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The Effect of 48 hr Glucose Administration on the Hepatic P-450 System

John N. Buchholz

Abstract

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The administration of glucose for 48 hrs to mice resulted in a shift of the dose response curve of pentobarbital, an indication that glucose administration significantly sensitized the animals to the effects of the drug. Glucose administration produced a reduction in the catalytic activity of hepatic microsomal P-450 as measured by the in vitro conversion of p-Nitroanisole to p-Nitrophenol.

The administration of glucose to rats for 48 hrs resulted in prolonged anesthesia after the administration of pentobarbital. The spectral binding of hexobarbital and methadone to hepatic microsomal P-450 was found to be significantly reduced following glucose administration. The changes in spectral binding were observed in the absence of any significant change in the microsomal P-450 content. The changes in the spectral binding of hexobarbital and methadone to P-450 in glucose treated animals might suggest that glucose administration is causing a qualitative or conformational change in hepatic microsomal P-450 enzymes. Glucose

administration resulted in an increase in the cytosolic protein content which may possibly indicate that glucose is inducing enzymes responsible for its metabolism and/or the synthesis of lipids from glucose.

Glucose treatment produced a decrease in hepatic glycogen content and an increase in total microsomal lipid, phospholipid, and fatty acid content. Specific fatty acid and phospholipid contents were also measured following glucose administration. The results showed that there was an increase in microsomal palmitic, palmitoleic, stearic and oleic acids as well as the phospholipids phosphatidylcholine and phosphatidylethanolamine. Changes in lipid content and specific fatty acid content have been shown to be associated with changes in microsomal P-450 activity and the binding of various substrates to P-450. The data reported in these studies suggest that glucose administration was associated with quantitative alterations in specific microsomal lipid content and that the lipid changes could have influenced the activity of microsomal P-450 and the binding of drug substrates to P-450.

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GRADUATE SCHOOL

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by

John N. Buchholz

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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Chapter 1

INTRODUCTION

The endoplasmic reticulum of the eukaryotic cell fulfills a number of vital roles in the regulation of carbohydrate and lipid metabolism and in the biosynthesis of biochemical entities such as cholesterol and steroids. The finding that this subfraction of the cell is also involved in the metabolism and hence detoxification of xenobiotics has made the endoplasmic reticulum a major focus in the pharmacological subspecialty known as drug metabolism. The bulk of drug metabolism appears to be carried out by enzymes located in the endoplasmic reticulum of hepatic cells (1).

The hepatic endoplasmic reticulum is an intracellular membrane which can be subdivided into two distinct subfractions known as the rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER). The RER contains ribonucleoprotein particles known as ribosomes which are involved in the synthesis of proteins secreted from the cell and of proteins destined for incorporation into the endoplasmic reticulum and other subcellular membranes. The SER although not capable of synthesizing proteins, is involved in numerous aspects of lipid metabolism and monooxygenations of steroids and xenobiotics.

Both the SER and RER contain the monooxygenase system which is made up of cytochrome P-450, NADPH-cytochrome P-450 reductase (a FAD- and FMN-containing flavoprotein) and

phosphatidylcholine as well as other phospholipids and neutral lipids. The lipid components are necessary for optimal monooxygenase activity (1).

The cytochrome P-450 system prevents the progressive accumulation of lipophilic xenobiotics. Thus detoxification appears to be one function of the P-450 system which is essential to the survival of humans and other animals alike (1).

There are a great multiplicity of substrates which undergo hydroxylations involving cytochrome P-450. This observation has raised doubts concerning the homogeneity of P-450. The theory of heterogeneous P-450's has been able to account for the fact that a single substrate such as testosterone can undergo several hydroxylations at different carbon atoms. Furthermore, the rates of each hydroxylation can be altered following pretreatment with different inducing agents. The existence of multiple forms of P-450 has been established on the basis of spectrophotometric, electrophoretic and immunological methods (2). These multiple forms of P-450 have different substrate selectivities with varying degrees of overlap, are genetically determined and they appear to be individually substrate-inducible. Induction of rat liver microsomal P-450 with phenobarbital and 3-methylchloanthrene appears to produce two spectrally different enzymes, having absorption maxima at 450 and 448 nm respectively (3). Later studies of this phenomenon have

shown that a total of seven cytochromes appear to be induced by the two inducing agents (4). The heterogeneity of the monooxygenase system has the virtue of great versatility with respect to its ability to meet the challenge of detoxifying many different chemicals utilized in medicine and industry today.

Phospholipids and Fatty Acids in the Microsomal System and Their Relationship to P-450

Cytochrome P-450 is an integral protein embedded in the membrane matrix of the endoplasmic reticulum. Consequently, the properties of these protein components and the rates of specific reactions they catalyze will likely be influenced by the nature of the lipid components of the membrane, in particular phospholipids. The membrane matrix provides a hydrophobic environment for P-450 enzymes, which mostly utilize lipophilic substrates. Thus the membrane constitutes a reservoir for the substrates of P-450 and the membrane composition may possibly influence the type of substrate-P-450 interactions that occur (1).

The phospholipid composition of the hepatic endoplasmic reticulum membrane is 55% phosphatidylcholine (PC), 20-25% phosphatidylethanolamine (PE), 8-10% phosphatidylserine (PS), 5-10% phosphatidylinositol (PI), and 4-7% sphingomyelin (SM), reveals that the membrane is negatively charged at neutral pH. The ratio of PC to PE is very constant (2.5) which suggests that the proper ratio of PC to PE may be very

important in the normal functioning of membrane bound P-450 (5,6). Analysis of the fatty acid composition of the rat hepatic endoplasmic reticulum showed that the predominant fatty acids (approx. 90%) esterified to the number 1 position of phosphatidylcholine and phosphatidylethanolamine are palmitic (C16:0) and oleic acid (C18:0). At the number 2 position of phosphatidylcholine and ethanolamine the predominant fatty acids (greater than 90%) are arachidonic (C20:4) and trilinolein (C18:2). The fluidity of the microsomal membrane appears to be determined by the fatty acid constituents on the phospholipids and can be altered with changes in dietary composition of lipids and fatty acids (6,7). Changes in fatty acid composition of the microsomal membrane which apparently produce alterations in membrane fluidity (6), also results in changes in the catalytic activity of microsomal P-450 (7). For example the feeding of 3%-10% corn oil diets to rats results in a decrease in the duration of hexobarbital induced anesthesia with an associated increase in the hepatic microsomal P-450 mediated metabolism of hexobarbital and ethylmorphine (7).

Isolation and reconstitution represents the classical biochemical approach to the study of multi-enzyme systems. In early studies the P-450 system was initially resolved into 3 components, P-450, NADPH cytochrome P-450 reductase and a heat stable factor (8). The latter component was eventually identified as a lipid, specifically PC (9).

Different types of phospholipids have been investigated for stimulatory properties of the P-450 system (9). It appeared that dilauryl phosphatidylcholine was the most efficient lipid in stimulating the activity of the P-450 system in reconstitution experiments (10).

The components of the microsomal P-450 system are integrated into the hydrophobic interior of the membrane and are in intimate contact with the fatty acyl chains of the phospholipids. It remains reasonable to expect that there is a pronounced interdependency between the function of P-450 and the structure of the phospholipid and the physical state of the lipid in the microsomal membrane. Extraction of the hepatic microsomes with organic solvents will remove all neutral lipids and 80% of the phospholipid from the membrane, and results in a nearly complete inactivation of P-450 dependent hydroxylase activity (11). The activity of the monooxygenase system was found to be restored by the addition of PC to the lipid free preparations (12). These studies seem to suggest that phospholipid has a role in determining the activity of the P-450 monooxygenase system and that added lipid could renature P-450, partially denatured by organic solvent extraction.

The importance of membrane phospholipid for substrate binding and catalysis was recognized over a decade ago. Treatment of microsomes with phospholipase A resulted in a conversion of P-450 to P-420 (13). Phospholipase C

treatment of solublized microsomes will also result in a conversion of P-450 to P-420 which reduces the binding of various substrates to P-450 and decreases the rate of drug metabolism (14,15). Isooctane extraction of liver microsomes, which removes PC and PE will significantly reduce the binding of drug substrates to P-450 and consequently their metabolism (16). There seems to be no simple explanation for the phenomena described above. However one possible explanation is that the lipid components of the membrane maintain the appropriate conformational states of P-450 which are necessary for their catalytic activity (16). The results do emphasize the interdependency between phospholipids and the ability of P-450 to bind and oxidize substrates.

The P-450 enzymes exhibit a characteristic spin state alteration when substrates bind to the enzymes. Cytochrome P-450 can be either in a low spin state or a high spin state. A transition from a low to high spin state and vice versa results in conformational changes in the P-450. The high spin transition (type 1) is characterized by an absorption maximum at 380-385 nm and a minimum at 415-420 nm. Low spin transition (type 2) is characterized by an absorption minimum at 380-385 nm and a maximum at 415-420 nm (17,18,19). The binding of cholesterol to specific P-450 (P-450cam) results in a change from the low spin state to a high spin state and the high spin form of the P-450 has a

higher affinity for cholesterol than the low spin form of P-450. Furthermore adrenodoxin (a synthetic steroid) activates the binding of cholesterol by enhancing the proportion of P-450 in the high spin state (20). It appears that some ligands such as cholesterol bind preferably to the high spin form of P-450 where as other ligands such as pregnenolone bind preferably to the low spin form of P-450 (20). From these observations it appears that the relative affinity of P-450 enzymes for similar substrates is dependent on the unique conformation of these enzymes associated with their spin states (21). In the absence of exogenous substrates there is an equilibrium between the high and low spin states of microsomal P-450 (22). Investigations considering what components of the microsomal membrane are important in maintaining spin state equilibria have revealed that phospholipids and fatty acid moieties on the phospholipids have significant effects on the spin state equilibria of P-450 (23,24).

One of the major roles of the membrane matrix in the P-450 mediated catalysis is to provide a hydrophobic environment suitable for the hydrophobic compounds which are substrates for P-450. A relatively low affinity of the substrate for P-450 can be compensated for by the partitioning of a substrate in the membrane, which concentrates hydrophobic substrates in the membrane interior (25). More recent data suggest that the substrate binding site for

P-450 is in intimate contact with the membrane lipid phase (26) and that the hydroxylation rate for a substrate such as cholesterol is sensitive to the fatty acid composition of the lipids (27).

The hepatic microsomal cytochrome P-450 system has been found not only to be involved in the oxidation of xenobiotic substances, but also oxidizes endogenous substrates such as steroids (28) and fatty acids on phospholipids in the microsomal membrane (29). More than a decade ago it was suggested that fatty acids and some drugs may be oxidized by a common hepatic NADPH-dependent mixed function oxidase system (30). Fatty acids such as oleic acid and saturated straight chain fatty acids also appear to be oxidized by the hepatic microsomal P-450 (31). The oxidation of fatty acids in microsomal lipids such as arachidonic acid by hepatic P-450 appears to facilitate the conversion of arachidonic acid to prostaglandins (32). Fatty acid oxidation by P-450 has been suggested as a mechanism which may facilitate lipid turnover in the microsomal membrane and furthermore fatty acids such as lauric acid seem to be preferred substrates for hydroxylation as compared to the hydroxylation of drugs (33).

Microsomal enzymes are involved in the synthesis of PC, PS, PE and the sphingolipids which suggests that the microsomal enzymes may play an important role in determining the optimal lipid environment for the P-450 enzyme system (34). From the preceding discussion it appears that the microsomal

P-450 system which metabolizes endogenous and xenobiotic substances is a membrane bound system, and that there are a number of factors that can apparently affect the activity of this system.

A major concern of contemporary medicine is the adverse effects resulting from the clinical use of pharmacologic agents. The metabolic clearance of drugs regulates in part the duration of action and adverse reactions involved in the use of pharmacological agents. Nutritional factors, including proteins, carbohydrates, fats, vitamins and minerals affect the efficiency of metabolic reactions and hence the clearance of various drugs. Since the majority of foreign compounds are metabolized by hepatic membrane bound enzymes, any factor that alters the binding of substrates to these enzymes is likely to cause a change in the rate of metabolism and hence the activity of a drug (35,36).

There have been a number of factors that have been shown to affect the duration of action of sedative hypnotics such as barbiturates. Starvation of an animal for a short period of time results in increased barbiturate induced anesthesia (37,39,40). It was suggested that starvation may cause a loss of hepatic microsomal protein which apparently results in decreased metabolism of the barbiturates and hence prolonged effect (39). Rats that are fed a diet high in carbohydrates exhibit a prolonged sleep time after the administration of pentobarbital and barbital (41).

Vitamin B deficient diets when fed to rats resulted in longer sleep times after the administration of thiopental and pentobarbital. The deficiency of nicotinic acid presumably leads to a nicotinamide adenine dinucleotide phosphate (NADPH) depletion which is a necessary component in the oxidation of these drugs and thus may be responsible for the observed effect (42,43).

Rats that are given an intraperitoneal (i. p.) injection of glucose or fructose over a 7 day period will exhibit decreased hepatic P-450 content and ethylmorphine N-demethylase activity (44). These data suggest that glucose or sucrose is altering the P-450 content of the hepatic cell and thus its ability to metabolize drugs. Mice that are allowed to drink a 30% glucose solution for 48 hrs have been studied in regards to barbiturate induced anesthesia, duration of opiate analgesia and the <u>in vitro</u> hepatic microsomal metabolism of various barbiturates and opiate analgesics (45,46,47). Animals treated with glucose were found to sleep significantly longer than controls when administered various barbiturates and the analgesic effect of representative opiates was extended (45,46,47). Furthermore the <u>in vitro</u> hepatic microsomal metabolism of representative opiates and barbiturates was found to be compromised in the glucose treated animals. Kinetic studies on the <u>in vitro</u> metabolism of hexobarbital and methadone in glucose treated mice showed significant changes in the kinetic parameters Km

and Vmax (46,47). The changes in the kinetic parameters could not be related to quantitative changes in P-450 content since the P-450 content was not significantly altered by 48 hr glucose treatment (46,47).

Other studies were designed to test the possibility that a metabolite of glucose or glucose itself might act as an inhibitor of P-450 activity (48). In these studies the cytosolic fraction from treated animals was reconstituted with the microsomes from the control animals to see if the in vitro metabolism of the substrate p-Nitroanisole (PNA) by control microsomes could be inhibited by some component in the cytosol from glucose treated animals. The results showed that following treatment with glucose the microsomes from the glucose treated animals were significantly impaired in their ability to metabolize PNA as compared to control microsomes. The results also showed that adding cytosol from treated animals to the microsomes from control animals did not change the rate of metabolism of PNA by the control microsomes. From the data presented above it seems clear that the metabolism of barbiturates and opiate analgesics is reduced as a result of 48 hr glucose treatment. Following the decrease in the metabolism of these compounds, they would then presumably accumulate in the blood which would account for the increase in the duration of action after glucose administration (48).

There are fundamental questions that could be asked concerning the glucose effect on the microsomal metabolism of barbiturates and opiate analgesics. The first question is, does glucose or one of its metabolites affect the activity of the P-450 system indirectly by causing a quantitative change in the amount of P-450 in the microsomes? The second question is, can glucose be converted to other substances such as fatty acids and lipids which can then be deposited into the microsomal membrane and produce a change in the activity of the microsomal P-450? The studies completed here were designed first, to further clarify the in vivo effects of glucose administration on pentobarbital induced anesthesia by examining the dose response curve of pentobarbital in mice; second, to examine the effect of glucose treatment on the relative activity of hepatic microsomal P-450 in mice utilizing reconstitution studies with microsomes and cytosolic components; third, to examine the effect of glucose administration on pentobarbital induced anesthesia in the rat and the effect of glucose administration on the spectral binding of hexobarbital and methadone to rat hepatic microsomal P-450; fourth, to observe possible changes in the content of hepatic cytosolic protein, glycogen, total microsomal phospholipid, total microsomal fatty acids, specific microsomal fatty acids and phospholipids following glucose administration.

Chapter 2

METHODS

The Effect of Glucose Administration on the Dose Response Curve of Pentobarbital in Mice and the In Vitro Metabolism of p-Nitroanisole by Mouse Liver Fractions

Male Swiss Webster mice (Simonsen Laboratories, Gilroy Ca.) weighing 25-28 g were maintained for one week after arrival and then randomized into control and glucose treated groups for each experiment. The control groups received water and food (ad libitum) while the glucose treated groups received a 30% glucose solution for 48 hrs and food (ad libitum). At 48 hrs the animals were injected i.p. with pentobarbital at dose levels of 35, 40, 45, 50, and 55 mg/kg. Induction of sleep was determined by the loss of the righting reflex. Dose response data was analyzed by the previously published method of Litchfield and Wilcoxin (38). Mice from the control and glucose treated groups were randomly selected, sacrificed by cervical dislocation and the livers removed. The 9000 xg and microsomal liver fractions were prepared as described by Dixon et al. (40). The demethylation of p-Nitroanisole was assayed by the previously published method of Kato and Gillette (49).

The Effect of 48 hr Glucose Administration on Pentobarbital Sleep Time in Rats

Male rats (Simonsen Laboratories, Gilroy Ca.) weighing 200-225 g were randomized into control and glucose treated groups. Both groups were treated in the same manner as the mice mentioned above. At 48 hrs the animals were injected i.p. with a dose of pentobarbital (70 mg/kg). Sleep time was determined as the time elapsed between the loss and re-acquisition of the righting reflex.

Assay of Hepatic Glycogen, Cytosolic Protein , Microsomal Protein, P-450, Microsomal Phospholipid, Microsomal Fatty Acid, Microsomal Cholesterol, and Total Microsomal Lipid Following 48 hr Glucose Administration

Hepatic glycogen content was measured by solubilizing a small piece of liver tissue (0.1 g) in 30% KOH at 50°C for one hour and then precipitating the glycogen in 95% ETOH at 100°C. The glycogen precipitate was resuspended in water and then reacted under alkaline conditions with anthrone (50). Hepatic cytosolic and microsomal protein was determined by the Lowry method (51).

Microsomal P-450 content was measured by the absorbance difference in dithionite reduced microsomal suspension and a dithionite reduced microsomal suspension mixed with carbon monoxide (46). Microsomal lipid was extracted with 1 part chloroform and 1 part methanol as described by Bligh and

Dyer (51). Lipid was stored at -80°C until each analysis was performed. An aliquot of the extracted microsomal lipid was removed and the phospholipid content was measured by determining total phosphorus content as previously described by Bartlett (53). Microsomal fatty acid content was determined by reacting an aliquot of extracted lipid with hydroxylamine and ferric chloride under alkaline conditions as described by Stern and Shapiro (54). Microsomal cholesterol content was measured colorometrically by reacting an aliquot of extracted microsomal lipid with ferric chloride under acidic conditions (55). Total microsomal lipid (phospholipid + neutral lipid) was measured by weighing the whole microsomal lipid extracts after the chloroform solvent was evaporated.

Quantitation of Specific Microsomal Fatty Acids by Gas Chromatography

Specific fatty acids were separated and quantitated by gas chromatography (GC) on a Suppelco Wax 10 column (Suppelco Inc., Belafont Pa.). The fatty acids in a 1 mg sample of extracted microsomal lipid were transmethylated in boron trifluoride-methanol (14/100 W/V) at 60°C for 60 min as previously described (53). The fatty acid methyl esters (FAME's) of palmitic, palmitoleic, stearic, and oleic acid were identified and quantitated using reference FAME's obtained from Sigma Chemical company (Sigma, St. Louis Mo.) (56). The GC operating conditions were set as follows;

helium gas flow 8 ml/min, detector temp 260°C, injector temp 250°C, column temp program was 200°C for 15 min then elevated to 235°C at 2°C/min and held for 10 min before re-equilibration at 200°C.

Separation and Quantitation of Microsomal Phosphatidylcholine and Phosphatidylethanolamine

The quantitation of phosphatidylcholine and ethanolamine was carried out by TLC. A 1 mg sample of microsomal lipid was spotted on silica gel coated thin layer plates (250 microns). The plates were developed in two solvent systems as previously described (57). The separated phospholipids were stripped from the plates and total phosphorus was determined (53).

Spectral Binding of Hexobarbital and Methadone to Hepatic Microsomal P-450

The binding of hexobarbital and methadone to microsomal P-450 was measured spectrally on a Beckman ACTA MVI dual beam spectrophotometer (Beckman Instruments, Irvine Ca.). A 0.1 ml aliquot of buffer (1.15% KCl in 0.025 M tris, pH 7.45) was added to disposable 4.0 ml polystyrene reference and sample cuvettes (pathlength 1.0 cm) which contained 2.9 ml of microsomal suspension at a final protein concentration of 3.0 mg/ml. The baseline was obtained by scanning the microsomal suspension from 500 to 360 nm in the split beam mode. The difference spectrum was obtained by adding 0.1 ml

of an appropriate amount of hexobarbital or methadone to 2.9 ml of microsomal suspension to obtain the concentrations given in the results. The cuvette containing the drug was placed in the sample chamber and scanned from 500 to 360 nm. After the spectral scan was obtained the baseline was subtracted and the difference spectra plotted. The data was subjected to a double reciprocal plot to obtain the maximal spectral binding (Δ Amax) and the spectral dissociation constant (Ks) as described by Schenkman and Kupfer (58,59).

Chapter 3

RESULTS

Figure 1 describes the effect of 48 hr glucose treatment on the dose response curve of pentobarbital in mice. Note that glucose treatment appears to shift the curve to the left which resulted in a reduction of the ED 50 by 5.85 mg/kg ($P<0.05$).

Table 1 describes the effect of 48 hr glucose treatment on the *in vitro* metabolism of p-Nitroanisole by the liver 9,000 xg supernatant and the microsomal fractions. With respect to the 9,000 xg fraction the glucose treated animals metabolized one half of the substrate (p-Nitroanisole) as did the controls. When the microsomal fraction was used the controls lost 37% of their activity but the glucose treated only lost approximately 12.5% of their activity (25.17 vs 18.74 Nmoles p-Nitroanisole metabolized/mg protein/30 min). However the difference in microsomal activity between the two groups was still highly significant ($P<0.01$). When the control microsomes were re-constituted with control cytosol they did not regain their original metabolic activity, but retained the microsomal level of metabolism. The microsomes from the treated animals reconstituted with cytosol from controls showed a slight increase in activity. The reconstitution of control microsomes plus cytosol from the treated animals and vice versa, did not change the activity. These data suggest that glucose treatment does not affect

factors in the cytosol other than the possibility of inducing enzymes that metabolize glucose and that glucose treatment appears to have a major influence on the metabolic activity of the microsomal fraction of the liver cell.

Table 1

NMoles p-Nitrophenol Formed/Mg Microsomal Protein/30 min

Group	9,000 xg	Microsomes	CM+CC	CM+TC
Control	40.4 ± 3.3	25.2 ± 1.3	26.4 ± 0.93	26.8 ± 0.9
			TM+TC	TM+CC
Glucose Treated	21.4 ± 0.22	18.7 ± 0.7	20.9 ± 0.2	21.9 ± 0.4
	P < 0.01	P < 0.01	P < 0.01	P < 0.01
	N = 20	N = 20	N = 20	N = 20

CM+CC = Control Microsomes + Control Cytosol
TM+TC = Treated Microsomes + Treated Cytosol
CM+TC = Control Microsomes + Treated Cytosol
TM+CC = Treated Microsomes + Control Cytosol

Spectral binding studies of hexobarbital and methadone to rat microsomal P-450 were carried out in order to examine further the effect of glucose treatment on the microsomal

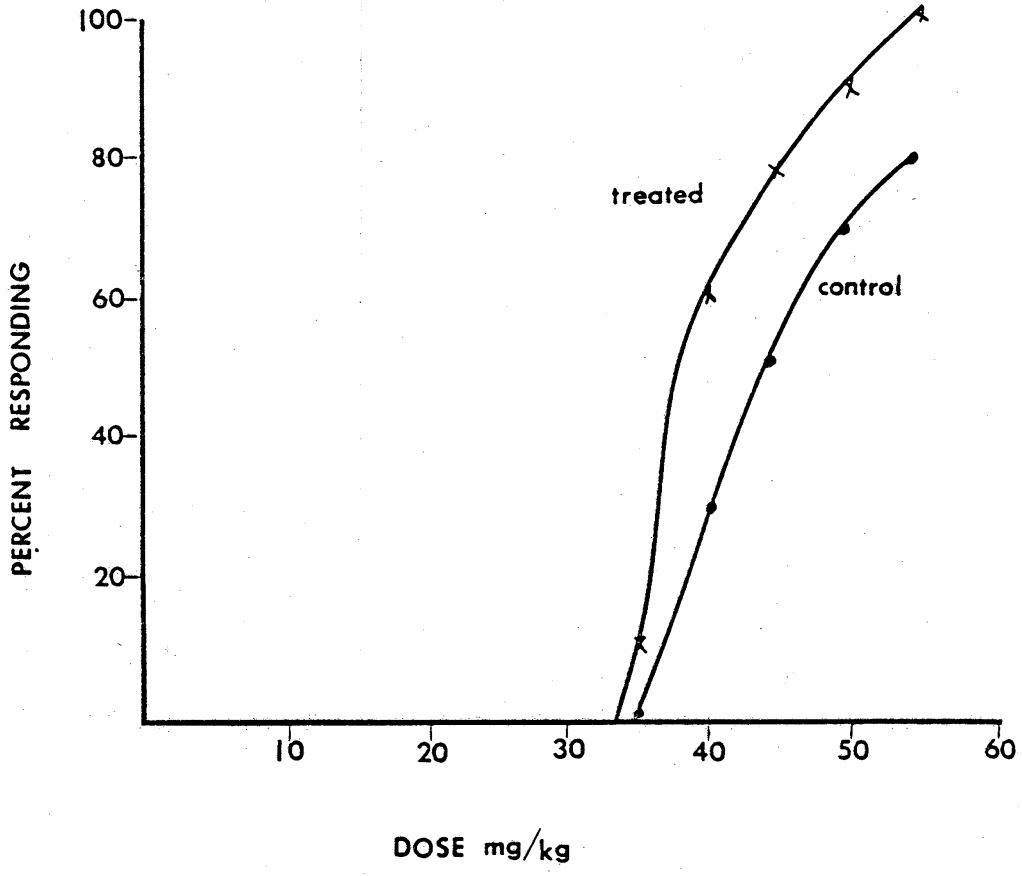

Figure 1. The effect of 48 hr glucose administration on the dose response curve of pentobarbital in mice. Animals in each group were injected I.P. with various doses of pentobarbital and the number of animals in each group that exhibited anesthesia (loss of righting reflex) was recorded.

P-450 system. The rat was selected as the species due to the fact that many of the spectral binding studies of various substrates to hepatic P-450 have been carried out in the rat (15,17,18,19,20,21,22,24,58,59), which provided an extensive data base for the comparison of control and treated animals. Since the binding was to be carried out in rats rather than mice, it was necessary to examine the effect of glucose treatment on the barbiturate sleep time, microsomal protein content and microsomal P-450 content as previously examined in mice. The effect of 48 hr glucose administration on the duration of pentobarbital induced anesthesia is shown in Figure 2. Glucose administration results in a highly significant increase ($P<0.005$) in barbiturate induced hypnosis which is consistent with previous studies showing an increase in barbiturate anesthesia after 48 hr glucose administration in mice. The effect of glucose administration on microsomal protein, cytosolic protein and microsomal P-450 content is shown in Figure 3. Glucose administration causes no significant changes in the microsomal protein or P-450 content but does induce a significant increase in the cytosolic protein content in the treatment group.

Effect of Glucose on Spectral Binding of Hexobarbital and Methadone to Microsomal P-450

The binding of hexobarbital to microsomal P-450 produced a blue shift (type 1 spectrum) as previously

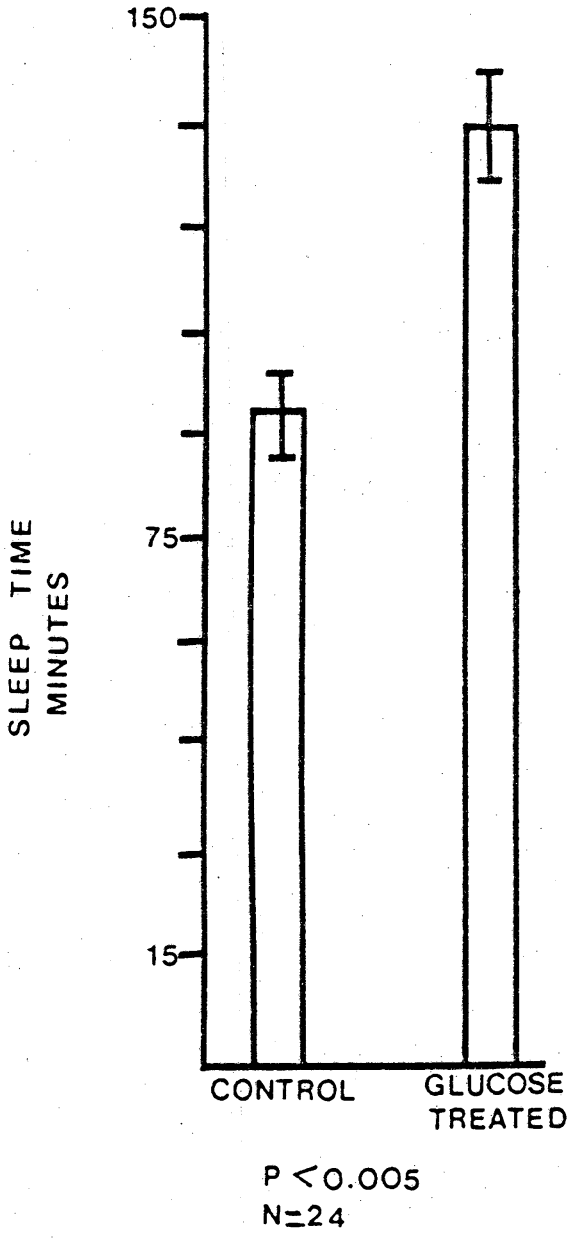

Figure 2. The effect of 48 hr glucose administration on the duration of pentobarbital induced anesthesia in the rat. Values are the mean ± the standard error for 24 animals.

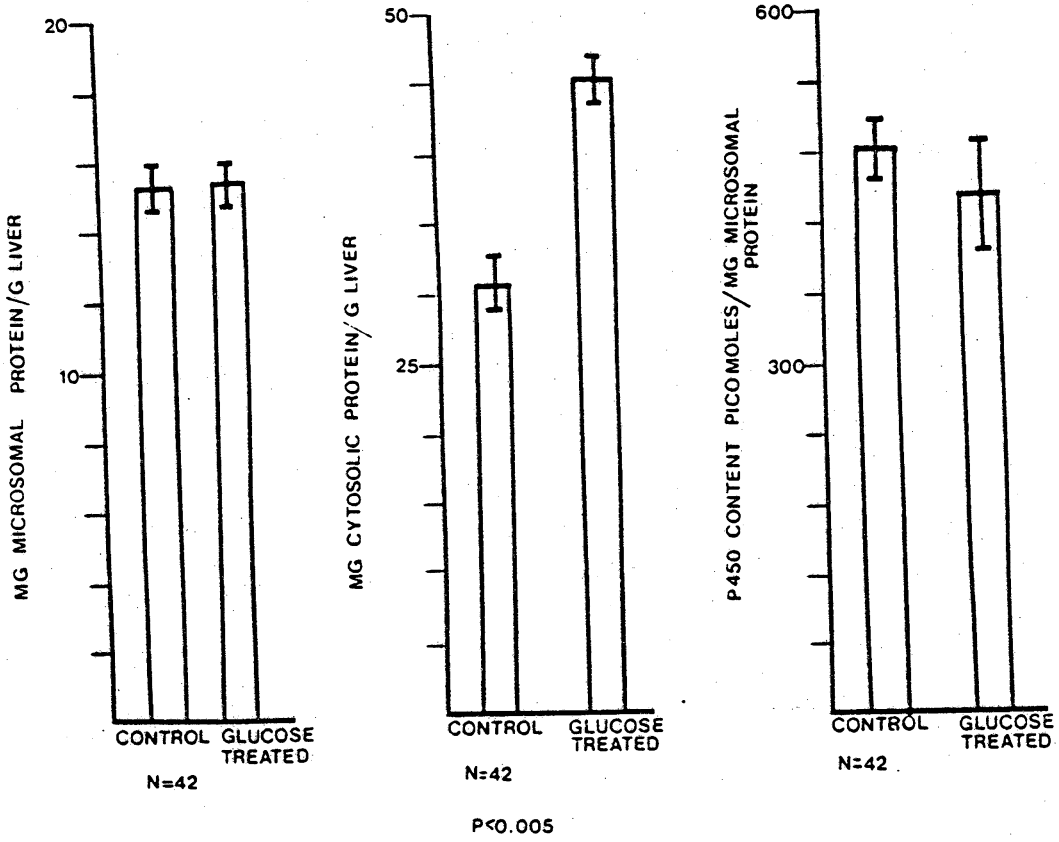

Figure 3. Measurements of rat hepatic microsomal protein, P-450 and cytosolic protein content following 48 hour glucose administration. Values represent the mean ± the standard error for 42 animals.

published (58). The binding of methadone to microsomal P-450 also produced a type 1 spectrum giving an absorption maximum at 384 nm and a minimum at 421 nm as shown in Figure 4. The spectral changes produced by the binding of these compounds were concentration dependent and showed saturability with increasing amounts of drug added to the microsomal suspension.

Figure 5 shows a plot of the (Δ A) absorbance changes at 420 nm versus the concentration of hexobarbital in the microsomal suspension. This figure shows a decrease in the binding of hexobarbital to hepatic microsomal P-450 after 48 hr glucose administration. Figure 6 is a plot of the Δ A values at 421 nm versus the concentration of methadone in the microsomal suspension. Glucose administration once again is associated with a reduction in the binding of a substrate to microsomal P-450. Figure 7 is a double reciprocal plot of Δ A at 420 nm versus the concentration of hexobarbital. This figure shows that the administration of glucose results in a decrease in the apparent spectral dissociation (Ks) and a decrease in the apparent maximal spectral binding (Δ Amax) of hexobarbital to hepatic P-450. These data are consistent with the previously observed decrease in apparent Km and Vmax for the microsomal metabolism of hexobarbital in mice following 48 hr glucose administration (46). Figure 8 is a double reciprocal plot illustrating the effect of 48 hr glucose administration on the

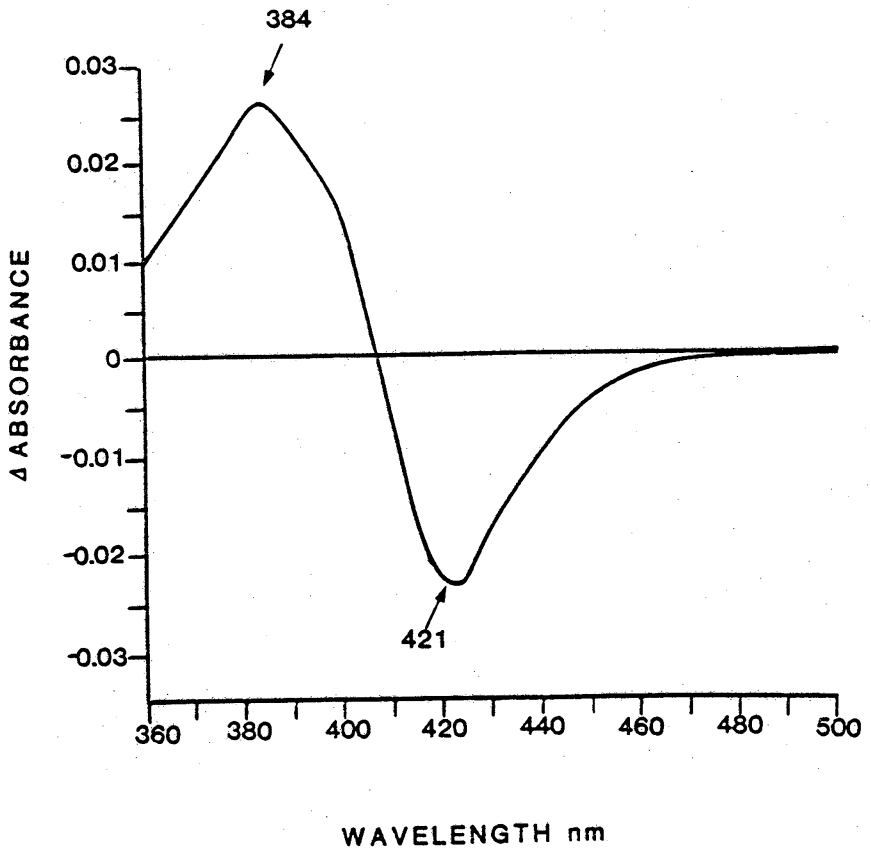

Figure 4. The spectral shift induced by methadone following the addition of methadone to a rat hepatic microsomal suspension. Microsomal protein concentration was 3.0 mg/ml and methadone concentration was 40 uM.

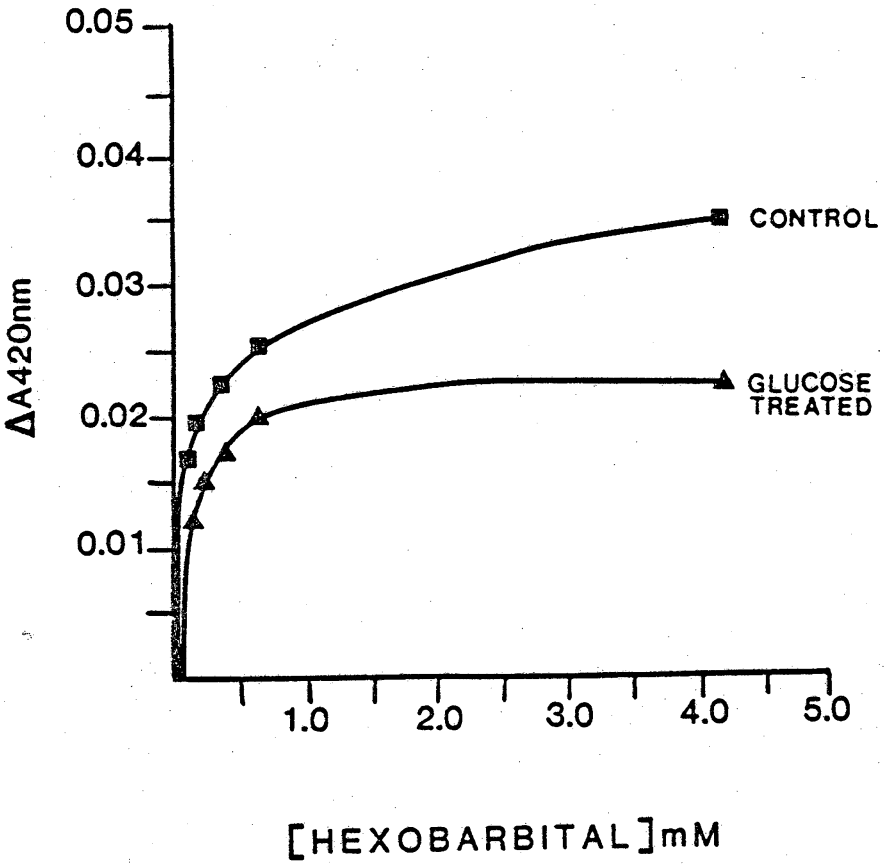

Figure 5. Plot of the absorbance changes (Δ A) at 420 nm versus the concentration of hexobarbital in a hepatic microsomal suspension from control and glucose treated rats. Each point represents the mean for 6 animals.

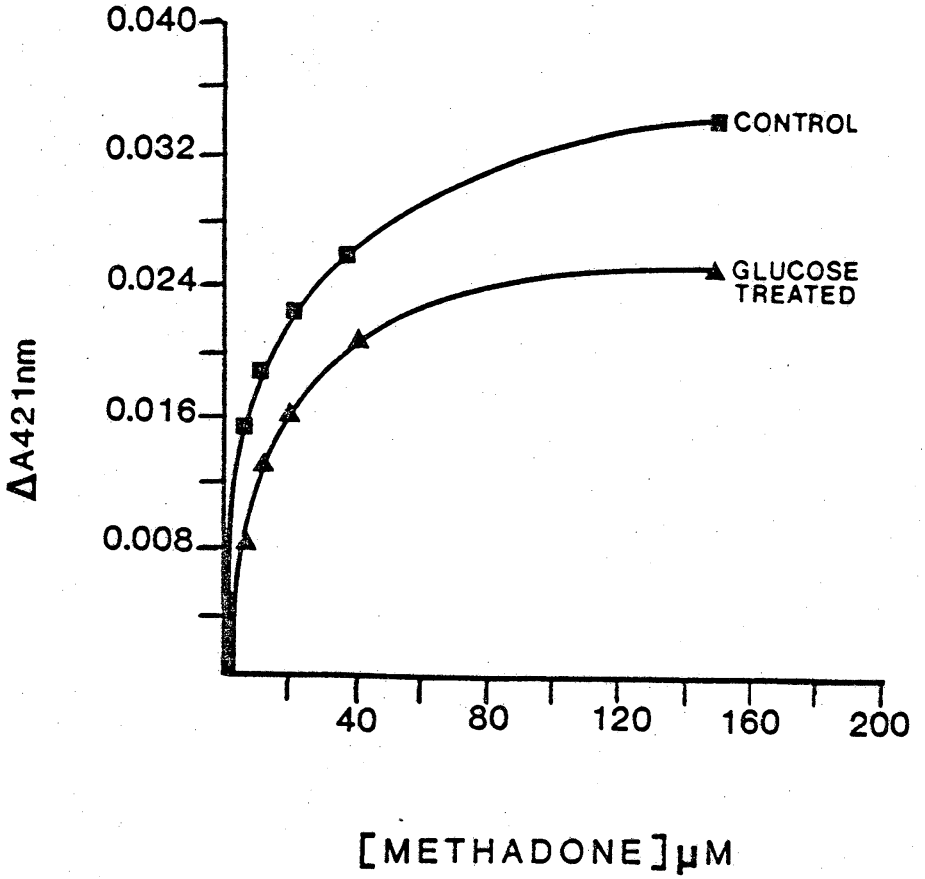

Figure 6. Plot of the absorbance changes (Δ A) at 421 nm versus the concentration of methadone in a hepatic micrsomal suspension from control and glucose treated rats. Each point represents the mean for 6 animals.

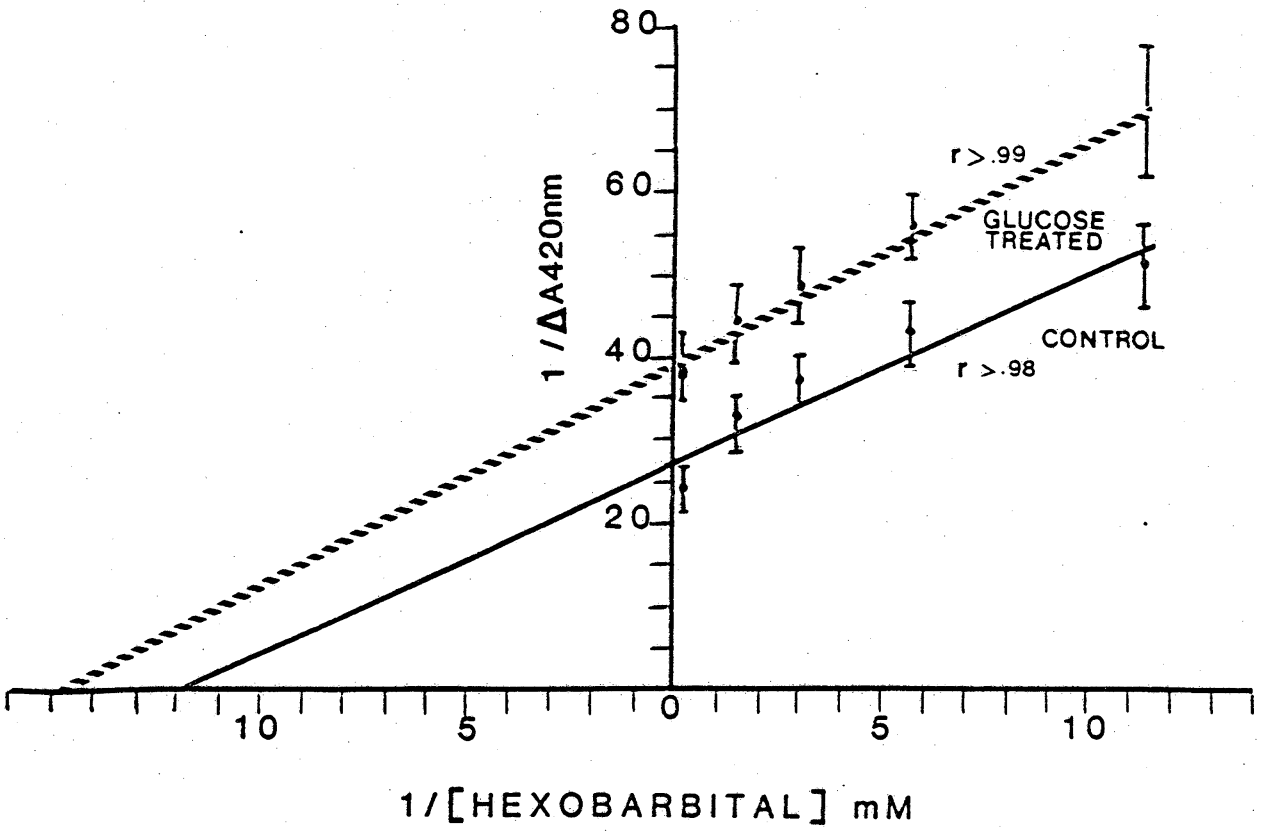

Figure 7. Double reciprocal plot of Δ A at 420 nm versus the concentration of hexobarbital in a hepatic microsmal suspension from control and glucose treated rats. Each point is the mean for 6 animals, vertical bars represent the standard error.

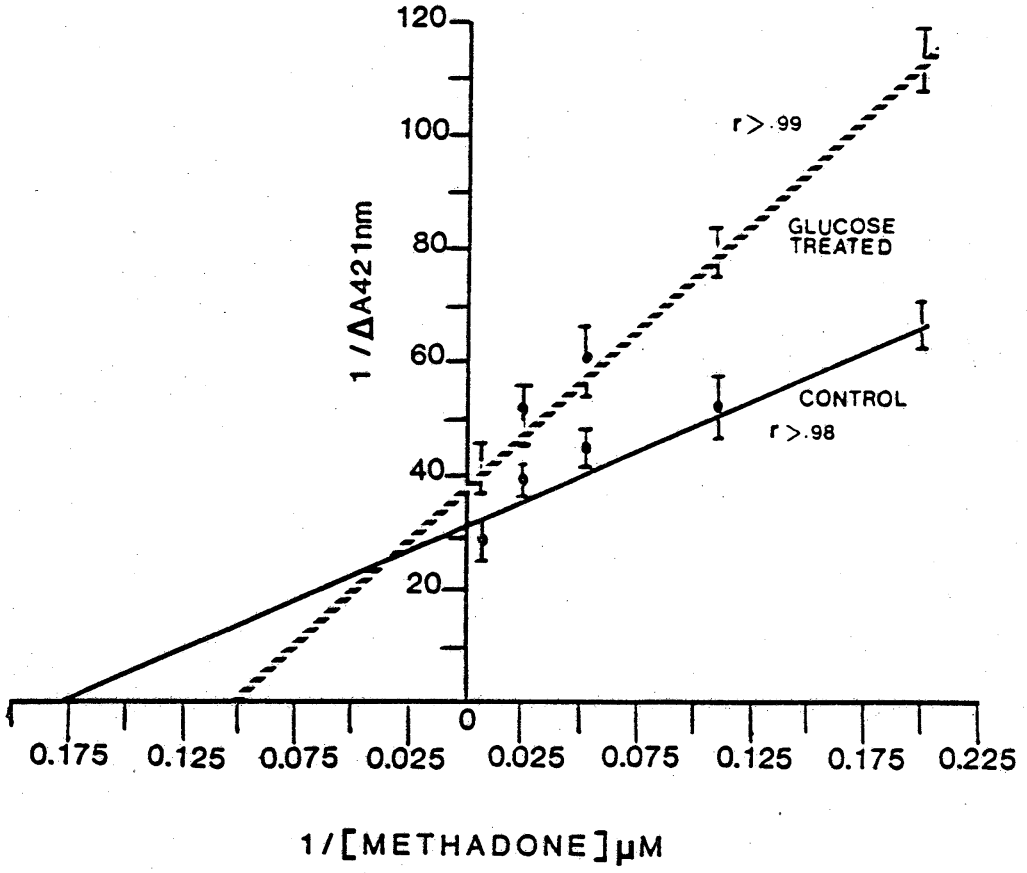

Figure 8. Double reciprocal plot of Δ A at 421 nm versus the concentration of methadone in a hepatic microsomal suspension from control and glucose treated rats. Each point is the mean for 6 animals, vertical bars represent the standard error.

binding of methadone to hepatic P-450. Note that glucose administration results in an increase in the apparent Ks and a decrease in the apparent Δ Amax. These data are also consistent with the previously observed increase in apparent Km and a decrease in the apparent Vmax for the hepatic microsomal metabolism of methadone in mice following 48 hr glucose administration (47).

The spectral binding of hexobarbital and methadone to rat hepatic microsomal P-450 are summarized in Figures 9 and 10. The two notable features of these figures are (a) the effect of glucose treatment on the apparent Ks (decreased Ks for hexobarbital and increased Ks for methadone) for both hexobarbital and methadone and (b) the decrease in the apparent Δ Amax per unit of P-450 for both hexobarbital and methadone in the glucose treated animals. The decrease in binding of hexobarbital to P-450 in the treated group is approximately 22% as compared to controls. The decrease in binding of methadone to P-450 in the treated group is approximately 12% as compared to the controls. The data from these studies suggest that glucose treatment alters the binding of hexobarbital and methadone to hepatic microsomal P-450.

Figure 11 illustrates the effect of glucose administration on liver, glycogen content, microsomal phospholipid content, total microsomal fatty acids and microsomal cholesterol content. Glucose treatment produces a reduction in

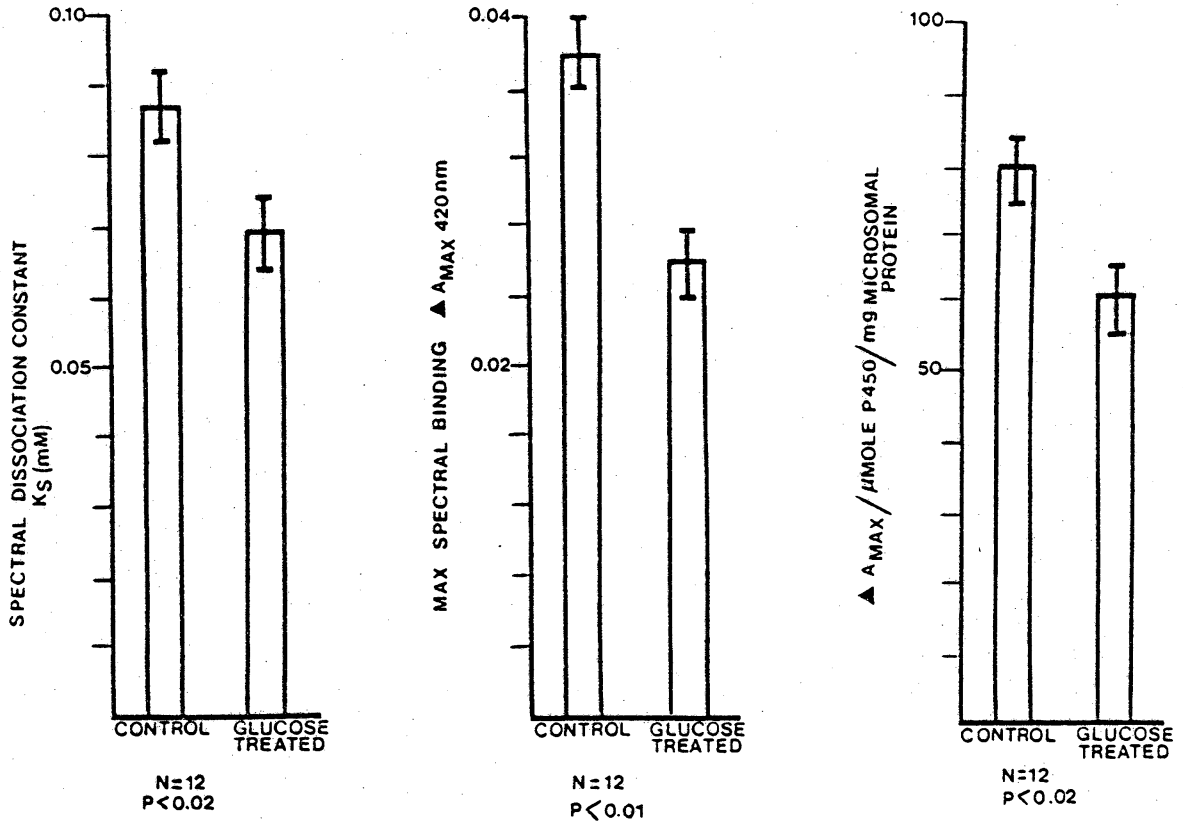

Figure 9. Summary of the effect of glucose treatment on the spectral binding parameters of hexobarbital to rat hepatic microsomal P-450. Each value represents the mean ± the standard error.

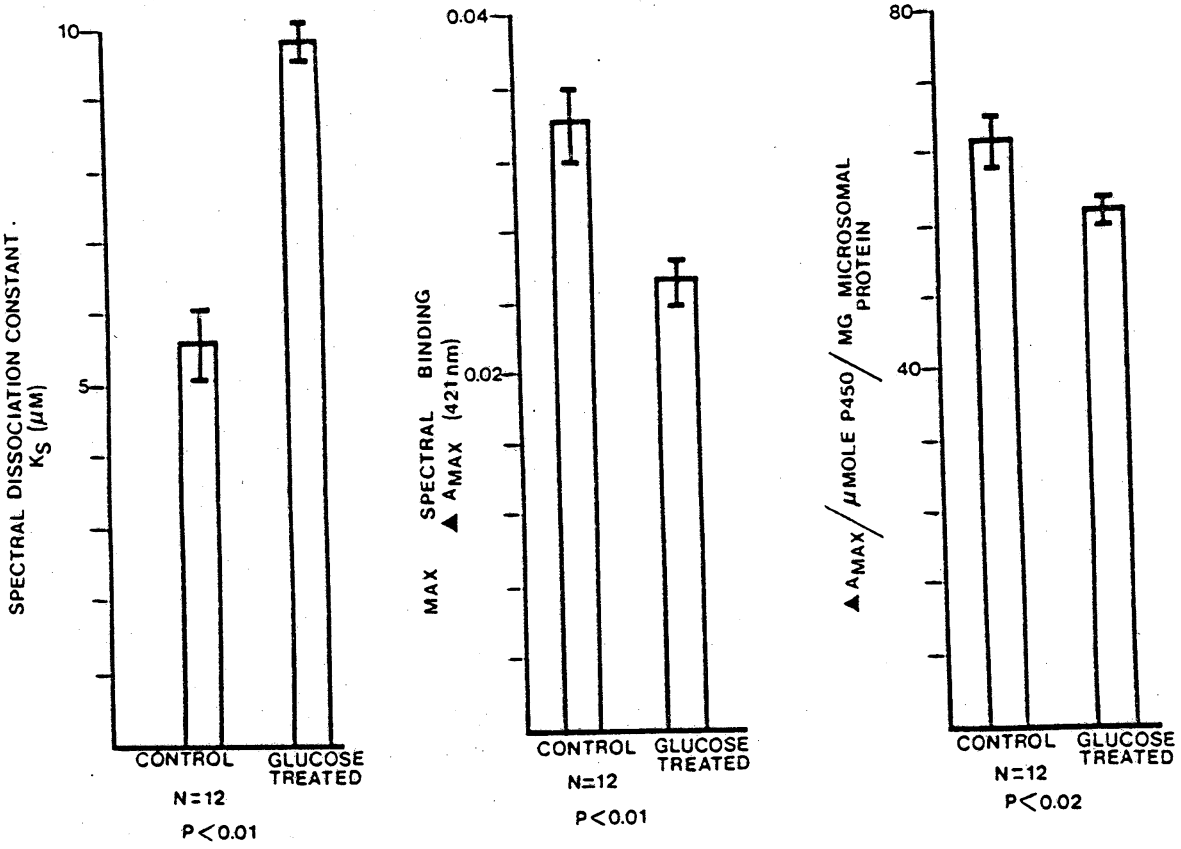

Figure 10. Summary of the effect of glucose treatment on the spectral binding parameters of methadone to rat hepatic microsomal P-450. Each value represents the mean ± the standard error.

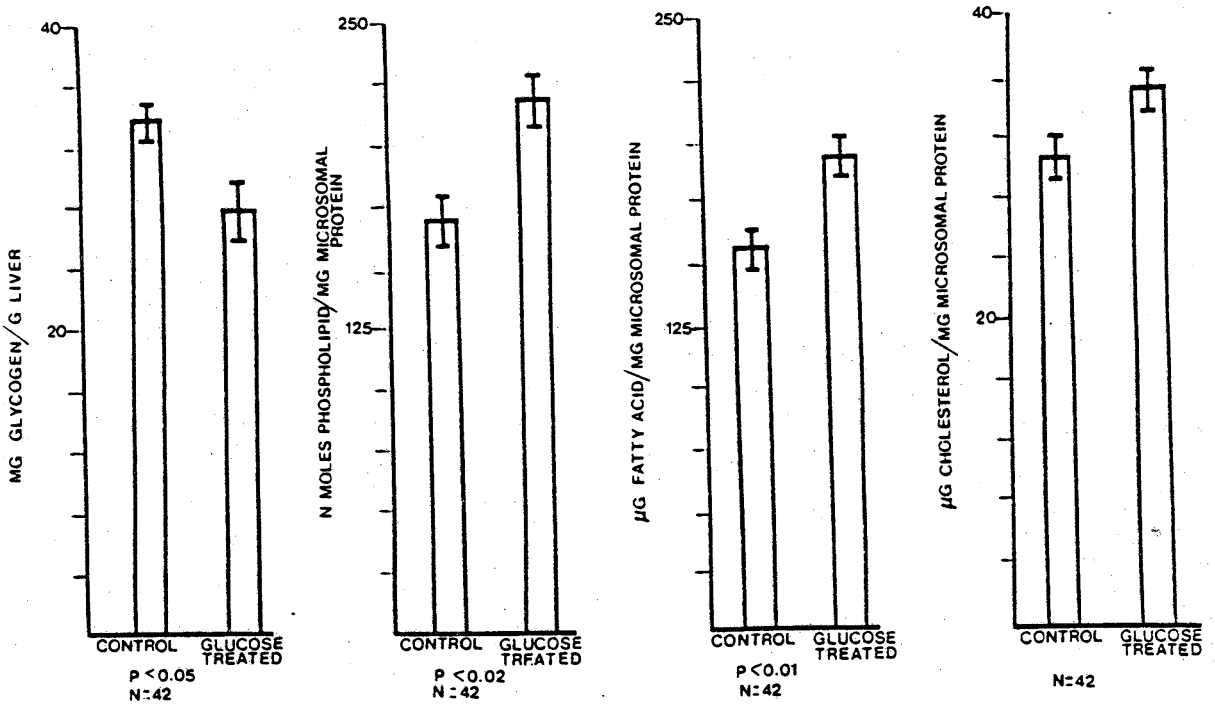

Figure 11. The effect of 48 hr glucose administration on rat hepatic glycogen content, microsomal phospholipid, fatty acid and cholesterol content. Values represent the mean ± the standard error.

glycogen content (16% reduction in treated animals as compared with controls). Phospholipid content and total fatty acid content in the microsomes increase significantly ($P<0.02$ and $P<0.005$ respectively) following glucose treatment. The increase in phospholipid content in the treated animals is approximately 26% as compared to controls and the increase in fatty acid content in the treated animals is approximately 22% as compared to controls. It is interesting to note that a 22% increase in microsomal fatty acid content is associated with a 22% decrease in the maximal spectral binding of hexobarbital to P-450 and a 12% reduction in the binding of methadone to P-450.

Glucose administration appears to have an effect on total microsomal lipid content and on the content of various fatty acid in the microsomal membrane (Figure 12). Glucose administration results in highly significant increases in palmitic, palmitoleic and oleic acid ($P<0.005$). There is also an increase in stearic acid, but the level of significance ($P<0.05$) is not as great as the other three fatty acids. These data may suggest that glucose is being converted to specific fatty acids and consequently lipid which can then be deposited in the microsomal membrane.

Quantitation of the two predominant lipids in the microsomal membrane, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), following glucose administration is shown in Figure 13. These data show that glucose

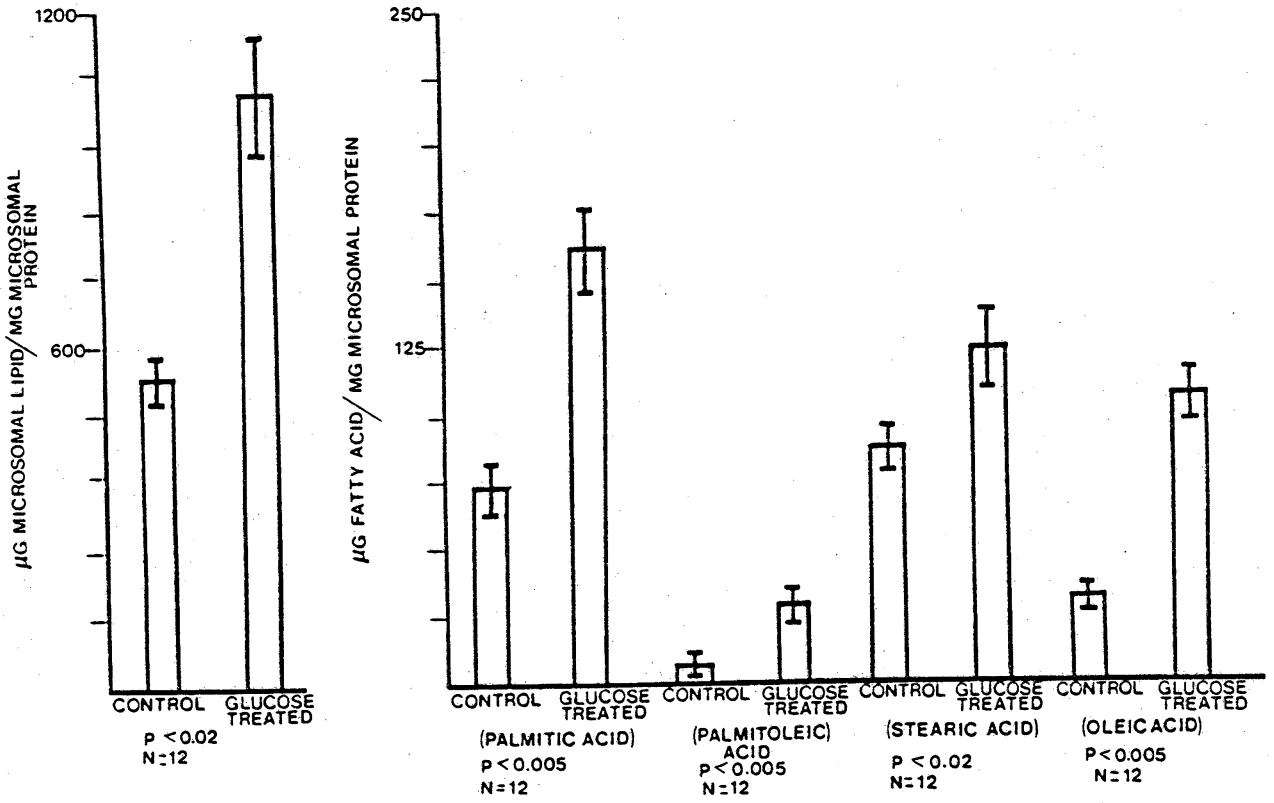

Figure 12. The effect of 48 hr glucose administration on rat hepatic microsomal lipid, palmitic acid, palmitoleic acid, stearic acid and oleic acid contents. Values represent the mean ± the standard error.

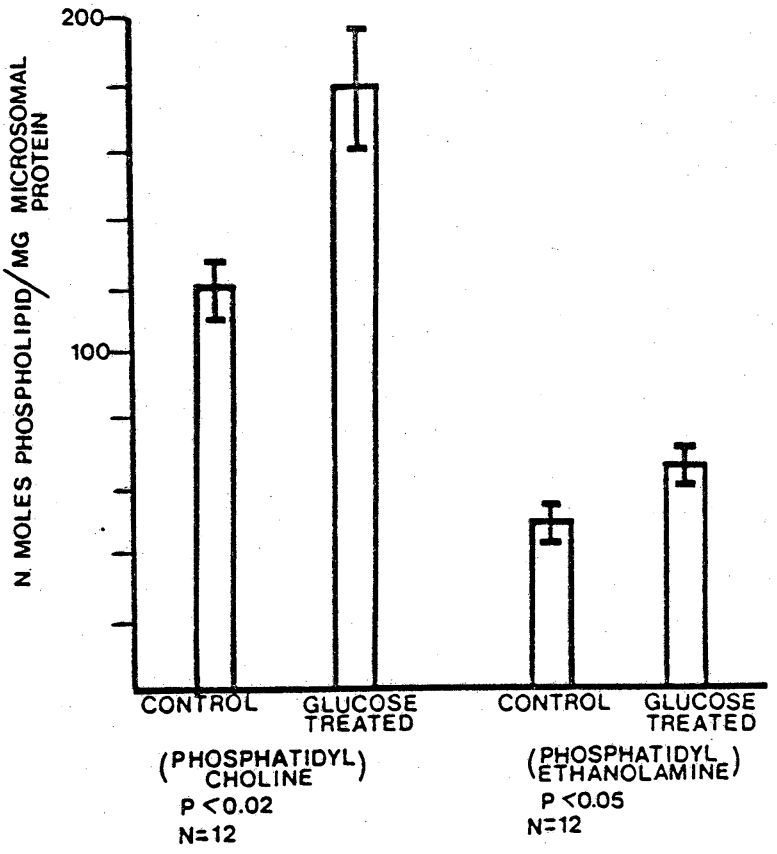

Figure 13. The effect of 48 hr glucose administration on rat hepatic microsomal phosphatidyl choline and phosphatidyl ethanolamine contents. Values represent the mean ± the standard error.

administration results in a significant increase in both PC and PE ($P<0.02$ and $P<0.05$ respectively). The increase in PC content was 33% and the increase in PE content was approximately 22% over the control values.

Chapter 4

DISCUSSION

Previous studies have shown that 48 hr glucose administration result in a significant increase in the duration of pentobarbital induced anesthesia and opiate induced analgesia (45,46,47). The data reported in these studies confirmed the glucose effect showing that glucose administration decreases the amount of pentobarbital necessary to achieve anesthesia (Figure 1). A possible theory explaining the effect of glucose administration on the duration of action of barbiturates and opiate analgesics is that glucose administration may somehow reduce the hepatic microsomal metabolism of these two groups of drugs. The reduced metabolism might then lead to higher blood levels of the drugs and hence prolonged effect. One study showed that mice treated for 48 hrs with glucose had higher blood levels of pentobarbital (46) and that the hepatic <u>in vitro</u> microsomal metabolism of compounds such as p-Nitroanisole, pentobarbital, hexobarbital and methadone were significantly reduced (45,46,47). The data presented in Table 1 confirm that glucose administration does indeed impair the <u>in vitro</u> hepatic microsomal metabolism of p-Nitroanisole in mice. The data also appear to indicate that a metabolite of glucose or glucose itself is not directly responsible for the reduction in metabolism as exemplified by the reconstitution of the cytosolic and microsomal fractions. What

appears to be clear from these data (Table 1) is that the microsomal system itself is altered in some way after glucose administration which results in reduced enzymatic activity. This information is consistent with previous observations showing that glucose administration appears to exert its effect on the hepatic microsomal system resulting in a significant reduction in P-450 enzyme activity (45,46).

Other animal species such as the rat have been shown to be affected by the administration of glucose or sucrose. In one previously published study the administration of glucose or sucrose in the diet resulted in prolonged sleep time after the administration of pentobarbital (35). The data presented in Figure 2 are consistent with this previous observation, showing that 48 hr glucose administration to rats results in highly significant increases in pentobarbital induced anesthesia. These data are also consistent with the previously observed prolonged pentobarbital anesthesia in mice after 48 hr glucose administration (45,46). The i.p. administration of glucose or sucrose to rats for 7 days will apparently result in a decreased hepatic microsomal P-450 content with a concomitant reduction of the *in vitro* microsomal P-450 mediated metabolism of ethylmorphine. The *in vivo* assessment of hepatic drug metabolic activity using the antipyrine test confirmed the parallel decrease in microsomal activity *in vitro* (44). The data reported in our studies (Figure 3) did not show any significant quantitative

change in microsomal protein or P-450 content following 48 hr glucose administration. The previously published studies by Hartshorn, et al. (44) reportedly saw no changes in rat hepatic microsomal protein or P-450 content within 2 days of initial treatment with glucose or sucrose which is consistent with the data reported in our laboratory (Figure 3). However significant changes in P-450 content did arise within 5 days after initial treatment (44). Peters and Strother showed that the 48 hr administration of glucose to mice apparently does not result in a significant quantitative change in hepatic microsomal protein or P-450 content (46) which is consistent with the data obtained in the rat as noted in Figure 3. These results may suggest that the effect of 48 hr glucose administration on pentobarbital anesthesia in mice (45,46) and rats is related to reduced hepatic metabolic activity. However the data reported here (Figure 3) and in other published studies (46) do not support the theory that the effect of glucose administration on drug action is related to quantitative changes in hepatic microsomal P-450 content.

Although hepatic microsomal protein content and P-450 content did not significantly change following glucose treatment, the cytosolic protein content did significantly increase in the glucose treated animals (Figure 3). The increased cytosolic protein content might suggest that the

increased glucose intake may be inducing enzymes that metabolize glucose or synthesize lipids.

In the absence of significant quantitative changes in hepatic microsomal P-450 content following 48 hr glucose treatment, binding studies were carried out to examine the effect of glucose treatment on the interaction between P-450 and the substrates hexobarbital and methadone. The binding of hexobarbital to microsomal P-450 produced a type 1 spectrum with an absorption maximum at 385 nm and minimum at 420 nm which is consistent with previous reports by Schenkman on the spectral binding of hexobarbital to rat hepatic microsomal P-450 (58). The binding interaction of methadone to rat hepatic P-450 also produced a type 1 spectral shift and is shown in Figure 4 since its spectrum has not been previously published. The absorption maximum was 384 nm and minimum at 421 nm. The type 1 spectral shifts produced by the binding of hexobarbital and methadone indicate a shift in the spin state of the heme moiety of cytochrome P-450 to the high spin state (58,60). Substrates that induce type 1 spectral shifts in microsomal P-450 have been shown to exhibit a preferential binding to the high spin form of P-450 (20,61).

The administration of glucose to rats for 48 hrs results in a significant reduction in the spectral binding of hexobarbital and methadone to P-450 (Figures 4,5). The reduced interaction between microsomal P-450 and these two

substrates (as indicated by the reduction in spectral binding) may offer a possible explanation for the previously observed reduction of the in vitro hepatic microsomal metabolism of hexobarbital in glucose treated mice (46,47). Plotting the spectral binding data (Figures 7,8) by the double reciprocal method showed a change in both the spectral dissociation constant (Ks) and the maximal spectral binding (Δ Amax) for hexobarbital and methadone. Changes in the spectral binding parameters have been suggested in some cases to be indicators of qualitative or conformational changes in P-450 (7,20,58,60,61). The data presented here (Figures 7,8) suggest that glucose administration may be effecting a qualitative change in the microsomal P-450 which would then be responsible for the decrease in the Δ Amax and the changes in the Ks. The increase in Ks for methadone indicates that the affinity of the microsomal P-450 has decreased following glucose administration. The data in Figure 3 show that P-450 content is not significantly changed by glucose administration, which provides evidence that the observed changes in the binding of hexobarbital and methadone (Figures 7,8) may be due to qualitative or conformational changes in the P-450 enzymes.

The decrease in Ks and Amax for the spectral binding of hexobarbital to P-450 is consistent with the data reported by Peters and Strother (46) showing a decrease in Km and Vmax for the microsomal metabolism of hexobarbital

following glucose administration in mice. The increase in Ks and decrease in Δ Amax (Figure 8) for the spectral binding of methadone to P-450 is consistent with the data published by Strother and Chau (47) showing an increase in Km and decreased Vmax for the microsomal metabolism of methadone following glucose administration in mice. In these published results (46,47) it was also noted that there was no significant change in microsomal P-450 content which suggested that the altered kinetics for the metabolism of hexobarbital and methadone was possibly due to some physical change in the microsomal P-450 system as opposed to a quantitative change in P-450 enzyme content.

The summary of the spectral binding of hexobarbital and methadone (Figures 9,10) showed that glucose administration to rats for 48 hrs significantly changes Ks and Δ Amax for hexobarbital and methadone. The most notable feature in these figures is that glucose administration results in a decrease in Amax per unit of P-450 for both hexobarbital (Figure 9) and methadone (Figure 10). The binding data for hexobarbital and methadone (Figures 7,8,9,10,) combined with the quantitative data on P-450 (Figure 3) show that glucose administration alters the ability of the P-450 system to bind substrates as compared to the controls.

The precise mechanism by which glucose administration alters the binding of hexobarbital and methadone to hepatic microsomal P-450 is not very clear. Two mechanisms have

been suggested. The first possibility is that glucose may be producing its effect on P-450 in some indirect manner possibly by altering the physical state of the microsomal P-450 system possibly through changes in lipid quantity and type. In order to consider an "indirect mechanism" for the glucose effect the fate of glucose in the liver needs to be considered. Previous studies by Touvinen and Bender (62) showed that the feeding of glucose or sucrose results in an increased lipid content in the rat liver. MacDonald and Roberts (63) showed that high glucose and fructose diets will result in the increased <u>in vivo</u> incorporation of ^{14}C-glucose into liver lipids. Hartshorn et al. (44) have also shown that the administration of glucose in the diet will result in the increased capacity of the liver to convert glucose to fatty acids and lipid, while Fitch and Chaikoff (64) showed that carbohydrate administration produced significant changes in enzymes responsible for the production of lipids.

The data reported in these studies examine some of the possible end products of glucose metabolism and the relationship between the end products of glucose metabolism and the microsomal P-450 system. The administration of glucose for 48 hrs resulted in a significant reduction in the hepatic glycogen content (16% reduction in glucose treated animals, Figure 11). Fouts et al. (65) have shown that reduced liver glycogen content after the administration of

epinephrine would result in a significant increase in hexobarbital induced sleep and a reduction in microsomal metabolic activity. However in the study reported by Fouts et al. (65) the hepatic glycogen content was reduced to 42% of the control value before any significant change in sleep time or microsomal metabolic activity occurred. Dixon et al. (66) showed that hepatic microsomal enzyme activity and glycogen content are not always correlated. Although the reduction in glycogen content reported here was significant, it is difficult in light of other studies (65,66) to make any inference as to the contribution of the observed change in glycogen content and the increase in pentobarbital sleep time.

Glucose administration for 48 hrs did not result in a significant change in hepatic microsomal cholesterol content (Figure 11) which is consistent with other published studies showing that sucrose enriched diets (62) and glucose enriched diets (67) do not alter hepatic cholesterol content.

Hepatic microsomal phospholipid content and fatty acid content apparently increase following 48 hr glucose treatment (Figure 11). These data may possibly indicate that glucose is being converted to lipids which are then deposited in the microsomal membrane. The increased microsomal lipid content is consistent with the increase in cytosolic protein content (Figure 3) which might suggest that glucose

administration is inducing enzymes that metabolize glucose and/or synthesize lipid. Other studies have shown increases in total hepatic lipid content (62,67) following glucose or sucrose administration. The data gathered here (Figure 11) show that lipid content is specifically increased within the endoplasmic reticulum. Studies in the past have shown that lipid is a necessary component in maintaining the appropriate physical state and hence activity of P-450 (9,12). Therefore it is possible that altered lipid content in the microsomal membrane may cause a physical change in P-450 as indicated by the altered binding of hexobarbital and methadone to microsomal P-450 following glucose administration (Figures 7,8,9,10).

Wade and Norred (68) showed that the feeding of 3%-10% corn oil to rats resulted in an increase in microsomal lipid and a corresponding increase of the <u>in vitro</u> microsomal metabolism of ethylmorphine and hexobarbital. Hexobarbital induced anesthesia was also found to be reduced in the animals fed the high fat diet. Gower and Willis (69) conducted a study in which rats were fed a fat diet rich in C20:5 and C22:6 fatty acids. These fatty acids were found to be incorporated into the intestinal microsomal lipids. The change in lipid content and specific fatty acid type resulted in an increased rate of P-450 mediated metabolism of benzo[a]pyrene. These two studies indicate that changes in microsomal lipid content (68) and lipid type (69) will

produce a change (increase) in P-450 activity. It is interesting to note that the administration of carbohydrate appears to produce the opposite effect on P-450 mediated metabolism of drugs, resulting in a decrease of P-450 activity and a corresponding increase in the duration of action of various drugs (35,45,46,47).

The administration of glucose was shown in these studies to have a significant effect on the specific fatty acid content within the microsomal membrane as shown in Figure 12 and suggest that the hydrophobic environment of microsomal P-450 may be altered. The lipid bilayer of the microsomal membrane has been shown to influence the conformation of microsomal cytochrome P-450 possibly through the iron spin state within the porphoryin ring (22,23,32). The binding of substrates to microsomal P-450 produces spectral changes which correspond to spin shifts in the iron moiety of the cytochrome P-450 enzymes. A shift to a high spin state is referred to as a type 1 spectral shift (λ min 420 nm, λ max 385 nm) and a shift to a low spin state is called a type 2 spectral shift (λ min 385 nm, λ max 419 nm) (22, 58,60). The magnitude of spectral change when a substrate interacts with P-450 is thus a measure of substrate binding (58,60). Since the addition of a substrate to microsomal P-450 can shift the spin equilibria of the ferric heme group there has been the suggestion that the magnitude of the observed binding of a substrate may depend on the relative

amounts of P-450 in the high or low spin state before a substrate is added (20,22,23). Thus any perturbation of the P-450 spin state equilibria could result in an alteration of the binding of a particular substrate. One example of the effect of spin equilibria on the binding of a substrate to microsomal P-450 is the effect of adrenodoxin on the binding of cholesterol to cytochrome P-450. The addition of adrenodoxin to a microsomal suspension will shift the spin equilibrium of P-450 to the high spin state which results in an enhanced binding of cholesterol to P-450 (20). The addition of pregnenolone to a microsomal suspension will shift the spin equilibrium of P-450 to a low spin state which then reduces the binding of adrenodoxin (20).

Gibson et al. (23) have attempted to examine the relationship between P-450 spin state equilibria and the microsomal lipid. If microsomal P-450 is reconstituted with phospholipid and free fatty acids there appears to be a shift in the spin equilibrium of P-450 from a low to high spin state. The shift in spin state resulted in the enhanced binding of type 1 substrates to P-450. Furthermore the report showed that the addition of oleic acid would shift the spin equilibrium of P-450 to the high spin state and that palmitic and stearic acid would shift the equilibria to the low spin state (23). These data suggests that the fatty acids on the phospholipid play a role in regulating the spin equilibria of P-450 which then affects the

binding of particular substrates. The data reported in Figure 12 show that specific fatty acid changes (especially palmitic and stearic acid) occurred in the microsomal membrane following glucose administration. The changes in lipid content and specific fatty acid types (Figures 11,12) within the microsomal membrane might be influencing the spin state and hence conformational states of membrane bound microsomal P-450. If indeed the lipid changes in the membrane are partly responsible for conformational changes in microsomal P-450 then it remains possible that conformational changes in P-450 may be responsible for the observed reduction in the binding of hexobarbital and methadone to P-450 following glucose administration (Figures 7,8,9,10).

Whole lipid extracts from the microsomal fraction were quantitated following glucose administration. The data showed that glucose administration resulted in a significant increase in total lipid content (Figure 12) and a significant increase in the content of the phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as shown in Figure 13. Quantitative changes in these two predominant lipids may possibly influence the conformational states of microsomal P-450 and thus the binding of particular substrates to P-450 as shown in Figures 7 and 8. However previous studies have shown that while whole lipid is important in maintaining the appropriate physical state of P-450 it is the fatty acid moiety of the phospholipid that

appears to be the predominant factor in the maintenance of P-450 spin state equilibria (23). Thus the alteration of PC and PE may not be all that important in the observed binding changes observed in these studies (Figures 7,8), but rather the quantitative changes in the fatty acid moiety of the lipid may be more important in terms of their influence on the conformational states of microsomal P-450.

The second hypothesis for the effect of glucose administration on the activity of hepatic microsomal P-450 is that glucose or some by product of glucose metabolism may cause a quantitative change in one or more of the P-450 enzymes. Although this is a reasonable hypothesis the data gathered in our laboratory (Figure 3) and published data (46,47) do not suggest that a significant change in the amount of P-450 is occurring. No reports have been located in the literature of a change in the proportions of the multiple forms of cytochrome P-450 following 48 hr glucose administration. If rats are treated for 5 to 7 days with fructose or glucose a significant reduction in P-450 content can be measured along with a significant reduction in the microsomal metabolism of substrates such as ethylmorphine, p-Nitroanisole and aniline (44). However 48 hr glucose administration will sensitize mice to the effects of pentobarbital and reduce the metabolic activity of hepatic microsomal P-450 as shown here (Figure 1, Table 2) and in other studies (45,46,47). Rats are also sensitized to the effects

of pentobarbital (Figure 2) by 48 hr glucose administration and changes in the binding of hexobarbital and methadone (Figures 7,8,9,10) to microsomal P-450 occur after the glucose exposure. The changes in microsomal activity in mice and the binding changes of drug substrates to rat microsomal P-450 observed after glucose administration all occur without significant changes in total microsomal P-450 content. Therefore the data presented here do not support the theory that 48 hr glucose administration is altering the hepatic microsomal P-450 in a quantitative manner.

Future studies that may further elucidate the effect of glucose administration on the hepatic microsomal P-450 system would involve the direct spectral examination of the heme spin states of microsomal P-450 in control and glucose treated animals. Possible changes in spin states and the conformational changes that altered spin equilibria produce may provide an explanation for the altered P-450 activity and binding of substrates to P-450 following glucose administration.

Chapter 5

SUMMARY

The administration of glucose for 48 hrs to mice resulted in a shift of the dose response curve for pentobarbital, an indication that glucose administration significantly sensitized the animals to the affects of the drug. The administration of glucose also reduced the microsomal P-450 catalytic activity as measured by the conversion of p-Nitroanisole to p-Nitrophenol. These results were consistent with previously published data and suggest that glucose administration may produce its effect on drug duration of action through a reduction in the hepatic metabolism of various compounds as previously suggested.

The administration of glucose to rats for 48 hrs also resulted in a prolonged effect of pentobarbital induced hypnosis. The spectral binding of hexobarbital and methadone was found to be significantly reduced following glucose administration. The changes in spectral binding were observed in the absence of any significant change in the microsomal P-450 content. The changes in spectral binding of hexobarbital and methadone to P-450 in glucose treated animals might suggest that glucose administration may be causing a qualitative or a conformational change in hepatic microsomal P-450 enzymes. The administration of glucose resulted in a significant increase in cytosolic protein content which might suggest that glucose is inducing enzymes

responsible for its metabolism and/or the synthesis of lipids from glucose.

In an attempt to understand the possible mechanism(s) by which glucose exerts its effect on the microsomal P-450 system, the effects of glucose administration were examined. Glucose treatment results in a significant reduction of hepatic glycogen content. Glucose treatment did result in significant increases in total microsomal lipid, phospholipid, and fatty acid content. Specific microsomal fatty acid and phospholipid contents were also measured following glucose administration. These results showed there was a significant increase in microsomal palmitic, palmitoleic, stearic, and oleic acids as well as the phospholipids phosphatidylcholine and phosphatidylethanolamine. Changes in lipid content and specific fatty acid content have been shown to be associated with changes in microsomal P-450 activity and the binding of various substrates to P-450. Changes in fatty acids within the membrane have been associated with alterations in the P-450 heme spin states which in turn produce qualitative or conformational changes in the P-450 enzymes as measured by spectral binding of substrates. Conformational changes in P-450 can enhance or reduce the binding of various substrates to P-450. The data reported in these studies suggest that glucose administration was associated with quantitative changes in specific microsomal lipids and that these lipid changes could have

influenced the activity of microsomal P-450 and the binding of drug substrates to P-450.

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