particularly useful model of human obesity and diabetes.

Other models of type II diabetes have been developed employing chemical and surgical means, or by manipulating the diet. Surgically damaging the ventromedial nucleus, a part of the brain involved with the endocrine system and feeding behaviour, results in an obesity syndrome similar to that of the genetically obese hyperglycaemic mouse. Chemicals such as gold thioglucose and monosodium glutamate injected intraperitoneally and into the brain respectively, can also damage the hypothalamus with similar results. In the case of monosodium glutamate, injection completely abolishes growth hormone secretion. This results in the induction of several P450 isoforms also induced in type I diabetes a disease associated with altered growth hormone levels.
1.2.2 Animal models of type I diabetes mellitus

The study of animals displaying spontaneous diabetes has shown that the majority are models of type II diabetes. The lack of models for type I diabetes resulted in the use of chemically induced diabetes and pancreatectomised animals. However, new models for this type of diabetes have been established in rodents and are now being used more widely to study this form of the disease.

Insulin-dependent diabetes is usually characterised by:

1. An abrupt onset of overt diabetes
2. Dependence on injected insulin to sustain life
3. Proneness to ketoacidosis
4. Massive lymphocyte infiltration around or in pancreatic islets
5. The appearance of islet antibodies and islet surface antibodies in the plasma

Chemically produced insulinopenic conditions are not always associated with dependence on insulin, lymphocytic infiltration or the production of islet cell antibodies in the plasma. Therefore, although these models display the main characteristics of type I diabetes, they are by no means completely representative of all the pathophysiological conditions.

To date only two spontaneously diabetic animal models of type I diabetes have been characterised for research. These are the Bio-Breeding (BB) Wistar rat (Marliss et al., 1982) and the non-obese diabetic (NOD) mouse (Tochino, 1987).
The spontaneously diabetic BB rat

The spontaneously diabetic BB rat was discovered in 1974 (Chappel, 1983), in a commercial breeding laboratory (the Bio-Breeding laboratories, Ottawa, Ontario). The strain appears very susceptible to pulmonary and other infections by common varieties of bacteria, mycoplasma and viruses. Two major factors are involved in the insulitis, a genetic and an immunological component, with multiple lines of evidence suggesting that altered immunity is involved in the aetiology of the syndrome.

Overt insulin-dependent diabetes occurs from 40-140 days with a mean age at onset of glucosuria of about 90 days. From onset the animals are dependent on exogenous insulin for survival. The dramatic feature of this form is the rapidity of progress from normal to grossly diabetic, with a time course measurable in hours to days (Nakhooda et al, 1978). Typically, hyperglycaemia (20-30mM), polyuria and polydipsia (100ml/day) and ketonuria (2-3mmol/day), weight loss and dehydration develop. The state is accompanied by hypoinsulinaemia (detectable within 4-8 days) and relative or absolute hyperglucagonaemia. Urea nitrogen and 3-methylhistidine excretion double, renal ammoniagenesis increases 6-fold (Nakhooda et al, 1980) and elevations in the levels of blood branched chain and other amino acids occur (Nakhooda et al, 1977). In contrast to human diabetes, blood alanine is normal and it is the glutamine levels that are elevated.

The BB rat also exhibits similar diabetic complications to those encountered in human diabetic patients such as retinopathy and neuropathy. The BB rat appears to be the most appropriate, currently available, model for the study of both acute and chronic metabolic alterations and complications associated with type I diabetes mellitus (Marliss et al., 1982; Shafirir et al., 1988)
Chemically-induced Type I diabetes mellitus

The relatively recent discoveries of the BB rat and the NOD mouse models of type I diabetes will prove invaluable models for investigating the pathogenesis of the disease in humans. Although more relevant to human insulin dependent-diabetes, the spontaneously diabetic models have not been extensively used. This has been largely due to the limited supply of the animals, their expense and the intensive care that they require. The more commonly used models are induced by chemical means. The two most frequently employed diabetogens are streptozotocin and alloxan, although alloxan is now infrequently used because of its extrapancreatic toxicity.

Alloxan

The diabetogenic effect of alloxan was first discovered by Dunn et al., (1943) while investigating the nephrotoxicity of a series of uric acid derivatives including alloxan. Alloxan (2,4,5,6, tetraoxohexahydropyrimidine) is an unstable and reactive compound that exists in several tautomeric forms (Figure 1.1). Alloxan is readily reduced to dialuric acid (Rerup, 1970).

Streptozotocin

Streptozotocin (Figure 1.1), the common name for 2-deoxy-2-(3-methyl-3-nitrosoureido)-D-pyranose, is an antibiotic isolated from Streptomyces achromogens. This agent has antibacterial and antitumor properties, as well as being carcinogenic and diabetogenic.
The mechanism of alloxan and streptozotocin diabetogenicity

The alloxan-dialuric acid coupling consumes oxygen and produces hydrogen peroxide when a reducing agent is present. The production of \( \text{H}_2\text{O}_2 \) attributed to the

Figure 1.1 The two most commonly used diabetogenic agents and their possible reactive intermediates.

ALLOXAN

\[
\begin{align*}
\text{HN} & \quad \text{CH}_2\text{OH} \\
\text{O} & \quad \text{OH} \\
\text{N} & \quad \text{NO} \\
\text{O} & \quad \text{CH}_3
\end{align*}
\]

2,4,5,6-tetraoxohexahydropyrimidine

DIALURIC ACID

\[
\begin{align*}
\text{HN} & \quad \text{CH}_2\text{OH} \\
\text{O} & \quad \text{OH} \\
\text{N} & \quad \text{NO} \\
\text{O} & \quad \text{CH}_3
\end{align*}
\]

STREPTOZOTOCIN

2-deoxy-2-(3-methyl-3-nitrosoureido)-D-pyranose

The quinone structure of alloxan (Dunlin et al., 1983), can be inhibited by superoxide dismutase, catalase and hydroxyl scavengers such as benzoate and ethanol, suggesting that the Haber-Weiss reaction is the source of the hydroxyl radicals (Dunlin et al., 1983). Alloxan-
mediated production of free radicals and its interaction with glutathione has been investigated by Winterbourne and Munday (1989), who demonstrated that alloxan undergoes redox cycling producing $\text{H}_2\text{O}_2$. Alloxan reacted in small quantities with glutathione producing an unidentified adduct which gradually accumulated. Thus, the mechanism of alloxan toxicity depends on its ability to produce free radicals, but the question remains as to why the $\beta$-cells of the pancreas are so highly susceptible to alloxan toxicity. One possibility is selective uptake of alloxan, although in vivo studies have shown that the liver takes up alloxan as rapidly and extensively as the $\beta$-cells but is resistant to its toxicity. There is evidence for higher concentrations of alloxan produced reactive oxygen species within $\beta$-cells than in other tissues. Malaisse (1982) concluded that both the capacity to take up alloxan and the sensitivity to oxygen species must be taken into account when examining tissue susceptibility to alloxan. Therefore, the $\beta$-cell susceptibility to alloxan may be due to the accumulation of alloxan in the cells and the poor ability of these cells to protect themselves against the deleterious effects of the generated reactive oxygen species (Malaisse-Lagae et al., 1981).

Streptozotocin contains a glucose moiety which is responsible for its selective uptake by the $\beta$-cell. Without this moiety little $\beta$-cell toxicity occurs (Anderson et al., 1976). Once within the $\beta$-cell it is believed that streptozotocin is metabolised by scission of the 2'-carbon and the methyl nitrogen. The N-nitrosoureido moiety is retained in the cell causing the cytotoxicity (Karunanayke et al., 1975a, 1975b). This moiety is believed to break down into the methyl carbonium ion (Calabresi and Parks, 1980) or methyl radical (Uchigata et al., 1982) which can alkylate and cross-link DNA. The DNA damage activates the enzyme poly (ADP-ribose) synthetase which is involved in DNA repair and requires $\text{NAD}^+$ (Wilson et al., 1984). Activation of this enzyme may deplete the cellular levels of $\text{NAD}^+$ leading to cytotoxicity. Inhibitors of this enzyme have prevented the decrease in cellular $\text{NAD}^+$ following streptozotocin administration and furthermore, treatment of animals with nicotinamide before and during streptozotocin treatment has also protected against the toxicity of the diabetogen (Dunlin et al., 1969; Schein et al.,
1967). Work by Okamato and Yamamoto (1983) has shown that poly (ADP-ribose) synthetase inhibitors did not prevent strand breaks from occurring, but that these inhibitors did prevent diabetes. Therefore, β-cells may survive with residual DNA damage within their genome and it has been reported that even after a year following the combined administration of alloxan or streptozotocin with poly (ADP-ribose) synthetase inhibitors, diabetes did not develop.

A variety of free radical scavengers have been reported to protect against the diabetogenic effect of streptozotocin, but not all of the findings are consistent (Boquist 1989; Oberley 1988). However, it is possible that if the target of free radicals or reactive intermediates generated by alloxan or streptozotocin respectively, is the DNA, then a common mechanism involving poly (ADP-ribose) synthetase activity could explain the toxicity of these compounds to the cell. This coupled with selective uptake of streptozotocin by the β-cell and the poor ability of the β-cell to protect itself against reactive species may explain the diabetogenicity of these compounds (Oberley, 1988).

1.3 Hepatic drug metabolism

The evolutionary success of all species is dependent on the ability to adapt to an ever changing environment. Since the first animals inhabited the land they have been exposed to a multitude of different environmental chemicals. The necessity to utilise some of these chemicals and effectively detoxify others has led to the development of highly complex enzyme systems within present day animals. Man in particular is exposed to a plethora of diverse chemical agents in the air he breaths, the food he eats and the water he drinks. Of major concern has been occupational exposure to many natural and man-made chemicals used routinely in industrial processes, some of which have been associated with human cancer.
All xenobiotics once introduced into the body, either deliberately or accidentally, undergo metabolism predominantly in the liver. The metabolism of xenobiotics can be largely divided into two distinct processes; those involving primarily oxidation of the compound (Phase I metabolism) and those associated with the conjugation of the Phase I metabolite with endogenous substrates such as glucuronic acid, sulphate, some amino acids or glutathione (Phase II metabolism). In general, the resulting metabolite conjugate is much more polar than the parent compound, thus facilitating its excretion in the urine and bile (Brenner, 1977).

Although xenobiotic metabolism primarily results in the detoxification or deactivation of compounds, there are occasions when metabolism can result in the production of reactive intermediates, a process termed "bioactivation". Many chemicals requiring bioactivation are quite often chemically inert per se and biologically innocuous but are rendered highly toxic by normal xenobiotic metabolism (Guengerich and Lieber, 1985). Most reactive intermediates possess an electrophilic character and readily attack the nucleophilic centers of cellular molecules by non-enzymic and covalent interaction (Miller and Miller, 1976). At particular risk are the sulphhydril groups of functional proteins and the purine and pyrimidine bases of DNA. Alternatively, these intermediates may react with molecular oxygen producing free radicals which can then initiate lipid peroxidation or attack the DNA. The interaction of these intermediates with critical cellular macromolecules can result in cytotoxicity, immunotoxicity, mutations and cancer if not prevented by the cell (Figure 1.2; Heidelberger, 1975).
The cell has several defence mechanisms towards reactive intermediates and free radicals including; deactivation by non-functional nucleophilic conjugating species, such as glutathione, catalysed by glutathione-S-transferases and the presence of catalase and superoxide dismutase to deactivate hydrogen peroxide and the superoxides respectively. Obviously, the levels of cellular conjugating species and enzymes will determine the ability of the cell to protect itself against the cytotoxic actions of such species (Guenthner and Oesch, 1981).

The reactive intermediates produced by the bioactivation of xenobiotics, although a serious problem, are not usually the predominant species resulting from metabolism. The reason is that a single compound may have many different possible metabolic products of which only a few may be biologically reactive. Therefore, the amount of reactive intermediate formed will be dependent on the rate of the competing pathways of bioactivation and detoxification. It can be concluded, that the toxicity of a compound is not simply a consequence of its molecular structure and physicochemical properties alone, but also of the enzyme complement of the exposed animal. These enzymes systems are predominantly under genetic control but can be modulated by a number of factors including; environmental compounds (Alvares, 1979), nutritional status (Campbell and Hayes, 1974) and disease (Kato, 1977). Such factors can disturb the delicate balance between the bioactivating and detoxifying systems and thus the response to chemical toxins and therapeutic agents. An impairment of drug metabolism can result in accumulation of the drug with adverse responses on repeated administration, whereas stimulation of metabolism can lead to a reduced response or complete abolition of the desired pharmacological effect.

Perhaps the most dramatic alterations in metabolism are encountered during disease, especially those which compromise hepatic function directly such as, hepatitis, cirrhosis of the liver and hepatocarcinoma. The complex interactions of various endogenous regulatory chemicals are disturbed producing pronounced modulation of xenobiotic
metabolism. Exposure to toxic chemicals during disease, even those of normally low toxicity, can have prolonged and sometimes fatal consequences. Even diseases which are not necessarily associated with liver dysfunction, such as diabetes mellitus have also been shown to perturb xenobiotic and endogenous metabolism. This is presumably due to the fact that the enzymes systems associated with xenobiotic metabolism are partly regulated by the circulating levels of endogenous substrates. One such enzyme system is the cytochrome P450 mediated mixed-function oxidase system, probably the single most important enzyme system involved in xenobiotic metabolism.

The cytochrome P450-dependent mixed-function oxidase system

The cytochrome P450 system is actively involved in the endogenous metabolism of a variety of substrates such as; fatty acids, vitamins, bile acids, prostaglandins and steroids (Yang and Lu, 1987). However, it is the involvement of this system in the metabolism of almost every xenobiotic that has initially aroused the most intense interest.

The key enzymic components of the system are the flavoprotein NADPH-cytochrome P450-oxidoreductase and the haemoprotein cytochrome P450. After the initial characterisation of the haemoprotein by Omura and Sato (1961), it became evident through further purification efforts that multiple forms of cytochrome P450 existed in mammals and other species. These different isoforms of cytochrome P450 had different, often overlapping substrate specificities. Most cytochrome P450 mediated metabolism results in the insertion of one atom of oxygen, from atmospheric O₂, into the substrate. Depending on the particular reaction and the nature of the various unstable intermediates, different reactions can occur (Table 1.)

The net result of the multiplicity of the cytochrome P450's and their diverse and overlapping substrate specificities is the ability to metabolise a multitude of chemicals,
thereby representing the major enzyme system involved in Phase I metabolism. The cytochrome P450 system also responds to environmental and endogenous substrates by being inducible by a wide variety of compounds including: phenobarbital, 3-methylcholanthrene, ethanol, clofibrate and pregnenolone-16α-carbonitrile (PCN). Each inducing agent is now known to exert a characteristic impact on both the induction of total cytochrome P450 and the levels of specific isoforms. Furthermore, with certain inducing agents, the overall amount of cytochrome P450 may not be altered because some of the isoforms may be induced as others are concomitantly repressed. In addition, some forms of cytochrome P450 are refractory to all known inducing agents, termed constitutive cytochrome P450, which are largely active in endogenous metabolism. The cytochrome P450 content can also be modified by age, sex, diet and state of health, and being so intricately involved in Phase I metabolism provides some explanation for the differences observed in xenobiotic metabolism associated with these factors.

The cytochrome P450 gene family so far consists of 13 gene families, including two bacterial, two yeast, one insect and eight vertebrate families (Nebert and Gonzalez, 1985). The gene families are designated by Roman numerals beginning with I and with gaps being left in the family numbers to allow for the addition of newly discovered gene families. Sub-families are denoted with sequential capital letters and genes within a sub-family by sequential Arabic numerals (Table 2).

Families I through IV primarily code for catabolic enzymes found in the liver and to some extent in extrahepatic tissues, whereas families XVII, XIX, XXI and XXII code for the cytochrome P450's involved in steroid biosynthesis. Gonzalez (1989) and Nebert and Gonzalez (1987) have recently reviewed the current concepts of cytochrome P450 evolution, structure, function and regulation.
Table 1. Reactions of the cytochrome P450 mixed-function oxidase system

<table>
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<tr>
<th>Reaction</th>
<th>Example</th>
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<td>Aromatic hydroxylation</td>
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<tr>
<td>Aliphatic hydroxylation</td>
<td>Phenobarbitone hydroxylation</td>
</tr>
<tr>
<td>Epoxidation</td>
<td>Epoxidation of benzo[a]pyrene</td>
</tr>
<tr>
<td>N-Dealkylation</td>
<td>N-demethylation of diazepam</td>
</tr>
<tr>
<td>O-Dealkylation</td>
<td>Deethylation of ethoxyresorufin</td>
</tr>
<tr>
<td>S-Dealkylation</td>
<td>Deamination of amphetamine</td>
</tr>
<tr>
<td>N-Oxidation</td>
<td>N-oxidation 2-acetylaminofluorene</td>
</tr>
<tr>
<td>S-Oxidation</td>
<td>S-oxidation of chlorpromazine</td>
</tr>
<tr>
<td>Phosphothionate oxidation</td>
<td>Oxidation of parathion</td>
</tr>
<tr>
<td>Dehalogenation</td>
<td>Dehalogenation of halothane</td>
</tr>
<tr>
<td>Alcohol oxidation</td>
<td>Oxidation of ethanol</td>
</tr>
</tbody>
</table>

Gibson and Skett (1986)

1.4 Alterations of hepatic drug metabolism by type I diabetes mellitus

1.4.1 Phase I metabolism

It is now well documented that diabetes mellitus induced by streptozotocin or alloxan is associated with alterations in the hepatic metabolism of xenobiotics. Dixon et al. (1961) first reported the prolonged sleeping time of animals to hexobarbital and the decreased metabolism of chlorpromazine and codein after treatment with alloxan.
<table>
<thead>
<tr>
<th>Family, Subfamily and gene designation</th>
<th>Other commonly used names</th>
<th>Typical inducing agents</th>
<th>Specifically catalysed reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450 I only one subfamily</td>
<td></td>
<td>PAH's</td>
<td>Ethoxyresorufin O-deethyldase</td>
</tr>
<tr>
<td>P450 I A1 Rat c, Mouse P1,</td>
<td></td>
<td>e.g. Benzo[a]pyrene</td>
<td></td>
</tr>
<tr>
<td>Rabbit form 6,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450 I A2 Rat d, mouse P3,</td>
<td></td>
<td>Isosafrole</td>
<td>Glu-P-1 N-hydroxylase</td>
</tr>
<tr>
<td>Rabbit form 4,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450 II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450 IIA subfamily</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450 IIA A1 Rat a</td>
<td></td>
<td>3-methylcholanthrene,</td>
<td>Testosterone 7α-hydroxylase</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450 IIB subfamily</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P450 IIB B1 Rat b, Rabbit form 2</td>
<td></td>
<td>Phenobarbital</td>
<td>Pentoxyresorufin O-depentyldase</td>
</tr>
<tr>
<td>P450 IIB B2 Rat e</td>
<td></td>
<td>Phenobarbital</td>
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<tr>
<td>P450 IIE subfamily</td>
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</tr>
<tr>
<td>P450 IIE B1 Rat j, Rabbit form 3a,</td>
<td></td>
<td>Ethanol, isoniaizid,</td>
<td>p-Nitrophenol hydroxylation</td>
</tr>
<tr>
<td>P450 alc, Human j</td>
<td></td>
<td>acetone.</td>
<td></td>
</tr>
<tr>
<td>P450 III only one subfamily</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450 III A1 Rat pcn 1</td>
<td></td>
<td>Pregnancy 16α-carbonitrile, phenobarbital</td>
<td>Ethylmorphine N-demethylase</td>
</tr>
<tr>
<td>P450 III A2 Rat pcn 2</td>
<td></td>
<td>Pregnancy-16α-</td>
<td>Testosterone 6β-hydroxylase</td>
</tr>
<tr>
<td>carbonitrile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450 IV subfamily</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450 IV A1 Rat LA</td>
<td></td>
<td>Clofibrate</td>
<td>Lauric acid ω-hydroxylase</td>
</tr>
</tbody>
</table>
Subsequent studies showed a general depression of the metabolism of numerous substrates including; the N-demethylation of aminopyrine (Chawalit et al., 1982; Reinke et al., 1978; Toda et al., 1987), benzphetamine (Watkins et al., 1988) and ethylmorphine (Peng et al., 1983); the hydroxylation of benzo[a]pyrene, phenylbutazone and zoxazolamine are also depressed (Ackerman and Leibman, 1977; Kato and Gillette, 1965; Dajani and Saheb, 1974).

Further studies revealed that in chemically-induced type I diabetes mellitus the metabolism of some compounds was enhanced by the diabetic state. These included; the hydroxylation of aniline and the O-demethylation of p-nitroanisole, O-deethylation of 7-ethoxycoumarin and the N-demethylation of dimethylnitrosamine (Toda et al., 1987; Luangkingkasoot et al., 1988; Al-Turk et al., 1980; Peng et al., 1983). Interestingly Eacho and Weiner (1980) found a decrease in aniline hydroxylation and p-nitroanisole demethylation in hepatocytes isolated from streptozotocin diabetic rats, although this may be due to the rapid conjugation of the phase I metabolites within the hepatocytes.

Chemically-induced type I diabetes has not only been associated with alterations of xenobiotic metabolism, but also endogenous steroid and vitamin metabolism. The serum levels of 1,25-dihydroxy vitamin D₃ are reduced but as the levels of 25-hydroxy vitamin D₃ are unaffected it appears that it is the 1-hydroxylase activity of the kidney that is impaired in diabetes. Steroid hydroxylation has also been shown to be impaired in both hepatocytes (Hussin and Skett, 1988) and microsomal preparations (Skett, 1986; Reinke et al., 1978).

Modulation of the cytochrome P450 mixed-function oxidase system by type I diabetes.

The involvement of the cytochrome P450 system in the metabolic modifications associated with type I diabetes was first documented by the studies of Past and Cook (1980,
1982) who isolated a diabetes-inducible cytochrome P450, subsequently termed P450i in the rat and P4503a in the rabbit (Ryan et al., 1986) but now termed P450 IIE1. This isoform of P450 has been shown to have a high turnover rate for aniline hydroxylation and dimethylnitrosamine demethylation. The isoform has been isolated and characterised from both alloxan and streptozotocin induced animals and is also inducible by a variety of small molecules such as ethanol (Ryan et al., 1986), isoniazid (Ryan et al., 1985) and acetone (Koop et al., 1985) as well as some physiological states such as fasting (Lorr et al., 1984).

There is evidence that other families of cytochrome P450 are modulated by type I diabetes mellitus including; P450 IA, P450 IIB, families (Ioannides et al., 1988; Flatt et al., 1989). Sex dependent alterations in the levels of specific constitutive forms of cytochrome P450 involved in steroid hydroxylase activity have also been characterised (Favreau and Schenkman, 1988a) and it has also been suggested that type I diabetes is associated with an increase in the microsomal FAD-monoxygenase (Rouer et al., 1987), an enzyme system also involved in xenobiotic metabolism.

In drug metabolism studies employing the BB rat, usually at 4 months of age, and removing their insulin therapy for four days, changes similar to those observed in streptozotocin- and alloxan-treated animals have been reported, including; increases in the activities of aniline p-hydroxylase and dimethylnitrosamine N-demethylase and a concomitant increase in the levels of cytochrome P450 IIE1 (Favreau and Schenkman, 1988b; Bellward et al., 1988; Dong et al., 1983). From the data available for the spontaneously diabetic rat it appears that there is a good correlation with the metabolic alterations induced by alloxan or streptozotocin.
1.4.2 Phase II Metabolism

The major conjugating reactions of phase II metabolism have been investigated in both alloxan and streptozotocin diabetic rats and mice. The effects appear to be substrate specific suggesting that there is specific induction of certain isoforms of the major conjugating enzyme families, as already indicated with respect to the mixed-function oxidase system.

UDP-Glucuronosyl transferase

The UDP-glucuronosyl transferase activity towards 1-naphthol, testosterone and phenacetin has been shown to be suppressed in type I diabetes, whereas the activity towards diethylstilboestrol and oestrone was unaffected (Watkins and Klaassen, 1988; Grant and Duthie, 1987; Younes et al., 1980). In the case of p-aminophenol there are conflicting reports, Hinohara (1974) found increased conjugation while Morrison and Hawksworth (1984) reported a decrease. Similarly, in hepatocytes isolated from streptozotocin- or alloxan-diabetic rats there was decreased glucuronidation of 1-naphthol and phenolphthalein. (Grant and Duthie, 1987) due to decreased levels of UDP-glucuronic acid. However, Eacho et al., (1981), also using isolated hepatocytes, reported that the accelerated formation of UDP-glucuronic acid was responsible for the increased UDP-glucuronic acid conjugation of p-Nitroanisole and p-Nitrophenol.
Glutathione-S-transferase

The role of glutathione conjugation in the deactivation of reactive intermediates has led to active investigation of both the levels and activities of glutathione-S-transferase. The activity towards ethracrynic acid and sulphobromophthalein conjugation was decreased (Watkins et al., 1987) as well as the conjugation of 1-chloro-2,4-dinitrobenzene in both hepatocytes and cytosolic preparations from streptozotocin-diabetic rats (Grant and Duthie, 1987; Watkins et al., 1988). In contrast Rouer et al. (1981) found that the streptozotocin-treated mouse displayed an increased conjugating capacity towards this substrate. However, this increase may have been diabetogen modulated since it was not prevented by simultaneous administration of nicotinamide and not induced in alloxan-treated animals (Agius and Gidari, 1985). No alteration was found when epoxy-p-nitrophenoxy propane was the substrate for glutathione-S-transferase in streptozotocin induced animals (Younes et al., 1980).

It would appear that specific isoforms of the glutathione-S-transferase are either induced or repressed by diabetes, or that the cellular levels of glutathione are affected. The levels of reduced glutathione have been investigated by Hassing et al. (1979) and Younes et al. (1980) who found a decrease in cellular levels of glutathione in streptozotocin-treated animals. When Grant and Duthie (1987) examined the ability of hepatocytes isolated from streptozotocin animals to resynthesise glutathione after cellular depletion, they observed that although the cellular levels were lower than normal, there was an increased ability to synthesise the tripeptide from L-methionine, indicating that the cystathionine pathway was more efficient in these animals. Hassing et al.; (1979) has also found that the plasma of uncontrolled human diabetics contains a lower concentration of glutathione than normal individuals.

Other phase II enzymes such as epoxide hydrolase and sulphate conjugation have
not been adequately studied. In streptozotocin-treated mice the hydrolysis of benzo[a]pyrene 4,5-oxide was increased (Rouer et al., 1981) whereas no alteration was evident when styrene oxide was employed. Thomas et al., (1989), investigating the cytosolic epoxide hydrolase activity in alloxan and streptozotocin-induced diabetic rats, found an increased activity and suggested that this may be due to the increased peroxisomal β-oxidation occurring during diabetes.

Sulphate conjugation of 1-naphthol was shown to be increased in hepatocytes from streptozotocin diabetic rats (Grant and Duthie, 1987).

Alterations of phase II metabolism in spontaneously diabetic animals has not been adequately studied to date, although on the evidence of phase I metabolism, it is probable that the alterations observed with streptozotocin and alloxan treated animals would be similar to those in the spontaneously diabetic animal.

1.4.3 Extrahepatic metabolism in diabetes mellitus

Extrahepatic metabolism in diabetic animals has attracted little attention over the years with only a few published studies available. In streptozotocin-treated animals the O-deethylation of 7-ethoxycoumarin and the hydroxylation of benzo[a]pyrene was increased in the gastrointestinal tract but in contrast the pulmonary activities were decreased indicating that the effects of diabetes are organ-specific (Stohs et al., 1979; Al-Turk et al., 1980).
1.4.4 Sex differences in the diabetes mellitus induced alterations of hepatic drug metabolism

Sex differences in drug metabolising enzymes in type I diabetes have been identified in both chemically-induced and spontaneously diabetic rats. The demethylation of aminopyrine is depressed in the male rat but elevated in the female and the activities of hexobarbital and aryl hydrocarbon hydroxylase activities are diminished only in males (Reinke et al., 1978; Reinke et al., 1979; Kato et al., 1970; Kato and Gillette, 1965; Warren et al., 1983). Skett and Joels (1985) investigating the sex-dependent demethylations of lignocaine and imipramine found that only the activity in the male was suppressed.

As is to be expected sex-differences are apparent in steroid metabolism, however, the effect of diabetes is to abolish the differences, with higher 7α-hydroxylase in the male resulting in activities similar to that of the female, whereas the 4α- and 6β-hydroxylases and 17-oxosteroid reductase are suppressed, again resulting in activities similar to those of the female (Skett, 1986). All of these differences may be correlated to sex-specific alterations of the cytochrome P450 proteins in response to diabetes. Sex-dependent responses to streptozotocin accounting for the alterations appear unlikely as the characteristics and severity of the diabetes is similar in both male and female animals (Skett and Joels, 1985).

1.4.5 Alteration of hepatic drug metabolism in human diabetics.

The influence of type I diabetes on hepatic drug metabolism has been poorly studied in humans. The disease is effectively controlled by insulin therapy which has been
extensively shown to alleviate the life-threatening symptoms and correct the metabolic alterations in animals. However, the metabolism of some compounds has been studied including; phenacetin and antipyrine which showed depressed metabolism although insulin corrected the alteration (Dajani et al., 1974; Murali et al., 1983). Surprisingly, even in insulin corrected diabetes the plasma half-life of antipyrine was prolonged when compared to controls (Daintith et al., 1976). *In vivo* studies showed that hepatic cytochrome P450 levels were significantly higher in diabetics with normal livers when compared to control patients with normal livers (Salmela et al., 1980). It would appear, therefore, that even in effectively controlled diabetics the cytochrome P450 compliment is altered so as to impair the metabolism of antipyrine.

1.4.6 The effect of type I diabetes mellitus on chemical toxicity

The involvement of cytochrome P450 in the bioactivation of chemicals and the fact that diabetes modulates the levels of isoforms, makes it highly probable that the toxicity of various chemicals will be altered.

It has been known for some time that diabetic rats are more susceptible to the hepatotoxic effects of some haloalkanes such as; bromobenzene, carbon tetrachloride, chloroform, 1,1,2,-trichloroethane and other hepatotoxins (Watkins et al., 1988, Hanasono et al., 1975). The induction of cytochrome P450 IIE1, capable of catalysing the production of the trichloromethyl radical from carbon tetrachloride (Watkins et al., 1988, Brady et al., 1989) may explain this increased toxicity. The inducing effects of ethanol on this isoform has suggested that it may be this isoform which, in part, explains the increased hepatotoxicity of these haloalkanes following ethanol pretreatment (Maling et al., 1975, Traiger and Plaa, 1972). This cytochrome has also been shown to metabolise fluorinated
anesthetics resulting in the release of fluoride ions which are known to be nephrotoxic (Pantuck et al., 1987; Rice et al., 1980; Hoffman et al., 1989). In contrast, Price and Jollow (1982) found that the streptozotocin diabetic rat was more resistant to the hepatotoxic effects of paracetamol. The half-life of the drug was shorter in the diabetic rat (Price and Jollow, 1983) suggesting increased overall metabolism. They concluded that the diabetic rat had a greater capacity to conjugate the compound with glucuronic acid and sulphate, the major pathways of paracetamol metabolism, thus preventing the generation of the reactive species. This is an unexpected finding since the P450IIE1 and P450 IA2 proteins have been shown to catalyse the formation of the toxic metabolite (Raucy et al., 1989) and both proteins are induced by diabetes (Ioannides et al., 1988). The effects of ethanol pretreatment on paracetamol toxicity has also been investigated in mice where, in contrast to diabetes, it was found to potentiate hepatotoxicity (Peterson et al., 1980).

Diabetes also increased the metabolism of benzene (Johansson and Ingelman-Sundberg, 1988), accelerated the cellular depletion of glutathione after menadione administration to hepatocytes from streptozotocin-diabetic rats (Grant and Duthie, 1988) and modified the metabolism of diethyl ether (Brady et al., 1988).

Perhaps the most significant effect of diabetes is the potentiation of the dimethylnitrosamine hepatotoxicity (Lorr et al., 1984). Cytochrome P450 IIE1 has been associated with the ability to N-demethylate dimethylnitrosamine, presumably through unstable intermediates of N-hydroxylation, causing bioactivation of the compound (Tu and Yang, 1985). The metabolism of several nitrosamines by P450 IIE1 has been investigated with the isoform displaying a range of activities towards the various substrates (Peng et al., 1983). The pretreatment of mice with ethanol has been associated with a marked enhancement of the mutagenicity of dimethylnitrosamine in in vitro mutagenicity assays (Glatt et al., 1981) and hepatic subcellular fractions from streptozotocin-diabetic rats have shown an increased propensity to bioactivate known chemical carcinogens (Flatt et al., 1989). These studies lend support to the suggestion that human diabetics may be at higher
risk to the toxicity of environmental chemicals because of the induction of the P450 IIE1 isoform of cytochrome P450.

1.5 Mechanism of the diabetes-induced modulation of chemical metabolism and toxicity

It would appear that the modulation of the cytochrome P450 isoforms can explain most of the metabolic alterations associated with diabetes mellitus. The mechanism of this modulation is still unclear although several theories have been postulated.

Type I diabetes is accompanied by many severe physiological abnormalities including:

1. Hyperglycaemia
2. Hypoinsulinaemia
3. Hyperketonaemia
4. Reduced growth hormone secretion
5. Alterations in endogenous androgens and other hormones.

Hyperglycaemia and its effects on drug metabolism were studied by Ackerman and Leibman (1977) who showed that prolonged infusion of glucose, failed to modify drug metabolism, and chronic hyperglycaemia actually resulted in a decrease of some activities such as aniline hydroxylase (Hartshorn et al., 1979). The investigation of compounds that induce cytochrome P450 IIE1 revealed that acetone was one of the most potent inducers and that other physiological states such as fasting were also accompanied by increases in P450
IIE1 (Mehendale et al., 1977; Lorr et al., 1984). This suggests that ketone bodies may be responsible for the modification in the cytochrome P450 isoforms. The major ketone bodies are acetoacetate and 3-hydroxybutyrate formed from the partial oxidation of free fatty acids in the mitochondria. Acetoacetate equilibrates with 3-hydroxybutyrate and both are released into the blood stream. Acetone is formed from the non-enzymic decarboxylation of acetoacetate. When carbohydrate utilisation is impaired and free fatty acid levels rise, due to increased lipolysis, ketones form the third most important blood borne energy fuel for extrahepatic tissues after glucose and free fatty acids. The primary physiological role of ketones is to serve as substrate in the brain, a tissue that does not take up free fatty acids. So during carbohydrate deprivation, as in type I diabetes, ketone bodies limit the expenditure of glucose in the brain and spare proteins which would otherwise be catabolised.

Bellward et al., (1988) suggested that ketones were probably the most important inducer of cytochrome P450 IIE1 although the modulation of other cytochrome P450 families by these compounds has not been investigated. The hypoinsulinaemic state also effects the levels of other hormones in the diabetic animal, some of which may themselves play a role in the regulation of cytochrome P450 levels. It is highly probable that the mechanism of modification of metabolism cannot be attributed to a single specific parameter but is the result of the concerted effects of many factors.

1.6 Alterations of hepatic drug metabolism by type II diabetes mellitus

The influence of type II diabetes on hepatic drug metabolism appears to be much less pronounced. No major differences in mixed-function oxidase activity or glutathione-S-transferase activity between genetically diabetic (db/db) mice, genetically obese
hyperglycaemic (ob/ob) mice and their respective controls were observed (Rouer et al., 1981; Rouer and Leroux, 1980; Herberg, 1988).

Aims of the Project

Previous work has suggested that insulin-dependent diabetes mellitus is associated with altered metabolism of various drugs predominantly metabolised in the liver. As a number of these drugs are metabolised by the cytochrome P450-dependent mixed-function oxidase system, initial research will investigate the hepatic levels of five inducible cytochrome P450 isoforms in various animal models of type I diabetes mellitus. These studies will also investigate the any sex-dependency for the alteration in these isoforms and also the propensity of hepatic fractions from these diabetic animals to bioactivate known chemical carcinogens using the Ames mutagenicity assay.

Further work will aim to establish the physiological mechanism responsible for these alterations in cytochrome P450 levels particularly the role of the elevated levels of ketone bodies accompanying type I diabetes mellitus.

Finally, other disease states where abnormal circulating insulin levels are present with be investigated in respect to hepatic drug metabolism. It is envisaged that these studies will concentrate on the effects of hyperinsulinaemia.
Chapter 2

Materials and Methods
2.1 Materials

Molecular Probes, Eugene, OR., USA
- Ethoxyresorufin, pentoxyresorufin, resorufin.

BDH Chemicals, Poole, Dorset.
- Ammonium acetate, Aniline HCl, Acetic acid
- Ammonium persulphate, Sodium dithionite
- Potassium cyanide, Hydrochloric acid, Sodium hydroxide, Copper sulphate (Pentahydrate)
- Potassium sodium tartrate, Sodium carbonate
- Sodium hydrogen carbonate, Magnesium chloride, Acetyl acetone, Hydrazine, Formaldehyde
- Glycerol, Mercaptoethanol, Glycine, Sodium chloride, Potassium dihydrogen orthophosphate
- Disodium hydrogen orthophosphate, Potassium chloride, Potassium hydroxide, 4-Nitrocatechol
- Agarose, isoamyl alcohol.

Sigma Chemicals, Poole, Dorset.
- Cytochrome c (Grade IV), NADPH (Grade I), NADP (Grade I), Tris buffer, NADH (Grade III), β-D-hydroxybutyrate dehydrogenase (Grade II)
- Glucose-6-phosphate (Grade I), Glucose-6-phosphate dehydrogenase (Grade XXIII), NAD+ (Grade IV), Lauryl sulphate, Bromophenol blue, N,N,N',N'-Tetramethylethylene diamine, Triton X-100, 3,3-Diaminobenzidine, Peroxidase labelled Donkey anti-sheep, Peroxidase labelled Goat anti-rabbit, MOPS buffer, Glutathione reductase (Grade IV), 5,5'-dithiobis-(2-nitrobenzoic acid), Lauric acid, p-Nitrophenol, Folin-Ciocalteau phenol reagent, Rabbit anti-human growth hormone antibody, Nitroso piperidine, Nitrosoppyrrolidine.

National Diagnostics, Aylesbury, Bucks.
- Acrylagel, Bis-Acrylagel.

Anderman & Co Ltd.
Kingston-on-Thames, Surrey.
- Nitrocellulose sheets.

London Analytical and Bacteriological Media Ltd, Salford, Lancs.
- Lab M Agar.

Gibco, Paisley, Scotland.
- Vogel Bonner E plates.

Oxoid, Ltd., Basingstoke, Hampshire.
- Oxoid No. 2 Nutrient broth.

Wako Fine Chemicals, 4040 Neuss 1, FRG.
- Glu-P-1, Trp-P-2, Trp-P-1

Antibodies to P450 IIB and P450 IV family were generously supplied by Dr. G.G. Gibson, University of Surrey, those to P450 IIIA by Dr. C.R. Wolf Imperial Cancer Research Fund, Hugh Robson Building, George Square, Edinburgh and antibodies to P450 IIE by Dr D Koop, Dept. Environmental Health Sciences, Case and Western School of Medicine, Ohio, USA.
2.2 Methods

2.2.1 Animals and animal pretreatment.

Male and female wistar albino rats (180 - 200g) were purchased from the Animal Breeding Unit, University of Surrey. The animals were housed in cages with softwood sawdust bedding, being allowed food (Sprats Animal Diet No1) and water ad libitum. A 12 hour light, 12 hour dark cycle was in operation (0700 - 1900) light at 22°C and 50% humidity.

2.2.2 Preparation of hepatic subcellular fractions

Hepatic subcellular fractions were prepared essentially as described previously (Ioannides and Parke, 1975); livers were immediately excised and washed in ice-cold 1.15% (w/v) KCl to remove excess blood. The livers were subsequently weighed, scissor-minced and homogenised in 3 volumes of ice-cold 1.15% (w/v) KCl using a motor driven Potter-Elvehjem glass teflon homogeniser. The homogenate was adjusted to 25% (w/v) by further addition of 1.15% (w/v) KCl and centrifuged at 9,000g for 20 minutes in a JA-17, 14x50ml aluminium angle-headed rotor in a Beckman J2-21 centrifuge. The supernatant (S9 fraction) was decanted off and stored at -20°C until required. The S9 fraction was never stored for periods longer than 3 months.

Microsomal suspensions (105,000g pellet resuspended) were prepared from the S9 fraction by centrifugation in a Beckman LS-7 ultracentrifuge with a 70Ti (12x10ml titanium angle-headed rotor) or a 65Ti (8x10ml titanium angle-headed rotor) at 105000g for 60 minutes. The resulting 105,000g supernatant (25% cytosolic fraction), or the resuspended pellet (25% microsomal suspension), were used immediately.
2.2.3 Microsomal Assays

Determination of microsomal and cytosolic protein

Microsomal and cytosolic protein were determined essentially as described by Lowry et al. (1951) using bovine serum albumin (fraction V) as the standard. Protein samples were diluted 1:20 for microsomes and 1:100 in the case of cytosol with 0.5M sodium hydroxide. Protein standards (0 - 500 μg bovine serum albumin per ml of 0.5M sodium hydroxide) were prepared. Aliquots (0.5ml) of protein standards, blanks and samples were further diluted with 0.5ml of 0.5M sodium hydroxide. To each tube was added 5ml of freshly prepared "copper reagent" (1:1:100, Copper sulphate (1% w/v), potassium sodium tartrate (2% w/v) and sodium carbonate (2% w/v), and after mixing allowed to incubate at room temperature for 10 minutes. Folin-Ciocalteau phenol reagent was diluted with an equal volume of 0.5M sodium hydroxide, 0.5ml added to each tube and the solution immediately mixed. After a further incubation of 30 minutes at room temperature the absorbance of the blue solution was read at 720nm. The amount of protein in each sample was calculated from a standard curve.

Total cytochrome P450 determination

Microsomal cytochrome P450 was determined essentially as described by Omura and Sato (1964). Total cytochrome P450 was measured as the difference spectrum of the reduced cytochrome P450-carbon monoxide bound complex versus the reduced cytochrome
P450 between 390-500nm using the Kontron Uvikon 860 spectrophotometer.

Microsomal suspension (25%) was diluted 1:6 with potassium phosphate buffer 0.1M, pH 7.6 to give a final volume of 3ml. Following reduction of cytochrome P450 by the addition of sodium dithionite (approximately 10mg) the diluted microsomes were divided between two 1.5ml disposable plastic cuvettes and a base line was recorded between 390-500nm. Carbon monoxide was then bubbled through the test cuvette (30 seconds at a rate of approximately 2 bubbles sec^-1) and the difference spectrum recorded. The total cytochrome P450 content was calculated from the absorbance difference between \( A_{450\text{ nm}} - A_{490\text{nm}} \) employing an extinction coefficient of 91mM^-1 cm^-1. The necessity to store the liver samples as post-mitochondrial fractions (S9) at -20°C rather than as microsomal suspensions at -80°C resulted in lower values for total cytochrome P450 levels. This was consistently observed throughout but was necessary in order to use the samples in the Ames test. It has also been shown that many of the chemicals used to stabilise microsomal P450 when freezing to -80°C, such as glycerol or dimethylsulphoxide are substrates for P450 IIE1 and could have affected the determination of activity of this isoform in the samples.

Cytochrome b\(_5\) determination

Microsomal cytochrome b\(_5\) was determined essentially by the method of Omura and Sato (1964). Cytochrome b\(_5\) content was determined as the difference spectrum between the dithionite-reduced and oxidised forms of the cytochrome.

Microsomes were diluted 1:6 with 0.1M potassium phosphate buffer, pH 7.6 to give a final volume of 3ml. The microsomes were divided between two cuvettes and a baseline obtained between 390-500nm. Sodium dithionite (approximately 5mg) was added to the test cuvette and the difference spectrum recorded under aerobic conditions. The cytochrome b\(_5\) content was determined from the absorbance difference \( A_{426\text{nm}} - A_{410\text{nm}} \) employing an extinction coefficient of 185mM^-1 cm^-1.

NADPH-cytochrome c reductase activity

NADPH-cytochrome P450 reductase (EC 1.6.2.4) was assayed by following the
reduction of the non-physiological electron acceptor, cytochrome c (Williams and Kamin, 1962), at 37°C using a Varion/Cary 2200 spectrophotometer. Into the reference and sample cuvettes the following reagents were added:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mM Potassium phosphate buffer, pH 7.6 containing 10mM potassium cyanide.</td>
<td>1.8</td>
</tr>
<tr>
<td>0.1mM cytochrome c</td>
<td>1.0</td>
</tr>
<tr>
<td>Microsomal suspension (25%)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

After a suitable preincubation period, to obtain a steady baseline, the reaction was initiated by the addition of 0.1ml of NADPH (30mM in 1% w/v sodium hydrogen carbonate) to the sample cuvette. The reduction of cytochrome c was followed at 550nm by recording the increase in absorbance. The concentration of the ferrous cytochrome c formed was calculated using an extinction coefficient of 18.5mM⁻¹cm⁻¹.

Aniline p-hydroxylase activity

The microsomal p-hydroxylation of aniline was determined essentially as described by Guarino et al., (1969). The following reaction mixture was prepared in duplicate and incubated at 37°C in a shaking water bath for 15 minutes. Standards containing 0-100μM p-aminophenol were also incorporated into the assay.
0.3M Tris-HCl Buffer, pH 7.6 0.5ml
40mM Aniline hydrochloride, pH 7.6 0.5ml
100mM Magnesium chloride 0.1ml
10mM NADP+ 0.1ml
100mM Glucose-6-phosphate 0.1ml
Glucose 6-phosphate dehydrogenase (10units/ml buffer) 100μl
Microsomal suspension (25%) 0.5ml

The reaction was terminated by the addition of solid sodium chloride which causes the protein to precipitate out of solution and the product, p-aminophenol, extracted into ether (12ml) containing 1.5% (v/v) isoamyl alcohol. Ether aliquots (10ml) were then added to 0.5M tri-potassium orthophosphate (4ml), to which phenol (1% w/v) was added prior to use, and extracted for 30 minutes on a rotary shaker. The absorbance of the resulting blue aqueous solution was read at 620nm and enzyme activity was calculated with reference to the standards.

**Dimethylnitrosamine N-demethylase activity**

The determination of dimethylnitrosamine N-demethylase activity was based on the method of Holtzman *et al.* (1969). Formaldehyde standards (0-100μM) and the following incubation mixtures were prepared:

50mM Potassium phosphate buffer, pH 7.2 0.6ml
150mM Magnesium chloride 0.1ml
NADPH generating system 0.1ml
Microsomal suspension (25%) 0.1ml

NADPH generating system: NADP+ (0.1mM), Glucose-6-phosphate (10mM), Glucose-6-phosphate dehydrogenase (10units/ml in buffer).
The reaction mixture was pre-incubated for 3 minutes and the reaction initiated by the addition of 0.1ml of dimethylnitrosamine (10mM) to each tube. After a further incubation of 10 minutes the reaction was terminated by the addition of ice-cold 12.5% (w/v) trichloroacetic acid. The tubes were left on ice for 5 minutes and then centrifuged for 10 minutes in a bench centrifuge to precipitate the protein. Supernatant (1ml) was added to freshly prepared Nash reagent (4M ammonium acetate containing 4ml/l acetyl acetone, 1ml; Nash, 1953) and heated in a water bath at 50°C for 10 minutes. After cooling absorbance at 412nm was recorded and the demethylase activity calculated with reference to the formaldehyde standards.

p-Nitrophenol hydroxylase activity

The hydroxylation of p-nitrophenol was followed essentially by the method of Reinke et al. (1985) as modified by McCoy and Koop (1988). The following were added to test tubes in duplicate.

- 0.2M Potassium phosphate buffer, pH 6.8 0.65 ml
- Ascorbate (1mM) 0.1 ml
- p-Nitrophenol (1mM) 0.1ml
- Microsomal suspension (25%) 50μl

The reactants were incubated for 3 minutes at 37°C in a shaking water bath and the reaction initiated by the addition of 0.1ml of NADPH (10mM). After a further 10 minute
incubation the reaction was terminated by the addition of 0.5ml of ice-cold 0.6N perchloric acid. The protein was precipitated by centrifugation at 3000rpm for 10 minutes. 10M Sodium hydroxide (0.1ml) was then added to 1ml of the supernatant and the solutions mixed. The absorbance was then read at 536nm using a Kontron Uvikon spectrophotometer. The p-nitrophenol hydroxylase activity was calculated from a standard curve (0-100μM 4-nitrocatechol).

Erythromycin N-demethylase activity

The microsomal erythromycin N-demethylase activity was performed as described by Wrighton et al., (1985b). To duplicate test tubes the following were added:

- 50mM Potassium phosphate buffer, pH 7.25 0.6ml
- Magnesium chloride (150mM) 0.1ml
- Erythromycin (10mM) 0.1ml
- Microsomal suspension (25%) 0.1ml

The tubes were pre-incubated for 3 minutes at 37°C and the reaction initiated by the addition of 0.1ml NADPH (10mM). After a further 10 minute incubation the reaction was terminated by the addition of 0.5ml of ice-cold 12.5 % (w/v) trichloroacetic acid. The tubes were then centrifuged at 3000rpm for 10 minutes to remove the protein. The supernatant (1ml) was then added to freshly prepared NASH reagent (4M ammonium acetate contain 4ml/L acetyl acetone). The tubes were heated in a water bath at 50°C for 30 minutes and after cooling the absorbance was read at 412nm using a Kontron Uvikon spectrophotometer. The erythromycin N-demethylase activity was calculated from standards (0-100μM formaldehyde) which were run in parallel.
Ethylmorphine-N-demethylase activity

Ethylmorphine N-demethylase was measured essentially by the method of Lu et al., (1972) and was performed as for the erythromycin N-demethylase assay except that 0.01M ethylmorphine was used instead of 0.01M erythromycin and that the tubes were incubated at room temperature with the NASH reagent.

Lauric acid hydroxylase activity

The microsomal activity of lauric acid ω-hydroxylase was assayed essentially as described by Parker and Orton, (1980). The following reagents were prepared and aliquoted into test tubes:

- Lauric acid (1mM) 200μl
- [14C] Lauric acid (10μCi/ml) 10μl
- 0.5M Tris-HCl Buffer, pH 7.4 1.65ml
- Microsomal suspension (25%) 100μl

The reaction was initiated by the addition of 40μl of NADPH (40mM) and the tubes incubated in a shaking water bath at 37°C for 10 minutes. The reaction was terminated by the addition of 3M HCl (0.2ml) and the lauric acid and its metabolites were extracted into ether (10 mls). The upper layer was removed and evaporated to dryness under nitrogen. The resulting residue was dissolved in 60 μl of methanol and 25μl spotted onto a silica gel G TLC plate. The plates were developed in a solvent system comprising hexane: diethyl ether: acetic acid (70:28:1) and the radioactivity determined using a
Berthold LB2842 plate scanner. This method measures the 12- and 11-hydroxy metabolites of lauric acid. Therefore, the interpretation of this data may reflect the activities of several P450 isoforms which can hydroxylate lauric acid at either the 12 or 11 positions. It will not be evident which of these metabolites are affected by the treatments employed on the basis of this data.

Determination of alkoxyresorufin O-dealkylase activity

The method of Burke and Mayer (1974) was essentially used for the measurement of resorufin formation from ethoxyresorufin and pentoxyresorufin by microsomes from their respective dealkylation reactions.

The following reagents were added to a 3ml fluorimetric cuvette:

- 0.1M Tris-HCl buffer, pH 7.8: 2.0ml
- Microsomal suspension: 50μl
- 0.53mM Ethoxyresorufin: 3μl
  - or
- 1.0mM Pentoxyresorufin: 5μl

All fluorimetric assays were performed using a Perkin Elmer LS5 luminescence spectrophotometer set to an excitation wavelength of 510nm and emission wavelength of 586nm with excitation and emission slit widths of 10nm and 2.5nm respectively. A baseline was recorded prior to initiation of the reaction by the addition of 10μl of NADPH (50mM in 1% w/v sodium hydrogen carbonate). The reaction was monitored continuously until a measurable gradient was obtained (approximately 2 minutes) and the initial rate of the reaction calculated from the slope. The instrument was calibrated using aliquotes (10μl) of resorufin (0.1mM)
2.2.4 Cytosolic enzyme activities

Total glutathione

Total glutathione was determined by the method of Akerboom and Sies (1981) at 37°C using a Varian/Cary 2200 spectrophotometer. The cytosol was further diluted 50 fold with 1.15% (w/v) potassium chloride. Diluted cytosol (1ml) was added to 1ml of perchloric acid (2M) containing 4mM EDTA. 2M Potassium hydroxide (1ml) containing 0.3M MOPS was then added and the resulting solution adjusted to approximately pH 7. Glutathione standards (0.1mM - 0.8mM) were prepared and carried through the same procedure.

In a 4.5ml cuvette the following were mixed:

0.1M potassium phosphate buffer, pH 7.0 and
containing 2.5mM EDTA  
1.0 ml

NADPH (4mg/ml in 1.0% (w/v) sodium hydrogen carbonate)  
0.05ml

Glutathione reductase (6units/ml buffer)  
0.02ml

Sample or standard  
0.1ml

The reaction was initiated by the addition of 100µl of 3.8mM 5,5-dithiobis(2-nitro)benzoic acid (DTNB) in 1% (w/v) sodium hydrogen carbonate and monitored at 412nm. The concentration of glutathione in the cytosol was calculated from a curve constructed using initial rates.
Cytosolic glutathione-S-transferase activity

Glutathione-S-transferase activity in the cytosolic fraction was determined by an adaptation of the method devised by Habig et al., (1974).

1,2-Dichloronitrobenzene (DCNB) is a good substrate for many of the glutathione-S-transferase enzymes, with conjugation resulting in the formation of S-(2-chloro,4-nitrophenyl) glutathione. This reaction can be measured spectrophotometrically as an increase in absorbance at 340nm and is proportional to the enzyme activity in the sample.

The reaction was performed at 25°C using a Varian/Cary 2200 split beam spectrophotometer with reference and sample cuvettes containing the following:

<table>
<thead>
<tr>
<th></th>
<th>Sample (ml)</th>
<th>Reference (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M Sodium phosphate buffer, pH 7.5</td>
<td>1.8</td>
<td>1.9</td>
</tr>
<tr>
<td>25mM DCNB (in absolute ethanol)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>5mM reduced glutathione</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

After recording a baseline, 0.1ml of cytosol, diluted 1:5 with buffer, was added to the sample cuvette, mixed and the change in absorbance measured at 340nm. All solutions were prepared immediately prior to use and kept on ice.

Glutathione-S-transferase activity was calculated from initial rate of the reaction, assuming a molar extinction coefficient of 8.5mM⁻¹cm⁻¹.

The glutathione-S-transferase activity towards 3,4-dinitrochlorobenzene (CDNB)
was also determined using the above protocol but substituting CDNB (25mM in ethanol) for DCNB and measuring the reaction at 340nm.

Glutathione reductase activity

The glutathione reductase activity of hepatic cytosol was performed as described by Carlberg and Mannervik (1975). Cytosol was diluted a further 1:50 with 0.1M sodium phosphate buffer, pH 7.5 containing 0.1% (w/v) EDTA.

The following reactants were placed into two cuvettes:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidised glutathione (17.5mM)</td>
<td>0.2ml</td>
</tr>
<tr>
<td>FAD (0.3mM)</td>
<td>0.1ml</td>
</tr>
<tr>
<td>Diluted cytosol</td>
<td>3.0ml</td>
</tr>
</tbody>
</table>

The reaction was initiated by the addition of 200μl of NADPH (2mM in 1% (w/v) sodium hydrogen carbonate) to the sample cuvette and the reaction monitored at 334nm using a Varian/Cary 2200 spectrophotometer. The glutathione reductase activity was calculated using the extinction coefficient of 4.3mM⁻¹cm⁻¹.
2.2.5 Determination of plasma parameters

During all studies involving diabetic animals blood samples were collected from the tail vein at regular time intervals, during experiments and at time of death. Blood was transferred into heparinised blood vials and placed on ice. Plasma was obtained following the centrifugation of the heparinised blood at 3000rpm and stored at -20°C until required.

Determination of plasma glucose concentration

Plasma glucose was determined using a glucose analyser II by the method of Stevens et al. (1971). The method is based on the glucose oxidase reaction where an oxygen electrode is employed to determine the concentration of glucose in the test sample. The apparatus is calibrated with a known glucose standard.

Determination of plasma 3-hydroxybutyrate and acetoacetate concentrations

Plasma 3-hydroxybutyrate and acetoacetate were determined by a modification of the method of Li et al. (1980). The method is based on the reversible reaction:

\[
\begin{align*}
\text{CH}_3\text{--CH--CH}_2\text{--COO}^- & \quad \text{NAD}^+ \quad \text{NADH} \\
\text{3-Hydroxybutyrate} & \quad \text{pH 9.5} \\
3\text{-hydroxybutyrate dehydrogenase} & \quad \text{pH 7.5} \\
\text{Acetoacetate} & \quad \text{NAD}^+ \quad \text{NADH}
\end{align*}
\]
The following reagents were added to cuvettes for the determination of 3-hydroxybutyrate:

- 0.1M Tris-HCl buffer, pH 9.5: 500μl
- 2.5mM NAD+ in buffer: 480μl
- Sample or test: 20μl

The reaction was initiated by the addition of 10μl of hydroxybutyrate dehydrogenase (61.5 U.) and the change in absorbance at 340 nm recorded for 30 seconds using a Kontron Uvikon spectrophotometer. Hydroxybutyrate or acetoacetate standards (sodium and lithium salts respectively) were prepared, 0-20 mM, and the rate of reaction determined. The concentration of the ketone body was calculated from the respective standard rate curve. For acetoacetate determination 500μl of 0.1M potassium phosphate buffer, pH 7.0 and 480μl of 180μM NADH were used instead of the Tris-HCl buffer and NAD+ respectively.

2.2.6 The Ames Salmonella mutagenicity test

Mutagenicity was determined by the procedure of Maron and Ames (1983) with slight modifications. All glassware and heat stable solutions were autoclaved at 120°C for 20 minutes prior to use, and all experimental manipulations were performed in a class 2 laminar flow cabinet.

The bacterial tester strains

Salmonella typhimurium strains TA98, and TA1530 were stored as permanent frozen cultures maintained at -80°C using dimethylsulphoxide as the cryoprotective agent.
(0.8% v/v). When required an overnight culture was prepared from the respective frozen stock. This was achieved by inoculating nutrient broth containing ampicillin (25μg/ml) for TA98 or nutrient broth without ampicillin for TA1530 with a platinum loop and incubating at 37°C for 10 hours in a shaking water bath. The resulting culture was then used in the Ames test.

Testing for strain properties

The Salmonella tester strains contain different types of histidine mutations, with TA98 able to detect frameshift mutations and TA1530 able to detect base-pair substitutions. In addition to this mutation each strain has two other mutations which greatly enhance their susceptibility to mutagens. These are the loss of the excision repair mechanism (uvrB) and the inability to synthesise the lipopolysaccharide cell wall (rfa). In some strains, such as TA98, sensitivity has been further increased by the introduction of plasmid (pKM101), which confers both ampicillin resistance and an error-prone repair system to the bacteria.

Each new bacterial culture was tested for viability and spontaneous mutation rate. Cultures were also routinely checked to ensure that all strain characteristics were maintained.

Histidine requirement

A solution containing histidine and biotin (0.1M histidine and 0.5mM biotin), 0.1ml, was spread onto the surface of a Vogel Bonner plate and allowed to dry. An overnight bacterial culture was then streaked onto the plate as well as onto a histidine-free plate and these plates were then incubated overnight at 37°C. The histidine mutation
was present if bacterial growth was observed only on the plate supplemented with histidine.

**rfa mutation and R-factor detection**

Bacterial culture (0.1ml) was added to 2ml of agar (agar 0.6%(w/v) and sodium chloride 0.5% w/v containing 5mM histidine and 25μM biotin) and immediately poured onto Vogel Bonner E plates. When the agar had solidified, a sterile filter paper disc (1cm diameter) was placed onto the center of the plate and either 100μl of crystal violet (1mg/ml), for the rfa test or 100μl of ampicillin (8mg/ml in 0.02M sodium hydroxide) for the presence of plasmid, pipetted onto the filter paper. The plates were then incubated overnight at 37°C and the presence of a zone of inhibition around the filter paper indicated the presence of either the rfa mutation or the plasmid.

**Bacterial viability**

The viability of an overnight culture was assessed by colony growth on agar (2ml) with a high histidine and biotin concentration (5mM histidine and 25μM biotin). The bacterial culture was serially diluted in nutrient broth to a final dilution of 1 in 10^6. One hundred microlitres of the final dilution were then added to 2ml of agar and poured onto a Vogel Bonner E plate and incubated overnight at 37°C. The total number of colonies were counted and the viability (number of bacteria/ml) of the original culture calculated.

**Spontaneous reversion rate**

The spontaneous reversion rate was tested during each Ames test by incorporating blanks (solvent replacing test compound) into the test. Typical spontaneous reversion rates
for TA98 and TA1530 with S9 activation system are 15 to 46 and 3 to 18 respectively.

The pre-incubation Ames test

Fresh overnight cultures were prepared as previously described and the following activation systems prepared:

<table>
<thead>
<tr>
<th></th>
<th>10% S9</th>
<th>25% S9</th>
</tr>
</thead>
<tbody>
<tr>
<td>S9 liver fraction</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>0.33M Potassium chloride</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>0.08M Magnesium chloride</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>0.02M Sodium phosphate buffer, pH 7.4</td>
<td>5.0</td>
<td>3.5</td>
</tr>
<tr>
<td>0.02M NADP+ and 0.025M Glucose-6-phosphate</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Depending on the mutagen being used either a 10%S9 or 25%S9 activation system was employed.

2.2.7 SDS-PAGE and Western blotting analysis of microsomal cytochrome P450 proteins

Solubilisation of hepatic microsomal preparations

Solubilisation of the microsomal suspensions (25%) was achieved by the addition
of 10µl of solubilisation buffer (0.1M potassium phosphate buffer, pH 7.4, containing 10% w/v sodium cholate and 2% w/v Emulgen 911) for every mg of microsomal protein and mixing for 40 minutes at 4°C. The preparations were then stored at -20°C until required.

Discontinuous sodium dodecyl sulphate polyacrylamide slab gel electrophoresis of hepatic microsomal proteins.

The method devised by Laemelli (1970) was used for the separation process performed at room temperature.

Reagents

- **Lower Gel Buffer:** 1.5M Tris-HCl, pH 8.8 containing 0.4% (w/v) sodium dodecyl sulphate (SDS)
- **Upper Gel Buffer:** 0.5M Tris-HCl, pH 8.3, containing 0.4% (w/v) SDS
- **Electrode Buffer:** 25mM Tris-HCl, pH 8.3, containing 192mM glycine and 0.1% (w/v) SDS
- **Sample Buffer:** 125mM Tris-HCl, pH 6.8, containing 4.6% (w/v) SDS, 30% (v/v) glycerol, 0.002% (w/v) bromophenol blue and 10% (v/v) β-mercaptoethanol.
- **Acrylagel:** Commercially available.
- **Bis-Acrylagel:** Commercially available.
Method

A vertical slab gel apparatus constructed in the Biochemistry Department workshop was used. The gel was cast in a glass cassette, with perspex side and bottom spacers and held together with bull-dog clips. The dimensions of the cast gel were 120mm x 110mm x 1.5mm.

The glass front and back plates were washed with Decon detergent, rinsed with tap water and then distilled water and finally cleaned with acetone and left to dry. The cassette was assembled and sealed with 2% (w/v) molten agar and then clamped in a vertical position. The lower running gel was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower Gel Buffer</td>
<td>6.25ml</td>
</tr>
<tr>
<td>Bis-Acrylagel</td>
<td>3.38ml</td>
</tr>
<tr>
<td>Acrylagel</td>
<td>8.11ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>7.14ml</td>
</tr>
<tr>
<td>Ammonium persulphate (10% w/v)</td>
<td>125μl</td>
</tr>
</tbody>
</table>

Polymerisation was initiated by the addition of 20μl of N,N,N',N'-tetramethylethylenediamine (TEMED). This amount was sufficient for one gel with final acrylamide concentration of 10% (w/v). The solution was poured into the cassette to a height of 8cm. A thin layer of distilled water was placed on top of the gel solution to ensure a flat interface between the lower and upper gels. Polymerisation of the lower gel was complete in approximately 30-40 minutes, at which time the layer of water was removed.
The upper stacking gel was then prepared:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper Gel Buffer</td>
<td>2.5ml</td>
</tr>
<tr>
<td>Bis-acrylagel</td>
<td>0.4ml</td>
</tr>
<tr>
<td>Acrylagel</td>
<td>1.0ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>6.0ml</td>
</tr>
<tr>
<td>Ammonium persulphate (10% w/v)</td>
<td>100μl</td>
</tr>
</tbody>
</table>

Again polymerisation was initiated by the addition of 20μl of TEMED and the upper stacking gel poured into the cassette above the lower running gel. A perspex comb was introduced into the upper gel to form the sample wells and after approximately 30 minutes the gel had polymerised. The final concentration of acrylamide in the upper stacking gel was 3% (w/v). The comb was removed and a small amount of electrode buffer was introduced into the sample wells to maintain their integrity. The bottom perspex spacer was removed and the cassette placed into the electrophoresis tank. The solubilised microsomes were diluted an equal volume of sample buffer to give a final protein concentration of 2mg/ml and boiled for 3 minutes in a water bath prior to loading into the sample wells. Electrode buffer was then placed in the upper and lower reservoirs of the tank, and any air bubbles trapped beneath the lower gel were removed with a clean syringe.

Electrophoresis was carried out immediately following sample loading at a constant current of 20mA using a Pharmacia EPS 500/400 power pack until the bromophenol blue band had migrated into the lower gel. The current was subsequently increased to 40mA until the bromophenol blue band had migrated to the lower edge of the lower running gel when the current was switched off. The cassette was removed from the electrophoresis tank and the glass plates separated. The lower gel was separated from the upper stacking gel and used for the transfer of proteins onto nitrocellulose paper for western blotting analysis.
Western blotting

The following buffers were prepared:

Transfer Buffer:
- Tris 20mM
- Glycine 150mM
- Methanol 15% (v/v)

Phosphate Buffered Saline:
- Sodium chloride 8% (w/v)
- Potassium dihydrogen orthophosphate 0.2% (w/v)
- Disodium hydrogen orthophosphate 2.9% (w/v)
- Potassium chloride 0.2% (w/v)

Wash Buffer:
- PBS 10% (v/v)
- Bovine serum albumin (fraction V) 1% (w/v)
- Triton X-100 0.2% (v/v)

Substrate Buffer:
- 0.1M Tris-HCl, pH 7.5
Method

The lower running gel was removed from the cassette and placed into transfer buffer (50mls) for 30 minutes to allow the buffer to permeate the gel and replace the electrode buffer. During this time the gel will also shrink as it equilibrates with the transfer buffer. After equilibration the gel was removed and placed into the transfer cassette (figure 2.3.3.1). The cassette is prepared under transfer buffer and all air bubbles removed by rolling with a test tube.

Once assembled the cassette was placed into the transfer tank with the nitrocellulose sheet closest to the positive electrode. The transfer was performed at a constant voltage of 60v for 1 hour after which time the cassette was opened and the nitrocellulose sheet placed into 50 mls of wash buffer and rocked for 2 hours. Depending on the cytochrome P450 proteins to be detected different primary and secondary antibodies were used (table 2.3.3.1). All primary antibodies were diluted to a final volume of 50 mls with wash buffer and placed onto the nitrocellulose sheet for 1 hour, after which time it was replaced with fresh wash buffer. Fifteen minutes later this was replaced with a second 50ml aliquot of wash buffer and the sheet washed for a further fifteen minutes. The
peroxidase linked secondary antibody was then diluted in 50 mls of wash buffer and added to the nitrocellulose sheet for 1 hour. The nitrocellulose sheet was then washed twice with 50mls of wash buffer for 15 minutes before the pre-substrate wash was added. The pre-substrate wash (10% v/v PBS) was left for 10 minutes before the substrate was added (50mg 3,3 diaminobenzidine in 100ml of 0.1M tris-HCl, pH 7.5 containing 20µl 30% H2O2) until the bands appeared. The reaction was terminated by washing the nitrocellulose sheet several times with copious quantities of ice-cold distilled water.

Table 2.3.3.1 Primary and secondary antibody information

<table>
<thead>
<tr>
<th>P450 Family</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450 IA</td>
<td>Sheep IgG</td>
<td>Peroxidase linked donkey anti-sheep IgG 1:2000 dilution</td>
</tr>
<tr>
<td></td>
<td>1:10000 dilution</td>
<td></td>
</tr>
<tr>
<td>P450 IIE</td>
<td>Sheep IgG</td>
<td>Peroxidase linked donkey anti-sheep IgG 1:2000 dilution</td>
</tr>
<tr>
<td></td>
<td>1:5000 dilution</td>
<td></td>
</tr>
<tr>
<td>P450 IVA</td>
<td>Sheep IgG</td>
<td>Peroxidase linked donkey anti-sheep IgG 1:2000 dilution</td>
</tr>
<tr>
<td></td>
<td>1:5000 dilution</td>
<td></td>
</tr>
<tr>
<td>P450 IIB</td>
<td>Rabbit IgG</td>
<td>Peroxidase linked Goat anti-rabbit IgG 1:2000 dilution</td>
</tr>
<tr>
<td></td>
<td>1:2000 dilution</td>
<td></td>
</tr>
<tr>
<td>P450 IIIA</td>
<td>Rabbit IgG</td>
<td>Peroxidase linked Goat anti-rabbit IgG 1:2000 dilution</td>
</tr>
<tr>
<td></td>
<td>1:2000 dilution</td>
<td></td>
</tr>
</tbody>
</table>

Antibodies to P450 IA1 recognises both 1A1 and 1A2 proteins. Antibodies to P450 IIB1 recognise both the B1 and B2 proteins as well as a third protein of lower molecular weight which is present in the liver of Wistar rats. Antibodies to P450IIE1 were a gift from Dr D Koop, Case and Western School of Medicine, USA and recognise a single protein, P450IIE1. Antibodies to cytochrome P450 IVA recognise two proteins of slightly differing molecular weight which correspond to P450IA1 and a secondary protein uncharacterised at present. Finally, antibodies to P450IIIA were supplied by Dr CR Wolf, Imperial Cancer Research Fund, Edinburgh and recognise several of the proteins in this family.
Chapter 3

Hepatic drug metabolism in Streptozotocin-induced type I diabetes mellitus
3.1 Modulation of hepatic mixed-function oxidase activity by streptozotocin-induced type I diabetes mellitus

Chemically-induced diabetes mellitus has been the primary model for the majority of investigations into the effects of the disease on drug metabolism. The use of streptozotocin as the diabetogenic agent has now replaced the use of the more toxic alloxan as the diabetogen of choice. Although, streptozotocin induces a form of diabetes that resembles the physiological alterations associated with human insulin-dependent diabetes, the effect of the diabetogen *per se*, as well as the disease state itself, on drug metabolism must be clearly established.

Many studies have established that streptozotocin-induced type I diabetes is associated with marked alterations of hepatic biotransformation (Dixon *et al.*, 1963; Reinke *et al.*, 1978). The metabolism of many of the compounds studied can be attributed to the cytochrome P450 dependent mixed-function oxidases and specifically to various families of that system. The identification of a diabetes-inducible isoform of cytochrome P450 (P450j in the rat or isozyme 3a in the rabbit) termed P450 IIE1 (Ryan *et al.*, 1986) has aroused much interest, especially as it has been associated with the activation of nitrosamines (Peng *et al.*, 1983) and the ability to bioactivate paracetamol to its hepatotoxic metabolite (Raucy *et al.*, 1989). The bioactivation of paracetamol and some nitrosamines, although catalysed by P450 IIE1, are reactions also catalysed by other P450 isoforms albeit at different rates and substrate concentrations (Raucy *et al.*, 1989; Gonzalez, 1989). Many of the previous studies have investigated alterations in drug metabolism by employing substrates metabolised by more than one isoform of cytochrome P450. This can present problems when evaluating the effects of diabetes on the cytochrome P450 oxidase system.
The use of specific diagnostic substrates whose metabolism is catalysed predominantly or exclusively by single isoforms will enable the effects of streptozotocin-induced diabetes on the activities of specific isoforms to be more adequately characterised. The use of exogenous insulin has already been shown to partially reverse the effects of the streptozotocin-induced alterations in hepatic drug metabolism and concurrent administration of nicotinamide with streptozotocin can block the diabetogenic effect of the compound (Ledoux et al., 1988). In the present study the effect of the diabetogen per se on drug metabolism and the alterations caused by the ensuing diabetes were investigated. In order to evaluate the effect and extent of reversal by insulin of the diabetes-induced alterations, one group of streptozotocin-induced diabetic rats received daily insulin injections throughout the study. Moreover another group that received streptozotocin was also injected with nicotinamide which is known to block the diabetogenic effect of the compound. In this way the effects of the diabetogen on drug metabolism may be discerned from those inherent to the chemical.

One of the inherent problems of using selective substrates is that other, as yet uncharacterised P450 isoforms may also metabolise the substrate. Therefore, immunological detection of specific P450 proteins was also employed using monospecific antibodies to selected P450 proteins.

Animal pretreatment

Male Wistar albino rats (180-200g) were randomly divided into four groups, each comprising four animals. One group served as control, the second received a single intraperitoneal injection of streptozotocin (65mg/kg) dissolved in 0.5M sodium citrate buffer, pH 4.5; the third group received, in addition to streptozotocin (65mg/kg), two intraperitoneal injections of nicotinamide (350mg/kg), one 10 minutes before and another 3 hours after the administration of streptozotocin; finally, the fourth group received, in addition to streptozotocin, single daily doses of insulin subcutaneously once overt diabetes was established (day 3). All animals were killed 21 days after treatment and 24
hours after the last administration of insulin. In all cases livers were immediately excised, hepatic microsomal fractions prepared and mixed-function oxidase activities determined (Chapter 2). Body weight gain, food and water intake were monitored daily and terminal blood samples were obtained from the animals by decapitation at time of death. Animals receiving insulin treatment were dosed daily with a single subcutaneous injection of 4 I.U. per day from day 4 until day 10 when the dose was increased to 8 I.U. In order to confirm the findings of the enzyme assays antibodies raised against specific cytochrome P450 proteins were employed for immunological analysis of the hepatic microsomal proteins (Chapter 2).

Insulin was long acting ultralente (40iu/ml) insulin from Novo Industrials. In comparison to the dose administered to human diabetics the streptozotocin-diabetic rat is resistant to the effects of this insulin and high doses must be given for adequate control to be obtained.

Results

Rats treated with streptozotocin alone exhibited hyperphagia, polydipsia, polyurea and reduced body weight gain (Figure 3.1). These effects could be partially reversed by the daily subcutaneous administration of insulin. They also exhibited markedly higher plasma glucose and ketone body concentrations when compared to control rats or streptozotocin-treated rats given nicotinamide or daily insulin injections (Table 3.1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose (mM)</th>
<th>Acetoacetate (mM)</th>
<th>3-Hydroxybutyrate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.6 ± 0.2</td>
<td>0.34 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>Streptozotocin diabetic</td>
<td>25.6 ± 4.1***</td>
<td>0.98 ± 0.16**</td>
<td></td>
</tr>
<tr>
<td>Streptozotocin and nicotinamide</td>
<td>5.8 ± 0.3</td>
<td>0.30 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin treated streptozotocin diabetic</td>
<td>14.9 ± 3.8**</td>
<td>0.40 ± 0.14</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean ± s.e.m. for four animals.
*** p<0.001, ** p<0.01 compared to control
Effects of streptozotocin-induced diabetes (STZ), combined streptozotocin and nicotinamide treatment (STZ + Nic) and insulin treated streptozotocin-induced diabetes (STZ + Ins) on body weight gain, water and food intake.

**Average body weight gain**

- **CONTROL**
- **STZ + Nic**
- **STZ**
- **STZ + Ins**

**Average water intake**

**Average food intake**

Insulin treated animals received 4 I.U. daily from day 4 until day 10 when the dose was increased to 8 I.U. daily.
The plasma glucose concentrations of the diabetic animals receiving daily insulin therapy were elevated over control at time of death, 24 hours after the last insulin injection, although the total ketone body concentration was only slightly elevated. Microsomal protein, total cytochrome P450 and cytochrome b$_5$ content showed no significant elevations in any of the groups when compared to controls. However, there was a significant increase in the NADPH-cytochrome c reductase activity in the streptozotocin-treated animals but not in those which received streptozotocin concurrently with nicotinamide, or those receiving daily insulin (Table 3.2).

Table 3.2  Effects of streptozotocin-induced diabetes (STZ), streptozotocin and nicotinamide treatment (STZ + Nic) and insulin-treated streptozotocin diabetes (STZ + Ins) on some microsomal parameters.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Microsomal protein (mg / g liver)</td>
<td>35.4±4.1</td>
</tr>
<tr>
<td>Cytochrome P450 (nmol / mg protein)</td>
<td>0.3±0.03</td>
</tr>
<tr>
<td>Cytochrome b$_5$ (nmol / mg protein)</td>
<td>0.25±0.05</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase (nmol / min / mg protein)</td>
<td>36±4</td>
</tr>
</tbody>
</table>

Values represent mean ± s.e.m for four animals. *** p<0.001 compared to control

Streptozotocin diabetic animals were associated with a marked increase in ethylmorphine and erythromycin N-demethylase activities (Figure 3.2) and also in ethoxy- and pentoxyresorufin O-dealkylases, dimethylnitrosamine N-demethylase and
lauric acid hydroxylase activities (Figure 3.3). These changes in microsomal activity could be reversed by the daily administration of insulin to the diabetic animals and concurrent administration of nicotinamide with streptozotocin prevented all the streptozotocin-induced increases. Immunodetection of specific isoforms of cytochrome P450 associated with the altered enzyme activities confirmed that the levels of these cytochrome P450 proteins were elevated as a result of the diabetic state.

Immunoblot analysis of the cytochrome P450 IA, IIB, IIE, IIIA and IVA sub-families supported the findings of the enzyme assays with the diabetic animals showing induction of the P450 proteins, an effect that was antagonised by the daily administration of insulin to the diabetic animals. Animals concomitantly treated with streptozotocin and nicotinamide did not show any induction of the P450 proteins (Figures 3.4 A-E).

**Figure 3.2** Ethylmorphine and erythromycin N-demethylase activities.

*Values represent mean ± s.e.m. for four animals. **p<0.01 ***p<0.001 when compared to controls*
Figure 3.3 The effects of streptozotocin-induced diabetes on specific cytochrome P450 catalysed reactions.

**Ethoxyresorufin O-deethylase activity**

![Graph showing the activity of Ethoxyresorufin O-deethylase for different groups: CONTROL, STZ, STZ+Nic, STZ+Ins.](image)

**Pentoxyresorufin O-depentyrase activity**

![Graph showing the activity of Pentoxyresorufin O-depentyrase for different groups: CONTROL, STZ, STZ+Nic, STZ+Ins.](image)

**Dimethylnitrosamine N-demethylase activity**

![Graph showing the activity of Dimethylnitrosamine N-demethylase for different groups: CONTROL, STZ, STZ+Nic, STZ+Ins.](image)

**Lauric acid hydroxylase activity**

![Graph showing the activity of Lauric acid hydroxylase for different groups: CONTROL, STZ, STZ+Nic, STZ+Ins.](image)

**STZ + Nic**: Streptozotocin and nicotinamide treated.

**STZ + Ins**: Insulin-treated streptozotocin-diabetic animals

Values represent mean ± s.e.m. of four animals **p<0.01 when compared to control**
Figure 3.4 Immunoblot analysis of microsomes from streptozotocin-induced diabetic rats using anti-cytochrome P450 IA, IIB1, IIE1, IIIA and IVA polyclonal anti-bodies

Figure A Lane 1 and 2 Control rat microsomes
P450 1A 3 and 4 Streptozotocin-diabetic rat microsomes
5 3-Methylcholanthrene-induced microsomes
6 and 7 Streptozotocin and nicotinamide treated rat microsomes
8 and 9 Insulin-treated streptozotocin-diabetic rat microsomes

Figure B Lane 1 and 2 Control rat microsomes
P450IIB1 3 and 4 Streptozotocin-diabetic rat microsomes
5 Phenobarbital-induced microsomes
6 and 7 Streptozotocin and nicotinamide treated rat microsomes
8 and 9 Insulin-treated streptozotocin-diabetic rat microsomes

Figure C Lane 1 Control rat microsomes 2 Isoniazid-induced rat microsomes
P450IIE1 3 Streptozotocin and nicotinamide treated rat microsomes
4 Insulin-treated streptozotocin-diabetic rat microsomes
5 Streptozotocin-diabetic rat microsomes

Figure D Lane 1 Control rat microsomes 2 Streptozotocin-diabetic rat microsomes
P450IIIA 3 PCN-induced rat microsomes
4 Streptozotocin and Nicotinamide treated rat microsomes
5 Insulin-treated streptozotocin-diabetic rat microsomes

Figure E Lane 1 Control rat microsomes 2 Streptozotocin-diabetic rat microsomes
P450IVA 3 Clofibrate-induced rat microsomes
4 Streptozotocin and nicotinamide treated rat microsomes
5 Insulin-treated streptozotocin-diabetic rat microsomes

Microsomal proteins were resolved by electrophoresis in a 10% (w/v) SDS-polyacrylamide gel and transferred to nitrocellulose. In all cases 20μg of solubilised microsomal protein was loaded except for the positive controls when only 10μg of protein was loaded.

Positive controls

Animal pre-treatment

3-methylcholanthrene (25mg/kg in corn oil) for 3 days.
Phenobarbital (80mg/kg in saline) for 3 days
Isoniazid (0.1% (w/v) in drinking water) for 7 days
PCN (100mg/kg in corn oil) for 3 days
Clofibrate (80mg/kg in saline) for 3 days
Discussion

Rats receiving a single intraperitoneal injection of streptozotocin displayed the characteristic physiological changes of hyperglycaemia, hyperketonaemia, polydipsia and hyperphagia associated with overt insulin-dependent diabetes mellitus. Concomitant administration of nicotinamide with streptozotocin prevented the onset of the diabetic syndrome possibly by the mechanism previously discussed (Chapter 1). Moreover, daily treatment of streptozotocin diabetic rats with insulin resulted in control of the diabetic state, reducing the plasma glucose and ketone concentrations.

Streptozotocin treated rats exhibited markedly higher metabolism of all substrates studied, as well as increased NADPH-dependent reduction of cytochrome c, as previously reported by other groups (Ackerman and Liebman 1977, Reinke et al., 1978, Ioannides et al., 1988, Thomas et al., 1987, Peng et al., 1983). The changes in the mixed-function oxidase activities in streptozotocin-treated rats are entirely attributable to the diabetic state rather than the diabetogen per se, as concurrent administration of nicotinamide with streptozotocin prevented the onset of diabetes as well as the alterations in mixed-function oxidase activity.

Daily insulin treatment of the streptozotocin diabetic animals also partially reversed the changes in activities. It must be noted that the insulin regime employed was based on water consumption versus insulin dose and therefore represents a very poorly controlled state with possibly both hyper- and hypoinsulinaemia occurring during each 24 hour period.

The induction of ethoxyresorufin O-deethylase activity, an enzyme activity predominantly associated with the P450 IA subfamily, is of particular interest as this subfamily has been implicated in the bioactivation of many carcinogens (Ioannides and
The confirmation of this finding by western blot analysis also reveals that it is the P450 IA2 isoform that appears to be more highly induced in the diabetic state. This isoform has already been shown to bioactivate some of the most potent mutagens such as Glu-P-1 (Ioannides et al., 1988). Why this isoform should be induced is unclear at present.

The increase in dimethylnitrosamine N-demethylase in chemically-induced diabetes has been attributed specifically to the induction of cytochrome P450 IIE1 which is confirmed by immunoblot analysis. The induction of this isoform of cytochrome P450 may have implications for the propensity of the diabetic to metabolise a range of nitrosamines encountered in the environment. Cytochrome P450IIE1 has already been shown to metabolically activate certain nitrosamines (Peng et al., 1983) and also to convert paracetamol to its reactive quinoneimine intermediate responsible for its hepatotoxicity (Raucy et al., 1989). The cytochrome P450 IIB subfamily has also been associated with the activation of some cyclic nitrosamines (Ayrton et al., 1987). The P450 IIB subfamily is also concerned with the effective deactivation of many therapeutic drugs and its induction may well affect the pharmacokinetics of drugs given to human diabetic patients to treat the multitude of complications to which they are prone.

The increases observed in the rates of N-demethylation of erythromycin and ethylmorphine, two drugs that have been used as diagnostic substrates for the P450 IIIA subfamily (Wrighton et al., 1985a) indicates induction. This subfamily is involved in the metabolism of many drugs such as midazolam (Fabre et al., 1988), mephenytoin (Shimada and Guengerich, 1985), cyclosporin (Bertauld-Peres et al., 1987) and the macrolide antibiotics, troleandomycin and erythromycin (Wrighton et al., 1985b) and will thus alter the pharmacologic effects of these drugs.

Streptozotocin-diabetic rats also displayed a higher activity of lauric acid hydroxylase, an activity associated with the P450 IVA subfamily of haemoproteins (Tamburini et al., 1984). This subfamily plays an important role in the metabolism of
endogenous substrates, being involved in the oxidation of fatty acids such as lauric and arachidonic acids (Bains et al., 1985, Sharma et al., 1988). Insulin-dependent diabetes mellitus is associated with high levels of fatty acids and it may be that the induction of the cytochrome P450 IVA subfamily is an adaptive response to the alterations in the levels of these lipids through fatty acid hydroxylation and subsequent β-oxidation.

In summary, streptozotocin-induced diabetes is associated with alterations in the levels of cytochrome P450 activities of the xenobiotic metabolising families. These effects are attributable to the diabetic state and not the diabetogen per se and can be partially reversed by daily administration of insulin. The observed increases in the cytochrome P450 families studied may have a profound effect on the ability of the diabetic individual to effectively metabolise and thus eliminate the many drugs used to treat the complications of this disease. Increased deactivation may reduce plasma levels of drugs so that the therapeutic effect is decreased or even totally abolished. On the other hand diabetics may be vulnerable to the toxicity of chemicals that rely on the cytochrome P450 proteins induced by diabetes for their activation. Since the streptozotocin-induced changes in mixed-function oxidase activity are similar to those occurring during starvation or following exposure of animals to ethanol or acetone it has been suggested that the hyperketonaemia, common to all the conditions, may mediate the alterations in cytochrome P450 activities in diabetes and some other conditions (Peng et al., 1983). It has also been suggested that the depressed body weight gain of streptozotocin-treated animals may be responsible for the alterations in hepatic drug metabolism. This is quite possible, as animals on restricted food intake also show some alterations of hepatic drug metabolism similar to that observed in diabetes. The common factors to both situations is depressed body weight gain and the production of ketone bodies. Streptozotocin-induced diabetes results in insulinopaenia causing an inability of the animal to assimilate absorbed nutrients. Therefore superficially, it may appear that the depressed body weight gain is responsible for the alterations, in all probability the depressed body weight gain is a consequence of the pathophysiological condition arising from β-cell destruction.
3.2 Sex-differences in streptozotocin-induced type I diabetes mellitus

Many factors can influence the metabolism of xenobiotics including: pathological conditions, physiological parameters and the effect of exogenous agents. The effect of diabetes mellitus on the cytochrome P450 system has already been discussed but it is important to investigate the effect of the disease in both sexes. Numerous studies have shown that sex-differences are observed in the metabolism of a variety of compounds (Reinke et al., 1978, 1979; Warren et al., 1983; Skett, 1986). Although in normal healthy individuals sex differences appear to be of little clinical relevance, it is possible that in certain disease states they may become pronounced and clinically relevant.

Sex-dependent metabolic differences in rats are believed to be determined primarily by events occurring during the neonatal period, when the secretion of testicular androgens in the male imprints a latent masculine potential onto the otherwise feminine pattern (Morgan et al., 1985; Jansson et al., 1985; Kato et al., 1986). Castration of males at birth results in a more feminine pattern of several sexually differentiated characteristics in adult life, including: gonadotropin secretion and sexual behaviour. However, castration of adult males does not alter, or only slightly effects, the already differentiated masculine characteristics.

Sex-related differences have been recognised in the rat hepatic microsomal cytochrome P450 dependent metabolism of steroids and many of the qualitative and quantitative differences in microsomal xenobiotic metabolism can be attributed to the expression of sex-specific P450 isoforms. On the regulation of hepatic cytochrome P450, studies have shown that endocrine hormones such as testosterone, oestradiol and growth
hormone participate in the regulation of constitutive forms in the adult animal including the expression of the male specific P450 IIC11 (rat P450b) and P450 IIC13 (rat P450g) and the female specific P450 IIC12 (rat P450i). In addition the phenobarbital-inducible forms of cytochrome P450, P450 IIB1 and IIB2 (P450b and e respectively) have also been shown to be under the influence of growth hormone. In contrast, the cytochrome P450 III family is not inducible by sex steroid hormones but the de novo synthesis is stimulated by a variety of glucocorticoids and other high molecular weight compounds (Wrighton et al., 1985a; 1985b). This subfamily does show sex-dependent differences in activity with the levels declining in female rats with age, such that adult male rats have higher constitutive levels of liver cytochrome P450 III proteins than adult females (Halvorson et al., 1988; Gonzalez et al., 1986). More recently the effect of growth hormone on other P450 isoforms has been investigated including P450 IIE1 (P450j) which suggest that the circulating levels of endogenous hormones may have an important role in determining the P450 complement of an individual (Yamazoe et al., 1989b).

Streptozotocin-induced diabetes results in a major perturbation of circulating insulin levels. The effect of this disturbance on other hormone secretory patterns has not been fully investigated although in male diabetic rats there is evidence of decreased testosterone (Baxter et al., 1981) and growth hormone secretion (Yamazoe et al., 1989a). The sex-specific P450 isoforms, being partially regulated by the levels of these hormones, will most probably be affected by streptozotocin-diabetes. The effects of streptozotocin-induced diabetes in male and female rats was investigated to evaluate whether the disease resulted in more pronounced sex-specific alterations of xenobiotic metabolism and P450 activities.

Animal pretreatment

Ten male and ten female Wistar albino rats (180-200g) were obtained from the
Experimental Biology Unit, University of Surrey. Each sex was divided into two groups consisting of five animals each. One group of each sex served as control while the other was rendered diabetic by the intraperitoneal administration of streptozotocin (65mg/kg), dissolved in 0.1M sodium citrate buffer, pH 4.5. Daily water and food intake were monitored together with body weight gain. All animals were killed 21 days after administration, terminal blood samples obtained and plasma glucose and ketone body concentrations determined. The livers were immediately excised and microsomes prepared as previously described (Chapter 2). Mixed-function oxidase activities were determined and immunological detection of specific cytochrome P450 apoproteins performed where appropriate.

Results

All streptozotocin animals displayed polydipsia, hyperphagia and depressed body weight gain when compared to their respective controls. Blood glucose and ketone body concentrations were significantly increased over the respective control animals but there was no significant differences between the severity of the hyperglycaemia or hyperketonaemia between the diabetic male or female groups (Table 3.2.1).

Although the diabetic female animals displayed a decrease in total microsomal protein, this was not significant when compared to the control group and there was no significant difference between the male diabetic animals or their controls. Total carbon monoxide discernible cytochrome P450 levels were significantly increased in the diabetic female group and there was a significant difference between the control male and control female group, the latter being lower, although not between the diabetic groups (Table 3.2.2). Cytochrome b₅ levels tended to be higher in the diabetic groups, but the difference was not significant. In contrast to our first study there was no increase in the NADPH dependent reduction of cytochrome c as a result of the diabetic condition the reason for this remains unclear (Table 3.2.2).
Table 3.2.1  Plasma glucose and ketone body concentrations in male and female diabetic rats

<table>
<thead>
<tr>
<th>Group</th>
<th>3-Hydroxybutyrate (mM)</th>
<th>Control Male</th>
<th>Diabetic Male</th>
<th>Control Female</th>
<th>Diabetic Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-Hydroxybutyrate (mM)</td>
<td>Glucose (mM)</td>
<td>Acetoacetate (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Male</td>
<td>6.8 ± 0.24</td>
<td>0.45 ± 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic Male</td>
<td>30.4 ± 5.0</td>
<td>1.40 ± 0.11***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Female</td>
<td>6.5 ± 0.54</td>
<td>0.51 ± 0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic Female</td>
<td>33.0 ± 7.4</td>
<td>1.21 ± 0.19**</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean ± s.e.m. for five animals

* p<0.001 **p<0.01 when compared to respective controls.

The effect of streptozotocin-induced diabetes on the ethoxyresorufin O-deethylase and pentoxyresorufin O-depentylase activity of both male and female groups was to significantly increase both activities when expressed per mg microsomal protein (Table 3.2.3) or per nmol P450 (Figure 3.2.1).

Table 3.2.2. Some microsomal parameters for male and female streptozotocin-diabetic rats

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Control Male</th>
<th>Diabetic Male</th>
<th>Control Female</th>
<th>Diabetic Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal Protein (mg/g liver)</td>
<td>42.3 ± 2.5</td>
<td>42.6 ± 3.8</td>
<td>40.0 ± 4.4</td>
<td>35.4 ± 1.6</td>
</tr>
<tr>
<td>Total cytochrome P450 (nmol/mg protein)</td>
<td>0.55 ± 0.03</td>
<td>0.62 ± 0.05</td>
<td>0.32 ± 0.04‡</td>
<td>0.54 ± 0.08*</td>
</tr>
<tr>
<td>Cytochrome b5 (nmol/mg protein)</td>
<td>0.68 ± 0.03</td>
<td>0.75 ± 0.04</td>
<td>0.67 ± 0.07</td>
<td>0.90 ± 0.10</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase (nmol/min/mg protein)</td>
<td>14.5 ± 0.3</td>
<td>16.5 ± 2.7</td>
<td>16.3 ± 2.7</td>
<td>13.5 ± 0.8</td>
</tr>
</tbody>
</table>

* p<0.05 for diabetic versus control †p<0.01 for male control versus female control

Values represent mean ± s.e.m. for five animals.
The microsomal metabolism of p-nitrophenol to 4-nitrocatechol was significantly increased in the male and female diabetic animals both when expressed per mg protein (Table 3.2.3) or per nmol P450 (Figure 3.2.1). Both diabetic male and female animals showed an increase over control animals in the hydroxylation of lauric acid (Table 3.2.3.), but only the diabetic male group displayed a significant difference when the activities were expressed per nmol P450 (Figure 3.2.1.). Similarly both male and female diabetic rats exhibited a significant induction of ethylmorphine N-demethylase activity when expressed per mg microsomal protein (Table 3.2.3). However, when expressed per nmol P450 only the male diabetic rats showed a significant increase (Figure 3.2.1). These findings were confirmed by immunoblot analysis (Figure 3.2.2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Male</th>
<th>Diabetic Male</th>
<th>Control Female</th>
<th>Diabetic Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethoxyresorufin O-deethylase</td>
<td>10.4 ± 2.5</td>
<td>24.6 ± 2.5**</td>
<td>8.7 ± 2.0</td>
<td>31.4 ± 9.1*</td>
</tr>
<tr>
<td>(pmol/ min/ mg protein)</td>
<td></td>
<td>(236)</td>
<td></td>
<td>(361)</td>
</tr>
<tr>
<td>Pentoxyresorufin O-depentylation</td>
<td>1.9 ± 0.3</td>
<td>10.4 ± 1.0***</td>
<td>1.5 ± 0.3</td>
<td>7.8 ± 1.4**</td>
</tr>
<tr>
<td>(pmol/ min/ mg protein)</td>
<td></td>
<td>(584)</td>
<td></td>
<td>(520)</td>
</tr>
<tr>
<td>p-Nitrophenol hydroxylation</td>
<td>0.5 ± 0.1</td>
<td>2.84 ± 0.3***</td>
<td>0.55 ± 0.04</td>
<td>1.46 ± 0.3**</td>
</tr>
<tr>
<td>(nmol/ min/ mg protein)</td>
<td></td>
<td>(588)</td>
<td></td>
<td>(265)</td>
</tr>
<tr>
<td>Lauric acid hydroxylase</td>
<td>0.99 ± 0.2</td>
<td>3.36 ± 0.18***</td>
<td>1.1 ± 0.12</td>
<td>2.05 ± 0.1***</td>
</tr>
<tr>
<td>(nmol/ min/ mg protein)</td>
<td></td>
<td>(339)</td>
<td></td>
<td>(186)</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>13.2 ± 0.18</td>
<td>28.6 ± 1.0***</td>
<td>12.5 ± 0.1</td>
<td>13.4 ± 0.2**</td>
</tr>
<tr>
<td>(nmol/ min/ mg protein)</td>
<td></td>
<td>(218)</td>
<td></td>
<td>(107)</td>
</tr>
</tbody>
</table>

Values represent mean ± s.e.m. for five animals. *p<0.05  **p<0.01  ***p<0.001 compared to respective control. Figures in parenthesis represent % of control activity.
Figure 3.2.1  Microsomal cytochrome P450 activities expressed per nmol of P450
for male and female streptozotocin-induced diabetic rats.

Ethoxyresorufin O-deethylase activity

Pentoxyresorufin O-depentylation activity

p-Nitrophenol hydroxylase activity

Lauric acid hydroxylase activity

Ethylmorphine N-demethylase activity

Values represent mean ± s.e.m. for five animals. *p<0.05  **p<0.01  ***p<0.001 compared to respective controls. Values in parenthesis are % of control activity.
Figure 3.2.2 Immunoblot analysis of microsomes from streptozotocin-induced diabetic male and female rats using anti-cytochrome P450 Ia, Iib1, Ie1, Iia and Iva polyclonal anti-bodies

Microsomal proteins were resolved by electrophoresis in a 10% (w/v) SDS-polyacrylamide gel and transferred to nitrocellulose. In all cases 20 µg of solubilised microsomal protein was loaded except for the positive controls when only 10 µg of protein was loaded.

Positive controls

Animal pre-treatment

3-methylcholanthrene (25 mg/kg in corn oil) for 3 days.
Phenobarbital (80 mg/kg in saline) for 3 days
Isoniazid (0.1% (w/v) in drinking water) for 7 days
PCN (100 mg/kg in corn oil) for 3 days
Clofibrate (80 mg/kg in saline) for 3 days
Fig. 3.2.2

A

B

C

D

E
Discussion

The induction of streptozotocin-diabetes in both male and female rats resulted in a comparable severity of diabetes-induced hyperglycaemia and hyperketonaemia. The levels of total cytochrome P450 in the control female were significantly lower than the levels in the control males. The effect of diabetes was to diminish this difference and therefore produce a more pronounced induction of total cytochrome P450 in the female animal, but there was no increase in the activity of the NADPH dependent reduction of cytochrome c.

The use of substrates whose metabolism is catalysed predominantly by a single P450 isoform revealed that there are native differences between the two sexes with respect to ethoxyresorufin O-deethylase, p-nitrophenol and lauric acid hydroxylase activities when expressed per nmol P450, although this cannot be substantiated for lauric acid hydroxylase activity due to the assay employed. The increased susceptibility of the female rat to the hepatotoxic effects of paracetamol has been attributed to the higher levels of the P450 IA2 protein in these animals, the proteins of which uniquely catalyse the deethylation of 7-ethoxyresorufin in the livers of untreated rats (Kelly et al., 1987). The effect of diabetes on the IA subfamily is to increase the activity of these proteins towards 7-ethoxyresorufin by approximately the same degree in both sexes when expressed per nmol P450. Similarly, the effect of the diabetic state on the activity of the P450 IIB subfamily of proteins (catalysing the depentylation of pentoxyresorufin) was also an increase and again by a similar fold induction.

The effect of the diabetic state on the levels of the P450 IIE subfamily as exemplified by hydroxylation of p-nitrophenol are comparable when expressed per mg protein with similar fold inductions occurring in both male and female diabetic animals. The P450 IIIA and P450 IVA subfamily display more overt sex-dependent differences with the female controls having slightly higher levels than the control males but these proteins
appear almost refractory to the diabetic state in the female while being significantly
induced in the male diabetic animals. These findings have been confirmed by immunoblot
analysis using monospecific antibodies to the respective cytochrome P450 families. Clearly
these results confirm that sex-dependent differences observed in untreated animals are also
present in the diabetic state.

From the enzyme activities, expressed per nmol P450, the induction of the
cytochrome P450 IA and IIB subfamilies do not show any sex-dependent differences in the
diabetic animals. If some endogenous metabolite, such as ketone bodies, was responsible for
the induction of these subfamilies then the severity of ketosis, being similar in both male
and female diabetic animals may provide some explanation for the observations (Table
3.2.1). Of the other families investigated, sex-dependent factors appear to be involved in
their regulation. The levels of the P450 IIE proteins differ in the male and female control
animals and appear to be induced to differing degrees by the diabetic state. The P450 IIE
subfamily has already been shown to be inducible by a variety of small molecules such as
acetone, ethanol and isoniazid (Ryan et al., 1986) and also in some physiological conditions
including; fasting and starvation (Tu and Yang, 1983). As acetone is a ketone body and
starvation and fasting also elevate the levels of circulating ketones it is possible that this
may explain the induction of the P450 IIE subfamily in the male and female diabetic
animals. However, it does not explain the difference in the degree of induction in the sexes
considering that the severity of hyperketonaemia was comparable in both. It has been
reported that the levels of circulating growth hormone and its pattern of secretion are
different in male and female diabetic animals and that diabetes causes a reduction in the
plasma levels of growth hormone (Tannenbaum et al., 1981, Serri and Brazeau, 1987). It has
been suggested that P450 IIE1 may be partially regulated by growth hormone and the
greater relative reduction in the levels of growth hormone in the diabetic male may be
involved in the sex-difference of P450 IIE1 induction in diabetic animals.
The P450 IIIA subfamily catalyses the demethylation of ethylmorphine and clearly shows sex-differences in the diabetic state. The diabetic female appears to be refractory to the inducing effect of diabetes on this subfamily of proteins while in the male they are significantly induced. This subfamily has been shown to be regulated by glucocorticoids and growth hormone and would possibly be affected if there were perturbations of the circulating levels of this hormone in the diabetic male animal only.

Finally, the P450 IVA subfamily also displayed differences in the levels of control animals and in the response to the diabetic state. The control female animals appeared to have higher levels than the control males and were almost refractory to the inducing effects of diabetes, whereas in the male diabetic animals the proteins were highly induced. Previous studies have shown that compounds causing increases in plasma triglycerides are capable of inducing the P450 IVA subfamily as are the peroxisomal proliferating agents (Hardwick et al., 1987). Diabetes is associated with the uncontrolled mobilisation of fatty acids from the peripheral adipose tissues and it is possible that these increased levels of circulating fatty acids are responsible for the induction. However, the reason why the female animals have higher levels than the males and why they are refractory to the inducing effect of diabetes requires further investigation.

In summary, the effect of streptozotocin-induced diabetes mellitus on the hepatic microsomal P450 system appears to be both sex-dependent and sex-independent depending on the P450 subfamily concerned. The results of this study suggest that some families may be enhanced directly by circulating levels of diabetes generated metabolites of intermediary metabolism, whereas other cytochrome P450 families may have more complex regulation involving a hormonal component. Whatever mechanism is responsible for the alterations, it is clear that pronounced sex-dependent changes of hepatic cytochrome P450 families

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occur in diabetes mellitus which, if also present in man may have implications for the diabetic patient during the prolonged drug therapy associated with this disease. It must be stressed that this study only considered the effects of sex on inducible families of cytochrome P450 and therefore did not address the possibility of the repressive effects of diabetes with respect to sex difference. Furthermore, we did not examine the metabolism of compounds which are specific for isoforms known to show pronounced sex-dependent expression. Further studies should address this area more fully.

3.3 Hepatic drug metabolism in long-term streptozotocin-induced type I diabetic rats.

Streptozotocin-induced insulin-dependent diabetes mellitus is considered a model for spontaneous diabetes mellitus. However, there are some important differences from the spontaneous form of the disease in these animals which must be addressed. When overt diabetes is apparent the streptozotocin-induced animal does not succumb to the hyperglycaemia and hyperketonaemia as observed in spontaneously diabetic animals. This ultimately presents questions as to how the streptozotocin-diabetic animal survives without exogenous insulin for so many weeks, and has led to the suggestion that there may be some β-cell regeneration in the streptozotocin animal. This model is also associated with an accelerate on set of the long-term complications such as glaucoma. For these reasons the period of time which the streptozotocin animal can be used as a model for spontaneous diabetes mellitus may be limited. In order to investigate the long-term effects of streptozotocin-induced diabetes mellitus on the hepatic P450 mixed-function oxidases we studied the diabetes-induced alterations over a period of twelve weeks.
Animal Pretreatment

Twenty four male Wistar albino rats (180-200g) were obtained from the Experimental Biology Unit, University of Surrey and divided into six groups. Three groups served as controls while the animals in the remaining three groups each received an intraperitoneal injection of streptozotocin (65mg kg) dissolved in 0.1M sodium citrate buffer, pH 4.5. One group of control and diabetic animals were killed after four, eight and twelve weeks post-treatment. Terminal blood samples were taken and the livers immediately excised and placed into ice-cold 1.15% KCl. Post-mitochondrial fractions (S9) were prepared as previously described (Chapter 2) and stored at -20°C until required. Plasma ketone body and glucose concentrations were determined and fresh microsomal preparations used for the determination of mixed-function oxidase activities (Chapter 2). Immunological analysis was performed to confirm the results of enzyme analysis where appropriate.

Results

All streptozotocin-treated animals displayed the characteristics of hyperphagia, polydipsia and polyuria throughout the study and continued depression of body weight gain. Analysis of total plasma ketone body concentrations revealed that all diabetic groups showed significantly elevated ketone body levels throughout the study. However, as the study progressed the severity of ketosis in the diabetic groups became less pronounced, although not significantly decreased (Figure 3.3.1). The plasma glucose levels of the diabetic animals were significantly increased over the control groups by week four and remained significantly elevated throughout the duration of the study (Figure 3.3.1).

During the course of the study several of the animals suffered from glaucoma and were killed at the next convenient time point. As the glaucoma was considered a consequence of the prolonged duration of elevated plasma glucose levels the animals were
included in the experimental data from the study.

**Figure 3.3.1** Plasma glucose and ketone body concentrations in long-term streptozotocin-diabetic rats.

**Plasma glucose concentrations**

Values represent mean ± s.e.m. for five animals.

*** p<0.001  ** p<0.01  * p<0.05 when compared to respective controls

There were no significant alterations of the total microsomal protein, cytochrome P450, or the NADPH dependent reduction of cytochrome c for any groups at any time point.
during the study (Table 3.3.1). However, the levels of cytochrome b5 were elevated in all diabetic groups but these elevations were not significant until 12 weeks (Table 3.3.1).

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 4wks</td>
</tr>
<tr>
<td>Microsomal protein (mg/ g liver)</td>
<td>29.2±1.20</td>
</tr>
<tr>
<td>Total cytochrome P450 (nmol / mg protein)</td>
<td>0.43±0.05</td>
</tr>
<tr>
<td>Cytochrome b5 (nmol / mg protein)</td>
<td>0.70±0.14</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase. (nmol/ min/ mg protein)</td>
<td>19.6±2.80</td>
</tr>
</tbody>
</table>

Values represent mean ± s.e.m. for five animals.
* p<0.05 when compared to respective control

The activities of the specific cytochrome P450 families investigated showed significant increases for all substrates investigated at the four week time point. The dealkylation of pentoxyresorufin and ethoxyresorufin remained significantly elevated throughout the study period, however, the hydroxylation of p-nitrophenol and lauric acid declined during the study with the increases becoming non-significant at the 12 week time point. Similarly the N-demethylation of ethylmorphine showed a similar decrease in activity with the increase at four weeks becoming non-significant by the eight and twelve week time points (Figure 3.3.2). Immunoblot analysis confirmed the findings in cytochrome P450 IA, IIE and IVA levels of apoproteins (Figure 3.3.3)

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Figure 3.3.2  Effect of long term streptozotocin-induced diabetes on some specific cytochrome P450 catalysed reactions.

- **Pentoxyresorufin O-depentylase activity**

- **Ethoxyresorufin O-deethylase activity**

- **p-Nitrophenol hydroxylase activity**

- **Lauric acid hydroxylase activity**

- **Ethylmorphine N-demethylase activity**

Data represent mean ± s.e.m for five animals. *p<0.05; **p<0.01; ***p<0.001 compared to respective controls.
Figure 3.3.3  Immunoblot analysis of long-term streptozotocin-diabetic rat microsomes using anti-cytochrome P450 IA, IIE1 and IVA1 polyclonal antibodies.

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>A P450IA</th>
<th>B P450IIIE</th>
<th>C P450IVIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LANE</td>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>D_{12}</td>
<td>D_{8}</td>
<td>D_{4}</td>
</tr>
<tr>
<td></td>
<td>C_{4}</td>
<td>C_{8}</td>
<td>C_{12}</td>
</tr>
<tr>
<td></td>
<td>D_{4}</td>
<td>D_{8}</td>
<td>D_{12}</td>
</tr>
</tbody>
</table>

D = Streptozotocin-diabetic rat  
C = Control rat  
Subscript number = Duration of diabetes (weeks)

Microsomal proteins were resolved by electrophoresis in a 10% (w/v) SDS-polyacrylamide gel and transferred to nitocellulose. In all cases 20µg of solubilised microsomal protein was loaded except for the positive controls when only 10µg of protein was loaded.

Positive controls

Animal pre-treatment

3-methylcholanthrene (25mg/kg in corn oil) for 3 days.
Isoniazid (0.1% (w/v) in drinking water) for 7 days
Clofibrate (80mg/kg in saline) for 3 days
Discussion

All animals treated with streptozotocin became diabetic as shown by the continued hyperglycaemia. The onset of diabetes resulted in all the expected alterations of water consumption, body weight gain and food intake. The resulting hyperketonaemia following streptozotocin-induced diabetes produced some unexpected findings, in that the severity of the ketosis decreased during the study period. Bellward et al., (1988) proposed that it is the ketone bodies which are responsible for the induction of cytochrome P450 IIE1. Our data shows that at four weeks there was a significant induction of \( p \)-nitrophenol hydroxylase, a characteristic activity of cytochrome P450 IIE1. However, by 12 weeks this activity, although still elevated, was not significantly induced. During this period the severity of ketosis was declining. It is possible that the induction of this isoform is tightly coupled to the circulating levels of ketone bodies, thereby explaining the decreasing activity. In contrast, the dealkylation of ethoxy- and pentoxyresorufin by the P450 IA and IIB subfamilies respectively, were consistently elevated throughout the study. This may represent induction of these proteins at lower concentrations of ketone bodies than the P450 IIE proteins.

Immunological detection of the cytochrome P450 IIE1 protein confirmed that there was a decrease in the levels of the protein as the duration of diabetes progressed. Consideration of the other cytochrome P450 families revealed that in the case of lauric acid hydroxylase and ethylmorphine N-demethylase activities, chronic diabetes was also associated with an initial induction of activity followed by a reduction towards the levels found in the control animals. In the case of ethylmorphine N-demethylase activity, characteristic of P450 IIIA induction, the initial increase was only bordering on significance at 4 weeks with the activity returning to control levels thereafter.

The induction of cytochrome P450 IVA is thought to be mediated partly by the circulating levels of plasma free fatty acids (Gibson et al., 1990). Initially, streptozotocin induced-diabetes is associated with a marked hyperlipidaemia (Prof P R Flatt, personal communication), but as the duration of uncontrolled diabetes
progresses the fat deposits of the diabetic animals become diminished and so the levels of plasma free fatty acids fall. If the hyperlipidaemia is responsible for the induction of P450 IVA then we would expect to see the observed results, namely an initial induction of P450 IVA followed by a fall in the levels of this protein as the hyperlipidaemia subsides.

This study reveals that prolonged streptozotocin-induced diabetes is associated with an initial elevation of all isoforms studied. However, as the disease progresses there appears to be a reduction in the severity of the ketosis which is accompanied by a decrease in the levels of three of the isoforms studied. In human diabetic patients type I diabetes is associated with severe hyperketonaemia and hyperglycaemia and if not corrected, death. It is possible that the reduction in the plasma ketones in the long-term helps protect the streptozotocin-diabetic animal from the fatal consequences of the disease. This may be due to a physiological adaption associated with increased clearance of the ketone bodies from the plasma, a theory finding support from the renal hypertrophy, severe polydipsia and polyuria associated with streptozotocin-induced diabetic animals (Marliss et al., 1982).

In summary, it would appear that in the short-term, streptozotocin-induced diabetes closely resembles the physiological alterations associated with type I diabetes. However, these animals appear to be more tolerant to the hyperketonaemia and hyperglycaemia, presumably by being able to adapt and possibly excrete the ketone bodies more efficiently. A role for acetone metabolism by cytochrome P450 IIE1 has been proposed (Casazza et al., 1984) but it is debatable whether the level of induction of this isoform would be able to reduce the levels of the circulating ketones sufficiently to allow the streptozotocin-induced animal to survive for prolonged periods. Further study of the role of the pancreas in long-term streptozotocin-induced diabetes with respect to β-cell regeneration and insulin release merits further investigation. Regeneration of β-cells, coupled to insulin secretion could, in part, explain the observed results, as insulin treatment has been shown to reverse all the diabetes-induced alterations (Chapter 3.1; Ioannides et al., 1988).
3.4 The bioactivation of chemical carcinogens in streptozotocin-induced type I diabetes mellitus

Much of human cancer is believed to result from exposure to chemicals, and numerous examples of chemically-induced carcinogenesis in experimental animals have been demonstrated. Most chemicals express their carcinogenicity through reactive intermediates and are themselves often chemically inert. The reactive intermediates are generated by metabolism and are usually electrophilic entities that interact with vital cellular macromolecules giving rise to toxicity / carcinogenicity. The most prominent enzyme system involved in the metabolic activation of chemical carcinogens is the cytochrome P450-dependent mixed-function oxidases. Some of the families, for example cytochrome P450IA, are closely associated with the bioactivation of carcinogens, while others play no role or direct metabolism primarily towards the formation of more readily excretable products (Ioannides and Parke, 1987).

Clearly, factors which modulate the levels of various cytochrome P450 proteins will also influence the metabolic activation of chemicals and presumably their carcinogenicity. Streptozotocin-induced diabetes is associated with alterations in the levels and composition of hepatic cytochrome P450 proteins, enhancing the synthesis of some at the expense of others (Ioannides et al., 1988; Favreau et al., 1987; Thomas et al., 1987; Yamazoe et al., 1988). The diabetes-induced changes influence the response of diabetic rats to a number of chemical toxins such as bromobenzene, carbon tetrachloride (Watkins et al., 1982) and paracetamol (Price and Jollow, 1982). In view of these reports and the presence of a nitrosamine activating cytochrome P450 induced by diabetes mellitus, the propensity of diabetic animals to bioactivate established chemical carcinogens, such as aromatic and heterocyclic amines and nitrosamines was investigated. The generation of reactive intermediates was monitored by the Ames, Salmonella / microsome assay, a test widely used in the detection of potential chemical carcinogens. (Maron and Ames, 1983).
Animal pretreatment

Male Wistar albino rats (180-200g) were obtained from the Experimental Biology Unit, University of Surrey and randomly divided into two groups. One group received a single intraperitoneal injection of streptozotocin (65mg kg), dissolved in 0.1M sodium citrate buffer, pH 4.5. All animals were killed 21 days after treatment by cervical dislocation and terminal blood samples obtained. Hepatic post-mitochondrial (S9) fractions were prepared as previously described (Chapter 2).

Metabolic activation of the chemical carcinogens by the hepatic fractions were determined using the Ames mutagenicity assay (Chapter 2) and employing Salmonella typhimurium strains TA98 and TA1530. Fresh cultures were prepared by inoculating nutrient broth and incubating overnight in a shaking water bath at 37°C. All carcinogens, with the exception of nitrosopiperidine and nitrosopyrrolidine were dissolved in dimethylsulphoxide. The S9 mixes contained 10% (v/v) of the hepatic fraction except in the case of the nitrosamines when it was increased to 25% (v/v) being equivalent to 0.3 and 0.75mg of microsomal protein respectively. Activation systems, carcinogen and bacteria were always pre-incubated for one hour at 37°C in a shaking water bath. A mutagenic response was considered positive only when there was at least a doubling of the spontaneous reversion rate and a concentration-dependent increase in the number of histidine revertants.

Results

The total plasma ketone body concentrations (3-hydroxybutyrate and acetoacetate) at the time of death were 0.33±0.06 and 1.1±0.10 mM in control and streptozotocin treated animals respectively (mean ± s.e.m), the plasma glucose concentrations in these groups being
5.2 ±0.1 and 20.8 ±1.5 mM respectively. The streptozotocin-treated animals displayed the expected polydipsia, hyperphagia, polyuria and depressed body weight gain, characteristics of insulin-dependent diabetes mellitus.

Activation systems derived from control animals were more efficient than those from streptozotocin-diabetic animals at activating the aromatic amine, 2-aminofluorene (Figure 3.4.1). The diabetic fractions were only slightly more efficient at activating Trp-P-1 than control activation systems (Figure 3.4.1). However, the mutagenic response to the two cyclic nitrosamines, nitrosopiperidine and nitrosopyrrolidine, were markedly enhanced in the activation systems derived from streptozotocin-diabetic rats (Figure 3.4.2). Similarly, streptozotocin-induced diabetes markedly enhanced the activation of the food precarcinogens Trp-P-1 and Glu-P-1 (Figure 3.4.3) to mutagenic intermediates in the Ames mutagenicity assay.

Discussion

2-Aminofluorene is very efficiently activated by control S9, in agreement with previous studies (Phillipson and Ioannides, 1983). 2-aminofluorene is bioactivated by the cytochrome P450 IA subfamily and especially P450 IA2 (Hammons et al., 1985) so the fact that diabetes induces P450 IA2, as confirmed by the results of Glu-P-1 activation is not in agreement with the observed decreased mutagenicity of this compound. Similarly, control S9 is a good activator of 2-aminofluorene (Luster et al., 1982) and yet has very low levels of P450 IA2. Therefore, other enzyme systems must be important and indeed the FAD-monoxygenases readily activates 2-aminofluorene (Hammons et al., 1985; Pelroy and Gandolf, 1980). Moreover, cytosol, in the absence of microsomes, can activate 2-aminofluorene (Ayrton and Ioannides, personal communication). These enzyme systems may be depressed by diabetes, and so explain the decreased mutagenicity of 2-aminofluorene.
Figure 3.4.1  The effects of streptozotocin induced diabetes on the bioactivation of 2-aminofluorene and Trp-P-2

The metabolic activation of 2-aminofluorene was carried out using S. typhimurium strain TA 1538. The spontaneous reversion rate of 11±3 has already been subtracted. Activation of Trp-P-1 was performed using strain TA 98. The spontaneous reversion rate of 10±2 has already been subtracted. Both precarcinogens were activated using 10% (v/v) S9 and pre-incubated with the bacteria for 1hr in a shaking water bath at 37°C.
The metabolic activation of two cyclic nitrosamines was performed using 25% (v/v) S9 activation and employing *Salmonella typhimurium* strain TA 1530. Activation system, nitrosamine, and bacteria were pre-incubated for 1 hr in a shaking water bath at 37°C. Results are expressed as mean ± s.e.m. The spontaneous reversion rate of 8±2 has already been subtracted.
Figure 3.4.3  The effects of streptozocin induced diabetes on protein pyrolysates

The metabolic activation was performed using 10%(v/v) S9 activation and employing *Salmonella typhimurium* strain TA 98. Activation system, pyrolysate and bacteria were pre-incubated for 1hr in a shaking water bath at 37°C. Results are expressed as mean ± s.e.m. The spontaneous reversion rate of 17±2 has already been subtracted.
Glu-P-1, Trp-P-1 and Trp-P-2 are activated through N-hydroxylation, catalysed by the P450 I family, particularly the P450 IA2 protein (Yamazoe et al., 1988; Kato, 1986). As the IA2 protein is induced in diabetes, it may be inferred that the increased levels of this protein is responsible for the activation of the above carcinogens.

Nitrosopiperidine and Nitrosopyrrolidine are activated by N-dealkylation catalysed by the cytochrome P450 IIE subfamily and also by the P450 IIB subfamily at higher concentrations. Both of these families are induced in diabetes resulting in increased levels of the respective proteins and therefore increased activation of these compounds.

The present study clearly indicates that the activation of chemical carcinogens is modulated by the diabetic state. The effect is dependent on the nature of the carcinogen and it appears that streptozotocin diabetes has a selective rather than general effect on drug metabolism enzymes. Our studies have clearly shown that chemical induction of diabetes increases the levels of some cytochrome P450 isoforms which may provide some explanation for the observed effects. However it must be stressed that the present studies involving the Ames test focus attention exclusively on the pathways of activation and as the activation system, being devoid of phase II cofactors, has a very limited phase II detoxication capability and therefore does not mimic the in vivo situation.
Chapter 4

Hepatic drug metabolism in the spontaneously diabetic BB rat
The streptozotocin-induced diabetic animal model has featured predominantly in the majority of studies investigating the effects of diabetes mellitus on hepatic drug metabolism. Numerous studies have shown that chemically-induced diabetes mellitus compares very well with the uncontrolled disease in humans on purely physiological grounds. However, the animals do not succumb to the hyperketonaemia and electrolyte imbalances seen in spontaneously diabetic individuals. Therefore, in terms of drug metabolism, the question remains as to whether the alterations associated with streptozotocin-induced diabetes reflect those of spontaneously diabetic animals or diabetic patients.

The diabetes which occurs spontaneously in the BB Wistar rat has many similarities with the human type I (insulin-dependent) diabetes. It occurs in a non-obese, laboratory rat derived from a non-inbred Wistar line. Both sexes are affected, with the onset of the disease occurring after the time of sexual maturation. Both genetic and immune factors are involved in the aetiology but their precise nature remains to be defined (Like et al., 1982). Evolution of the overt clinical syndrome occurs over a period of hours to a few days and an intense insulitis is observed, accompanied by selective destruction of the β-cells. Although insulitis may precede diabetes by a few weeks, within 7 - 21 days following the appearance of glycosuria the β-cells are completely destroyed and the islets are few, small and with little residual inflammation. If untreated, marked wasting of body tissues, including fat and muscle protein, dehydration and ketosis supervene (Marliss et al., 1982).

Livers from diabetic BB rats demonstrated increased cyclic AMP levels but subnormal protein and glycogen content (Appel et al., 1981). Isolated perfused livers of BB Wistar rats demonstrate increased rates of gluconeogenesis and impaired glycogenesis.
The BB rat has not been extensively used in drug metabolism studies. Bellward et al., (1988) investigated the induction of cytochrome P450 IIE1 (P450j) in the BB rat. After insulin withdrawal there was a significant increase in the activity of this isoform within four days. The uncontrolled animals displayed severe ketosis and there was a significant correlation between the plasma levels of hydroxybutyrate and immunologically detectable cytochrome P450 IIE1 apoprotein.

Chemically-induced diabetes has already been shown to modulate the levels and activities of various cytochrome P450 isoforms (Chapter 3.). The purpose of the present study was two fold:

a) to compare the alterations in hepatic drug metabolism in chemically-induced diabetes with the alterations occurring in uncontrolled diabetes in the spontaneously diabetic BB rat.

b) As human diabetic patients control the disease by single daily injections of insulin, it is probable that during any 24 hour period they display a range of plasma insulin concentrations from hyperinsulinaemic through normoinsulinaemic to hypoinsulinaemic. Therefore, it was of relevance to establish whether poorly insulin-controlled diabetes was still associated with changes of hepatic drug metabolism.

4.1 The effect of uncontrolled diabetes on hepatic drug metabolism in the spontaneously diabetic BB rat

Diabetes prone BB rats can exhibit a spectrum of severity of diabetic syndromes ranging from full-blown insulin dependent type I diabetes to an impaired glucose tolerance. The majority of animals however, develop overt type I diabetes and show the following symptoms in approximate chronological order of appearance;
1. Depression of body weight gain
2. Mild glucosuria
3. Increased blood sugar level
4. Severe glucosuria
5. Markedly increased water consumption
6. Loss in body weight and rough coated appearance
7. Depletion of body fat with severe ketosis
8. Coma and death

Progression from symptom 1 to symptom 8 varies greatly, ranging from three days to two weeks. The onset of the disease is not reversible but the symptoms can be controlled by the subcutaneous administration of insulin.

The BB rat animal model has been predominantly used to investigate the diabetes-induced changes in only one cytochrome P450 protein, namely P450 IIE1. Moreover, it appears that the effects of uncontrolled diabetes in the BB rat are associated with increases in the hydroxylation of aniline and the demethylation of dimethylnitrosamine (Favreau and Schenkman 1988), both activities having been attributed to P450 IIE1. The purpose of the present study is to establish whether the other inducible forms of cytochrome P450 are also perturbed in the diabetic BB rat.

Animal Pretreatment

Twelve diabetic BB/S and twelve diabetes-resistant BB/S rats were obtained from Dr Adrian Bone, Southampton General Hospital, Southampton. The animals had been diabetic for differing periods of time and were thus divided into three groups; one at 4 weeks ± 1 week following detection of overt diabetes; one at 12 weeks ± 2 weeks and one group at 24 weeks ± 3 weeks following detection of diabetes. Each group contained four diabetic animals and four age matched diabetes-resistant controls. All diabetic animals
were received with full medical records of body weight gain, date of detection of diabetes and daily insulin dose. The insulin dose ranged from 1.81 I.U to 3.6 I.U daily for the diabetic animals. The insulin dose administered was dependent on the body weight of the individual rat, with the underlying principle being sufficient insulin to maintain the body weight of the animal. Periodically the urine was tested for glucose and the level of glycated haemoglobin in the blood determined. These tests were used to monitor the effectiveness of the insulin regime employed and make any necessary adjustments.

The animals were housed under normal laboratory conditions (Chapter 2.) and allowed 48 hours to acclimatise. The animals were injected daily with long acting ultratard insulin (40 I.U./ml, Novo biochemicals) given subcutaneously. Following acclimatisation the insulin therapy for the diabetic animals was gradually reduced employing the following regime:

| Day 1  | 50% of normal insulin dose |
| Day 2  | 25% of normal insulin dose |
| Day 3  | No insulin dose            |
| Day 4  | No insulin dose            |

The regime employed was designed to cause a gradual decrease in circulating insulin with very low levels resulting after day 4. These animals are very susceptible to the hyperketonaemia and complete withdrawal from day 1 could have resulted in death of some of the animals by day 4. In view of the numbers of diabetic animals employed in the study no loss could be afforded. All animals were killed on the fifth day, terminal blood samples obtained and plasma glucose and ketone body concentrations determined. The livers were immediately excised, microsomal supernatants prepared and mixed-function oxidase activities determined (Chapter 2). Immunoblot analysis of specific cytochrome P450 proteins was performed to confirm any alterations in enzyme activity associated with the diabetic group.
Results

On reduction of the insulin dose the diabetic animals began to display the characteristic signs of uncontrolled type I diabetes mellitus. As previously reported the degree of severity of diabetes varied from one animal to the next (Like et al., 1982) but by day four all animals displayed polyuria and polydipsia although hyperphagia was not apparent.

Analysis of blood plasma samples revealed that all uncontrolled animals were hyperglycaemic and markedly hyperketonaemic (Table 4.1.1). Total microsomal protein was not significantly altered in the diabetic groups and there was no age dependent alteration. Similarly, the levels of total carbon monoxide discernible cytochrome P450 or activity of the NADPH dependent reduction of cytochrome c were not altered in the diabetic groups. The level of cytochrome b$_5$ was elevated in all the diabetic groups but was statistically significant only in the 12 week and 24 week diabetic groups (Table 4.1.2).

Table 4.1.1  Plasma glucose and ketone body concentrations in the spontaneously diabetic BB rat

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Time from detection of diabetes</th>
<th>Plasma glucose</th>
<th>3-hydroxybutyrate + Acetoacetate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4 weeks</td>
<td>6.8±0.54</td>
<td>0.51±0.1</td>
</tr>
<tr>
<td>Diabetic</td>
<td>4 weeks</td>
<td>28.2±4.1***</td>
<td>11.4±0.5***</td>
</tr>
<tr>
<td>Control</td>
<td>12 weeks</td>
<td>7.1±0.8</td>
<td>0.43±0.08</td>
</tr>
<tr>
<td>Diabetic</td>
<td>12 weeks</td>
<td>26.6±5.0***</td>
<td>11±2.3***</td>
</tr>
<tr>
<td>Control</td>
<td>24 weeks</td>
<td>5.9±0.9</td>
<td>0.49±0.2</td>
</tr>
<tr>
<td>Diabetic</td>
<td>24 weeks</td>
<td>32.0±5.8***</td>
<td>8.4±1.2***</td>
</tr>
</tbody>
</table>

Data represents mean ± s.e.m for three animals  *** p<0.001 compared to respective controls
### Table 4.1.2 Some hepatic microsomal parameters in the spontaneously diabetic BB rat

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Microsomal Protein (mg/g liver)</th>
<th>Cytochrome P450 (nmol/mg Pt)</th>
<th>Cytochrome b5 (nmol/mg Pt)</th>
<th>NADPH cytochrome c reductase (nmol min⁻¹ mg Pt⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (4 weeks)</td>
<td>37.1±0.9</td>
<td>0.40±0.07</td>
<td>0.63±0.05</td>
<td>18.1±1.8</td>
</tr>
<tr>
<td>Diabetic (4 weeks)</td>
<td>38.1±1.2</td>
<td>0.34±0.10</td>
<td>0.91±0.19</td>
<td>15.0±2.4</td>
</tr>
<tr>
<td>Control (12 weeks)</td>
<td>29.6±0.7</td>
<td>0.38±0.10</td>
<td>0.53±0.04</td>
<td>25.1±2.4</td>
</tr>
<tr>
<td>Diabetic (12 weeks)</td>
<td>30.3±2.5</td>
<td>0.43±0.05</td>
<td>0.82±0.16**</td>
<td>20.9±3.1</td>
</tr>
<tr>
<td>Control (24 weeks)</td>
<td>32.0±0.4</td>
<td>0.35±0.06</td>
<td>0.53±0.05</td>
<td>17.3±1.0</td>
</tr>
<tr>
<td>Diabetic (24 weeks)</td>
<td>35.1±1.2</td>
<td>0.52±0.07</td>
<td>0.73±0.10**</td>
<td>15.0±2.1</td>
</tr>
</tbody>
</table>

Data represents mean ± s.e.m. for three animals

** p<0.01 compared to respective controls

(Mg Pt = Mg protein)

The dealkylations of both ethoxy- and pentoxyresorufin were elevated in all diabetic animals (Figure 4.1.1). The demethylation of ethylmorphine was significantly elevated at all time points in the diabetic groups but less pronounced in the older animals (Figure 4.1.2). The hydroxylations of both p-nitrophenol (Figure 4.1.2) and lauric acid (Figure 4.1.3) were significantly increased in the diabetic animals and did not show any decrease in activity with respect to the duration of diabetes (Figure 4.1.3).

Immunoblot analysis of the P450 IA, IIE and IVA sub-families showed elevated levels of these apoproteins in the diabetic animals (Figure 4.1.4)
Figure 4.1.1  The effect of uncontrolled diabetes in the spontaneously diabetic BB rat on ethoxyresorufin and pentoxyresorufin O-dealkylase activity

**Ethoxyresorufin O-deethylase activity**

![Graph showing ethoxyresorufin O-deethylase activity](image)

**Pentoxyresorufin O-depentyrase activity**

![Graph showing pentoxyresorufin O-depentyrase activity](image)

Data represents mean ± s.e.m for three animals

** p<0.01 compared to control
* p<0.05 compared to control
Figure 4.1.2  The effect of uncontrolled diabetes in the spontaneously diabetic BB rat on ethylmorphine N-demethylation and p-Nitrophenol hydroxylation

Ethylmorphine N-demethylase activity

![Graph showing ethylmorphine N-demethylase activity](image)

Data represent mean ± s.e.m for three animals. *p<0.05; ** p<0.01; ***p<0.001 compared to respective control.

p-Nitrophenol hydroxylase activity

![Graph showing p-Nitrophenol hydroxylase activity](image)
Figure 4.1.3  The effect of uncontrolled diabetes in the spontaneously diabetic BB rat on lauric acid hydroxylation

Lauric acid hydroxylase activity

<table>
<thead>
<tr>
<th>Group</th>
<th>Data represents mean ± s.e.m for three animals</th>
<th>** p&lt;0.01 compared to control</th>
<th>*** p&lt;0.001 compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 4 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic 4 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 12 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic 12 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 24 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic 24 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

The spontaneously diabetic BB rat is subdivided into two different populations of animals namely, the diabetic resistant sub-population and the diabetes prone sub-population which, when the disease becomes overt, must be maintained on insulin in order to survive.
Figure 4.1.4  Immunoblot analysis of microsomes from uncontrolled spontaneously diabetic BB rats

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>LANE</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>A P450IA</td>
<td>D₆</td>
<td>D₃</td>
<td>D₆</td>
<td>3MC</td>
<td>C₆</td>
<td>C₃</td>
<td>C₁</td>
<td></td>
</tr>
<tr>
<td>B P450IIE</td>
<td>D₆</td>
<td>D₃</td>
<td>ISON</td>
<td>D₁</td>
<td>C₁</td>
<td>C₃</td>
<td>C₆</td>
<td></td>
</tr>
<tr>
<td>C P450IVA</td>
<td>D₆</td>
<td>D₃</td>
<td>D₁</td>
<td>CLO</td>
<td>C₁</td>
<td>C₃</td>
<td>C₆</td>
<td></td>
</tr>
</tbody>
</table>

D = Spontaneously diabetic BB rat  
C = Control rat  
Subscript number = Duration of diabetes (months)

Microsomal proteins were resolved by electrophoresis in a 10% (w/v) SDS-polyacrylamide gel and transferred to nitocellulose. In all cases 20µg of solubilised microsomal protein was loaded except for the positive controls when only 10µg of protein was loaded.

Positive controls

Animal pre-treatment

3-methylcholanthrene (25mg/kg in corn oil) for 3 days.  
Isoniazid (0.1% (w/v) in drinking water) for 7 days  
Clofibrate (80mg/kg in saline) for 3 days
When insulin was withdrawn, the animals rapidly develop signs of uncontrolled diabetes although the severity varied markedly between animals. The animals became lethargic and displayed polyuria, although they did not develop such severe polydipsia as observed with the streptozotocin-diabetic animals, and by day four following insulin withdrawal their water intake and body weight were decreasing. The severity of uncontrolled diabetes in the BB rat was comparable to that in the uncontrolled streptozotocin-diabetic rat. However, duration of diabetes was not associated with amelioration of enzyme activity in the BB rat. This is in contrast to the situation observed in the long-term streptozotocin-diabetic animals (Chapter 3) where prolonged uncontrolled diabetes was associated with less marked alterations of drug metabolism. However, in the case of the BB rat, the diabetes had been controlled by daily insulin injection from onset and so these animals were chronically insulin-controlled diabetic animals until commencement of the study. Once insulin was withdrawn from these animals there appeared to be a pronounced variation in the onset of the characteristic symptoms of uncontrolled diabetes, with some animals displaying polydipsia after 24 hours while others only begin to show polydipsia after 48 to 72 hours. If this was also the case with the alteration of hepatic drug metabolism it would provide some explanation for the inter-animal variation observed with our studies, although when dealing with spontaneous diabetes, as with many other genetically determined diseases, this is to be expected and larger numbers of animals would be required to make the study more statistically relevant.

It would appear that the duration of diabetes does not have any major influence on the severity of diabetes in the BB rat and once insulin is withdrawn from these animals they become markedly hyperglycaemic and hyperketonaemic at any age. One of the major differences between the uncontrolled diabetic BB rat and the uncontrolled streptozotocin-diabetic animal is the length of time the animals can survive without insulin. In the case of the BB rat it is a matter of days whereas the streptozotocin-diabetic animals survive without insulin for several months. Our present studies reveal that the severity of ketosis
is far greater in the uncontrolled diabetic BB rat than in the uncontrolled streptozotocin-diabetic animal, and this difference may explain the survival rate differences. As most of the changes in cytochrome P450 activity are comparable between the two models it is possible that physiological adaptation may provide some explanation as to the difference in survival time between the two models. It has been suggested that there is islet regeneration in the streptozotocin animal which helps it to survive although we have shown that the severity of hyperglycaemia is comparable at 4 and 12 weeks post-streptozotocin treatment which suggests that this is not the case (Chapter 3). One marked difference between the two models is the severity of polydipsia and polyuria. The BB rat does not show such marked polydipsia and possibly becomes dehydrated much more quickly than the streptozotocin-treated animal which increases its water intake in excess of 15 times that of non-diabetic animal (Chapter 3). Therefore a possible explanation for the differences in survival time may be the clearance rate of the ketone bodies from the two models, with the streptozotocin animal being much more efficient at eliminating the toxic metabolites when compared to the diabetic BB rat. This may also explain why the plasma levels of ketone bodies are also markedly higher in the diabetic BB rat than its streptozotocin-diabetic counterpart (Chapter 3).

Uncontrolled diabetes in the BB rat represents an extreme condition. Although this study has confirmed that the diabetes-induced modifications in hepatic drug metabolism are comparable to the streptozotocin-diabetic animal, a further study to investigate the possible situation occurring in human diabetics would be of interest. In the following study the BB rat was again used but the animals were poorly insulin-controlled. In this way it was hoped that the diabetic BB rat may better represent the situation in human diabetics following a daily insulin regime which, although controlling the overt complications of the disease may itself represent a poorly insulin-controlled state.
4.2 Hepatic drug metabolism in the poorly insulin-controlled spontaneously diabetic BB rat

The spontaneously diabetic BB rat provides one of the most appropriate models of human type I diabetes mellitus, the syndrome being both genetically determined and due to an autoimmune response. The acute life-threatening complications of this disease; hyperglycaemia and hyperketonaemia, are effectively controlled in humans by the administration of insulin and a strict diet regime. However, even with the most strict regime employed most human type I diabetics present a poorly insulin-controlled state rather than complete normality. Therefore, studies involving complete insulin withdrawal from the BB rat cannot provide effective information concerning the possible effects of prolonged insulin controlled type I diabetes mellitus on hepatic drug metabolism. To investigate the effect of a more poorly insulin-controlled state upon drug metabolism we employed further groups of spontaneously diabetic BB rats, of differing duration of overt diabetes which, over a period of two weeks had their daily insulin injections altered so that they received both sub-optimal and excess insulin therapy than necessary to maintain their correct body weight.

Animal pretreatment

Twelve diabetes prone and twelve diabetes resistant BB rats were obtained from Dr A. Bone, Southampton General Hospital, Southampton, U.K. The duration of diabetes varied so the animals were divided into three groups; one group of diabetic animals displaying overt diabetes for 4 weeks ± 1 week, one group for 12 weeks ± 3 weeks and finally one group for 24 weeks ± 3 weeks. All animals were allowed 48 hours to acclimate before
commencement of treatment. The diabetic animals of each group were treated daily with long acting ultratard insulin (40 I.U./ml, Novo biochemicals) given subcutaneously, the dose was calculated from data sheets of animal body weight gain since the detection of diabetes using a protocol designed by Dr A. Bone at Southampton General Hospital. On commencement of the study the diabetic animals received 10% decrease of their daily insulin dose for seven days followed by 10% excess insulin dose for a further seven days. All animals were killed on day 15, 16 hours after the last insulin injection and terminal blood samples obtained. Plasma glucose and ketone body concentrations were determined as previously described (Chapter 2). The livers were immediately excised, microsomal fractions prepared and hepatic mixed-function oxidase activities determined. Immunodetection of specific cytochrome P450 apoproteins were performed where appropriate.

Results

All animals being controlled by insulin injection, with dose determined from their medical history data, did not display severe polydipsia, hyperphagia or alteration in body weight. Analysis of the plasma ketone body and glucose concentrations revealed that all animals were hyperketonaemic and hyperglycaemic at the time of death. This is to be expected as the animals were killed some 16 hours after their last insulin injection (Table 4.2.1).

There was no significant difference in microsomal protein, total cytochrome P450 or NADPH cytochrome c reductase activity in any of the groups when compared to their controls. However, there was an increase in the levels of cytochrome b5 content in the 4 week diabetic animals but this increase was not found to be significant (Table 4.2.2).
Table 4.2.1  Plasma glucose and ketone body concentrations for the poorly controlled spontaneously diabetic BB rat.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Glucose (mM)</th>
<th>3-hydroxybutyrate</th>
<th>Acetoacetate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.7 ± 0.75</td>
<td>0.38 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Diabetic 4 weeks</td>
<td>23.1 ± 1.4***</td>
<td>2.40 ± 0.80**</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.90 ± 0.15</td>
<td>0.24 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Diabetic 12 weeks</td>
<td>24.1 ± 0.45***</td>
<td>1.90 ± 0.10**</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.40 ± 0.6</td>
<td>0.48 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Diabetic 24 weeks</td>
<td>33.6 ± 8.3***</td>
<td>2.28 ± 0.09***</td>
<td></td>
</tr>
</tbody>
</table>

Data represents mean ± s.e.m. for three animals

***p<0.001 compared to control

**p<0.01 compared to control

The hydroxylation of lauric acid and the demethylation of ethylmorphine were also increased in the diabetic animals although the latter was not significantly increased (Figure 4.2.1). Lauric acid hydroxylation was markedly increased in all diabetic animals being highly significant at all time points (Figure 4.2.2). The deethylation of ethoxyresorufin was enhanced in the 12 and 24 weeks diabetic animals but the effect was significant only in the latter diabetic animals. In contrast, the dealkylation of pentoxyresorufin was significantly increased in the 4 week and 12 week diabetic groups but not in the 24 week diabetic animals (Figure 4.2.2). The hydroxylation of p-nitrophenol, characteristic of P450 IIE1 was increased in all diabetic animals, being significantly increased at all time points.
Table 4.2.2  Microsomal parameters in the poorly controlled spontaneously diabetic BB rat.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Microsomal Protein (mg/g liver)</th>
<th>Cytochrome P450 (nmol/mg Pt)</th>
<th>Cytochrome b5 (nmol/mg Pt)</th>
<th>NADPH cytochrome c reductase (nmol/min/mg Pt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (4 weeks)</td>
<td>32.3±4.1</td>
<td>0.48±0.04</td>
<td>0.61±0.03</td>
<td>19.8±3.0</td>
</tr>
<tr>
<td>Diabetic (4 weeks)</td>
<td>28.4±2.5</td>
<td>0.50±0.09</td>
<td>0.74±0.05</td>
<td>17.1±2.1</td>
</tr>
<tr>
<td>Control (12 weeks)</td>
<td>34.1±3.8</td>
<td>0.42±0.04</td>
<td>0.58±0.07</td>
<td>18.4±1.8</td>
</tr>
<tr>
<td>Diabetic (12 weeks)</td>
<td>27.9±3.1</td>
<td>0.37±0.07</td>
<td>0.69±0.04</td>
<td>17.6±2.1</td>
</tr>
<tr>
<td>Control (24 weeks)</td>
<td>36.0±2.8</td>
<td>0.46±0.08</td>
<td>0.55±0.08</td>
<td>15.4±3.8</td>
</tr>
<tr>
<td>Diabetic (24 weeks)</td>
<td>33.0±4.1</td>
<td>0.51±0.08</td>
<td>0.81±0.10</td>
<td>14.7±3.0</td>
</tr>
</tbody>
</table>

Data represent mean ± s.e.m. for three animals (Pt = mg protein)

Figure 4.2.1  The effect of poorly controlled diabetes in the BB rat on Ethylmorphine N-demethylase activity.

**Ethylmorphine N-demethylase activity**

Data represents mean ± s.e.m. for four animals
Figure 4.2.2  Cytochrome P450 catalysed reactions in the poorly controlled spontaneously diabetic BB rat.

**Ethoxyresorufin O-deethylase activity**

![Graph showing ethoxyresorufin O-deethylase activity with data points for Control, Diabetic at 4 weeks, 12 weeks, and 24 weeks.]

**Pentoxyresorufin O-depentylase activity**

![Graph showing pentoxyresorufin O-depentylase activity with data points for Control, Diabetic at 4 weeks, 12 weeks, and 24 weeks.]

**p-Nitrophenol hydroxylase activity**

![Graph showing p-nitrophenol hydroxylase activity with data points for Control, Diabetic at 4 weeks, 12 weeks, and 24 weeks.]

**Lauric acid hydroxylase activity**

![Graph showing lauric acid hydroxylase activity with data points for Control, Diabetic at 4 weeks, 12 weeks, and 24 weeks.]

Data represent mean ± s.e.m. for three animals

*p<0.05; **p<0.01; ***p<0.001 compared to respective control
Discussion

As expected, the continued administration of insulin to the diabetic BB rats helped ameliorate the characteristic physiological effects of polydipsia and polyuria associated with the uncontrolled disease. Daily insulin therapy also reduced the severity of ketosis compared to the previous study (Chapter 4.1). However the plasma glucose concentrations were comparable, clearly showing that all animals were diabetic and hyperglycaemic at the time of death, some 16 hours after their last insulin injection. Hyperketonaemia is a consequence of excessive fatty acid oxidation by the liver mitochondria and is dependent on the mobilisation of lipid stores around the body. When administered daily insulin, these animals would not be expected to become as severely ketotic as the uncontrolled animals because the fluctuating levels of plasma insulin would not be reduced for a sufficient period of time to allow uncontrolled ketogenesis by the liver. Furthermore, the next injection of insulin would inhibit further ketogenesis allowing clearance of the plasma ketones. However, the animal would be hyperglycaemic as the lower levels of insulin causes rapid glycogenolysis and gluconeogenesis by the liver thereby increasing the plasma glucose levels.

In the previous study (Chapter 4.1) uncontrolled diabetes was associated with similar changes in the cytochrome P450 families as observed in the streptozotocin-induced diabetic animals (Chapter 3). The present findings correlate well with the alterations encountered in the insulin-treated streptozotocin-diabetic animals in most instances. However, the level of insulin control appears much more effective than that in the streptozotocin-diabetic animal. All of the diabetic BB rats displayed the expected effects in the cytochrome P450 activities reported in previous studies (Chapter 3, 4.1), except that the level of induction is markedly reduced reflecting the control associated with even the poor insulin therapy. The dealkylation of ethoxy- and pentoxyresorufin, activities attributed to the IA and IIB subfamilies respectively, were not as extensively induced as found in the uncontrolled study. It is possible that if these subfamilies are partly induced
by the hyperketonaemia associated with the diabetic state, then the reduced plasma ketones in the poorly controlled animals may provide some explanation for these findings. Insulin therapy failed to reduce the activity of the IIE subfamily towards p-nitrophenol or the IVA subfamily towards lauric acid. However, although the diabetic animals were not as severely ketotic as the uncontrolled animals they still displayed quite a high level of ketosis which may explain the observation. Similarly, in the poorly controlled animals it is quite probable that the levels of circulating free fatty acids are still elevated providing a possible stimulus for the induction of cytochrome P450 IVA1.

It is clear from these studies that poorly insulin-controlled diabetes is associated with changes in hepatic cytochrome P450 families. This suggests that further consideration should be given to the effects of poorly insulin-controlled human type I diabetes on drug metabolism during the prolonged and multi-drug therapy associated with the long term complications of this disease.

4.3 Bioactivation of chemical carcinogens in the uncontrolled spontaneously diabetic BB rat

From our previous studies using the streptozotocin-induced diabetic animal model (Chapter 3) it was demonstrated that the diabetic state was associated with an increased ability to bioactivate known chemical carcinogens. We have further shown that uncontrolled diabetes mellitus in the BB rat is associated with similar alterations in the levels of cytochrome P450 families as previously observed with the chemically-induced model. The propensity for hepatic fractions isolated from these animals to bioactivate chemical carcinogens is therefore of particular relevance. Of special interest is the induction of the P450 IA2 and the P450 IIE1 proteins as these proteins have already been shown to play a significant role in the bioactivation of many chemical carcinogens (Ioannides and Parke, 1987; Peng et al., 1983).
In our earlier study (Chapter 3) using the streptozotocin-diabetic animals we investigated their ability to bioactivate nitrosamines, aromatic amines and pyrolysate products. In this study we chose more selective chemical carcinogens, namely Glu-P-1 and two cyclic nitrosamines; N-nitrosopiperidine and N-nitrosopyrrolidine. These carcinogens are activated by specific isoforms of cytochrome P450 namely, P450 IA2 in the case of Glu-P-1 and P450 IIE1 in the case of the cyclic nitrosamines.

Animal pretreatment

Hepatic fractions derived from the previous studies already described (section 4.1) were used for this study. Fresh overnight cultures of Salmonella typhimurium strains TA98 and TA1530 were prepared by inoculating nutrient broth and incubating overnight at 37°C in a shaking water bath. The activation systems used contained 10%(v/v) microsomal suspension (105000g pellet resuspended in 1.15% w/v KCl) in the case of Glu-P-1 and 25%(v/v) microsomal suspension for the two nitrosamines, being equivalent to 0.35 and 0.8mg of microsomal protein respectively. Activation system, carcinogen and bacteria were always pre-incubated for one hour at 37°C in a shaking water bath before being plated onto minimal nutrient plates. A mutagenic response was considered positive only when there was at least a doubling of the spontaneous reversion rate and a concentration dependent increase in the number of histidine revertants.
Results

The data presented in Table 4.3.1 and 4.3.2 demonstrate that uncontrolled insulin-dependent diabetes mellitus in the spontaneously diabetic BB rat was associated with enhanced bioactivation of both nitrosamines and the pyrolysate product Glu-P-1. The duration of diabetes did not influence the bioactivation of the chemical carcinogens employed.

Table 4.3.1 The bioactivation of Glu-P-1 by microsomal fractions from uncontrolled spontaneously diabetic BB rats employing *Salmonella typhimurium* strain TA98.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>HISTIDINE REVERTANTS PER PLATE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0 µg/plate</td>
</tr>
<tr>
<td>Control (4 weeks)</td>
<td>1010±110</td>
</tr>
<tr>
<td>Diabetic (4 weeks)</td>
<td>3019±96</td>
</tr>
<tr>
<td>Control (12 weeks)</td>
<td>891±41</td>
</tr>
<tr>
<td>Diabetic (12 weeks)</td>
<td>2179±64</td>
</tr>
<tr>
<td>Control (24 weeks)</td>
<td>911±32</td>
</tr>
<tr>
<td>Diabetic (24 weeks)</td>
<td>3047±104</td>
</tr>
</tbody>
</table>

Data represent mean±s.e.m. for triplicates. Spontaneous reversion rate of 58±8 has already been deducted. All data for diabetic animals was significant (p<0.001) compared to respective controls.

Table 4.3.2 The ability of hepatic microsomal fractions from uncontrolled spontaneously diabetic BB rats to bioactivate two cyclic nitrosamines.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>HISTIDINE REVERTANTS PER PLATE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mg/plate</td>
</tr>
<tr>
<td>Control (4 weeks)</td>
<td>43±2</td>
</tr>
<tr>
<td>Diabetic (4 weeks)</td>
<td>78±9</td>
</tr>
<tr>
<td>Control (12 weeks)</td>
<td>28±5</td>
</tr>
<tr>
<td>Diabetic (12 weeks)</td>
<td>77±5</td>
</tr>
</tbody>
</table>
**Table 4.3.2 cont...**

<table>
<thead>
<tr>
<th></th>
<th>Control (24 weeks)</th>
<th>Diabetic (24 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>54±19</td>
<td>125±4</td>
</tr>
<tr>
<td></td>
<td>89±6</td>
<td>169±20</td>
</tr>
<tr>
<td></td>
<td>40±12</td>
<td>205±5</td>
</tr>
</tbody>
</table>

**N-nitrosopiperidine Strain TA 1530**

<table>
<thead>
<tr>
<th></th>
<th>Control (4 weeks)</th>
<th>Diabetic (4 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>159±12</td>
<td>174±16</td>
</tr>
<tr>
<td></td>
<td>202±16</td>
<td>258±15</td>
</tr>
<tr>
<td></td>
<td>286±11</td>
<td>308±13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Control (12 weeks)</th>
<th>Diabetic (12 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>45±3</td>
<td>87±15</td>
</tr>
<tr>
<td></td>
<td>142±6</td>
<td>353±54</td>
</tr>
<tr>
<td></td>
<td>241±6</td>
<td>409±24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Control (24 weeks)</th>
<th>Diabetic (24 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>47±11</td>
<td>69±9</td>
</tr>
<tr>
<td></td>
<td>63±8</td>
<td>128±24</td>
</tr>
<tr>
<td></td>
<td>107±31</td>
<td>125±31</td>
</tr>
</tbody>
</table>

Data represent mean±s.e.m for triplicates. Spontaneous reversion rate never exceeded 11±4. All data was significant (p<0.001) compared to respective control.

**Discussion**

Immunoblot analysis of cytochrome P450 IA in streptozotocin-induced (Chapter 3.) and uncontrolled diabetes in the spontaneously diabetic BB rat (Chapter 4.1) showed that it was the P450 IA2 protein that is induced by diabetes. This observation is confirmed by the increased propensity for hepatic fractions from uncontrolled diabetic BB rats to bioactivate Glu-P-1. The bioactivation of this compound has been shown to be exclusively catalysed by the cytochrome P450 IA2 protein (Kato, 1986).

The activation of nitrosamines has been attributed to the induction of cytochrome P450 IIE proteins in diabetes (Peng et al., 1983). Clearly, the increased activation of the two nitrosamines by hepatic fractions derived from the uncontrolled diabetic BB rat correlates with the increased hydroxylation of p-nitrophenol, an activity specifically catalysed by cytochrome P450 IIE1.
The increased ability for the bioactivation of known carcinogens by both chemically-induced and spontaneously diabetic animals raises the probability that similar effects may be observed in human diabetic patients. Although, human diabetic patients are effectively controlled by insulin therapy, our study of poorly insulin-controlled diabetes (Chapter 4.2) demonstrates that alterations of these cytochrome P450 proteins are still apparent. Thus the human diabetic patient may show a greater pre-disposition towards chemical carcinogen activation. However, it must be stressed that the present study employing the Ames mutagenicity test focuses attention exclusively on the pathways of activation. The activation system, being devoid of phase II cofactors, has a very limited phase II detoxification capability and therefore cannot mimic the \textit{in vivo} situation. Furthermore, streptozotocin-induced diabetes has been associated with alteration of phase II enzyme activity, especially glutathione S-transferase, which effectively deactivates many metabolically formed reactive intermediates.

This study further confirms that it is the diabetic state that is responsible for the increased propensity of both the streptozotocin-diabetic and the spontaneously diabetic rats to bioactivate known chemical carcinogens. The induction of Glu-P-1 bioactivation has been previously shown to be exclusively performed by P450 IA2, a protein that is induced in uncontrolled insulin-dependent diabetes mellitus (Chapter 3 and Chapter 4).

There does not appear to be any discernible difference between the duration of insulin controlled diabetes and the ability of the uncontrolled animals to bioactivate the carcinogens used in this study. This suggests that individuals with insulin-dependent diabetes mellitus of any age may show a increased propensity of carcinogen bioactivation, independent of the duration of the disease and also suggests that poorly controlled diabetes where similar alterations of the cytochrome P450 family does occur, albeit not to the same extent as in uncontrolled diabetes, may also show a greater pre-disposition towards chemical carcinogen bioactivation. However it must be stressed that the present studies involving the Ames test focus attention exclusively on the pathways of activation and as
the activation system, being devoid of phase II cofactors, has a very limited phase II detoxication capability and therefore does not mimic the *in vivo* situation.
Chapter 5

The modulation of hepatic mixed-function oxidase activity by ketone bodies
The ability of the average individual to survive long periods of food deprivation is made possible through a complex series of hormonal and biochemical signals that ensure the appropriate inter-organ redirection and utilisation of major fuels. In the early stages of a fast, there is a shift in liver metabolism from glucose storage to glucose production, coupled with other adaptations that conserve circulating glucose for maintenance of brain and CNS function. Crucial is the ability of most tissues in the body to switch from carbohydrate to a lipid-based catabolism, utilising free fatty acids mobilised from fat depots as their main source of energy. The liver responds uniquely by enhancing its capacity to convert incoming free fatty acids into ketone bodies, which serve as auxiliary fuels of respiration for non-hepatic tissues. As starvation progresses the blood ketone body concentration gradually rises to the region of 5mM, allowing these compounds to make an increasing contribution to the energy requirements of the brain.

Physiological ketosis, although initiated by a rise in circulating glucagon and fall in circulating insulin, is kept in check by the β-cells which remain responsive to stimulation by rising concentrations of free fatty acids and ketones, even in the presence of non-stimulatory concentrations of glucose in the plasma. Low-level secretion of insulin throughout starvation modulates free fatty acid release and prevents progression to full-blown ketoacidosis. However, this delicately balanced interplay of critical hormones and fuels is subject to breakdown in a variety of diseases including diabetes mellitus.

The hallmark of all ketotic-states, whether physiological or pathophysiological, is insulin deficiency. For many years it was believed that a fall in insulin was sufficient to initiate ketogenesis, but it now appears that although a fall in circulating insulin is the major 'triggering' event for accelerated ketogenesis, there is a requirement for a relative or absolute excess of the pancreatic α2-cell hormone, glucagon.
The work of Gerich et al., (1975) and others confirmed that ketogenesis is under bihormonal control. The first requirement is increased fatty acid delivery to the liver, fulfilled by a decrease in circulating insulin and the activation of fatty acid oxidation brought about by an elevated glucagon/insulin ratio. Therefore, acute on-off control of ketogenesis is mediated primarily by the pancreatic α2- and β-cells.

5.1 The role of ketone bodies in the modulation of hepatic mixed function oxidase activity.

During type I diabetes the delicate balance of ketogenesis becomes disturbed. The destruction of the β-cells results in low or undetectable levels of plasma insulin, which prevents the secretion of insulin in response to the increasing levels of plasma fatty acids. The decreased insulin levels result in an increased glucagon/insulin ratio leading to uncontrolled ketogenesis. If this condition is not corrected by the administration of exogenous insulin then full-blown ketoacidosis will occur with fatal consequences. The alterations of hepatic cytochrome P450 activities in streptozotocin-induced diabetes (Chapter 3), particularly the induction of P450 IIE1 (Past and Cook, 1982; Thomas et al., 1987), are often observed during starvation or when animals are administered acetone, or large doses of ethanol. It has been postulated, therefore, that the alteration in P450 activities may be mediated by the circulating levels of ketone bodies (Peng et al., 1983). This hypothesis has already been supported by a good correlation between the plasma ketone body levels and the induction of cytochrome P450 IIE1 (Thomas et al., 1987).

In order to investigate the role of hyperketonaemia in the diabetes-induced alterations of the hepatic mixed-function oxidase activities, rats were rendered ketotic by the daily administration of medium chain triacylglycerols (Flatt et al., 1987). The
activities of selected substrates for the mixed-function oxidases, shown to be influenced by
diabetes, were investigated in comparison to streptozotocin-diabetic and control rats.
Immunodetection of cytochrome P450 proteins was also performed using monospecific
antibodies to confirm the findings of the diagnostic substrates employed.

Animal pretreatment

Male Wistar albino rats (170-200g) were randomly divided into three groups. The
first group received a single intraperitoneal injection of streptozotocin (65mg/kg) in 0.5M
sodium citrate buffer, pH 4.5; the second group received daily administration of medium
chain triacylgllycerols (fractionated coconut oil comprising; C₆ not more than 2%, C₈ = 56%,
C₁₀ = 40%, C₁₂ not more than 1.5%) by gastric intubation (8g/kg). The third group served as
control. All animals were killed 21 days after the commencement of treatment. Hepatic
microsomal fractions were prepared and mixed-function oxidase activities determined.

Body weight, food and water intake were monitored daily and blood samples from
the cut tip of the tail of conscious rats were used for the determination of plasma glucose,
acetoacetate and 3-hydroxybutyrate.

Immunodetection by SDS polyacrylamide gel electrophoresis and Western blotting
to identify specific cytochrome P450 proteins was performed as previously described
(Chapter 2).

Results

The diabetic animals displayed the normal physiological characteristics of
depressed body weight gain, polydipsia and hyperphagia (Figure 5.1.1) while the
animals receiving the medium chain triacylglycerols resembled the control animals except for a slight decrease in body weight gain and food intake (Figure 5.1.1).

Figure 5.1.1 Effect of streptozotocin-induced diabetes (STZ) or medium chain triacylglycerol treatment (MCT) on body weight gain, food and water intake.

Data represent mean body weight gain for five animals. Food and water intake are average intake of cage per day divided by five animals.
Plasma analysis revealed that the diabetic animals were markedly hyperketonaemic and hyperglycaemic throughout the study. Animals receiving the medium chain triacylglycerols, were hyperketonaemic but in contrast to the diabetic animals they were normoglycaemic (Figure 5.1.2) and displayed ketone body levels lower than the diabetic animals at day four (p<0.05) but comparable levels thereafter.

Figure 5.1.2 Plasma glucose and ketone body concentrations in control, streptozotocin-diabetic (STZ) and medium chain triacylglycerol treated animals (MCT)

Values represent mean ± s.e.m. for five animals. * p<0.001 when compared to day 0
Microsomal cytochrome b₅, cytochrome P450 and microsomal protein levels were not significantly affected by streptozotocin and medium chain triacylglycerol treatment (Table 5.1.1). The NADPH-dependent reduction of cytochrome c was, however, markedly increased in both the medium chain triacylglycerol and especially the streptozotocin-treated animals (Table 5.1.1). The O-dealkylations of ethoxyresorufin and pentoxyresorufin were significantly elevated in both the streptozotocin- and medium chain triacylglycerol-treated rats (Figure 5.1.3). A similar pattern emerged in the p-hydroxylation of aniline, and the N-demethylation of dimethylnitrosamine (Figure 5.1.3). Both streptozotocin and medium chain triacylglycerol treatments significantly increased the hydroxylation of lauric acid (Figure 5.1.4). However, the N-demethylation of ethylmorphine was not affected by treatment with medium chain triacylglycerols in contrast to the significant induction of this activity in the streptozotocin-diabetic animals (Figure 5.1.4). Immunoblot analysis confirmed that the increased enzymes activities associated with both streptozotocin-induced diabetes and medium chain triacylglycerol treatment correlated with increased levels of specific cytochrome P450 apoproteins (Figure 5.1.5).

Table 5.1.1 Effect of treatment of rats with streptozotocin or medium chain triacylglycerols on hepatic microsomal parameters.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>Control</th>
<th>Streptozotocin</th>
<th>Medium chain triacylglycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450 (nmol/mg protein)</td>
<td>0.28±0.04</td>
<td>0.37±0.05</td>
<td>0.36±0.04</td>
</tr>
<tr>
<td>Cytochrome b₅ (nmol/mg protein)</td>
<td>0.25±0.05</td>
<td>0.31±0.05</td>
<td>0.32±0.02</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase (nmol/min/mg protein)</td>
<td>33±3</td>
<td>139±5*</td>
<td>128±4*</td>
</tr>
<tr>
<td>Microsomal protein (mg/g liver)</td>
<td>36±4</td>
<td>38±2</td>
<td>35±5</td>
</tr>
</tbody>
</table>

Data represents means±s.e.m for five animals *p<0.001 compared to control
Figure 5.1.3 The effect of streptozotocin-diabetes (STZ) and medium chain triacylglycerol (MCT) treatment on some hepatic mixed-function oxidase activities.

Ethoxyresorufin O-deethylase activity

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>STZ</th>
<th>MCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol resorufin min⁻¹ mg protein⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL Group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ Group</td>
<td>**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCT Group</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pentoxyresorufin O-depentylase activity

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>STZ</th>
<th>MCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol resorufin min⁻¹ mg protein⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL Group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ Group</td>
<td>**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCT Group</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dimethylnitrosamine N-demethylase activity

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>STZ</th>
<th>MCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol formaldehyde min⁻¹ mg protein⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL Group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ Group</td>
<td>**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCT Group</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Aniline p-hydroxylase activity

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>STZ</th>
<th>MCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol p-aminophenol min⁻¹ mg protein⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL Group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ Group</td>
<td>**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCT Group</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean ± s.e.m. for five animals. ** p<0.001 *p<0.05 All findings are significant when compared to controls. Similarly differences between the two treatment groups were always statistically significant (p<0.05).
Figure 5.1.4  The effect of streptozotocin-diabetes (STZ) and medium chain triacylglycerol (MCT) treatment on some hepatic mixed-function oxidase activities.

**Lauric acid hydroxylase activity**

**Ethylmorphine N-demethylase activity**

Values represent mean ± s.e.m. for five animals. **p<0.01; ***p<0.001 compared to control**
Microsomal proteins were resolved by electrophoresis in a 10% (w/v) SDS-polyacrylamide gel and transferred to nitocellulose. In all cases 20µg of solubilised microsomal protein was loaded except for the positive controls when only 10µg of protein was loaded.

**Positive controls**

Animal pre-treatment

- 3-methylcholanthrene (25mg/kg in corn oil) for 3 days.
- Isoniazid (0.1% (w/v) in drinking water) for 7 days
- Clofibrate (80mg/kg in saline) for 3 days
Discussion

Rats treated with medium chain triacylglycerols or with streptozotocin exhibited marked hyperketonaemia within four days of treatment with the ketosis being sustained throughout the duration of the study. Streptozotocin-treated rats exhibited markedly higher activities in the metabolism of all substrates studied, as well as in the NADPH-dependent reduction of cytochrome c as previously reported by other groups (Ioannides et al., 1989).

Hyperketonaemia induced by the daily administration of medium chain triacylglycerols gave rise to similar increases in the various mixed-function oxidase activities, providing direct evidence for the role of ketosis in the diabetes-induced changes in microsomal drug metabolism. The generally higher increases observed in the streptozotocin treated animals may reflect the fact that these animals suffered a more severe ketosis than was produced by the medium chain triacylglycerol treatment. These results clearly demonstrate that the changes in circulating plasma glucose concentrations are not responsible for the diabetes-induced increases in hepatic drug metabolism. In fact previous studies have already shown that chronic hyperglycaemia actually decreased mixed-function oxidase activity (Hartshorn et al., 1979).

The increases in dimethylnitrosamine N-demethylase and aniline p-hydroxylase activities in chemically induced diabetes have been attributed to the induction of cytochrome P450 IIE1, and it is clear from this study that the same isoform is being induced by the administration of medium chain triacylglycerols. The O-deethylation of ethoxyresorufin is a reaction exclusively catalysed by the P450 IA subfamily, and this protein appears to be induced by both treatments. The results of the metabolic activation of Glu-P-1 demonstrate that it is the P450 IA2 protein that is being induced in both cases, an observation confirmed by immunodetection (Figure 5.5). Immunodetection of the respective
cytochrome P450 proteins confirms that streptozotocin and medium chain triacylglycerols induce the same P450 isoforms with the exception of P450 IIA which does not appear to be responsive to medium chain triacylglycerol treatment. This is not necessarily a surprising finding since inducers of this isoform are generally very large molecules such as the macrolide antibiotics. The cytochrome P450 IIB proteins have an important role in the detoxication of many drugs, the induction of this subfamily by both treatments may have clinical implications for diabetic patients being administered a variety of drugs to combat the long-term complications of the disease.

5.2 The modulation of carcinogen activation by hyperketonaemia

In the previous study our observations clearly demonstrate that the hyperketonaemia accompanying uncontrolled type I diabetes mellitus has an important role in the alterations in hepatic drug metabolism associated with the disease. In view of the fact that hyperketonaemia can occur as a result of various physiological conditions such as fasting or starvation and that the induction of the cytochrome P450 IA and IIE subfamilies has been associated with the increased metabolic activation of known chemical carcinogens (Phillipson et al., 1984; Thomas et al., 1987; Garro et al., 1981) it was decided to investigate the ability of hepatic fractions from these animals to metabolically activate the chemical carcinogens previously investigated with the streptozotocin diabetic and spontaneously diabetic BB rat hepatic fractions.

Animal pretreatment

The hepatic fractions from control, streptozotocin and hyperketonaemic animals
were obtained from the animals used in the previous study. Metabolic activation of the precarcinogens to their mutagenic intermediates was determined using the Ames test and employing fresh overnight cultures of the various *Salmonella typhimurium* strains as previously described (Chapter 3). All carcinogens were dissolved in dimethylsulphoxide and 100μl used on each plate. Activation systems contained 10% (v/v) of the S9 fraction (post-mitochondrial supernatant), except in the case of the nitrosamines when the concentration was increased to 25% (v/v) (Ayrton *et al.*, 1987).

Activation system, pre-carcinogen and bacteria were pre-incubated for 1 hour at 37°C in a shaking water bath. The number of induced histidine revertants increased linearly with time, at least up to one hour of preincubation.

**Results**

The mutagenic response to the two cyclic nitrosamines N-nitrosopiperidine and N-nitrosopyrrolidine was markedly enhanced in the presence of activation system derived from the animals pretreated with the medium chain triacylglycerols than from control animals and streptozotocin-induced diabetes gave rise to an even higher response (Table 5.2.1). Similarly, administration of medium chain triacylglycerols markedly enhanced the activation of the food precarcinogens Trp-P-2 and Glu-P-1 and to a lesser extent Trp-P-1 (Table 5.2.2) and again the effect of streptozotocin-induced diabetes was to produce a more pronounced effect. In contrast, administration of medium chain triacylglycerols gave rise to a small, but significant decrease, in the mutagenicity of 2-aminofluorene at higher concentrations with the streptozotocin-diabetic fraction showing an even more pronounced decrease in the activation of this carcinogen (Table 5.2.3).
Table 5.2.1  Metabolic activation of two cyclic nitrosamines by streptozotocin (STZ), and medium chain triacylglycerol (MCT) treated animals

N-nitrosopiperidine  Strain TA 1530

<table>
<thead>
<tr>
<th>Concentration (mg/plate)</th>
<th>Control</th>
<th>Histidine revertants/plate</th>
<th>MCT</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>8±1</td>
<td>75±10</td>
<td>162±9</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>10±2</td>
<td>110±8</td>
<td>202±11</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>14±3</td>
<td>125±5</td>
<td>209±7</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>16±3</td>
<td>173±7</td>
<td>297±16</td>
<td></td>
</tr>
</tbody>
</table>

N-nitrosopyrrolidine  Strain TA 1530

<table>
<thead>
<tr>
<th>Concentration (mg/plate)</th>
<th>Control</th>
<th>Histidine revertants/plate</th>
<th>MCT</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>4±1</td>
<td>104±10</td>
<td>191±7</td>
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</tr>
<tr>
<td>1.0</td>
<td>12±2</td>
<td>156±9</td>
<td>226±18</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>18±3</td>
<td>173±11</td>
<td>279±15</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>23±4</td>
<td>180±10</td>
<td>350±24</td>
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</table>

Data represents mean±s.e.m for triplicates  
p< 0.001 for all MCT and STZ values compared to control. Spontaneous reversion rate of 6±2 and has already been subtracted.

Table 5.2.2  Metabolic activation of the food precarcinogens Trp-P-1, Trp-P-2 and Glu-P-1 by fractions from streptozotocin-diabetic (STZ) and medium chain triacylglycerol (MCT) treated animals.

Glu-P-1  Strain TA 98

<table>
<thead>
<tr>
<th>Concentration (µg/plate)</th>
<th>Control</th>
<th>Histidine revertants/plate</th>
<th>MCT</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>9±2</td>
<td>2480±121</td>
<td>2980±96</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>20±4</td>
<td>3604±214</td>
<td>4200±186</td>
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<tr>
<td>7.5</td>
<td>30±6</td>
<td>5001±397</td>
<td>5480±276</td>
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<tr>
<td>10.0</td>
<td>47±9</td>
<td>6364±487</td>
<td>7290±401</td>
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129
Table 5.2.2  continued...

<table>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td><strong>Trp-P-2</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>7±2</td>
</tr>
<tr>
<td>5.0</td>
<td>5±3</td>
</tr>
<tr>
<td>75</td>
<td>9±2</td>
</tr>
<tr>
<td>10.0</td>
<td>11±3</td>
</tr>
<tr>
<td><strong>Strain TA 98</strong></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>53±13</td>
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<tr>
<td>5.0</td>
<td>247±56</td>
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<td>75</td>
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<td>25</td>
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<td>5.0</td>
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<td>75</td>
<td>521±84</td>
</tr>
<tr>
<td>10.0</td>
<td>694±75</td>
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Data represents mean ± s.e.m. for triplicates. p<0.001 for all MCT and STZ values compared to control. The spontaneous reversion rate of 17±2 and has already been subtracted.

Table 5.2.3  The metabolic activation of 2-aminofluorene by streptozotocin-diabetic (STZ) and medium chain triacylglycerol treated (MCT) animals.

<table>
<thead>
<tr>
<th>2-Aminofluorene</th>
<th>Strain TA 1538</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>Control</td>
</tr>
<tr>
<td>(µg / plate)</td>
<td>MCT</td>
</tr>
<tr>
<td></td>
<td>STZ</td>
</tr>
<tr>
<td>25</td>
<td>960±270</td>
</tr>
<tr>
<td>5.0</td>
<td>1680±138</td>
</tr>
<tr>
<td>75</td>
<td>1940±87</td>
</tr>
<tr>
<td>10.0</td>
<td>2604±50</td>
</tr>
<tr>
<td></td>
<td>1280±101</td>
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<td></td>
<td>1340±60</td>
</tr>
<tr>
<td></td>
<td>1560±74*</td>
</tr>
<tr>
<td></td>
<td>1970±62**</td>
</tr>
<tr>
<td></td>
<td>1294±101</td>
</tr>
<tr>
<td></td>
<td>1310±60</td>
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<tr>
<td></td>
<td>1468±74**</td>
</tr>
<tr>
<td></td>
<td>1801±62***</td>
</tr>
</tbody>
</table>

Data represents mean±s.e.m. for triplicates. The spontaneous reversion rate of 7±2 has already been subtracted. *p<0.05; **p<0.01; ***p<0.001 compared to control.
Discussion

Hyperketonaemia induced by the oral administration of medium chain triacylglycerols profoundly modulated the metabolic activation of major groups of carcinogens such as nitrosamines and both aromatic and heterocyclic amines. This is in concert with our previous findings that hyperketonaemia modulated the activities of the cytochrome P450 dependent hepatic mixed-function oxidases (Barnett et al., 1988). However, the effect of hyperketonaemia in the activation of chemical carcinogens is very much dependent on the nature of the carcinogen involved.

It is generally accepted that the initial step in the bioactivation of nitrosamines is an α-hydroxylation catalysed primarily by the cytochrome P450-dependent mixed-function oxidases (Hecker et al., 1979). The α-hydroxynitrosamines are unstable and rapidly decompose, giving rise to alkylating agents and aldehydes. Of the various cytochrome P450 proteins, the cytochrome P450 IIE1 form plays a major role in the activation of many nitrosamines (Yang et al., 1985) including N-nitrosopyrrolidine (McCoy et al., 1979) and presumably the increased activation by the medium chain triacylglycerol-treated preparations reflects the elevated levels in this protein.

The initial and rate limiting step in the activation of the heterocyclic amines Glu-P-1 and Trp-P-2 is an α-hydroxylation which is specifically catalysed by the cytochrome P450 IA subfamily and especially the P450 IA2 isoform (Kato 1986). The aromatic amine 2-aminofluorene is also activated by N-hydroxylation but in contrast to Glu-P-1 and Trp-P-2 its N-hydroxylation is catalysed not only by P450 IA proteins but also by isozymes belonging to the P450 IIB sub-family, constitutive forms of the cytochrome as well as the flavine monooxygenase system (Hammons et al., 1985) and cytosolic enzyme systems (Ayrton and Ioannides, personal communication) and so interpretation of this decreased mutagenicity is difficult.
In summary, the effect of hyperketonaemia on the activation of chemical carcinogens closely resembles that displayed by streptozotocin-induced and spontaneously diabetic derived hepatic fractions. This observation together with the fact that hyperketonaemia gives rise to similar changes in hepatic mixed-function oxidase activities, as observed in uncontrolled type I diabetes, provides compelling evidence that the diabetes-induced effects are mediated, at least partly, through the increased production of ketone bodies and that similar alterations would be expected in other physiological or pathological conditions associated with elevated ketone body production.

5.3 The effect of acetone, 3-hydroxybutyrate and 1,3 butanediol on hepatic drug metabolism.

The studies investigating the effects of hyperketonaemia on drug metabolism clearly demonstrate that it is a major factor influencing hepatic drug metabolism mediated by the cytochrome P450-dependent mixed-function oxidases. Numerous studies have shown that acetone is one of the most potent inducers of cytochrome P450 IIE1 (Koop et al., 1985), and is elevated during uncontrolled type I diabetes in animals. The inducibility of cytochrome P450 IIE1 by acetone prompted the investigation of the possible catalytic activity of P450 IIE1 towards this substrate. Casazza et al., (1984) showed that this isoform was capable of hydroxylating acetone to acetol, a possible precursor for gluconeogenic enzymes. They proposed that this isoform was capable of redirecting acetone back into the gluconeogenic pathways rather than being excreted from the body. Acetone is produced by the spontaneous decarboxylation of acetoacetate produced in the liver from excess acetyl CoA. However, the liver also produces 3-hydroxybutyrate in equimolar concentrations with acetoacetate yet, in our studies of both spontaneous and chemically-induced diabetes, the plasma acetoacetate concentration does not appear to be as elevated as the 3-hydroxybutyrate concentration. This raises the question as to whether acetoacetate spontaneously decarboxylates at a rate significantly high enough to account
for the discrepancy or that acetoacetate is being actively removed from the plasma. Furthermore, our previous studies with dietary-induced hyperketonaemia suggested that ketone bodies may be the inducing agents for the cytochrome P450 IVA subfamily. However during prolonged streptozotocin-induced diabetes, the duration of induction of this isoform was limited even though the plasma ketone body concentration was still elevated.

To investigate the effect of individual ketone bodies on the induction of cytochrome P450, animals were treated with either acetone, 3-hydroxybutyrate or 1,3-butanediol, which is metabolised to yield all three ketone bodies (Mehlman et al., 1975).

**Animal pretreatment**

Sixteen male Wistar albino rats (150-200g) were obtained from the Experimental Biology Unit, University of Surrey and divided into four groups. One group served as control and received 2ml of water daily by gavage. Of the other groups; one group received acetone (15mmol/kg); one group 3-hydroxybutyrate, sodium salt (15mmol/kg) and the last group 1,3 butanediol (15mmol/kg). All doses were given in a final volume of 2ml of water and administered daily by gavage for three days. All animals were killed 24 hours after the final dose and terminal blood samples obtained. The livers were immediately excised and microsomal fractions prepared as previously described. Mixed-function oxidase activities were determined and immunological detection of specific cytochrome P450 proteins performed as previously described (Chapter 2).

**Results**

All animals were normoglycaemic and the levels of plasma 3-hydroxybutyrate were slightly elevated in the animals treated with 3-hydroxybutyrate but significantly elevated (p<0.01) in those treated with 1,3-butanediol (Table 5.3.1). There were no significant alterations of the plasma acetoacetate concentrations in any of the treated
animals. None of the treatment groups showed any significant difference in microsomal protein, total carbon monoxide discernible cytochrome P450, cytochrome b$_5$, or the NADPH dependent reduction of cytochrome c compared to control levels (Table 5.3.2).

**Table 5.3.1** Plasma glucose and ketone body concentrations in acetone, 3-hydroxybutyrate and 1,3-butanediol treated animals.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Acetoacetate (mM)</th>
<th>3-Hydroxybutyrate (mM)</th>
<th>Glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.26±0.01</td>
<td>0.27±0.02</td>
<td>5.8±0.15</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.30±0.03</td>
<td>0.24±0.003</td>
<td>5.9±0.20</td>
</tr>
<tr>
<td>3-hydroxybutyrate</td>
<td>0.16±0.04</td>
<td>0.34±0.05</td>
<td>5.6±0.4</td>
</tr>
<tr>
<td>1,3-butanediol</td>
<td>0.23±0.05</td>
<td>0.47±0.02***</td>
<td>6.1±0.2</td>
</tr>
</tbody>
</table>

Values represent mean±s.e.m. for five animals. ***p<0.001 compared to control

**Table 5.3.2** Some hepatic parameters in rats treated with various ketones.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Protein (mg/g liver)</td>
<td>28.7±1.12</td>
</tr>
<tr>
<td>Cytochrome P450 (nmol/mg Protein)</td>
<td>0.43±0.02</td>
</tr>
<tr>
<td>Cytochrome b$_5$ (nmol /mg protein)</td>
<td>0.73±0.05</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase (nmol/min/mg protein)</td>
<td>16.8±2.2</td>
</tr>
</tbody>
</table>

Values represent mean±s.e.m. for five animals
Similarly there was no treatment dependent alteration in the hydroxylation of lauric acid or in the N-demethylation of ethylmorphine. However, acetone treatment resulted in a significant increase in the deethylation and depentylation of ethoxy- and pentoxyresorufin respectively. These increases were also observable with 3-hydroxybutyrate treatment, although the increases were not significant. Treatment with 1,3 butanediol treatment did not cause any alteration of activity. The hydroxylation of p-nitrophenol was significantly induced by acetone and 1,3 butanediol treatment but not by 3-hydroxybutyrate treatment (Table 5.3.3). Immunological detection confirmed that the increases in the various cytochrome P450 activities were accompanied by increases in the levels of specific cytochrome P450 apoproteins (Figure 5.3.1).

**Table 5.3.3** Cytochrome P450 catalysed activities in the liver of rats treated with various ketones.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethoxyresorufin-O deethylase (pmol/min/mg protein)</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>9.7±2.0</td>
</tr>
<tr>
<td>Pentoxyresorufin-O depentylase (pmol/min/mg protein)</td>
<td>2.5±0.4</td>
</tr>
<tr>
<td>p-Nitrophenol hydroxylase (nmol/min/mg protein)</td>
<td>0.9±0.15</td>
</tr>
<tr>
<td>Lauric acid hydroxylase (nmol/min/mg protein)</td>
<td>0.76±0.08</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase (nmol/min/mg protein)</td>
<td>16.0±1.4</td>
</tr>
</tbody>
</table>

Values represent mean±s.e.m for five animals. *p<0.05; **p<0.01; ***p<0.001 compared to control.
Figure 5.3.1 Immunoblot analysis of microsomes from acetone, 3-hydroxybutyrate and 1,3-butanediol treated rats

LANE

FIGURE   a   b   c   d   e
A P450IA  A   H   1,3B  3MC  C
B P450IIIE A   ISON  H   1,3B  C
C P450IVA  C   1,3B  CLO  A   H

C = Control rat
A = Acetone
H = 3-hydroxybutyrate
1,3B = 1,3-butanediol

Microsomal proteins were resolved by electrophoresis in a 10% (w/v) SDS-polyacrylamide gel and transferred to nitocellulose. In all cases 20μg of solubilised microsomal protein was loaded except for the positive controls when only 10μg of protein was loaded.

Positive controls
Animal pre-treatment

3-methylcholanthrene (25mg/kg in corn oil) for 3 days.
Isoniazid (0.1% (w/v) in drinking water) for 7 days
Clofibrate (80mg/kg in saline) for 3 days
Discussion

Hyperketonaemia has previously been shown to cause similar alterations in the cytochrome P450 mixed-function oxidase system as observed in uncontrolled type I diabetes mellitus of both spontaneous and chemically-induced origin (Barnett et al., 1988). The role of specific ketones in the modulation of this system has not been fully investigated, although many studies have shown that acetone is a potent inducer of the cytochrome P450 IIE1 protein (Koop et al., 1985).

In agreement with the above studies, acetone was shown to be capable of inducing the cytochrome P450 IIE1 protein but our data also demonstrates that the molecule is capable of inducing both the P450 IA2 protein and the P450 IIB protein. However, acetone did not affect the activities of the P450 IIIA or the P450 IVA subfamilies. Similarly, the treatment of animals with 3-hydroxybutyrate caused a more modest increase in the activity of the P450 IA and IIB subfamilies as determined by both enzyme analysis and immunological techniques. In contrast to acetone, the induction of the cytochrome P450IIE subfamily by this molecule was minimal, although a high degree of inter-animal variation was apparent. In common with acetone, 3-hydroxybutyrate had no effect on the levels of the cytochrome P450 IIIA and IVA subfamilies.

The inclusion 1,3-butanediol in this study provided a compound which has been shown to be metabolised to acetoacetate and 3-hydroxybutyrate (Mehlman et al., 1975). However, this compound did not alter the levels of the P450 IA, IIB, IIIA or IVA subfamilies but did cause an elevation of the P450 IIE subfamily. The reason for this remains unclear at the present time.

All three treatment regimes failed to affect the activities of the cytochrome P450 IIIA subfamily. This is not necessarily an unexpected result when the chemical structures of
typical inducers of this subfamily are considered. In the case of P450 IIIA inducers, most are large molecules such as the macrolide antibiotics and the immunosuppressant drug cyclosporin and therefore these small molecules would not be expected to cause induction of this subfamily. These results do however provide further evidence to support our previous suggestions that the induction of the P450 IIIA subfamily is due to other changes associated with the disease state and not by ketosis.

The effect of hyperketonaemia induced by the administration of medium chain triacylglycerols on the P450 IVA subfamily suggested that circulating ketone bodies may be responsible for the induction. The current study indicates that this is not the case and that other factors in diabetes mellitus may be responsible. Many studies have already shown that diabetes mellitus is associated with hyperlipidaemia (Dunn, 1990) an effect that would have occurred when animals were treated with medium chain triacylglycerols. When considering our previous study of prolonged uncontrolled diabetes in the streptozotocin-diabetic animal (Chapter 3) we showed that as the duration of disease progressed the levels of the P450 IVA protein began to decrease to levels similar to those of control animals. These observations suggest that it may be the increased levels of circulating fatty acids, associated with acute uncontrolled type I diabetes, that are responsible for the induction of this isoform of cytochrome P450.

In agreement with previous studies, acetone is a potent inducer of cytochrome P450 IIE1 (Ryan et al., 1986). Previous investigations have shown a correlation between the levels of plasma 3-hydroxybutyrate and the levels of cytochrome P450IIE catalysed reactions (Bellward et al., 1988). However in our study 3-hydroxybutyrate did not affect the activity of P450 IIE1. Uncontrolled diabetes mellitus is associated with a severe ketosis which can show marked inter-animal variation in severity. It is possible that a correlation between the levels of cytochrome P450IIE1 and plasma 3-hydroxybutyrate concentration does exist but that this is not the major inducing species for P450 IIE1.
Casazza et al., (1984) have already shown that cytochrome P450 IIE1 is capable of hydroxylating acetone to acetal, presumably the first step in shunting acetone back into the gluconeogenic pathways. Our results would tend to suggest that the levels of acetone in the plasma are more important than those of 3-hydroxybutyrate in terms of inducing potential. In our previous studies it was observed that the levels of 3-hydroxybutyrate in the plasma are usually several fold higher than those of acetoacetate. The one compound not investigated in this study was acetoacetate due to problems obtaining sufficient quantities in the form of a non-toxic salt.

Finally, this study suggests that the various ketone bodies have differing effects on the cytochrome P450 families, with acetone being the most effective inducer of the P450 IA, IIB and IIE subfamilies and it will be necessary to investigate the role of acetoacetate in the modulation of cytochrome P450 families in view of our present findings. It has been suggested that as 3-hydroxybutyrate caused very modest changes in the parameters examined it may be due to the fact that this compound is not readily absorbed. However, when Mehlman et al., (1975) examined the effects of oral administration of 3-hydroxybutyrate to animals they found significant elevations in the plasma levels of this compound. As our data for plasma levels represents terminal samples, ie 24 hours after the last administration, it is probable that the levels of this ketone body had returned to within normal plasma ranges.
Chapter 6

The effect of other insulin-aberrant conditions on hepatic drug metabolism
6.1 The effect of type II diabetes mellitus on hepatic drug metabolism

Although type I diabetes is the most severe form of diabetes mellitus, maturity onset or type II diabetes represents the most common form of the disease. It is characterised by a relative lack of insulin due to a resistance of the target cells to insulin combined with faulty secretion of insulin by the β-cells of the pancreas. This type of diabetes is associated with hyperglycaemia but rarely with ketosis. The condition is usually effectively controlled by diet and drug therapy. Complications associated with type II diabetes include hypertension and obesity and the disease can represent a mild hyperinsulinaemic condition as insulin resistance can result in increased secretion by the β-cells. In view of the dramatic changes in the hepatic cytochrome P450-dependent mixed-function oxidase system observed with type I diabetes, the investigation of the effects of type II diabetes on hepatic drug metabolism is necessary to complete our investigations into the effects of diabetes mellitus on drug metabolism. Previous studies by Rouer and Leroux (1981) have shown that spontaneously hyperglycaemic mice show some slight quantitative differences in their mixed-function oxidase activities although only limited investigations were performed.

Animal pretreatment

Six male spontaneously obese hyperglycaemic (ob/ob) mice and six normal lean littermates, 16 weeks of age, were obtained from Aston university. After an initial period of acclimatisation the animals were killed by cervical dislocation. The livers were immediately excised and the gall bladder removed before microsomal fractions were prepared and mixed-function oxidase activities determined (Chapter 2).
Results

The obese mice were hyperglycaemic but not hyperketonaemic. The hyperglycaemia was not as marked as seen in the insulin-dependent diabetic animals (Table 6.1.1). The obese animals did not show any alteration of microsomal protein, cytochrome b₅, total cytochrome P450 or the NADPH-dependent reduction of cytochrome c (Table 6.1.2).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Lean</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mM)</td>
<td>5.6±0.2</td>
<td>11.2±1.4**</td>
</tr>
<tr>
<td>Acetoacetate + 3-hydroxybutyrate (mM)</td>
<td>0.40±0.01</td>
<td>0.38±0.1</td>
</tr>
</tbody>
</table>

Data represents mean±s.e.m. for six animals. ** p<0.01 compared to control

Determination of specific cytochrome P450 activities did not reveal any significant differences from the activities encountered in the lean animals (Table 6.1.3). However, investigation of enzymes involved in phase II conjugation reactions revealed that the obese mice did have significantly lower glutathione S-transferase activity and levels of total
glutathione (Table 6.1.3). This was coupled with a slight elevation in the ability to reduce oxidised glutathione.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>Lean</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/g liver)</td>
<td>26.0±0.9</td>
<td>28.1±1.2</td>
</tr>
<tr>
<td>Cytochrome P450 (nmol / mg protein)</td>
<td>0.21±0.05</td>
<td>0.19±0.01</td>
</tr>
<tr>
<td>Cytochrome b5 (nmol / mg protein)</td>
<td>0.45±0.1</td>
<td>0.51±0.1</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase (nmol/min/mg protein)</td>
<td>5.5±0.7</td>
<td>7.1±2.0</td>
</tr>
</tbody>
</table>

Data represents mean±s.e.m. for six animals

Discussion

Type II diabetes is associated with a moderate hyperglycaemia which is effectively controlled by a dietary regime supplemented in some cases with oral hypoglycaemic drug therapy. The obese mice in this study were not severely hyperglycaemic and were not ketotic. Previous studies undertaken by Rouer and Leroux (1981) have shown that the effect of type II diabetes on the hepatic mixed-function oxidase system is minimal. In the present study we have investigated the effects of this form of diabetes on a more extensive range of cytochrome P450 activities. This confirmed that type II diabetes does not appear to influence the mixed-function oxidase system to any great
extent. It is possible that the absence of ketosis in these animals may explain the lack of induction of specific isoforms of cytochrome P450, whose induction, in some cases has already been correlated with circulating levels of ketone bodies (Bellward et al., 1988).

Table 6.1.3 The effects of type II diabetes on hepatic cytochrome P450 activities, glutathione S-transferase, glutathione reductase and cellular glutathione levels

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>GROUP</th>
<th>Lean</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethoxyresorufin O-deethylase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pmol resorufin / min/ mg protein)</td>
<td></td>
<td>9.9±2.1</td>
<td>8.6±2.8</td>
</tr>
<tr>
<td>Pentoxyresorufin O-depentylase</td>
<td></td>
<td>0.4±0.2</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td>(pmol resorufin/ min/ mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Nitrophenol hydroxylase</td>
<td></td>
<td>0.8±0.07</td>
<td>0.91±2.4</td>
</tr>
<tr>
<td>(nmol/min/ mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td></td>
<td>14.0±1.1</td>
<td>15.1±2.4</td>
</tr>
<tr>
<td>(nmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lauric acid hydroxylase</td>
<td></td>
<td>1.84±0.7</td>
<td>2.31±0.6</td>
</tr>
<tr>
<td>(nmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total glutathione (mM)</td>
<td></td>
<td>7.8±0.5</td>
<td>4.8±0.7**</td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td></td>
<td>1.48±0.06</td>
<td>0.34±0.06***</td>
</tr>
<tr>
<td>(µmol/ min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td></td>
<td>644±24</td>
<td>782±72</td>
</tr>
<tr>
<td>(nmol GSSG/ min/ mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represent mean±s.e.m. for six animals. **p<0.01; ***p<0.001 compared to lean mice.

Grant and Duthie (1987) investigated the effects of streptozotocin-induced diabetes in the rat on the activities of conjugating enzymes in isolated rat hepatocytes.
Their studies revealed that chemically-induced diabetes caused a decrease in the cellular levels of reduced glutathione but that the resynthesis of glutathione was faster in the diabetic animals. They also showed that the conjugation of 1-chloro-2,4-dinitrobenzene and 3,4-dichloronitrobenzene, two substrates for glutathione S-transferases, was deficient in the diabetic animals. This indicates that these animals may be more susceptible to xenobiotic induced toxicity. In the present study similar findings have been found with the obese mice.

In summary, it would appear that type II diabetes mellitus has little influence on hepatic drug metabolism although the alterations in the glutathione conjugating species and enzymes are of interest and merit further investigation.

6.2 The effect of hypertension on hepatic drug metabolism

It has been recognised for many years that hypertension and diabetes (or impaired glucose tolerance) frequently occur together. However, the pathogenetic relationship between the two common disorders remains elusive. The time course and natural history of hypertension differs markedly between patients with type I diabetes mellitus and those suffering from the type II form of the disease. In the former blood pressure is normal at the onset of diabetes and frequently remains normal during the first 5 - 10 years of diabetes. Hypertension typically develops with the onset of renal insufficiency. In patients who had type I diabetes for more than 30 years, approximately 50% will have hypertension.
(Christleib et al., 1981). This group is almost entirely represented by patients that have developed diabetic nephropathy.

In contrast to type I diabetes, those with type II diabetes are often hypertensive at the time of diagnosis of the disease. The increase in blood pressure is correlated with the increased prevalence of obesity and the greater age associated with sufferers (approximately 60% of this population is over 60 years old).

In both type I and type II diabetes, the impact of hypertension on excess morbidity and mortality is substantial. It is well known that diabetes is associated with an increased risk of death from renal failure, coronary artery disease and cerebrovascular disease. When hypertension is superimposed, the risk from all causes may be exacerbated by as much as four fold (Dupree and Meyer, 1976).

Our previous studies have examined the direct effects of the diabetic state on hepatic drug metabolism and established unequivocally that insulin-dependent diabetes can markedly alter the activities of various cytochrome P450 isoforms. As hypertension is probably the most common complication of diabetes mellitus it was of interest to investigate whether hypertension could itself directly influence the levels of hepatic cytochrome P450 isoforms.

Animal pretreatment

Five male WKY non-hypertensive and five male SHR hypertensive rats were obtained from Charles River Laboratories, England at nine weeks of age. After two weeks of acclimatisation and to allow the hypertension to develop (usually at nine weeks of age)
the animals were killed. The livers were immediately excised, microsomal preparations prepared and mixed-function oxidase activities determined as previously described (Chapter 2). The effect of hypertension on the phase II enzyme, glutathione transferase was investigated with two substrates; 1-chloro-2,4-dinitrobenzene and 3,4-dichloronitrobenzene. All assays and conditions employed are described in detail in Chapter 2. Immunological detection of specific cytochrome P450 proteins employing monospecific antibodies were used to confirm enzyme results where appropriate.

Results

The hypertensive animals had normal appearance, and no adverse effects due to the condition were evident. The hypertensive condition did not influence microsomal protein, cytochrome P450 or the NADPH-dependent reduction of cytochrome c. However, the hypertensive animals did show a significant decrease in the levels of cytochrome b5 (Table 6.2.1).

With respect to the diagnostic substrates employed to investigate specific cytochrome P450 activities, the hypertensive animals did show a slight, but non-significant decrease in the ability to hydroxylate lauric acid. There was no alteration of the activity towards the dealkylation of pentoxyresorufin or the hydroxylation of p-nitrophenol. The hypertensive animals did however show a significant increase in the deethylation of ethoxyresorufin indicative of alteration of the activities of the P450 IA subfamily (Table 6.2.2).
Table 6.2.1  The effect of hypertension on some hepatic microsomal parameters in spontaneously hypertensive rats.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Control</th>
<th>Hypertensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal protein (mg / g liver)</td>
<td>26.4±0.46</td>
<td>26.6±0.5</td>
</tr>
<tr>
<td>Cytochrome b$_5$ (nmol / mg protein)</td>
<td>0.92±0.04</td>
<td>0.59±0.07**</td>
</tr>
<tr>
<td>Cytochrome P450 (nmol / mg protein)</td>
<td>0.39±0.04</td>
<td>0.34±0.02</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase activity (nmol / min / mg protein)</td>
<td>25.6±2.4</td>
<td>25.8±3.4</td>
</tr>
</tbody>
</table>

Data represent mean+s.e.m. for five animals. **p<0.01 compared to control

Previous studies have reported that the hypertensive state was associated with the induction of the P450 IIIA proteins (Schenkman et al., 1989) although the activity of the hypertensive microsomes towards ethylmorphine, a substrate of this isoform, showed no difference from control levels.

Discussion

Spontaneous hypertensive rats (SHR) have previously been shown to have shorter hexobarbital sleeping times and enhanced rates of microsomal ethylmorphine N-
demethylase activities (Greenspan and Baron, 1981; Merrick et al., 1976). Such effects are indicative of alteration of the microsomal mixed-function oxidase activity. However, our data shows that at 10 weeks of age the SHR animals did not display an increase in the activity of ethylmorphine N-demethylase, an activity specifically associated with the cytochrome P450 IIIA subfamily. It is possible that the induction of this isoform takes longer to manifest itself and would not be apparent at onset of hypertension, since in their studies Schenkman et al. (1989), used animals which had been hypertensive for many weeks.

Table 6.2.2  The effects of hypertension on specific cytochrome P450-catalysed reactions in spontaneously hypertensive rats.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>Control</th>
<th>Hypertensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethoxyresorufin-O-deethylase</td>
<td>12.9±1.4</td>
<td>22.6±2.0**</td>
</tr>
<tr>
<td>(pmol/min/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentoxynresorufin-O-depentylase</td>
<td>5.3±1.3</td>
<td>5.13±0.8</td>
</tr>
<tr>
<td>(pmol/min/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Nitrophenol hydroxylase</td>
<td>1.91±0.1</td>
<td>1.98±0.2</td>
</tr>
<tr>
<td>(nmol/min/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lauric acid hydroxylase</td>
<td>10.0±0.5</td>
<td>7.91±0.7</td>
</tr>
<tr>
<td>(nmol/min/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>27.7±0.8</td>
<td>27.3±1.0</td>
</tr>
<tr>
<td>(nmol/min/mg protein)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represents mean±s.e.m. for five animals  ** p<0.01 compared to control

149
Schenkman et al., (1989) have shown that there was essentially no observable difference in the activity of aniline hydroxylase a reaction predominantly catalysed by cytochrome P450IIE1 which is supported by our data using the more specific substrate p-nitrophenol. Similarly, no alteration was observed in the activity of lauric acid hydroxylation or the depentylation of pentoxyresorufin (cytochrome P450 IVA and P450IIB subfamilies respectively).

Of possibly greater interest is the fact that hypertension was associated with a modest elevation in cytochrome P450IA activity. This subfamily has already been associated with the bioactivation of numerous compounds (Ioannides and Parke, 1985) and is also induced during uncontrolled type I diabetes mellitus. It is not clear from the present study whether one or both proteins in this subfamily were increased.

In conclusion, the effect of short-term hypertension on hepatic drug metabolism appears to be a modest, but selective induction of the P450IA subfamily at the onset of the disease may be followed by further alterations in other cytochrome P450 families as the condition progresses. Clearly measurements of blood pressure in the SHR animals would have been advantageous in determining the severity and onset of hypertension.

6.3 The modulation of hepatic mixed-function oxidase activity by hyperinsulinaemia

Hypoinsulinaemia induced by the administration of diabetogenic compounds such as streptozotocin in rats has been associated with marked alterations of the hepatic
As an absolute lack of circulating insulin is associated with such marked alterations of hepatic cytochrome P450 levels it was of interest to investigate the effect of hyperinsulinaemia on the cytochrome P450 system. Enteropancreatic endocrine cancer is associated with hyperinsulinaemia, due to excessive secretion of insulin by an islet cell tumour. Hyperinsulinaemia occurs as insulin secretion is not regulated by circulating nutrient levels (Friesen, 1982). The model of hyperinsulinaemia used was the serially transplantable radiation-induced rat insulinoma (Chick et al., 1977). When transplanted into NEDH rats, the tumour is characterised by rapid growth and the insulinoma-bearing rats display marked hyperinsulinaemia, severe hypoglycaemia and neuroglycopenic comas within one month following transplantation (Flatt et al., 1986). The metabolic effects consequent to transplantation of the tumour are similar to those associated with spontaneous insulinoma in man (Friesen, 1982), rendering these rats an appropriate model (Flatt et al., 1986, 1987).
Animal pretreatment

Male Wistar albino (150-200g) and New England Deaconess Hospital (NEDH, 150-180g) rats were obtained from the Experimental Biology Unit, University of Surrey. The NEDH rats were transplanted with radiation-induced tumour from a donor male NEDH rat. The tumour and surrounding capsule were rapidly removed from the donor and the minced tumour (0.15ml/rat) was implanted subcutaneously into the subscapular region of lightly anaesthetised rats using a 16 gauge needle. Plasma glucose levels were determined following withdrawal of blood samples from the tail tip of conscious rats. The animals were sacrificed when they displayed severe hypoglycaemia (plasma glucose levels approximately 2mM). In a second study the Wistar albino rats received daily subcutaneous administration of insulin (Novo Industries, long acting monocomponent human insulin, 40 I.U./ml) at doses of 10 I.U./kg or 20 I.U./kg for four days and were killed 24 hours after the last administration.

In both studies livers were immediately excised and hepatic microsomal fractions prepared as previously described (Chapter 2). Microsomal protein, total carbon-monoxide discernible cytochrome P450 and cytochrome b5 were determined as well as the NADPH dependent reduction of cytochrome c. The activity of cytochrome P450 IA and IIB proteins towards ethoxy- and pentoxyresorufin, respectively were investigated and in addition the hydroxylations of p-nitrophenol and lauric acid (catalysed predominantly by the P450 IIE and P450 IVA subfamilies respectively). The N-demethylation of ethylmorphine was employed to monitor the activity of the P450 IIIA subfamily (Chapter 2). Immunological detection of specific cytochrome P450 proteins employing monospecific antibodies was used to confirm results were appropriate.
The metabolic activation of Glu-P-1 to mutagens was monitored using the Ames mutagenicity test (Chapter 2). The activation system contained 10% (v/v) of microsomal preparation (25% w/v) and was supplemented with glucose-6-phosphate dehydrogenase (1 unit/plate). Glu-P-1 (2μg/plate) was dissolved in dimethylsulphoxide. Activation system, bacteria (Salmonella typhimurium strain TA 98) and Glu-P-1 were pre-incubated for 1 hour at 37°C in a shaking water-bath.

Results

As expected the insulinoma bearing rats exhibited markedly lower glucose levels when compared to control rats, but no significant differences were observed in the plasma ketone body levels (Table 6.3.1A).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Animal group</th>
<th>Glucose (mM)</th>
<th>3-hydroxybutyrate (mM)</th>
<th>Acetoacetate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>6.1±0.3</td>
<td>0.36±0.05</td>
<td>0.14±0.04</td>
</tr>
<tr>
<td></td>
<td>Insulinoma</td>
<td>2.8±0.4*</td>
<td>0.42±0.02</td>
<td>0.21±0.04</td>
</tr>
<tr>
<td>B</td>
<td>Control</td>
<td>5.1±0.2</td>
<td>0.31±0.02</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td></td>
<td>Insulin (10 IU/kg)</td>
<td>4.6±0.2</td>
<td>0.32±0.01</td>
<td>0.12±0.04</td>
</tr>
<tr>
<td></td>
<td>Insulin (20 IU/kg)</td>
<td>4.5±0.3</td>
<td>0.16±0.04</td>
<td>0.09±0.01</td>
</tr>
</tbody>
</table>

Data represents mean ±s.e.m. for five animals. *p<0.01 compared to control.

The hepatic O-deethylation of ethoxyresorufin and the N-demethylation of ethylmorphine were significantly higher in the insulinoma-bearing rats whereas, in
contrast, p-nitrophenol oxidation and lauric acid hydroxylation were significantly lower when compared to controls (Table 6.3.2). Hepatic microsomal preparations from insulinoma-bearing rats were markedly more efficient than preparations obtained from control animals at converting Glu-P-1 to mutagenic intermediates in the Ames test (Table 6.3.3A).

Immunoblot analysis employing antibodies to the P450 I family revealed that the insulinoma-bearing animals had higher levels of the P450 IA2 apoprotein (Figure 6.3.1A).

| Table 6.3.2 Hepatic microsomal mixed-function oxidase activity in insulinoma-bearing rats. |
|---------------------------------|---------------------------------|
| PARAMETER                      | Control                      | Insulinoma-bearing          |
| Ethoxyresorufin O-deethylase (pmol/min/mg protein) | 6.6±0.6                | 19.1±2.9**                 |
| Pentoxyresorufin O-depentylase (pmol/min/mg protein) | 3.4±0.9                | 4.2±0.4                 |
| p-Nitrophenol hydroxylase (nmol/min/mg protein) | 1.46±0.09             | 0.93±0.03***               |
| Ethylmorphine N-demethylase (nmol/min/mg protein) | 2.2±0.3                | 4.6±0.4**                 |
| Lauric acid hydroxylase (nmol/min/mg protein) | 2.1±0.2                | 1.4±0.2*                  |
| Cytochrome b5 (nmol/mg protein) | 0.38±0.03             | 0.38±0.02                |
| Cytochrome P450 (nmol/mg protein) | 0.38±0.03             | 0.38±0.02                |
| Microsomal protein (mg /g liver) | 27.3±0.5              | 28.2±1.6                |

Data represents mean±s.e.m for five animals  *p<0.05; **p<0.01; ***p<0.001
compared to control.

Table 6.3.3  Bioactivation of Glu-P-1 to mutagens by microsomal preparations from insulin-treated and insulinoma-bearing rats.

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Mutagenic response (Histidine revertants/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Control</td>
<td>477±79</td>
</tr>
<tr>
<td>Insulinoma-bearing</td>
<td>488±204</td>
</tr>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td>Control</td>
<td>2305±147</td>
</tr>
<tr>
<td>Insulin (10 IU/kg)</td>
<td>3905±237</td>
</tr>
<tr>
<td>Insulin (20 IU/kg)</td>
<td>5967±272</td>
</tr>
</tbody>
</table>

Data represent mean±s.e.m. for five animals. Spontaneous reversion rate of 45±10 histidine revertants/plate has already been subtracted. All data is significant (p<0.001) compared to control.

Rats pretreated with insulin, at two dose levels, had slightly lower levels of plasma glucose but this was not significant (Table 6.3.1B). Of the mixed-function oxidase activities studied only the O-deethylation of ethoxyresorufin was higher in the hormone-receiving animals (Table 6.3.4). Moreover, insulin administration gave rise to a dose-dependent increase in the bioactivation of Glu-P-1 to mutagens in the Ames test (Table 6.3.3B). A dose-dependent increase following insulin administration was also seen in the P450 IA2 microsomal apoprotein level (Figure 6.3.1B).
Table 6.3.4  The effect of insulin administration on hepatic microsomal cytochrome P450 catalysed reactions.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>Control</th>
<th>Insulin (10 I.U./kg)</th>
<th>Insulin (20 I.U./kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethoxyresorufin O-deethylase</td>
<td>13±2</td>
<td>19.1±1**</td>
<td>20±3*</td>
</tr>
<tr>
<td>(pmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Nitrophenol hydroxylase</td>
<td>1.2±0.1</td>
<td>0.9±0.1</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>(nmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>8.2±1.4</td>
<td>10.3±2.1</td>
<td>11.1±1.8</td>
</tr>
<tr>
<td>(nmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>0.40±0.05</td>
<td>0.51±0.01</td>
<td>0.46±0.04</td>
</tr>
<tr>
<td>(nmol/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>35.0±3.6</td>
<td>34.5±2.0</td>
<td>39.0±2.0</td>
</tr>
<tr>
<td>(mg /g liver)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represents mean±s.e.m for five animals  *p<0.05; **p<0.01 compared to control.

Discussion

The insulinoma-bearing animals displayed a significantly higher ethoxyresorufin O-deethylase activity, a reaction catalysed exclusively by the P450 IA subfamily and in particular the P450 IA1 (Phillipson et al., 1984). In order to discern between the two P450 IA isoforms, the ability of hepatic microsomal preparations from insulinoma-bearing and control rats to convert the pre-mutagen Glu-P-1 to active mutagens
Figure 6.3.1 Immunoblot analysis of microsomes from insulinoma-bearing and insulin-treated rats using polyclonal antibodies to cytochrome P450

**FIGURE**

<table>
<thead>
<tr>
<th>A P450IA</th>
<th>Lane</th>
<th>Control rat microsomes</th>
<th>4</th>
<th>3-Methylcholanthrene-induced rat microsomes</th>
<th>5,6,7</th>
<th>Insulinoma-bearing rat microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>B P450IA</td>
<td>Lane</td>
<td>Control rat microsomes</td>
<td>4</td>
<td>3-Methylcholanthrene-induced microsomes</td>
<td>5,6,7</td>
<td>10 I.U/kg insulin treated rats</td>
</tr>
</tbody>
</table>

Microsomal proteins were resolved by electrophoresis in a 10% (w/v) SDS-polyacrylamide gel and transferred to nitocellulose. In all cases 20μg of solubilised microsomal protein was loaded except for the positive controls when only 10μg of protein was loaded.

**Positive controls**

Animal pre-treatment

3-methylcholanthrene (25mg/kg in corn oil) for 3 days.
in the Ames test was examined as this reaction is catalysed exclusively by the P450IA2 protein (Kato, 1986). Activation of Glu-P-1 to mutagens was markedly more efficient in microsomal preparations derived from the insulinoma-bearing animals when compared to controls, indicating that the rise in the O-deethylation of ethoxyresorufin may have been due to the increase in the A2 protein. Immunoblot analysis confirmed this finding in that microsomes from the insulinoma-bearing animals had higher levels of the P450 IA2 apoprotein than the control animals and that the P450 IA2 protein was induced rather than the P450 IA1 protein.

Insulinoma-bearing rats also exhibited significantly higher levels of ethylmorphine N-demethylase activity compared to the control animals, an enzyme closely associated with the P450 IIA subfamily (Wrighton et al., 1985a). In contrast to the above findings hyperinsulinaemia reduced microsomal p-nitrophenol and lauric acid hydroxylations, two activities associated with the P450 IIE and IVA subfamilies. The O-dealkylation of pentoxyresorufin, a reaction used to monitor P450 IIB activity (Lubet et al., 1985) was unaffected by the hyperinsulinaemic condition. Since the levels of total cytochrome P450 were unchanged by treatment it may be inferred that the observed increases in P450 IA2 and IIA levels may occur at the expense of other cytochrome P450 subfamilies including the P450 IIE and IVA. It has already been established that some agents may selectively induce the levels of one family of cytochrome P450 while decreasing others (Favreau and Schenkman, 1988a). Finally, the NADPH-dependent reduction of cytochrome c was unaffected by hyperinsulinaemia indicating that the flow of electrons to cytochrome P450 is unlikely to be impaired.

It is logical to argue that the observed changes in hepatic cytochrome P450 proteins in the insulinoma bearing animals may not be the result of the hyperinsulinaemia and the ensuing hypoglycaemia, but is the consequence of the physiological changes brought about by the presence of an invasive tumour. In order to address this point and to evaluate the
contribution of hypoglycaemia, Wistar albino rats were treated with insulin in order to ensure hyperinsulinaemia is the causative factor in these changes and that the effects are not limited to the NEDH strain of rat. A slight drop in the plasma glucose levels following insulin administration was noted but the effect was not statistically significant indicating that hypoglycaemia was not responsible for the changes observed. A modest but statistical increase was observed in the O-deethylation of ethoxyresorufin. The increased ability of hepatic microsomes from insulin-treated rats in activating Glu-P-1 to mutagens indicates that the enhanced O-deethylation of ethoxyresorufin is due to the P450 IA2 protein and this is further substantiated by the immunoblot analysis. Although a modest increase was also seen in the N-demethylation of ethylmorphine the effect did not reach statistical significance. Thus, in general, the effects induced by insulin administration resemble those brought about by the subscapular transplantation of the insulinoma but the effects are much less pronounced. This is not surprising since in the insulinoma-bearing rats, not only is the degree of hyperinsulinaemia much higher, as exemplified by the severe hypoglycaemia, but also the hyperinsulinaemia and hypoglycaemia are maintained by continuous tumour secretion (Flatt et al., 1986) whereas, following daily subcutaneous administration of insulin, the plasma levels insulin would fluctuate widely as the excess plasma insulin in removed.

The effects of hyperinsulinaemia on drug metabolism, in contrast to hypoinsulinaemia, has not received much attention, and the hormonal and other biochemical changes that may occur have not been explored. In streptozotocin-induced hypoinsulinaemia, the modulation of cytochrome P450 changes have been attributed to the high levels of ketones (P450 I, IIB, IIE) that function as inducing agents, and to modulation of growth hormone levels that play a major role in the regulation of some cytochrome P450 proteins such as P450 III (Barnett et al., 1989, 1990a and b). In the present study no significant change was noted in the circulating levels of ketone bodies so that the first mechanism may be excluded. Moreover, hyperketonaemia causes a marked increase in the
microsomal levels of the P450 IIB proteins (Barnett et al., 1990a), which was not induced in the hyperinsulinaemic condition. It is possible that hyperinsulinaemia triggered the release of an endogenous planar substrate that can act as a selective inducer of P450 IA2 although other mechanism may also be operative. It should be noted that the physiological function of the P450 I family has yet to be established.

In summary, the present study demonstrates that hyperinsulinaemia causes a modest, but selective induction in the hepatic levels of P450 IA2, an enzyme closely associated with the activation of chemical carcinogens, particularly those containing an exocyclic amino group (Ioannides and Parke, 1990).

It has been suggested that the trauma caused by the injection of 0.15ml of minced tumour into the subscapular region of lightly anaesthetized animals may have caused the observed changes some 21 days post-treatment. Although, this was not examined by sham-injection of the control animals, previous work performed on plasma parameters, which are usually altered during stress, displayed no alteration other than those expected from the presence of an insulinoma (Flatt et al., 1986).
Chapter 7.

Discussion
7.1 Alteration of hepatic drug metabolism in streptozotocin-induced type I diabetes mellitus

It is now recognised that by far the most important microsomal system in the metabolism of xenobiotics is the cytochrome P450-dependent mixed-function oxidase system, an enzyme system characterised by broad substrate specificity. It achieves this by existing as a number of distinctly different families, each consisting of two or more proteins with differing substrate specificity. The diabetes-induced changes in the metabolism of endogenous and exogenous substrates reflects the alteration in the levels of the specific cytochrome P450 families.

The recognition that type I diabetes mellitus is associated with changes in cytochrome P450 proteins followed the studies of Past and Cook (1982) who isolated a diabetes-inducible form with high turnover towards aniline, the hydroxylation of which is consistently increased in type I diabetes mellitus. This isoform was later characterised as P450 IIE1, a protein induced in rats and rabbits by a variety of chemicals (Koop et al., 1985). Subsequent studies by ourselves and others have revealed that streptozotocin-induced diabetes causes alterations of many other cytochrome P450 families involved in the biotransformation of xenobiotics. The polycyclic aromatic hydrocarbon-inducible P450 I family is induced in both chemically-induced and spontaneous type I diabetic animals (Ioannides et al., 1988; Yamazoe et al., 1988; Barnett et al., 1990). The same workers also demonstrated increases in the apoprotein and activities of the phenobarbital-inducible P450 IIB subfamily. Increased levels and activities were also observed in the steroid inducible P450 III family (Barnett et al., 1990). Moreover, streptozotocin-induced diabetes has been shown to influence the hepatic levels of sex-specific constitutive forms of cytochrome P450 endowed with steroid hydroxylase activity (Favreau and Schenkman, 1988). Finally, streptozotocin-induced diabetes and spontaneous type I diabetes enhanced the levels of the cytochrome P450 IV family (Barnett et al., 1990).
In our studies of the streptozotocin-induced diabetic animal (Chapter 3), we found that the alterations of hepatic drug metabolism was due to the diabetic state itself and not a consequence of the administered diabetogen. Furthermore, insulin therapy of streptozotocin-diabetic animals has revealed that although insulin is capable of reversing the diabetes-induced changes in drug metabolism they do not always return to normal levels. This suggests that even strictly insulin-controlled diabetics may still be influenced by the disease as far as xenobiotic metabolism is concerned.

Sex-differences in the diabetes-induced changes in hepatic drug metabolism

Sex-differences in the diabetes-induced alterations have been observed in the streptozotocin-induced diabetic animal. Although the increase in aniline hydroxylase activity is not sex-specific, the demethylation of aminopyrine is depressed in the male but elevated in the female, and hexobarbital and aryl hydrocarbon hydroxylase activities decreased only in the male (Reinke et al., 1978; 1979; Warren et al., 1983).

In our investigations of sex-differences in streptozotocin-induced diabetes mellitus we found that when expressed per nmol of total cytochrome P450, the induction of cytochrome P450IA and IIB subfamilies did not show sex-specific differences. Of the other families investigated sex-dependent factors appeared to be involved in the level of induction of cytochrome P450 IIE, IIIA and IVA subfamilies. In all cases the control females had higher levels than the control males, but the diabetic males showed more pronounced induction of these isoforms. Clearly, sex-specific differences in the levels of various cytochrome P450 families may have pharmacological and toxicological implications for drug therapy regimes.
Long-term diabetes mellitus and hepatic drug metabolism

Most studies using either chemically-induced or spontaneously diabetic animals used the animals one month after the onset of diabetes and the long-term effects have been totally neglected. There is experimental evidence that the effect of chronic diabetes on xenobiotic metabolism may differ from that seen in the early stages of diabetes despite the fact that circulating glucose levels may be similar (Dixon et al., 1963; Skett and Joels, 1985). The degree of inhibition of the microsomal metabolism of diazepam, lignocaine and imipramine was much more pronounced in rats 3 days after streptozotocin administration than after 20 days, although hepatotoxic effects of the streptozotocin causing changes in metabolism cannot be ruled out at 3 days (Skett and Joels, 1985). In studies of chronic streptozotocin-induced diabetes, we investigated hepatic mixed-function oxidase activities at 4, 8 and 12 weeks following streptozotocin treatment of male rats. Our data shows that after 4 weeks the initial, severe hyperketonaemia began to subside, although still being significantly elevated at 12 weeks. This correlated with a decrease in the activities of the some of the cytochrome P450 families investigated. In particular, the induction of cytochrome P450 III became less apparent after 4 weeks and became comparable to control animals from week 8 onwards. The induction of the cytochrome P450 IVA subfamily also decreased and was only slightly elevated by 12 weeks following streptozotocin treatment. As streptozotocin-induced diabetes progressed the diabetic animals lost much of the fat stored in their adipose tissues. This would have led to a fall in the circulating levels of free fatty acids which, if responsible for the induction of the cytochrome P450 IVA1 activity, as previously suggested (Gibson et al., 1990), would result in a fall in the level of induction of P450 IVA1. These observations further support the role of hyperketonaemia and hyperlipidaemia in the alterations of hepatic drug metabolism.
7.2 Hepatic drug metabolism in the spontaneously diabetic BB rat

Of significant interest is the fact that the streptozotocin-induced diabetic animals can survive for more than 12 weeks without insulin. This suggests that either the animals do not develop diabetes as severely as genetically susceptible animals, which survive for only a few days without insulin, or that the streptozotocin-induced diabetic animals undergo a physiological adaptation in order to withstand the high levels of plasma ketone bodies.

Examination of uncontrolled diabetes in spontaneously diabetic BB rats at 1, 3 and 6 months after onset, revealed that when insulin therapy was withdrawn, the animals quickly became hyperglycaemic and hyperketonaemic and showed all of the characteristic changes in mixed-function oxidase activity as observed in the streptozotocin-induced diabetic animals. Furthermore, these changes were not age-dependent, with similar affects being evident at 1 month and 6 months following onset of diabetes. The severity of the ketonaemia in these animals was several fold higher than that observed in the streptozotocin-induced diabetic animals, and withdrawal of insulin therapy could only be tolerated for a few days before the animals succumbed to the life-threatening effects of the disease. In these animals the plasma levels of ketone bodies was always several fold higher than that observed in the streptozotocin-induced diabetic animal. However, this was not associated with a greater induction of cytochrome P450 IIE than that found in the chemically-induced animals. This finding raises the possibility that 3-hydroxybutyrate may be a poor inducer of cytochrome P450 IIE1, and that acetone, and possibly acetoacetate, are the physiologically important inducers of this isoform. This would support the role of cytochrome P450 IIE in the hydroxylation of acetone to aceto, and the possibility for a physiological role for cytochrome P450 IIE in the salvage of acetone for use as a substrate for gluconeogenesis (Casazza et al., 1984).
Alterations of hepatic drug metabolism in insulin treated spontaneously diabetic BB rats

Our investigations of poorly insulin controlled spontaneously diabetic BB rats demonstrated that modulation of the mixed-function oxidases does occur in these animals, albeit less pronounced than when the disease is uncontrolled. These findings suggest that even when strictly controlled by insulin therapy, human diabetic patients may display some of the alterations of drug metabolism associated with the uncontrolled condition. Clearly, these findings have pharmacological as well as toxicological implications when considering the multi-drug therapy used to treat the complications of this disease. However, one must always be cautious when evaluating the implications to humans from responses observed in animal models. Our studies, using both chemically-induced and spontaneously diabetic rats, suggests that the BB rat is a more relevant model for human diabetes, apart from the fact that it is spontaneously occurring. Long-term streptozotocin-diabetic rats began to display alterations of drug metabolism which were not observed in the long-term diabetic BB rats. Furthermore, the ability of the streptozotocin-induced diabetic animals to survive without insulin therapy for more than 12 weeks, raises questions as to whether this model is appropriate.

7.3 Diabetes and chemical toxicity

Since type I diabetes modulates the levels of various cytochrome P450 isoforms, it is inevitable that the response of the diabetic individual to the toxicity/carcinogenicity of chemicals will be altered. In studies using the Ames mutagenicity assay to study the activation of chemical carcinogens, hepatic preparations from both chemically-induced and spontaneously diabetic animals were more effective at activating carcinogens such as
nitrosamines and heterocyclic amines (Ioannides et al., 1988; Flatt et al., 1989; Barnett et al., 1990), but in contrast, preparations from diabetic animals were less efficient at activating 2-aminofluorene.

Many studies have shown that type I diabetic animals are more susceptible to the hepatotoxicity induced by haloalkanes such as carbon tetrachloride, chloroform and 1,1,2-trichloroethane and other hepatotoxins (Watkins et al., 1988; Hanasono et al., 1975). The increased susceptibility to carbon tetrachloride may be due to the higher levels of the cytochrome P450 IIE subfamily which is induced in diabetic animals and catalyses the generation of hepatotoxic trichloromethyl radical (Watkins et al., 1975).

The induction of the cytochrome P450 IIB subfamily by type I diabetes suggests that there will be alteration in the pharmacokinetics of many drugs administered to the diabetic, as this subfamily is associated with the deactivation of many therapeutic compounds. Similarly, alterations in the levels of the P450 III subfamily, which metabolises large molecular weight compounds including drugs such as the macrolide antibiotics and the immunosuppressant cyclosporin, could pose further complications.

### 7.4 Possible mechanisms of the diabetes-induced changes in the metabolism and toxicity of chemicals.

The onset of diabetes is accompanied by numerous and marked changes in the physiological function of the living organism, one or more of which may be responsible for the observed alterations in the metabolism of xenobiotics. Type I insulin-dependent diabetes is associated with severe hyperglycaemia and hyperketonaemia, either or both of which might be responsible for the changes in the bio-transformation of chemicals. Early studies showed that prolonged intravenous infusion of glucose did not modify the mixed-function oxidase activities (Ackerman and Liebman, 1977) and chronic hyperglycaemia decreased activities such as aniline hydroxylase (Hartshorn et al., 1979),
an activity readily induced by diabetes mellitus. Furthermore, when animals where treated with N-methylacetamide, a compound which produced an insulin-resistant condition similar to type II diabetes mellitus, the ensuing hyperglycaemia did not alter mixed-function oxidase activity (Peters et al., 1966), further demonstrating that it is not the hyperglycaemia which is responsible for the diabetes-induced effects on drug metabolism.

The role of ketone bodies

It has been suggested that compounds which promote ketosis stimulate mixed-function oxidase activities, including aniline hydroxylase (Mehendale et al., 1977), raising the possibility that the ketone bodies may have a prominent role in the diabetes-induced changes. The major ketone bodies are 3-hydroxybutyrate and acetoacetate formed from the partial oxidation of fatty acids within the hepatic mitochondria. Acetoacetate equilibrates with 3-hydroxybutyrate and both ketone bodies are released into the blood. Acetone is believed to be formed non-enzymically through the decarboxylation of acetoacetate. When carbohydrate metabolism is impaired and free fatty acid levels rise as a result of increased lipolysis, ketone bodies form the third most important blood-borne energy source for extrahepatic tissues after glucose and free fatty acids. The primary physiological role of the ketone bodies is to serve as substrates for the brain, a tissue that cannot take up free fatty acids. Therefore, during carbohydrate deprivation the ketone bodies limit the expenditure of glucose in the brain and thus spare proteins which would otherwise be catabolised.

In our study using rats rendered hyperketonaemic, but remaining normoglycaemic, mixed-function oxidase activities were increased. This correlated with induction of the same cytochrome P450 isoforms as observed in streptozotocin-induced diabetes (Barnett et al., 1988), thereby implicating the increased ketone body levels seen in diabetes (Barnett et al., 1990 and 1990). As in diabetes, increased levels of P450 I and P450 IIIB proteins were
noted. Moreover, a good correlation between plasma ketone levels and the hepatic levels of P450 IIE has been reported (Bellward et al., 1988). Hyperketonaemia also occurs during fasting (Tu and Yang, 1983; Miller and Yang 1984) and excessive alcohol consumption, although alcohol-induced ketoacidosis in man is rare, and all of these conditions are associated with the induction of cytochrome P450 IIE (Koop et al., 1987; Hong et al., 1987). It appears that this protein is active in the metabolism of acetone, being the only isoform of six purified isoforms capable of oxygenating acetone to acetal (1-hydroxyacetone). Furthermore, this isoform was the most active protein in the further hydroxylation of acetal (Koop and Casazza, 1985). It is reasonable to assume that the increase in the levels of cytochrome P450 IIE is an adaptive response to deal with the high levels of ketone bodies present in diabetes mellitus (Casazza et al., 1984).

Further work examining the effects of acetone, 3-hydroxybutyrate and 1,3-butanediol revealed that acetone was capable of inducing the cytochrome P450 IA, IIB and IIE subfamilies. 3-Hydroxybutyrate caused less pronounced elevations of cytochrome P450 IA and IIB and did not significantly effect the levels of P450 IIE. None of the ketones administered had any effect on the cytochrome P450 III or P450 IV subfamilies. 1,3-Butanediol did show induction of cytochrome P450 IIE but not of any other isoforms. These observations confirm that ketone bodies can cause similar alterations of hepatic cytochrome P450 proteins as observed in both chemically-induced and spontaneously occurring diabetes. However, there was no induction of the cytochrome P450 IVA subfamily in contrast to the significant induction of this subfamily in diabetes and medium chain triacylglycerol treated animals (Barnett et al., 1990c). It has been proposed that the P450 IVA subfamily is induced by hyperlipidaemia, a condition associated with diabetes (Dunn, 1990). Therefore, the observations from this study support the theory that the increased levels of plasma free fatty acids associated with type I diabetes is responsible for the increased cytochrome P450 IVA activity.

Ketoacidosis would also be expected to mediate the diabetes-induced changes in
the susceptibility to the toxicity of chemicals since these are brought about by changes in the complement of hepatic cytochrome P450 proteins. The ability of preparations from the livers of diet-induced hyperketonaemic rats to activate various precarcinogens was very similar to that encountered in the streptozotocin-induced diabetic animals (Barnett et al., 1990). Moreover, pretreatment of rats and mice with ketogenic substances, like diabetes, exacerbated the toxicity of carbon tetrachloride (Traiger et al., 1972; Hewitt and Plaa, 1979). However, ketone bodies cannot account for all the diabetes-induced changes in mixed-function oxidase activity and chemical toxicity. One of the diabetes-induced isoforms of cytochrome P450, namely P450 IIIA, was not inducible by dietary hyperketonaemia suggesting that other factors must be involved. Insulin-dependent diabetes impairs growth hormone secretion in the rat and as a result growth hormone levels are markedly suppressed (Tannenbaum, 1981; Yamazoe et al., 1989a). Low levels of growth hormone have been associated with increased levels of P450 III subfamily (Lemoine et al., 1988; Kamataki et al., 1985). Similarly, growth hormone can modulate the levels of male-specific form P450 IIIC2 (Morgan et al., 1985; Kamataki et al., 1985).

A mechanism for the diabetes-induced alterations of hepatic drug metabolism can therefore be proposed. Ketone bodies are the predominant inducing species, being responsible for the induction of the cytochrome P450 IA, IIB and IIE subfamilies (Figure 7.1). However, the hyperlipidaemia accompanying type I diabetes has a direct inducing effect on the cytochrome P450 IVA subfamily (Figure 7.1). The complex interactions between insulin and other hormones is perturbed during type I diabetes. This causes many alterations in the levels and secretory patterns of other hormones including growth hormone (Tannenbaum, 1981; Yamazoe et al.; 1989b). Growth hormone has been implicated in the regulation of many isoforms of cytochrome P450 including; P450 IIIA proteins and maintenance of the sex-specific constitutive isoforms (Wrighton et al., 1985a; 1985b; Figure 7.1). This suggests that the alteration of the cytochrome P450 IIIA proteins may be a consequence of the reduced levels of growth hormone in diabetic rats (Thummel and Schenkman, 1990).
Figure 7.1 Summary of the effects of type I diabetes on hepatic cytochrome P450 proteins.

Type I Diabetes Mellitus

INSULIN DEFICIENCY

Defective carbohydrate metabolism

Perturbation in the levels and secretory patterns of other hormones including; growth hormone.

HYPERLIPIDAEMIA

HYPERGLYCAEMIA

Induction/repression of various P450 isoforms

HYPERKETONOAEMIA

P450 IIIA

P450 IVA

P450 IIA

P450 IIE

P450 IIB

INDUCTION?

INDUCTION

INDUCTION

INDUCTION

INDUCTION

Defective arachidonic acid metabolism?

Increased propensity to bioactivate chemicals

Altered drug pharmacokinetics

Toxicity/carcinogenicity

Ineffective drug therapy/drug toxicity
7.5 The effects of other insulin-aberrant conditions on hepatic drug metabolism

The effects of type II (non insulin-dependent) diabetes on hepatic drug metabolism are much less pronounced than those of type I diabetes. As type II diabetic mice are not hyperketonaemic, this may not be an unexpected finding. However, they are hyperglycaemic, providing further evidence that hyperglycaemia is not responsible for the observed changes in hepatic drug metabolism in type I diabetes. Similarly, hypertension was not associated with any significant alterations of hepatic drug metabolism in the spontaneously hypertensive rat. Hypertension is frequently a complication of type II diabetes and therefore type II diabetic mice may have also been hypertensive. Thus it may have been predicted that hypertension in the rat would not be associated with marked alterations of drug metabolism.

Hyperinsulinaemia was associated with the induction of the cytochrome P450 IA subfamily in both insulinoma-bearing rats and rats injected with monocomponent long-acting insulin. This finding is of interest and suggests that the endogenous interactions of insulin and the effects of these interactions on drug metabolising enzymes merits further investigation.

7.6 Conclusion

There can be no doubt that the influence of insulin-dependent diabetes mellitus on the biotransformation of xenobiotics is marked and of clinical and toxicological importance. Insulin-dependent diabetes mellitus modifies the cytochrome P450 composition of the liver and possibly other tissues and in this way perturbs the metabolic pathways of chemicals. It
is therefore not surprising that the response of diabetic animals to the toxicity of chemicals is altered. However, the lack of any systemic study of the effects of diabetes on human drug metabolism precludes any extrapolation of the animal data to man. It may be argued that since human diabetes is corrected by a strictly controlled insulin regime, no hormonal changes or ketosis usually occur. However, the possibility that the diabetic patient, even when on daily insulin therapy, is still influenced by the disease, as far as the ability to cope with chemicals is concerned, cannot be ruled out and can only be answered by in vivo studies in humans.
HORMONAL REGULATION OF CYTOCHROME P450

The intricate interplay between hormones and cytochrome P450 regulation is most clearly demonstrated by sex-imprinting of gene expression. Most studied are the male-specific and female-specific P450 isoforms, P450IIC11 and P450IIC12 respectively. The expression of P450IIC11 is dependent on androgen exposure during the neonatal period and adulthood. Thus, castration of neonates abolishes P450IIC11 expression while castration of adults only partially decreases expression of this isoform. This partial effect is eliminated if rats are administered testosterone during adulthood, directly demonstrating a role for adult testosterone (Gonzalez, 1989). Similarly, testosterone injection to castrated rats during the first three days of life also results in significant P450IIC11 expression at adulthood, consistent with the proposal that this enzyme is one of the hepatic proteins subjected to neonatal imprinting or programming by androgen exposure.

The role of pituitary hormones in the regulation of sex-specific P450 isoforms has been extensively investigated (Kato et al., 1986; Morgan et al., 1985). Adult male rats have pulsatile secretions of pituitary growth hormone, while adult females have more constant levels, and these patterns are determined by both neonatal androgen exposure and adult testosterone levels (Jansson et al., 1985). Growth hormone secretion is also involved in the regulation of the female specific P450IIC12. Continuous infusion of growth hormone re-establishes levels of female-specific P450IIC12 in hypophysectomised female rats (Kato et al., 1986). Growth hormone also has suppressive effects on gene expression. For example, hypophysectomy of male and female rats results in a 5-fold to 10-fold respective increase in P450IIIE1 which can be partially reversed when these rats are treated with growth hormone.

Effect of Diabetes on P450 Regulation

The secretion of several hormones are known to be modulated directly and indirectly by diabetes. Thus diabetic rats are considered to be a good models to understand the regulatory influence of endocrine hormones on hepatic cytochrome P450. The hepatic content of cytochrome P450IIC11 is known to be increased by pulsatile secretion of growth hormone, whereas the level of P450IIC12 is shown to be increased by continual presence of serum growth hormone levels in adult rats. In diabetes mellitus serum growth hormone levels are reduced and Yamazoe et al., 1989b recently indicated that the constitutive and inducible levels of expression of phenobarbital-inducible P450IIIB1 and P450IIIB2 was regulated suppressively by this hormone. Therefore, the reduced levels of this hormone in diabetes may explain the induction of these isoforms. Furthermore, growth hormone has been implicated in the suppressive regulation of cytochrome P450IIIA2 and P450IIIE1 regulation. However, this hormone has not been associated with the regulation of the cytochrome P450I or P450IVA subfamily of proteins. Obviously, diabetes affects the levels of the cytochrome P450 proteins by a variety of mechanisms. Recently, the regulation of cytochrome P450IIIE1 activities in diabetes has been extensively studied providing evidence for several regulatory sites in the expression of this protein.

Regulation of P450IIIE1 in Diabetes

In spontaneously diabetic rats or in rats made diabetic by treatment with diabetogens, P450IIIE1 levels are induced to 6-fold above the levels seen in control rats. This increase is accompanied by up to a 10-fold increase in P450IIIE1 mRNA in the absence of an increase in transcription of the IIIE1 gene (Koop and Tierney, 1990). When diabetic rats are given insulin the effect is reversed. Therefore, in the diabetic rat either the IIIE1 mRNA s stabilised or the efficiency of pre-mRNA is markedly enhanced. P450IIIE1 is not detected in neonatal liver but significant levels of P450IIIE1 RNA are detectable within a few hours. The increase in MRNA is coincident with transcriptional activation of the P450IIIE1 gene.
The onset of transcription is accompanied by the demethylation of cytosine residues upstream from the transcription start site in the P450IIE1 gene 1 day after birth with additional demethylation detectable at 1 and 10 weeks after birth (Koop and Tierney, 1990). The transcriptional activation of P450IIE1 gene at birth is in sharp contrast to the regulatory mechanisms employed in the adult where mRNA and protein stabilisation are the most important factors in the regulation of enzyme level. In almost all cases, elevated mRNA is absent in situations where hormonal changes are involved. The induction of P450IIE1 by the diabetic state is variable. The increase in both the enzyme concentration and the mRNA is reversed by insulin administration. Initial suggestions were made that the induction of P450IIE1 by the diabetic state was due to an increase in the ketone body levels in the circulation. However, fasting conditions do not produce high enough ketone levels to account for the induction observed.

Growth hormone levels are depressed in diabetic animals and may have an important role in P450IIE1 induction. However, Thummel and Schenkman, 1990 found that while both male and female rats have elevated P450IIE1, the diabetic state has no effect on the circulating growth hormone concentration in females. These investigations also reported that administration of growth hormone to diabetic males failed to reverse the effect of diabetes. These results suggest that there are probably more than one mechanism important in the induction of P450IIE1 in diabetic rats. On involves the stabilisation of the mRNA while the other operates through increased mRNA translation or decreased protein degradation. There have been few studies examining stabilisation of P450IIE1 mRNA and further work needs to be performed before conclusive statements can be made. For all chemical inducers examined in adult animals there is no significant increase in the hepatic concentration of P450IIE1 mRNA. Therefore, it is suggested that P450IIE1 is primarily induced by post-transcriptional mechanisms. Support for this mechanism involving a decrease in P450IIE1 degradation as a result of protein stabilisation is provided by a number of investigations. In diabetes mellitus two mechanism are probable; i) Direct stabilisation of the protein resulting in decreased degradation. This is performed by interactions of ketone bodies, predominantly acetone, with P450IIE1 protein thereby preventing degradation. ii) Hormonal influence. Gene transcription, possibly receptor mediated which may be due to the lack of growth hormone suppression of gene transcription in diabetes.

The regulation of expression of P450IIE1 is extremely complex and involves both transcription and post-transcriptional mechanisms. In adult animals and presumably man, the control of degradation of the enzyme is one of the principal components in the regulatory pathway. The figure below outlines the possible regulation of P450IIE1 by diabetes mellitus.

The regulation of cytochrome P450IIE1 (Gonzalez, 1989)

![Regulation diagram](attachment:image.png)
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