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THE EFFECTS OF STREPTOZOTOCIN-INDUCED DIABETES ON RESPONSES TO OPIATES AND OTHER CENTRALLY-ACTING PHARMACOLOGIC AGENTS

Glenn Stuart Simon

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by

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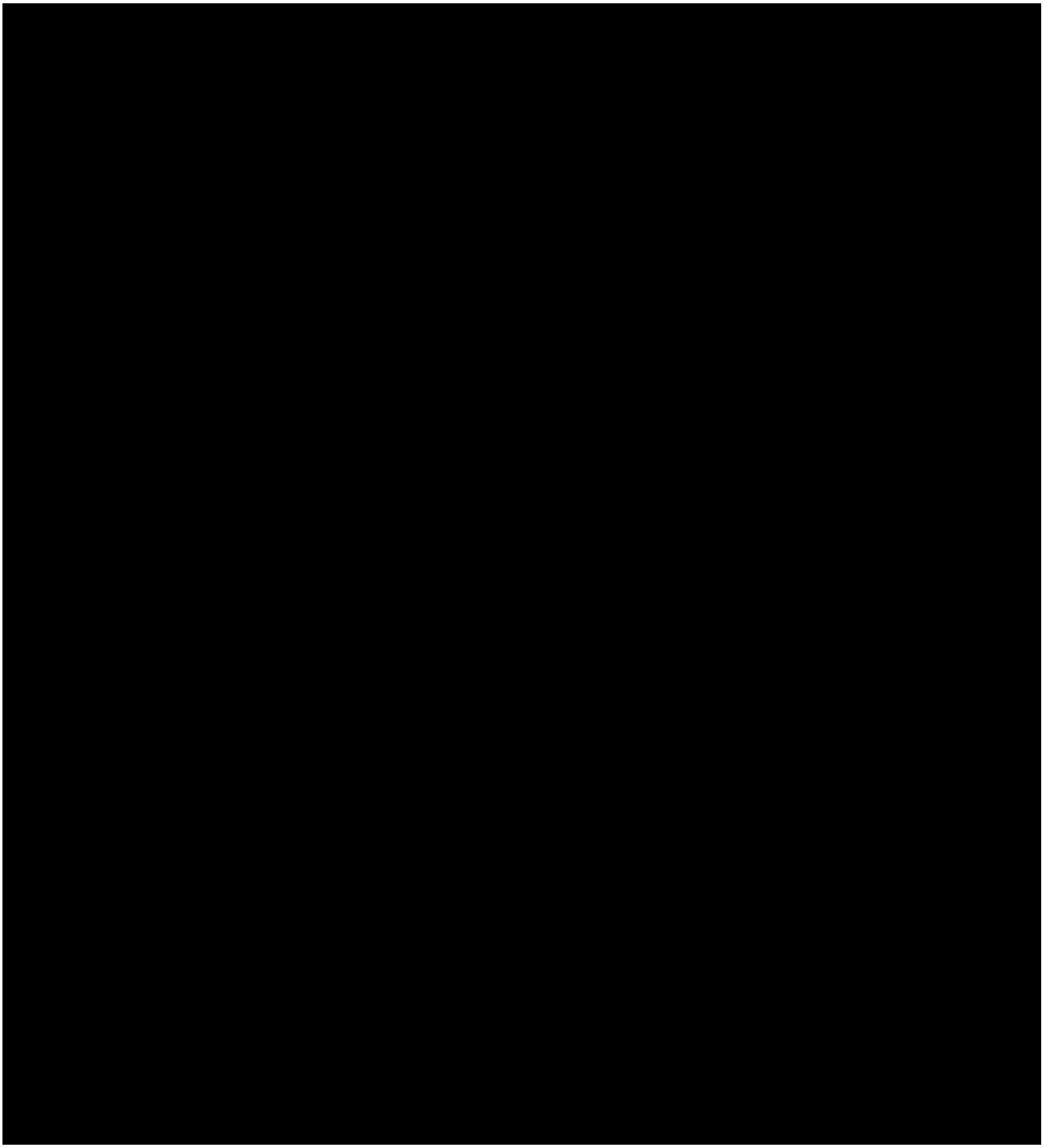

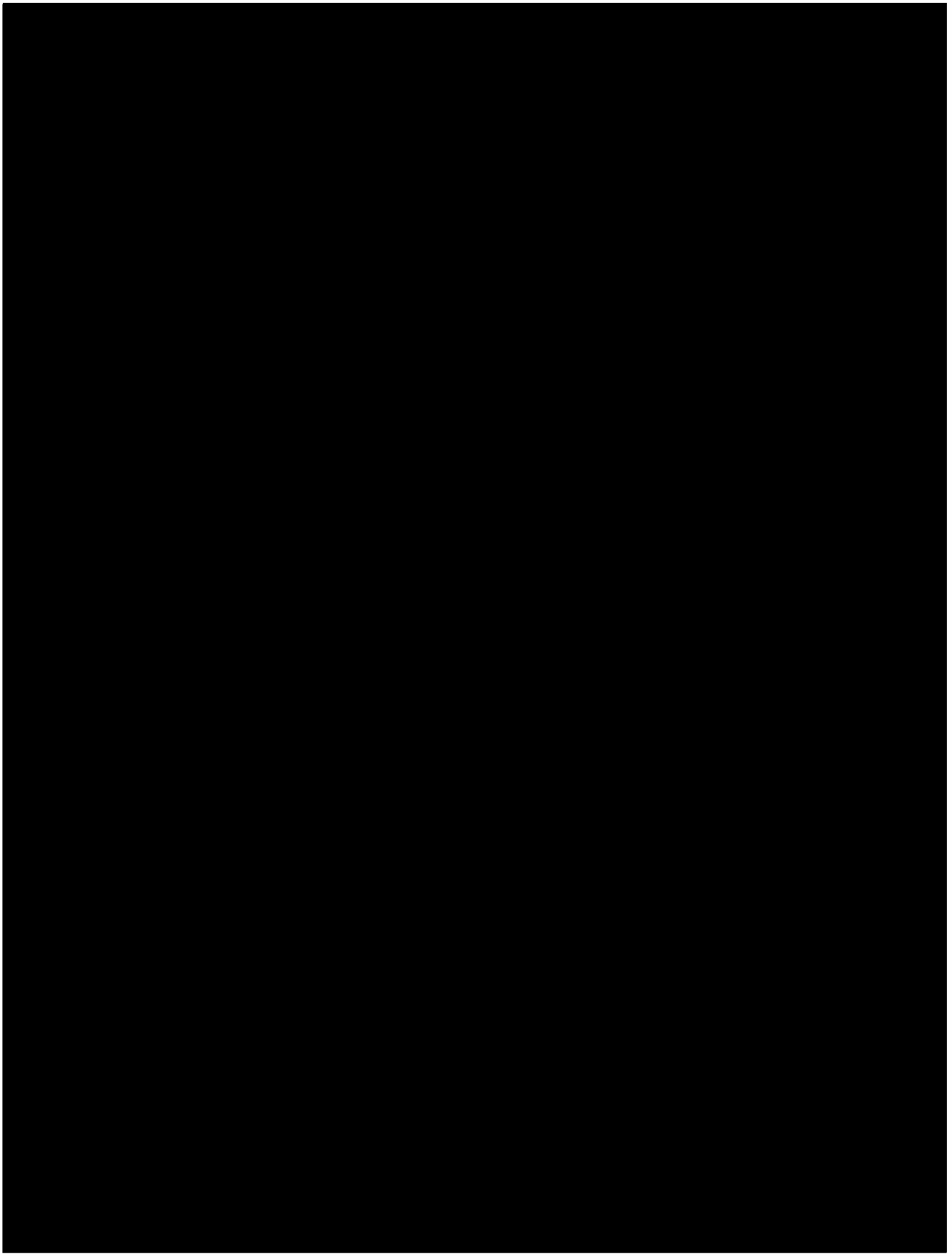

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DEDICATION

This thesis is dedicated to my wife, Sue, who has inspired me with her love and encouragement since the day we met; and to my daughter, Cori, whose rays of love and sunshine brighten all my days.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
°C	degrees centigrade
Ci	Curie
cm	centimeter
CNS	central nervous system
cpm	counts per minute
dl	deciliter
dpm	disintegrations per minute
DRC	dose-response curve
ED_{50}	dose expected to produce 50% of maximum possible response
EDTA	ethylenedinitrilotetraacetic acid
Eq	equivalent
g	gram
GO	glucose oxidase
G-6-P	glucose-6-phosphate
G-6-PDH	glucose-6-phosphate dehydrogenase
^{3}H	tritium
HCl	hydrochloric acid
$HClO_4$	perchloric acid
HK	hexokinase
H_2O	water
hr	hour
INT(INTH)	2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium-chloride (reduced)
i.p.	intraperitoneal

IRI	immunoreactive insulin
i.v.	intravenous
kg	kilogram
$KHCO_3$	potassium bicarbonate
LD_{50}	dose expected to cause 50% lethality
m	milli
M	molar
Mg++	magnesium ion
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mol	moles
MPE	maximum possible effect
N	normal
n	number in sample
N_2	nitrogen
NADP(NADPH)	nicotinamide-adenine-dinucleotide phosphate (reduced)
NaI	sodium iodide
ng	nanogram
nm	nanomole
pA_2	negative logarithm of dose required to decrease effect by 50%
6-PGA	6-phosphogluconic acid
pH	negative logarithm of hydrogen ion concentration
PMS (PMSH)	phenazine methosulfate (reduced)
PPQ	p-phenyl quinone
rpm	revolutions per minute

s.c.	subcutaneous
S.E.	standard error
sec.	second
STZ	streptozotocin
U	unit
uCi	microCurie
ul	microliter
uM	micromolar
uU	microunit
vs.	versus
$\bar{X}$	mean
+	plus

ABSTRACT

THE EFFECTS OF STREPTOZOTOCIN-INDUCED DIABETES ON RESPONSES TO OPIATES AND OTHER CENTRALLY-ACTING PHARMACOLOGIC AGENTS

Glenn Stuart Simon, Ph.D.

Medical College of Virginia-Virginia Commonwealth University, 1979

Major Professor: Dr. William L. Dewey

Diabetes mellitus affects millions of people. Although diabetics can lead relatively normal lives, all treatments for the disease are symptomatic and not curative. The purpose of this investigation was to determine whether the sensitivity to opiates and other selected centrally-acting drugs in animals is altered by streptozotocin (STZ)-induced diabetes. A second objective was to determine which aspect of the diabetic syndrome primarily was responsible for the altered sensitivity. Other experiments were performed in an attempt to elucidate the mechanism whereby this altered sensitivity occurred.

STZ-induced diabetes or dextrose-induced transient hyperglycemia did not have a significant effect on the duration of hexobarbital-induced anesthesia. Similarly, following 5 days treatment with phenobarbital, the duration of hexobarbital-induced anesthesia was reduced equally in both control and STZ-induced diabetic mice. STZ-induced diabetes did not alter the acute oral LD_{50} of nicotine.

The antinociceptive potency of morphine as determined by the tail-flick test was significantly decreased ($p < 0.05$) in STZ-induced diabetic mice and mice pretreated with equimolar doses of hypertonic dextrose or fructose. STZ-induced diabetic rats and spontaneously diabetic mice were also significantly less sensitive to the antinociceptive effects of mor-

phine as quantitated by the tail-flick test. The ability of morphine to inhibit phenylquinone-induced writhing was attenuated in STZ-induced diabetic mice. Hypoglycemic mice were significantly more sensitive to morphine in the tail-flick test. Insulin reversal of dextrose-induced and STZ-induced diabetic hyperglycemia returned sensitivity to morphine-induced antinociception in the tail-flick test to control values. Pretreatment with the non-metabolizable sugar 3-O-methylglucose at a dose equimolar to the doses of dextrose and fructose had no effect on morphine potency. The antinociceptive potencies of phenazocine and levorphanol were altered similarly to that of morphine, but the potencies of methadone, propoxyphene and meperidine were not altered by changes in blood glucose levels.

The LD_{50} of morphine but not methadone was significantly decreased in STZ-induced diabetic mice. These results confirm the selectivity of the STZ-induced diabetes to alter the sensitivity of morphine and not methadone, and are provocative since they show that the lethal effect of morphine is altered in the opposite direction from the antinociceptive potency.

Levels of morphine in the brains of STZ-induced diabetic and insulin-treated STZ-induced diabetic mice were not significantly different from control mice. The durations of action of morphine in STZ-induced diabetic and control mice were similar, although the level of antinociception in the diabetic mice was lower at all time points.

STZ-induced diabetes in mice did not alter serum osmolarity and brain water content. Mice receiving various pretreatments (STZ-induced diabetes, STZ-induced diabetes plus insulin, dextrose, fasting or fasting plus insulin) were subjected to analyses of their serum glucose levels, serum insulin levels, and brain glucose levels. From these data only

blood glucose levels correlated (inversely) with the antinociceptive potency of morphine.

The results of these experiments led to the hypothesis that the hyperglycemia was the aspect of diabetes principally responsible for selectively affecting the potency of certain opiate-like pharmacologic agents.

INTRODUCTION

The reasons for studying the pharmacologic and toxicologic effects of chemicals in laboratory animals are numerous. They include the determination of molecular mechanisms of action, kinetics of drug action, structure-activity relationships, etc. In many cases, the ultimate rationale for testing the actions of a chemical in animals is to provide information regarding its potential usefulness and hazards to man.

Most testing occurs in normal, healthy animals. Data obtained from these experiments are useful in predicting the effects of chemicals in humans. Therapeutic agents are designed to treat a specific disease; therefore, animal models have been developed which can, to some extent, mimic the characteristics of the disease seen in man. Thus, there is considerable information available concerning the effects of antihypertensive agents on hypertensive animals, hypoglycemic agents on diabetic animals, etc., and the effects of drugs like antihistamines, barbiturates, narcotics and non-narcotic analgesics, etc. on normal, healthy animals. But people with chronic diseases may take drugs not ordinarily associated with their particular disease state. Diabetics can suffer from insomnia, hypertensive individuals may also have allergies, and people with asthma can get headaches. Anyone may require surgery, and then suffer from postoperative pain. The objectives of this thesis are to determine whether animals with a particular pathophysiologic condition (experimental diabetes) have an altered sensitivity to selected centrally-acting pharmacologic agents and to determine the mechanism of this altered sensitivity.

Diabetes mellitus[1] was chosen for several reasons as the pathophysiologic condition to be investigated. It is one of the most common ailments in the United States. Over 10 million Americans suffer from diabetes, and the number of diabetics is rising by 6% each year (American Diabetes Association, 1976). The estimated life expectancy for diabetic humans is shorter than for nondiabetics (Bale and Entmacher, 1977). Diabetes mellitus, its causes, symptomology, treatment, and consequences have been the subject of hundreds of books, symposia and articles.

When severe diabetes mellitus occurs, many body tissues (e.g. muscle) are less able to absorb and utilize glucose from blood. The body reacts as if it is starving, initiating several processes (e.g. glycogenolysis, gluconeogenesis) with the net result being hyperglycemia. Glucose passes into the urine, carrying water with it, causing polyuria, dehydration and subsequently, polydipsia. The sensation of starvation also leads to polyphagia. Due to glycogenolysis, liver glycogen falls to very low levels, and muscle glycogen is lowered, but to a lesser extent. The ingested and newly formed endogenous glucose contributes to even greater hyperglycemia, glucosuria, polyuria, etc., creating a vicious cycle. In order to maintain the body's energy requirements, tissue-protein breakdown occurs (for gluconeogenesis) causing muscle wasting and increased urinary nitrogen excretion. Body fat depots are broken down, causing production of large quantities of ketone bodies which cannot be oxidized by muscles. This causes metabolic acidosis which can lead to coma and death. During acidosis, the excretion of large amounts of glucose and ketone bodies in

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[1]Diabetes mellitus, as discussed in this dissertation, is defined as a chronic disorder of carbohydrate metabolism characterized by hyperglycemia, resulting from an inadequate production or utilization of insulin (Taber, 1973).

the urine results in salt loss (Tepperman, 1973; Bhagavan, 1978). In diabetics where insulin deficiency is the cause of these conditions, administration of insulin promptly corrects all the above mentioned metabolic disturbances (Bhagavan, 1978). However, even with rigid control over blood glucose levels by insulin management, many of the long-term consequences of diabetes mellitus (neuropathy, retinopathy, atherosclerosis, microangiopathy and nephropathy) still occur (Hockaday, 1974).

There are a number of different animal models that have been developed which exhibit to varying degrees many of the characteristics of diabetes mellitus in humans (Meier, 1960; Renold, 1968; Rerup, 1970; Renold and Burr, 1970; Stauffacher et al., 1971; Renold et al., 1971; Renold et al., 1972; Cameron et al., 1972; Renold et al., 1974; Karl, 1975; Herberg and Coleman, 1977). These models may be categorized by the mechanism in which the diabetes-like syndrome is induced[2]. For example:

A. Virally-induced Diabetes. In this model (Craighead, 1975) the M variant of encephalomyocarditis virus is injected s.c. into mice. Virus-exposed mice display hyperglycemia, beta cell degranulation and destruction, and hypoinsulinemia. Susceptability to the virus varies with the strain of mouse used. There is limited epidemiologic evidence for a viral link in the etiology of human diabetes mellitus (Maugh, 1975, 1979).

B. Immunologically-induced Diabetes. In this model, animals (usually guinea pigs) are sensitized to insulin. Their antibodies to insulin are

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[2]Use of the terms "juvenile-onset" and "adult-onset" was avoided because of the overlap in symptomology aften seen in patients described as having one type or the other. Rather, some of the individual signs that are present in each animal model will be discussed, with no attempt to classify a model as being juvenile-onset or adult-onset in nature.

collected and then injected into a different species of animal. Wright (1961) has reviewed this model, and comments that often the amount of guinea-pig serum required to induce the diabetes-like syndrome (hyperglycemia, glycosuria and varying degrees of ketonuria) can also induce other severe immunologic reactions, which detract from the desirability of the model.

C. Surgically-induced Diabetes. In this model, animals are subjected to a complete pancreatectomy. Rats, for example, become hyperglycemic, glycosuric, and ketonuric within 18 hr (Scow, 1957). Rats not maintained with insulin die within 48 hr. Unfortunately, complete pancreatectomy removes the exocrine as well as the endocrine functions of the pancreas. Impaired intestinal absorption of nutrients also occurs, a characteristic not generally seen in human diabetes.

D. Spontaneous-diabetes. A spontaneously occurring diabetes-like syndrome has been reported to occur in many species, including dogs, cats, cattle, sheep, horses, pigs, monkeys, shrews, hippopotomi, foxes, dolphins, sekoke carp, ayu-fish and several rodents (Renold et al., 1974). Rodents have become the animals of choice as models of human diabetes because of their size, ease of handling, relatively low cost and relatively high frequency of occurrence of the diabetes-like syndrome. Rodent models may be divided into two categories, ketotic and non-ketotic. Until recently, the only rodent model of ketotic diabetes was the Chinese hamster (Gerritsen and Dulin, 1967). These animals are non-obese, hyperglycemic, glycosuric, severely ketotic, insulin-responsive and have increased fasting liver glycogen and circulating free fatty acid levels. The characteristics, etiology and genetics of this model have been painstakingly studied (Shirai et al., 1967; Sims and Landau, 1967; Malaisse et al., 1967;

Carpenter et al., 1967; Gundersen et al., 1967; Sirek and Sirek, 1967; Schmidt et al., 1970; Carpenter et al., 1970; Gerritsen and Blanks, 1970; Chang and Schneider, 1970; Gerritsen and Blanks, 1974; Chang, 1974; Chang et al., 1977a and b; Petersson et al., 1977). However, their poor temperament and breeding peculiarities have prevented Chinese hamsters from becoming a widely used model for studying human diabetes mellitus (Schwentker, 1957). In 1977, Nakhooda et al. reported a spontaneous diabetic syndrome in outbred Wistar rats of both sexes. Like the hamsters, these animals are non-obese, insulin-responsive, hyperglycemic, and show varying degrees of ketonemia. Further characterization and breeding of these animals is currently underway.

Of the several non-ketotic spontaneously-diabetic animal models of diabetes mellitus, the two most popular are the yellow KK mice, and the db/db mice. The KK mice are obese, hyperglycemic, etc., but not insulin-responsive. They have been shown to be hyperinsulinemic, and presumed to have a defect in insulin utilization. These animals are not generally available in the United States (Nakamura and Yamada, 1967; Yamada and Nakamura, 1969; Iwatsuka et al., 1970; Iwatsuka and Shino, 1970; Wehner et al., 1972). In addition, the diabetes-like condition undergoes remission by four months of age (Iwatsuka et al., 1974a and b). The recessive gene "db" (currently linked to the misty color "m" gene -- "dbm") was first reported in the C57BL/Ks mouse strain in 1966 (Hummel et al., 1966). Like the KK mice, the homozygous (dbm/dbm) mice are obese, hyperglycemic, non-ketotic and not insulin-responsive. Their genetics, characteristics, metabolism, etc. have been extensively studied and reviewed (Hummel et al., 1966; Coleman and Hummel, 1967; Like et al., 1972; Hummel et al., 1972; Staats, 1975; Chan et al., 1975; Chan and Exton, 1977; Coleman, 1979).

Because the dbm/dbm mice (both sexes) are sterile, heterozygotic pairs must be mated, yielding, on the average, one diabetic (determined at four weeks of age) of each sex per eight offspring. Their availability is limited and their cost is currently $11/mouse.

E. Chemically-induced Diabetes. The use of chemicals to alter insulin production, secretion and/or utilization is for several reasons the most popular means for causing diabetic syndromes in animals. Virtually any species of animal can be rendered diabetic. The cost of a diabetogenic agent is far less than that for spontaneously-diabetic animals. Different degrees of severity can be produced by adjusting the dose of the diabetogenic agent. Many different chemicals have been discovered to be diabetogenic agents, including 5,5-diphenyl-2-thiohydantoin (Mackerer et al., 1977), dehydroascorbic acid (Patterson, 1949), several chelating agents including 1-hydroxyacridine, potassium xanthate and Styrlquinoline 90 (Rerup, 1970) and even Vacor, a currently used rodenticide (Pont et al., 1979). The two most popular diabetogenic agents are alloxan and streptozotocin. Alloxan-induced diabetes was first reported to occur in 1943 in rats (Dunn and McLetchie, 1943), rabbits (Bailey and Bailey, 1943) and dogs (Goldner and Gomori, 1943). The only animal reported to be resistant to alloxan diabetogenesis was the guinea pig (West and Highet, 1948). In addition to hyperglycemia and glucosuria, alloxan-diabetic animals show a weight loss, hyperlipemia, ketonuria, and acidosis (Rerup, 1970). The metabolic consequences of alloxan-induced diabetes have been extensively studied (Dixon et al., 1961; Maurer et al., 1972; Ithakissios et al., 1974; Reaven et al., 1977; Peavy et al., 1978; Rinaudo et al., 1978; Awadallah et al., 1979). Unfortunately, alloxan administration can produce toxic effects not directly related to its diabetogenic action.

The kidney is most seriously affected. Vacuolation or hydropic changes, necrosis and desquamation of the tubular cells can occur, whereas the glomeruli appear normal. Toxic liver changes have not been reported consistently, but include fatty infiltration and central lobular necrosis. Some investigators have reported necrosis in the adrenal medulla and anterior pituitary gland. All these effects have been reviewed by Rerup (1970). It was the lack of specificity of alloxan that has led to the current popularity of streptozotocin (STZ) as a diabetogenic agent. STZ was fortuitously recognized as a diabetogenic agent in 1963 (Rakieten et al.) during preclinical trials as an anticancer chemotherapeutic agent. It is highly soluble but labile in water, and must be prepared at a pH = 4.0 and used within 60 sec of dissolution. It was found to be diabetogenic in all species tested (Rerup, 1970). Several investigators have compared and contrasted STZ- and alloxan-induced diabetes (Mansford and Opie, 1968; Rerup and Tarding, 1969; Veleminsky et al., 1970). Unlike alloxan-induced diabetic animals, animals injected with STZ are not generally ketotic and never return to their age-normal weight unless insulin is administered. The mechanisms by which alloxan and STZ cause diabetes are also thought to be different since the manifestations and consequences of their respective diabetic states differ. Almost 1000 papers have been published on the characteristics of STZ-induced diabetes and its effects on the body.

The mechanism(s) by which STZ causes diabetes is (are) not clear, although some information is available. Severe necrosis of the β-cells of the pancreas has been observed as has islet atrophy (Wilander, 1975). Nuclear enlargement and nucleolar loss in the β-cells have also been reported (Lazarus and Shapiro, 1972). Nicotinamide can prevent STZ-induced diabetes by preventing the STZ-induced decrease in pyridine nucleotide

content of the pancreatic islets (Schein et al., 1967) which accounts for the prevailing hypothesis that an interaction between STZ and DNA causes diabetes (Hinz and Pfeiffer, 1974). However, the antitumor effect of STZ is not modified by nicotinamide. This theory of STZ-DNA interaction is further supported by the observation that STZ-induced diabetes can be prevented by pretreatment with pertussis vaccine (Katada and Ui, 1977), where only a transient hyperglycemic state is seen. Also, STZ is a nitrosamine derivative, and so may act as an alkylating agent (Rakieten et al., 1963). Because of its specificity of effect in causing diabetes, and the large data base available, STZ was chosen as the agent to be used in producing diabetes.

Several pharmacologic agents have been shown to affect insulin secretion and/or blood glucose levels. Some agents can produce opposite effects on blood glucose levels due to different conditions of administration. Diphenylhydantoin administration inhibits insulin secretion in the isolated perfused rat pancreas (Levin et al., 1970). Diazoxide, an antihypertensive agent, causes hyperglycemia in dogs (Speight and Avery, 1971), and chlordiazepoxide can aggravate diabetic hyperglycemia in humans (Zumoff and Hellman, 1977). Ethanol can cause hypoglycemia, as can propranalol, steroids, salicylates, several diuretics and phenothiazines (Hansten, 1971). Hyperglycemia can be induced by salicylates, caffeine, phenothiazines, steroids, epinephrine, furosemide, heparin, imipramine, morphine and other drugs (Hansten, 1971).

It has been known for some time, that intraventricularly administered insulin can cause a prompt, profound hypoglycemic response in laboratory animals (Chowers et al., 1966; Szabo and Szabo, 1972). Controversy exists however, as to whether this effect is due to leakage into the systemic

circulation and/or a centrally mediated stimulation of pancreatic insulin release (Woods and Porte, 1975) or to a centrally mediated increase in glucose uptake by the liver without hyperinsulinemia (Szabo and Szabo, 1975a, b, c). Circumstantial evidence suggested that the presumed insulin "receptors" were localized in the ventromedial hypothalamus (Storlien et al., 1975). Havrankova et al. (1978a), determined where the specific binding of ^{125}I-insulin to rat brain homogenates occurred, and demonstrated the wide though uneven distribution of binding sites throughout the rat CNS. No area of the brain was without these binding sites. Later, these investigators reported levels of insulin in the brain from 10 to 100 times the serum level (Havrankova et al., 1979). Insulin binding sites were demonstrated on synaptosomes, leading these investigators to postulate a role for insulin as a neurotransmitter (Havrankova et al., 1979; Pacold et al., 1979). This hypothesis has yet to be proven. In genetically obese mice, where serum insulin levels are high, and insulin receptor levels in liver, fat and other tissues are low (Stauffacher et al., 1967; Kahn et al., 1973; Soll et al., 1975), normal concentrations of insulin and insulin binding sites are present in the brain (Havrankova et al., 1978b). The same is true for STZ-induced diabetic rats (Havrankova et al., 1978b; W.G. Blackard, personal communication). The observation that the levels of insulin and numbers of insulin binding sites are normal in the brains of STZ-induced diabetic rats does not necessarily mean that the system(s) in which these two components have functions operate properly. For example, it has been reported that the resting serotonergic levels and turnover in STZ-induced diabetic rats are normal (Mackenzie and Trulson, 1978c, e) but the serotonergic system does not respond normally to pharmacologic manipulation (Fernando and Curzon, 1978). Evidence which supports

the position that the central glucoregulatory system in diabetic animals is abnormal was recently published by Coimbra et al. (1979). They reported the following: In non-diabetic rats, if glucose or insulin is injected intraventricularly, plasma free fatty acid levels are markedly reduced. In alloxan-induced diabetic rats, plasma free fatty acid levels were not affected by intraventricularly administered glucose, but were decreased by intraventricularly administered insulin. Thus, the CNS glucoregulatory system appears to be functionally altered in diabetic rats. Experimentally-induced diabetes has already been shown to affect carbohydrate metabolism in the brain (Prasannan and Subramanvan, 1968; Flock et al., 1969; Ruderman et al., 1974). These alterations are reversed by insulin administration (Rinaudo et al., 1978). The diabetes-induced changes in brain carbohydrate metabolism could interact with drugs that have effects on brain carbohydrate metabolism, e.g. morphine (King et al., 1977, 1978).

There are conflicting reports in the literature concerning the effects of diabetes (or other conditions that alter blood glucose levels) in altering the sensitivity of animals to drugs. As early as 1951, Lamson et al. demonstrated that the injection of glucose i.v. in a dog awakening from pentobarbital-induced anesthesia caused a prompt return to narcosis. This could be repeated several times in the same animal, with the duration of anesthesia decreasing after each glucose injection until these injections no longer had any effect. This effect was subsequently shown to occur in several other species (hamster, rabbit, guinea pig) but not the rat or mouse. This effect of glucose was seen only in animals anesthetized with each of the five different barbiturates tried, but not with any of seven volatile anesthetics tested. Borzelleca and Manthei (1957) have shown that dehydration and changes in acid-base balance can alter the duration

of action of phenobarbital. Both of these are characteristics of human diabetes mellitus (Tepperman, 1973). Strother et al. (1971) and Strother (1979) reported that the intake of large amounts of glucose in the drinking water for 48 hr increased the duration of anesthesia induced by several barbiturates, and decreased the in vitro metabolism of barbiturates and p-nitroanisole in mice. Ackerman and Leibman (1975) and Ackerman (1976) reported that STZ-induced diabetes in rats reduced the in vitro activity of hexobarbital hydroxylation, and had no effect on aniline hydroxylation. In addition, they reported that continuous infusion of glucose had no effect on the hydroxylation of hexobarbital. In contrast Reinke et al. (1977 and 1978) reported marked increases in aniline hydroxylase activity in STZ-induced diabetic rats.

Hanasono et al. (1975a and b) have reported that carbon tetrachloride-induced hepatotoxicity (determined morphologically and by liver function testing) was increased in STZ- and alloxan-induced diabetic rats compared with nondiabetic rats. Insulin-treated diabetic rats were affected by exposure to carbon tetrachloride only to the same extent as nondiabetic rats.

Some investigations (Armstrong and Doll, 1975; Walsh et al., 1977) have been concerned with the combined effects of diabetes and cigarette smoking upon the incidence of bladder cancer and with alterations by cigarette smoke on blood sugar levels, and serum insulin and non-esterified fatty acid levels, respectively, in humans. No apparent interactions were observed. No other reports on the toxicity of nicotine in diabetic animals or humans are available.

Diabetes mellitus has many adverse effects on the autonomic nervous system and reactions to stress (Faerman et al., 1974; Stewart et al., 1976; Fraser et al., 1977; Bennett et al., 1977; Head and Berkowitz, 1977;

Eden et al., 1977; Fukuma et al., 1978). De Nicola et al. (1977) have reported that in STZ-induced diabetic rats, resting ACTH and corticosterone levels were significantly higher than in nondiabetic rats, mimicking a condition of chronic stress. These animals also secreted significantly more corticosterone than nondiabetic controls when stressed with an injection of cold water. They concluded that the threshold for steroid feedback was less sensitive to variations in plasma corticosterone levels. Zapf et al. (1975) have shown that STZ-induced diabetic rats were hyposensitive to the lipolytic effects of ACTH and glucagon, but hypersensitive to the lipolytic effects of epinephrine. Insulin administration reversed the hypersensitivity to epinephrine. High plasma levels of corticosterone accompanied by abnormally high ACTH secretion has been seen in newly diagnosed cases of human diabetes mellitus (W.G. Blackard, personal communication) which could result from a "starvation"-induced stress reaction. These high levels of ACTH in human and experimental diabetes are important when one considers that ACTH has been shown to inhibit the antinociceptive potency of morphine (Takemori, 1976) and inhibit the stereospecific binding of dihydromorphine (Terenius, 1975).

There have been other reports in the literature which suggest a direct interaction between experimental diabetes and an animal's sensitivity to centrally-acting drugs. Marshall et al. (1976) have shown that alloxan-induced diabetic rats have a decreased sensitivity to the anorexic and locomotor-stimulating actions of d-amphetamine. This effect could be partially reversed by feeding the diabetic rats a diet containing a high fat/protein ratio, which also partially ameliorates the diabetic-hyperglycemia (Marshall, 1978b). Mackenzie and Trulson (1978d) have reported that STZ-induced diabetic rats had a reduced sensitivity to L-tryptophan and p-chloroampheta-

mine. The occurrance of a behavioral syndrome consisting of resting tremor, rigidity or hypertonicity, hind limb abduction, Straub tail, lateral head weaving and reciprocal forepaw treading was used in these studies to assess the rats' sensitivity to the drugs (Jacobs, 1976).

Morphine has been shown to cause hyperglycemia in rats (Borison et al., 1962) by interacting at a site in the subfornical region of the brain (Borison et al., 1964; Moore et al., 1965). Apomorphine and etorphine produce marked hyperglycemia, but meperidine does so only weakly (Dey and Feldberg, 1975). Endogenous opiate-like peptides and morphine have been shown to affect several neuroendocrine functions at the hypothalamic level in rats (Meites et al., 1979). Thyrotropin releasing hormone caused "wet dog shakes" in rats, similar to those observed during morphine withdrawal (Martin et al., 1977). Morphine administration blocked this effect. Reed and Ghodse (1973) have reported that heroin addicts showed a delayed and attenuated increase in blood sugar levels following an oral glucose tolerance test. Resting insulin levels were higher in addicts than non-addicts, and the increase in plasma insulin levels in response to the glucose challenge was delayed and attenuated. Brambilla et al. (1976) have confirmed these findings, except that they reported an increase in plasma insulin levels in addicts following a glucose challenge. Ghodse (1977) subsequently confirmed all the results of Brambilla et al. (1976). Leslie et al. (1979) have postulated that non-insulin-dependent diabetes mellitus in humans might be due to an innate hypersensitivity to endogenous opiate-like peptides, although the evidence to support this hypothesis is circumstantial.

These many observations indicate that pharmacologic intervention and endocrine function in general, and effects secondary to diabetes mellitus

in particular, can play an important role in drug responses.

There are, in addition, many factors that can affect the potency of narcotics. Several of these factors may be important in diabetes. Glucagon can inhibit morphine-induced motor wave activity of the distal colon and rectum (Chowdhury and Lorber, 1977). Harris et al. (1975) have reported that intraventricular injections of calcium, magnesium or manganese ions can antagonize the antinociceptive effects of morphine. Lujan et al. (1978) confirmed the results with calcium and extended the work to include sodium. Electrolyte imbalance is not uncommon in diabetes (Tepperman, 1973), suggesting that the antinociceptive potency of narcotics could be altered in diabetic individuals.

Webster et al. (1976) have shown that old mice are significantly less sensitive to morphine than are young adults. Actinomycin D, steroids, promethazine, atropine, aspartic acid, prostaglandin E, and barbiturates have been shown to antagonize narcotic-induced analgesia, whereas sulphonamides, daunomycin and ouabain have been reported to potentiate analgesia (Takemori, 1976). Many neurotransmitters, and drugs which affect neurotransmission have been reported to affect narcotic potency. Classes of drugs reported to decrease narcotic potency include biogenic amine depletors, dopaminergic stimulators, α-adrenergic blockers, anticholenergics, monamine oxidase inhibitors, neurotransmitter precursors, anxiolytic drugs, cyclic nucleotides, antipsychotics and hallucinogens; while cathecholamines, amphetamines, cocaine, L-dopa, dopaminergic blockers, α-adrenergic blockers, antidepressants, antipsychotics, monoamine oxidase inhibitors, serotonin and parasympathomimetics have been reported to potentiate narcotic action (Dewey et al., 1970; Calcutt et al., 1971; Hansten, 1971; Major and Pleavry, 1971; Sparkes and Spencer, 1971; Scheel-Kruger, 1973;

Tulunay et al., 1973; Takemori, 1976; Maickel et al., 1976; Carenzi, 1978; Sprague and Takemori, 1978).

In a paper providing limited procedural detail, Davis et al. (1956) reported that insulin-induced hypoglycemia potentiated the antinociceptive action of morphine in the rat tail-flick test. Using two different doses of glucose and two different routes of administration, they found no dose-related effects of glucose administration on the antinociceptive potency of morphine were observed. They did not attempt to correlate blood glucose levels with changes in the potency of morphine. Since hyperinsulinemia will persist after, and hypoglycemia will often follow glucose-induced hyperglycemia (Tepperman, 1973; Matschinsky et al., 1975), information concerning blood glucose levels at all time points where antinociception was assessed is essential for a proper interpretation of the data. It is for this reason that morphine-induced antinociception in STZ-induced diabetic animals was investigated together with an assessment of key biochemical indices.

The tail flick test (D'Amour and Smith, 1941), as modified by Dewey et al. (1970), was used to compare the antinociceptive potency of morphine in diabetic and control mice. This procedure is a very specific test for analgesics of the morphine-type. Although several other methods which involve responses other than the tail-flick reflex are available for assessing narcotic-induced antinociception (Randall and Selitto, 1957; Pearl and Harris, 1966; Fennessy and Lee, 1975), the tail-flick test has been the most widely used. Nociceptive stimuli such as hot water (Ben-Bassat et al., 1959), radiant heat of fixed intensity and variable duration (D'Amour and Smith, 1941) or radiant heat of fixed duration and variable intensity (Hardy et al., 1940) have been used in this test. Radiant heat of fixed

intensity was used in this dissertation. Generally, mechanical techniques, electrical stimulation and behavioral tests either require sophisticated equipment or long preparatory time, or have limited reproducibility (Fennessy and Lee, 1975).

The purpose of these investigations was to determine which portion of the diabetic syndrome primarily was responsible for the altered sensitivity to morphine observed in pilot experiments. Additional experiments were designed in attempt to elucidate the mechanism of how this portion of the pathophysiological syndrome in turn altered the potency in diabetic mice. It is hoped that these results might provide some insight into the pathology of diabetes mellitus and/or the mechanism of action of morphine.

METHODS

I. General Methods and Pretreatments

Female animals were used, since in humans, diabetes mellitus is reported to be more severe in females than males (Amer. Diab. Ass., 1976; Bale and Entmacher, 1977). Using female animals might increase the probability of detecting diabetes-induced changes in sensitivity to the drugs used in these studies. The species and strains of animals used were: Outbred ICR Swiss mice (weighing 15-30 g); outbred Sprague-Dawley rats (200-250 g); and C57BL/KsJ (dbm/++ or dbm/dbm) mice (20-45 g). All drugs were dissolved in distilled water except STZ which was dissolved in 0.01 M sodium citrate, adjusted to a pH of 4.0 with HCl. In all statistical analyses, $p < 0.05$ was considered to be significant.

The following pretreatments were administered to animals to induce diabetes or to modify their blood glucose levels (BGL's). Data from preliminary studies were used to determine the optimum dosages and pretreatment times. BGL's at the appropriate times are shown in section IV A. of the Results. All animals were allowed access to food and water ad libitum unless otherwise indicated.

A. Swiss Mice

1. Control. No pretreatment.

2. STZ-diabetic. STZ was supplied by the Upjohn Co. and administered (200 mg/kg i.v.) not less than 6 days prior to the experimental day. The actual number of days is reported in the Results section for each experiment. Observations were recorded on the effects of STZ-induced diabetes on body weight and survival for 126 days following STZ administration.

3. Insulin-managed STZ-diabetic. Lilly regular porcine insulin (3 U/kg s.c.) was administered to STZ-diabetics (as in 2. above) 20 min before drug treatment. BGL's were determined in each animal during each experiment to verify the return of BGL's to normal. This dose was necessary to achieve normal blood glucose levels.

4. Sugars. Either dextrose, fructose or 3-O-methyl glucose (Sigma) was administered (0.028 mol/kg i.p.) 25 min prior to drug treatment. Administration of dextrose produced hyperglycemia which persisted for about 90 min.

5. Fasted. Animals were allowed access to water only for 18 hr prior to drug treatment.

6. Fasted plus (+) insulin. Lilly regular porcine insulin (1 U/kg s.c.) was administered to fasted animals (as in 5. above) 20 min before drug treatment. This dose was sufficient to cause hypoglycemia for > 120 min.

B. Rats

1. Control. No pretreatment.

2. STZ-diabetic. STZ (60 mg/kg i.v.) administered 6 days prior to the experimental day.

C. C57Bl/KsJ Mice

No pretreatments were administered. Animals were 6-7 weeks old.

II. Narcotic-Induced Antinociception and Related Studies

A. Tail-Flick Procedure

A reliable method for assessing the antinociceptive potency of drugs in animals is the tail-flick test.

1. Experimental procedure. The procedure used was that of D'Amour and Smith (1941), as modified by Dewey et al. (1970). The tail-

flick apparatus consisted of a radiant heat source, photocell, timer and power supply. The heat source was a 100 watt projection lamp mounted in a reflector and situated above the photocell. The intensity of the lamp was controlled by a rheostat on the power supply. The animal's tail was placed in a slit over the photocell, preventing light from reaching the photocell. A common switch activated the lamp and the timer. When the animal flicked its tail, light fell on the photocell, automatically stopping the timer. The lamp intensity was adjusted to give a control flick latency of 2-4 sec. Animals whose control latency was outside this range were rejected. Maximum antinociception following administration of an analgesic (test latency) was defined as 10 sec (mice) or 20 sec (rats). Each animal was tested before a pretreatment was administered, immediately before narcotic administration, and 20 min after narcotic administration.

The antinociceptive effect was calculated as the percentage of the maximum possible effect using the formula:

$$\frac{\text{Test latency-control latency (sec)}}{\text{10 (mice) or 20 (rats) sec}} \times 100 = \text{\% Maximum possible effect (\% MPE)}$$

At least three doses of each narcotic per pretreatment were tested. For statistical calculations, doses were converted to log doses.

2. Statistical evaluation. An analysis of covariance was used to test for a statistically significant shift of the dose-response curve (DRC) for each pretreatment group vs control (Barr et al., 1976). The % MPE for each animal of all pretreatment groups was entered into a SAS computer. The computer generated a single linear regression which best fit all data points (log narcotic dose, % MPE). If the raw data from any pretreatment group did not fit the regression line generated, the program

would be rejected. This never occurred. The y-intercept (b) for the regression line and each pretreatment's DRC was determined by the computer, as were the slope of the curves (m), the mean square error (S^2), the number of degrees of freedom (N_t) and the corrected sum of squares (ΣS_{xx}). The log of the potency ratio (log M) of a narcotic in a pretreatment group vs control is described by: log M $(\bar{X}_c - \bar{X}_p) - \frac{(\bar{Y}_c - \bar{Y}_p)}{b}$ where $\bar{X}_c$ and $\bar{X}_p$ are the mean doses administered the control and pretreatment groups, respectively, and $\bar{Y}_c$ and $\bar{Y}_p$ are the mean % MPE for the control and pretreatment groups, respectively. The 95% confidence limits of the log of the potency ratio is described by: $M \pm (\log R)\ (t)$ where log R = standard deviation of log M and t = the critical value of t for N_t degrees of freedom. Log R is defined as the square root of the variance (v) of log M, determined by $v = \frac{S^2}{m^2}\left(\frac{1}{n_c} + \frac{1}{n_p}\right) + \frac{(\log M - X_c + X_p)^2}{\Sigma S_{xx}}$, where n_c and n_p are the number of animals tested in the control and pretreatment groups respectively. When log M and log R have been calculated, the antilogs of each (M) and (R) are calculated. The interval $M \pm (R)(t)$ must not include 1.00 for there to be a significant shift in the log dose-response curve of a pretreatment group vs control.

3. Narcotics tested. The analgesics tested in the tail-flick test were morphine sulfate, phenazocine hydrobromide, levorphanol tartrate, methadone hydrochloride, meperidine hydrochloride, and propoxyphene hydrochloride.

B. Phenylquinone-Induced Writhing Procedure

This test also quantitates narcotic potency in animals, and was used to confirm some of the results seen with the tail-flick test. The procedure used was a modification of that of Pearl and Harris (1966). Following the appropriate pretreatment, mice were injected s.c. with graded

doses of morphine (test). Ten minutes later the mice were injected i.p. with 0.1 ml/10 g of 0.03% p-phenylquinone (Sigma) made up as a 10% ethanolic solution. Beginning 10 minutes later, the number of writhes (stretching, twisting of torso or contraction of abdomen) for each mouse was counted for 1 min, and then 5 min later for 1 min again. Thus, the number of writhes in each mouse was counted for 2 min. For each pretreatment tested, one group of mice received the appropriate pretreatment and phenylquinone, but no morphine (control). The % protection against writhing was calculated as: $\%\ \text{Protection} = 1 - \frac{(\text{test \# writhes})}{(\text{control \# writhes})} \times 100.$ The % protection for each group was plotted on log-probit paper, and the ED_{50}'s determined by interpolation. Then, the 95% confidence limits of the ED_{50}'s, slope functions and potency ratios were calculated by the log-probit method of Litchfield and Wilcoxon (1949).

C. Duration of Action of Morphine

If the absorption, metabolism or excretion of morphine were altered by the induction of STZ-diabetes, this might be reflected as a change in the duration of antinociceptive action in the tail flick test unless two or more of these parameters were altered in different directions yielding no net difference. STZ-induced diabetic and control mice were injected s.c. with 4 mg/kg morphine and then tested on the tail-flick apparatus 5, 10, 20, 40, 80 or 120 min later. At each time point the % MPE was calculated as described on page 18. Due to the possibility of cumulative heat-induced nerve damage to the tails of the mice, different mice were tested at each time point.

D. Naloxone Antagonism of Morphine-Induced Antinociception

A study to determine the pA_2 value for naloxone antagonism of morphine-induced antinociception in the tail-flick test (control vs

STZ-induced diabetic mice) was conducted in the manner described by Takemori et al. (1969) with modification. After the appropriate pretreatment mice were administered naloxone hydrochloride at either 0, 0.03, 0.1 or 0.3 mg/kg s.c. Ten min later, 6.0 mg/kg morphine sulfate was administered by the subcutaneous route. Twenty minutes following morphine, the animals were tested for tail-flick latency. Statistical evaluation of the data was conducted by the analysis of covariance (Barr et al., 1976) as described in the section on tail-flick methods (page 19) and the method of Litchfield and Wilcoxon (1949). The negative logarithm of the dose of naloxone hydrochloride that reduced the antinociceptive effect of morphine alone by 50% = pA_2. If the pA_2 values were different for diabetic and control mice, this would suggest that different receptors were being bound by naloxone in the two groups.

E. Radiotracer Studies

1. Preparation of samples. Tritiated morphine (1-^{3}H) with a specific activity of 20-30 Ci/mM was purchased from Amersham Corporation. One hundred uCi of tritiated morphine was added to a solution of 4 mg/10 ml unlabeled morphine sulfate. Control, STZ-induced diabetic and insulin-injected STZ-induced diabetic mice were administered the ^{3}H-morphine solution. The antinociceptive potency of morphine was quantitated using the tail-flick test as described on page 18. Immediately after the post-morphine tail-flick determination, each mouse was decapitated, and trunk blood collected into micro-centrifuge tubes. The blood was allowed to clot for 10 min, and then centrifuged. The supernatant serum was transferred to small vials and stored at -20°C. The brains were dissected out, cleansed of adherant blood and dried. A mid-sagittal incision was made, and each brain half was weighed and placed in a paper cone for oxidation.

Serum, tritium standard and ^{3}H-morphine solution samples were pipetted onto paper cups inside paper cones to facilitate combustion in the oxidizer.

2. Tissue oxidation. Brain sections and serum samples were combusted in a Packard Tri-Carb model #B306 tissue oxidizer. This procedure allowed the ^{3}H present in the samples to be carried by H_2O into Packard Monophase-40 scintillation cocktail for counting. Brain samples with known amounts of radioactivity added and brain samples with no radioactivity (blank) also were combusted to determine % recovery and % memory (retention) in the oxidizer. All standard and blank samples were counted in a Beckman model #LS-3133T liquid scintillation counter before any other samples were combusted to assure proper functioning of the oxidizer (virtually 100% recovery and 0% memory). Samples of the drug solution administered to the mice were combusted and counted to determine the number of cpm/mg of morphine. This value was used in the determination of morphine levels in brain and serum.

Sample sizes for combustion were 25 ul for serum (in duplicate), and drug solutions, standard solutions and blank solutions (in triplicate). The right and left halves of each brain were oxidized separately. The amount of time necessary for complete combustion was 10 sec/liquid sample and 1 min/brain sample.

3. Liquid scintillation counting. The cocktail from the oxidizer was dispensed into plastic scintillation vials which were capped, shaken vigorously for 10 sec and then let stand overnight to eliminate chemiluminescence. Vials were then placed in the Beckman counter and programmed for 10 min counting or not greater than 2% counting error, whichever occurred first. The counter also was programmed to determine the ex-

ternal standards ratio (ESR) for each sample (a relative measure of the amount of quench by water in each vial). The mean number of counts per minute (cpm) from at least 10 blank samples was subtracted from the number of cpm determined for each sample vial, yielding a net number of cpm.

A quench curve for the counter had previously been generated by adding known amounts of ^{3}H and water to Packard Monophase-40 cocktail, and then plotting the % counting efficiency (obtained from the number of cpm reported by the counter) vs the ESR determined by the counter for each sample. The % counting efficiency was defined as:

$$\frac{\text{\# cpm detected}}{\text{\# cpm added}} \times 100 = \text{\% counting efficiency}$$

From the ESR determined by the counter for each unknown sample the counting efficiency (in these experiments 25-32%) was determined by reading directly from the curve. The number of disintegrations per minute (dpm) was determined by the equation:

$$\text{dpm} = \frac{\text{cpm} \times 100}{\text{\% counting efficiency}}$$

The number of dpm in each mouse's serum sample was averaged yielding dpm/5 ul serum. In brain halves, dpm/brain-half were added yielding total number of dpm/total brain weight. The number of dpm/ml serum or /g brain was then determined by simple proportions. From the known specific activity (# dpm/ng morphine) of the drug solution; the number of ng Eq morphine/ml serum or /g brain were determined. The term "Eq" is used to signify that all ^{3}H present was assumed to be in the form of ^{3}H-morphine.

Student's t-test was used to detect statistically significant differences in serum or brain morphine Eq levels.

III. Other Pharmacologic Studies

A. LD_{50} Determinations

Changes in the antinociceptive potency of narcotics due to altered blood glucose levels may not reliably predict changes in lethality, if the mechanisms by which antinociception and lethality occur are not the same. The log-probit method of Litchfield and Wilcoxon (1949) was used to construct dose-response curves for the acute lethality of subcutaneously administered morphine or methadone in STZ-induced diabetic and control mice. Nicotine was also tested since many diabetics smoke. Groups of at least six animals were administered each dose of the test drug. The percentage of animals dead by 24 hr following drug administration was used as the endpoint in the statistical calculations. The LD_{50} of each drug in each pretreatment group was determined from the curves constructed, and the 95% confidence limits of the LD_{50}, slope functions and potency ratios were calculated according to the log-probit method (Litchfield and Wilcoxon, 1949).

B. Duration of Hexobarbital-Induced Anesthesia

Other investigators have published conflicting reports on the effects of altering BGL's on the subsequent metabolism of barbiturates in vitro. We decided to investigate the effect of altering BGL's on the action of barbiturates in vivo. Control, STZ-induced diabetic and dextrose-pretreated mice were injected i.v. with 100 mg/kg hexobarbital sodium. Anesthesia occurred immediately, the mice were laid on their sides and the duration of anesthesia in each animal was quantitated. Awakening was defined as the time of return of the righting reflex. (To be considered awake, each mouse had to right itself three times in 10 sec.)

Beginning the next day, the control and diabetic mice were in-

jected with phenobarbital sodium each day for five consecutive days at 60 (1st day), 80 (2nd day, etc.), 100, 100 and 100 mg/kg i.p. The following day, 100 mg/kg hexobarbital sodium was again administered i.v. and the duration of anesthesia ("post-phenobarbital") determined. A nonpaired Student's t-test was used to determine the level of significant differences between the pre- and post-phenobarbital durations of anesthesia in each pretreatment group vs control, and the pre- and post-phenobarbital durations of anesthesia within each pretreatment group. A paired Student's t-test was used to compare the effects of phenobarbital pretreatment among pretreatment groups.

IV. Analyses of Biochemical Changes Induced by Pretreatments

A. Glucose Level Determinations in Whole Blood or Serum

Two methods were used. Both are free from interference by sugars other than glucose. BGL's were determined when screening for diabetes (> 250 mg/dl glucose in STZ-treated mice) since this method is economical but only semi-quantitative. For precise determinations, serum glucose levels (SGL's) were tested.

1. BGL's were determined by a test-strip glucose-oxidase (GO) method (Dextrostix^{R}, Ames Co., Elkhart, Indiana). Animals were placed under light ether anesthesia, and 50-60 ul of blood was drawn into a capillary pipet inserted into the animal's retro-orbital sinus. A large drop was placed over the entire reagent area on a test strip. Exactly 60 sec later, the blood was washed off with a stream of water. A semiquantitative (within 25 mg/dl up to 175 mg/dl, or simply > 250 mg/dl) measurement was made by comparing the color of the test strip with a standard color chart.

2. SGL's were determined by the hexokinase (HK) method (General Diagnostics Gluco Strate, General Diagnostics, Morris Plains, NJ). Blood was collected as described above and evacuated into a 250 ul centrifuge tube, allowed to clot for 10 min, centrifuged, and the supernatant serum used for the assay. Ten ul of serum was added to 0.5 ml of enzyme reagent containing > 0.32 I.U. of HK, > 0.06 I.U. of G-6-PDH, 0.75 M ATP, 0.2 uM NADP, 25 uM Mg^{++}, tris buffer, EDTA, protein and methylparaben. Samples were placed in a 37°C water-bath and preincubated for 3 minutes. One hundred ul of color developer containing 50 mg PMS, 200 mg INT and methylparaben was added to the samples. The tubes were vortexed, and then incubated at 37°C for 10 min. Diluent (2.5 ml of 0.1 N HCl) was added forcibly. The tubes were vortexed, and the optical density of each sample was determined at 500 nm in a Bausch and Lomb Spectronic 20 spectrophotometer. Glucose levels (mg/dl) were calculated from simultaneously run standards. Calibration curves retained linearity through 400 mg/dl (data not shown). The reactions involved were as follows:

$$\text{Glucose} + \text{ATP} \xrightarrow[Mg^{++}]{\text{HK}} \text{ADP} + \text{G-6-P}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6-PGA} + \text{NADPH}$$

$$\text{NADPH} + \text{PMS} \longrightarrow \text{NADP} + \text{PMSH}$$

$$\text{PMSH} + \text{INT} \longrightarrow \text{PMS} + \text{INTH (red formazan)}$$

Simultaneously drawn and processed blood and serum samples from control and diabetic mice tested for their glucose levels verified the reliability of the test-strip method (data not shown).

B. Glucose Level Determinations in Brain

Changes in BGL could alter the levels of glucose in the brain, which, in turn, could affect responses to centrally-active pharmacologic agents. The method of Lowry et al. (1964) with the modifications described

by King et al. (1977) was used to quantitate brain glucose levels. At the appropriate time after pretreatment, mice were plunged into liquid N_2 (-150°C) and agitated vigorously for 1 min to facilitate rapid freezing. About 100 mg of cortex was removed in a cold chamber at -20°C to prevent thawing. Frozen animals and dissected cortices were kept at -80°C, except for the few minutes necessary for dissection. The cortex was powdered in a mortar chilled in liquid N_2. The powder was added to a tube containing 0.3 ml of 3 M $HClO_4$ and agitated until the acid had completely penetrated the powder and removed the H_2O (3 to 5 min). To each sample was added 1.25 ml of 1 mM EDTA in H_2O, and after mixing at 4-5°C for 10 min, the tubes were centrifuged, and 1 ml of supernatant was mixed with 0.33 ml of 2 M $KHCO_3$. The pH of the solution was adjusted to between 7.5 and 8.0 by the addition of approximately 25 ul of 2 M Tris base. Samples were kept at -80°C until they were analyzed for their glucose levels.

The determination of glucose levels in brain was similar to the determination of SGL's (HK method), except that the final reaction yielded a fluorescent material measured in an Aminco spectrofluorometer (Lowry et al., 1964). Brain glucose levels were interpolated from a curve generated by adding known amounts of glucose to the extraction reagents, and assayed simultaneously with the brain samples. Statistical comparisons of brain glucose levels in pretreatment groups vs control were made by Student's t-test.

C. Insulin Level Determinations in Serum

Sugar-induced hyperglycemia, insulin injection or diabetes could affect serum insulin levels, disrupting the body's metabolic profile, and affecting responses to drugs. A modification of the ^{125}Iodine (^{125}I) radioimmunoassay technique of Morgan and Lazarow (1963) was used to de-

termine the concentration of immunoreactive insulin (IRI) in serum samples of mice from the various pretreatment groups.

1. Preparation of columns and equilibrating solution. Isotonic saline was poured on dry sephadex G25 and G75 (in separate vessels) and left to equilibrate overnight. Burettes (25 ml) were cut off at the 11 ml mark and the bottoms packed with glass wool. With 2-5 ml of saline already added to the burettes, the gel-saline mixture was poured into the columns with the stopcock open. The gel was thus packed at the liquid flow rate. The dimensions of the packed columns were 1 x 12 cm. The G25 column was used just after the iodination of the insulin tracer for the separation of free and insulin-bound ^{125}I as described below. The G75 column was used before the actual assay was performed to separate damaged (impure) tracer from the usable tracer as described below. Before use, both columns were equilibrated with a modified phosphate buffer with the following constituents: 36 g sodium chloride (NaCl), 11.88 g anhydrous dibasic sodium phosphate, 3.48 g mono-basic sodium phosphate, 0.4 g thimerosal, and 8.0 g albumin, which were dissolved in 2000 ml H_2O, and then water was added to make 4000 ml. The final solution was equivalent to 0.025 M phosphate buffer with 0.9% NaCl, 0.1% thimerosal and 0.2% albumin. (All phosphate buffers were at a pH of 7.5.)

2. Diluting fluid preparation. Equilibrating solution (100 ml) was added to 0.8 g albumin (increasing the albumin content to 1.0%). This solution was used to dilute all unknowns, to formulate standards, and to dilute the tracer.

3. Brom-phenol blue-stained diluting fluid preparation. Fifteen mg of brom-phenol blue was added to 100 ml of diluting fluid. This was used as a marker on the columns since it is eluted coincidentally with the

crude ^{125}I-insulin tracer and just prior to purified, usable tracer.

4. Production of anti-sera I. One-half ml of isotonic (0.9%) saline was added to 0.5 mg of pork insulin (Lilly): an equal volume of the insulin solution was added to complete Freund's adjuvant (Difco). The adjuvant-insulin mixture was emulsified by repeatedly being drawn into a 20 gauge needle attached to a syringe. Guinea pigs were immunized by the s.c. injection of 2 mg insulin (in the adjuvant-insulin emulsion) every 3 weeks. Ten days after the third injection, the guinea pigs were exsanguinated by cardiac puncture. The blood was allowed to clot for 10 min, centrifuged, and the supernatant serum stored at -80°C until use.

5. Anti-Sera II. This was purchased from Arnel Laboratories. It contains rabbit antibodies to guinea pig sera. It was diluted 1:2 with the diluting fluid described in Section 2, and 12.5 mg/ml EDTA was added as a preservative.

6. ^{125}Iodination of insulin tracer. A modification of the method of Greenwood *et al*. (1963) was used to prepare the ^{125}I insulin tracer.

Sodium ^{125}Iodide ($Na^{125}I$) was purchased from Amersham Corp. (specific activity 1 mCi in 0.1 ml). Fifty ul of 0.1 mg/ml pork insulin in 0.05 M phosphate buffer, 20 ul of 0.5 M phosphate buffer, and 20 ul of 1 mg/ml chloramine-T in 0.05 M phosphate buffer was added to the ^{125}I. Eleven to 13 sec later 20 ul of 5 mg/ml sodium metabisulfite in 0.05 M phosphate buffer, 50 ul of 50 mg/ml potassium iodide in 0.05 M phosphate buffer and 50 ul of the brom-phenol blue stained albumin solution were added. Two hundred ul of the resulting mixture were put on the sephadex G25 column and collected in 1 ml fractions. Useable tracer was eluted coincident with the blue dye.

7. Purification of crude ^{125}I-insulin. Fifty ul of the bromphenol blue stained albumin, 75 ul of the crude tracer and enough normal guinea pig sera to yield a total volume of 210 ul were put in a small test tube. This mixture was incubated at 4°C for 24 hours. 200 ul of the mixture was placed on the sephadex G75 column and 1 ml fractions were collected. Useable, purified tracer was eluted in the first 2 ml after elution of the blue dye.

8. Preparations of insulin standards. Crystalline pork insulin, assayed at 25.9 U/mg was used to prepare standard solutions. A stock solution containing 500 uU/ml in diluting fluid was prepared. Working standards of 160, 120, 80, 40 and 10 uU/ml were prepared from the stock solution.

9. Insulin radioimmunoassay. This procedure is a modification of the method of Morgan and Lazarow (1963). The following tubes were set up: Blank (triplicate) consisting of 200 ul diluting fluid and 50 ul tracer; Anti-sera Blank (triplicate) containing 100 ul diluting fluid, 50 ul tracer and 100 ul anti-sera I; Standards (triplicate) containing 100 ul insulin standard, 50 ul tracer and 100 ul anti-sera I; and unknown sera (6 samples/pretreatment in duplicate) consisting of 100 ul of serum (which was diluted 1:2, 1:3 or 1:4 so insulin concentration is < 160 uU/ml), 50 ul tracer and 100 ul anti-sera I. All tubes were incubated for 2 days at 4°C. 100 ul of anti-sera II was added to each tube. All tubes were then incubated at 4°C for one additional day. Tubes were then centrifuged at 4°C, 3000 rpm for 20 min, and the supernatant aspirated and discarded. The precipitates (containing antibody-bound ^{125}Insulin) were resuspended and washed with 0.3 ml of cold equilibrating solution. Following centrifugation at 4°C, 3000 rpm for 20 min, the washing fluid was aspirated and

discarded. The tubes were placed in counting tubes, and counted in a Gamma counter for 5 minutes. The number of cpm recorded by the counter for the standards and unknowns, was used to calculate the % bound immunoreactive insulin (IRI) as:

$$\% \text{ bound IRI} = \frac{\text{cpm in standard or unknown}}{\text{cpm in anti-sera blank (100\% bound)}} \times 100$$

A standard curve of % bound IRI vs insulin (uU/ml) was prepared and the actual insulin concentrations in unknown samples were determined by interpolation from the curve. Significant differences in serum insulin levels from pretreatment groups vs control were determined by Student's t-test.

D. Brain Water Content and Serum Osmolarity

The hyperglycemia caused by diabetes could increase serum osmolarity sufficiently to induce dehydration of the brain, altering drug distribution in the brain.

1. Brain water content. Mice were decapitated, and their brains removed, cleansed of adherant blood, blotted and weighed. They were then placed in an oven and dried to a constant weight (overnight). The next morning they were weighed again, and the % H_2O by weight was calculated as:

$$\frac{\text{Wet Weight - Dry weight}}{\text{Wet weight}} \times 100 = \% \ H_2O$$

2. Serum osmolarity. Trunk blood was collected into centrifuge tubes from 6 mice following decapitation and allowed to clot for 10 min[3]. The tubes were centrifuged and the supernatant serum transferred into smaller stoppered tubes. Serum osmolarity was then determined in a Precision Scientific osmometer by comparing serum freezing point with

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[3]The assumption is being made that changes in the osmolarity of the combination of serum and other tissue fluids obtained by decapitation reflect changes in serum alone.

standard solutions of known osmolarity. Values were expressed as m Osmolar U/kg serum. Statistically significant differences in brain water content or serum osmolarity were determined by Student's t-test.

RESULTS

I. General

A. Streptozotocin-Induced Diabetes in Swiss Mice

1. Body weight. Injection of STZ (200 mg/kg i.v.) in randomly selected mice caused a prompt, marked weight loss (Figure 1). Mean body weights decreased from a pre-STZ mean of 24.9 g to 19.3 g ($p < 0.05$) within one week of the injection. This weight loss did not appear to result from decreased food consumption, but could have been due to increased water loss. These animals did not gain significant amounts of weight until 5 weeks after the injection. Although the mice continued to gain weight throughout the rest of the 18 week observation period, and eventually returned to their pre-injection weights, their body weights never caught up to those of the control group, and hyperglycemia persisted throughout the 18 week observation period.

2. Survival. The effects of a single i.v. dose of STZ (200 mg/kg) on the animals' survival is shown in Table 1. Three days after the injection of STZ into nine mice, one died. No other deaths occurred in the diabetic animals until week nine, when one additional mouse died. Necropsies revealed no gross pathologic signs. During the routine preparation of STZ-diabetic animals, throughout the entire research project, it was observed that when deaths occurred following STZ injection, they occurred during days 2-4 following injection and at approximately the same rate (1 dead/10 injected). For this reason no experiment was begun less than 6 days following STZ injection, the period after which any deaths would occur (until several weeks later) and during which the animals' loss of weight was greatest. No moribund animals were observed on the experi-

Figure 1

The effects of a single injection of Streptozotocin (STZ) at 200 mg/kg i.v. on body weights in randomly selected female mice. Control mice received vehicle only. At each time point, $n \geq 7$. Serum glucose levels ($\bar{X} \pm$ S.E.) are shown in parentheses at each time point along the STZ curve.

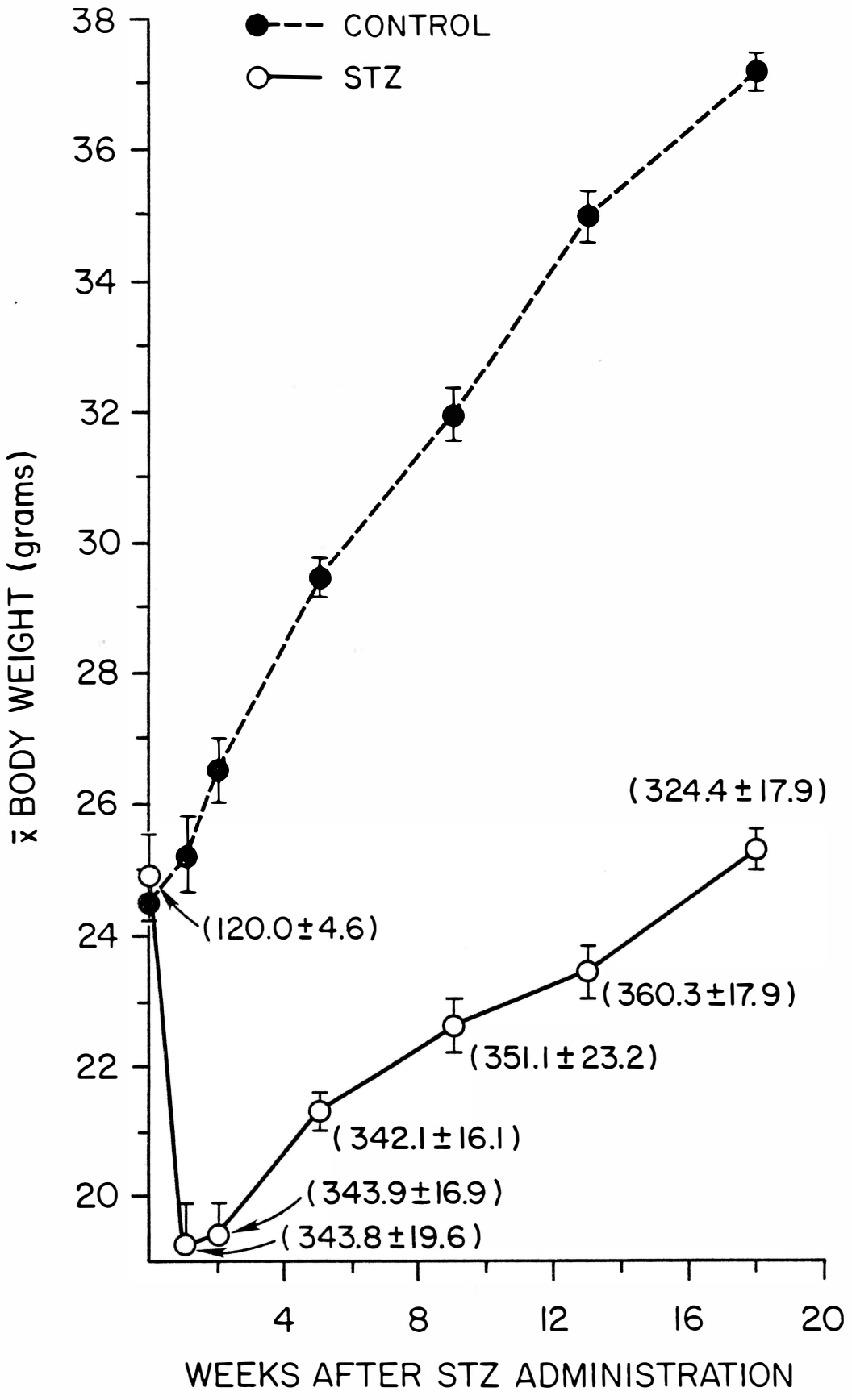
CONTROL
STZ
38
36
34
32
30
28
26
24
22
20
x̄ BODY WEIGHT (grams)
(324.4 ± 17.9)
(120.0 ± 4.6)
(360.3 ± 17.9)
(351.1 ± 23.2)
(342.1 ± 16.1)
(343.9 ± 16.9)
(343.8 ± 19.6)
4
8
12
16
20
WEEKS AFTER STZ ADMINISTRATION

Table 1

Comparison of Survival in Streptozotocin (STZ)-Induced Diabetic Mice with Vehicle-Injected Control Mice

Pretreatment	Weeks After STZ or Vehicle						
	0	1	2	5	9	13	18
Vehicle Control							
Number Surviving/ Number Injected	10/10	10/10	10/10	10/10	10/10	10/10	10/10
% Surviving	100	100	100	100	100	100	100
STZ 20 mg/kg i.v.							
Number Surviving/ Number Injected	9/9	8/9	8/9	8/9	7/9	7/9	7/9
% Surviving	100	88.9	88.9	88.9	77.9	77.8	77.8

mental days.

II. Narcotic Induced Antinociception and Related Studies

A. Tail-Flick

1. Swiss mice. General observations. The pretreatment group that was fasted and then received insulin s.c. was lethargic and hyposensitive to touch. No behavioral changes were observed in any of the other pretreatment groups nor did any of the various pretreatments have a significant effect on tail-flick latency.

a. Morphine. In a pilot set of experiments, a single dose of morphine (5 mg/kg s.c.) was administered to mice with various pretreatments, and the increase in tail flick latency was compared with control mice. In experiments with STZ-induced diabetic mice and dextrose-pretreated mice (both causing hyperglycemia)[4] significantly attenuated responses to morphine in the tail-flick test were observed compared with control mice (Table 2). Pretreatment with 1 U/kg insulin following an 18 hr fast (sufficient to cause hypoglycemia) significantly potentiated the antinociceptive effects of morphine (3 mg/kg s.c.) compared to control mice (Table 3). Subsequently, log-dose-response curves were generated for the effects of morphine in the tail-flick test using each of the following pretreatment groups: control; STZ-induced diabetic; STZ-diabetic and insulin; dextrose; fasted; and fasted and insulin. In each case, there was an inverse relationship between morphine potency and serum glucose levels (SGL's) reported in Table 24. The potency ratios vs control were significantly different for all pretreatment groups except the fasted

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[4]Alterations in BGL produced by the various pretreatments are presented on page 73.

Table 2

Effect of Streptozotocin (STZ)-Induced Diabetes or Pretreatment with Dextrose on the Antinociceptive Action of a Single Dose of Morphine[a] Compared with Control Mice as Quantitated in the Mouse Tail-Flick Test

Pretreatment	Baseline Reaction (sec)[b]	Test Reaction (sec)[b]	Difference (sec)[b]	Level of Significance[c] $p <$
Control	2.83 ± 0.15	9.73 ± 0.27	6.90 ± 0.31	
STZ[d]	3.08 ± 0.18	6.13 ± 0.90	3.05 ± 0.92	0.001
Control	3.16 ± 0.14	9.04 ± 0.49	5.88 ± 0.51	
Dextrose[e]	2.77 ± 0.14	5.98 ± 0.83	3.21 ± 0.83	0.001

[a] Morphine 5 mg/kg (s.c.) 20 minutes prior to Test Reaction determination

[b] $\bar{X}$ ± S.E., n = 12

[c] Student's t-test

[d] 200 mg/kg i.v. 6 days earlier

[e] 0.028 mol/kg i.p. 25 min before morphine

Table 3

Effect of Fasting + Insulin Pretreatment on the Antinociceptive Action of a Single Dose of Morphine[a] Compared with Control Mice as Quantitated in the Mouse Tail-Flick Test

Pretreatment (n)	Baseline Reaction (sec)[b]	Test Reaction (sec)[b]	Difference (sec)[b]
Control (12)	2.75 ± 0.15	4.62 ± 0.60	1.86 ± 0.60
Fasting + Insulin[c] (13)	2.95 ± 0.13	8.08 ± 0.65	5.13 ± 0.70[d]

[a] Morphine 3 mg/kg (s.c.) 20 minutes prior to Test Reaction determination

[b] $\bar{X}$ ± S.E.

[c] 18 hr fast + insulin 1 U/kg s.c. 20 min before morphine

[d] $p < 0.005$ vs control group difference using Student's t-test

group. The data from this experiment are presented in Table 4. Both the dextrose-pretreated and STZ-induced diabetic mice (both groups were hyperglycemic) were significantly less sensitive to the antinociceptive effects of morphine, suggesting that the hyperglycemia caused the altered sensitivity. Insulin treatment (3 U/kg s.c.) of other STZ-induced diabetic mice lowered serum glucose levels and returned the sensitivity to morphine to normal. However, the diabetic animals administered insulin were not the same mice as those which were tested without insulin. In another experiment the potency of morphine was tested using the same STZ-diabetic mice with and without insulin injections on separate days. On day 1, they were administered morphine but not administered insulin and tested in the tail-flick assay. After a four day rest (day 5), they were tested in the tail-flick test again, but were injected with 3 U/kg insulin s.c. 20 min prior to morphine. These results are presented in Table 5. On day 1, the STZ-diabetic mice were significantly less sensitive to morphine than the control mice, but on day 5 when administered 3 U/kg insulin 20 min before morphine, the diabetic animals' sensitivity to morphine's antinociceptive effect returned to normal. Thus the effects of STZ-induced diabetes on morphine potency are reversible by insulin administration. Insulin-induced hypoglycemia in fasted mice caused a significant increase in morphine potency (Table 4).

The effects of pretreatment with fructose or 3-O-methylglucose (a non-metabolizable sugar) on morphine-induced antinociception were determined in an attempt to elucidate the mechanism of how STZ-diabetes altered morphine potency. Pretreatment with 3-O-methyl glucose did not have a significant effect on morphine potency whereas pretreatment with fructose significantly decreased sensitivity to morphine (Table 6). This

Table 4

Effect of Various Pretreatments on the ED_{50} of Morphine in the Mouse Tail-Flick Test

Pretreatment (n/dose)	ED_{50}[a]	Potency Ratio[b]	95% Confidence Limits of Potency Ratio
Control (36)	2.73	1.00	---
STZ[c] (12)	3.91	0.70[h]	0.53 - 0.93
STZ + Insulin[d] (12)	2.37	1.17	0.67 - 2.02
Dextrose[e] (12)	3.81	0.72[h]	0.58 - 0.89
Fasting[f] (12)	2.69	1.02	0.88 - 1.17
Fasting + Insulin[g] (12)	1.87	1.47[h]	1.08 - 1.99

[a] mg/kg s.c.

[b] vs control

[c] 200 mg/kg Streptozotocin (STZ) i.v. 6 days earlier

[d] 200 mg/kg STZ i.v. 6 days earlier + 3 U/kg insulin s.c. 20 min before morphine

[e] 0.028 mol/kg i.p. 25 min before morphine

[f] 18 hr before morphine

[g] 18 hr fast + 1 U/kg insulin s.c. 20 min before morphine

[h] $p < 0.05$ vs control (Barr et al., 1976)

Table 5

Return to Normal of Sensitivity to Morphine in Streptozotocin (STZ)-Induced Diabetic Mice by Treatment with Insulin in the Tail-Flick Test

Day	Pretreatment	ED_{50}[a]	Potency Ratio[b]	95% Confidence Limits of Potency Ratio
	Control	2.94	1.00	
1	STZ[c]	6.21	0.56[f]	0.34 - 0.91
5	STZ + Insulin[d,e]	1.61	1.83	0.88 - 3.73

[a] mg/kg s.c., n = 6/dose of morphine

[b] vs control

[c] 200 mg/kg i.v. 6 days earlier

[d] same animals as Day 1 STZ

[e] 200 mg/kg STZ i.v. 10 days earlier + 3 U/kg insulin s.c. 20 min before morphine

[f] $p < 0.05$ vs control (Barr et al., 1976)

Table 6

Effect of Fructose or 3-O-Methyl Glucose Pretreatment on the ED_{50} of Morphine in the Mouse Tail-Flick Test

Pretreatment	ED_{50}[a]	Potency Ratio[b]	95% C.L. of Potency Ratio
Control	4.38	1.00	---
Fructose[c]	15.4	0.28[d]	0.15 - 0.53
3-O-methyl glucose[c]	3.90	1.12	0.56 - 2.23

[a] mg/kg s.c., n = 6/dose of morphine

[b] vs control

[c] 0.028 mol/kg i.p. 20 min before morphine

[d] $p < 0.05$ vs control (Barr et al., 1976)

decrease in morphine potency caused by fructose pretreatment was more marked than that caused by dextrose pretreatment. It was found (data not shown) that an injection of insulin (5 U/kg s.c.) 15 min prior to the administration of dextrose could prevent the occurrance of hyperglycemia. This regimen was also found to return the sensitivity to morphine to control levels (Table 7). When this same insulin treatment was administered to mice pretreated with fructose, these animals' sensitivity to morphine was still significantly lower than controls, but also significantly greater than with fructose alone.

The next question to be answered was whether the effect of these pretreatments on morphine potency would also affect the potency of other narcotic analgesics.

b. Phenazocine. The effects of various pretreatments administered to Swiss mice on the antinociceptive potency of phenazocine are shown in Table 8. Without exception, pretreatments that significantly altered morphine potency (Table 4) also significantly changed the potency of phenazocine in the same direction. The potency ratio for each pretreatment group tested vs control for phenazocine was similar to that for morphine.

c. Levorphanol. The various pretreatments administered caused changes in the antinociceptive potency of levorphanol similar to those seen with morphine and phenazocine with the exception of the group that was fasted and administered insulin (Table 9). In this group, the 95% confidence limits of the potency ratio just included 1.00. This indicates that the fasted + insulin pretreatment group is not significantly more sensitive to levorphanol than the control group, although it is more sensitive to morphine and phenazocine.

Table 7

Effect of Insulin in Reversing the Decreased Potency of Morphine in Mice Pretreated with Dextrose or Fructose in the Tail-Flick Test

Pretreatment	ED_{50}[a]	Potency Ratio[b]	95% Confidence Limits of Potency Ratio
Control	3.31	1.00	---
Dextrose[c]	5.40	0.61[e]	0.49 - 0.78
Insulin + Dextrose[d]	3.13	1.05	0.85 - 1.31
Fructose[c]	18.94	0.17[e]	0.14 - 0.22
Insulin + Fructose[d]	9.02	0.37[e,f]	0.29 - 0.46

[a] mg/kg s.c., n = 6/dose of morphine

[b] vs control

[c] 0.028 mol/kg i.p. 25 min before morphine

[d] 5 U/kg insulin s.c. 15 min before dextrose or fructose + 0.028 mol/kg dextrose or fructose 25 min before morphine

[e] $p < 0.05$ vs control (Barr et al., 1976)

[f] $p < 0.05$ vs fructose (Barr et al., 1976)

Table 8

Effect of Various Pretreatments on the ED_{50} of Phenazocine in the Mouse Tail-Flick Test

Pretreatment (n)	ED_{50}[a]	Potency Ratio[b]	95% Confidence Limits of Potency Ratio
Control (12)	0.25	1.00	---
STZ[c] (6)	0.38	0.65[g]	0.45 - 0.94
Dextrose[d] (12)	0.46	0.67[g]	0.50 - 0.90
Fasting[e] (6)	0.22	1.12	0.79 - 1.61
Fasting + Insulin[f] (6)	0.10	2.49[g]	1.74 - 3.56

[a] mg/kg s.c.

[b] vs control

[c] 200 mg/kg Streptozotocin (STZ) i.v. 10 days earlier

[d] 0.028 mol/kg i.p. 25 min before phenazocine

[e] 18 hr before phenazocine

[f] 18 hr fast + 1 U/kg insulin s.c. 20 min before phenazocine

[g] $p < 0.05$ vs control (Barr et al., 1976)

Table 9

Effect of Various Pretreatments on the ED_{50} of Levorphanol in the Mouse Tail-Flick Test

Pretreatment (n/dose)	ED_{50}[a]	Potency Ratio[b]	95% Confidence Limits of Potency Ratio
Control (12)	0.65	1.00	---
STZ[c] (6)	1.03	0.63[g]	0.41 - 0.98
Dextrose[d] (6)	1.08	0.61[g]	0.39 - 0.95
Fasting[e] (8)	0.45	1.44	0.93 - 2.24
Fasting + Insulin[f] (6)	0.42	1.57	1.00 - 2.49

[a] mg/kg s.c.

[b] vs control

[c] 200 mg/kg Streptozotocin (STZ) i.v. 8 days earlier

[d] 0.028 mol/kg i.p. 25 min before levorphanol

[e] 18 hr fast before levorphanol

[f] 18 hr fast + 1 U/kg insulin s.c. 20 min before levorphanol

[g] $p < 0.05$ vs control (Barr et al., 1976)

d. Methadone. None of the various pretreatments administered had a significant effect on the antinociceptive effect of methadone in the tail-flick test (Table 10). This provided an indication that STZ-induced diabetes or the other pretreatments does not alter the potency of all narcotics.

e. Propoxyphene. Like methadone, the antinociceptive potency of propoxyphene was not significantly altered in STZ-induced diabetic mice or in mice with various other pretreatments (Table 11). But because propoxyphene is structurally similar to methadone, it was decided to test a narcotic structurally dissimilar to those previously tested.

f. Meperidine. The antinociceptive potency of meperidine was not significantly changed by any of the pretreatments as shown in Table 12.

It thus appears that the various pretreatments can alter the sensitivity of mice to some narcotics, but not all, as measured in the tail-flick test. Morphine was selected as the prototype narcotic to be used in all subsequent experiments.

2. Rats. In an attempt to determine whether the effects on morphine antinociception seen in Swiss mice with STZ-induced diabetes occurred in other species, the anticociceptive potency of morphine was evaluated in control vs STZ-induced diabetic rats. The data from this experiment are presented in Figure 2. As was the case with STZ-induced diabetic mice, the diabetic rats were significantly less sensitive to the antinociceptive effects of morphine as measured by the tail-flick test. The ED_{50}'s for morphine were 4.6 and 8.4 mg/kg in control and STZ-induced diabetic rats, respectively. The analysis of covariance procedure determined a potency ratio of 0.54, with 95% confidence limits of 0.40 to 0.74,

Table 10

Effect of Various Pretreatments on the ED_{50} of Methadone in the Mouse Tail-Flick Test

Pretreatment	ED_{50}[a]	Potency Ratio[b]	95% Confidence Limits of Potency Ratio
Control	2.42	1.00	---
STZ[c]	2.72	0.89	0.57 - 1.76
Dextrose[d]	2.48	0.98	0.68 - 1.42
Fasting[e]	2.35	1.03	0.71 - 1.50
Fasting + Insulin[f]	2.36	1.03	0.71 - 1.49

[a] mg/kg s.c., n = 6/dose of methadone

[b] vs control (Barr et al., 1976)

[c] 200 mg/kg Streptozotocin (STZ) i.v. 6 days earlier

[d] 0.028 mol/kg i.p. 25 min before methadone

[e] 18 hr fast before methadone

[f] 18 hr fast + 1 U/kg insulin s.c. 20 min before methadone

Table 11

Effect of Various Pretreatments on the ED_{50} of Propoxyphene in the Mouse Tail-Flick Test

Pretreatment	ED_{50}[a]	Potency Ratio[b]	95% Confidence Limits of Potency Ratio
Control	12.4	1.00	---
STZ[c]	12.2	1.02	0.67 - 1.54
Dextrose[d]	12.7	0.98	0.64 - 1.48
Fasting[e]	12.3	1.01	0.66 - 1.51
Fasting + Insulin[f]	12.1	1.02	0.67 - 1.54

[a] mg/kg, n = 6/dose of propoxyphene

[b] vs control (Barr et al., 1976)

[c] 200 mg/kg Streptozotocin (STZ) i.v. 8 days earlier

[d] 0.028 mol/kg i.p. 25 min before propoxyphene

[e] 18 hr fast before propoxyphene

[f] 18 hr fast + 1 U/kg insulin s.c. 20 min before propoxyphene

Table 12

Effect of Various Pretreatments on the ED_{50} of Meperidine in the Mouse Tail-Flick Test

Pretreatment (n/dose)	ED_{50}[a]	Potency Ratio[b]	95% Confidence Limits of Potency Ratio
Control (12)	13.8	1.00	---
STZ[c] (6)	12.1	1.14	0.81 - 1.61
Dextrose[d] (6)	13.6	1.01	0.72 - 1.42
Fasting[e] (6)	10.7	1.29	0.87 - 1.92
Fasting + Insulin[f] (6)	9.7	1.42	0.94 - 2.13

[a] mg/kg

[b] vs control

[c] 200 mg/kg Streptozotocin (STZ) i.v. 10 days earlier

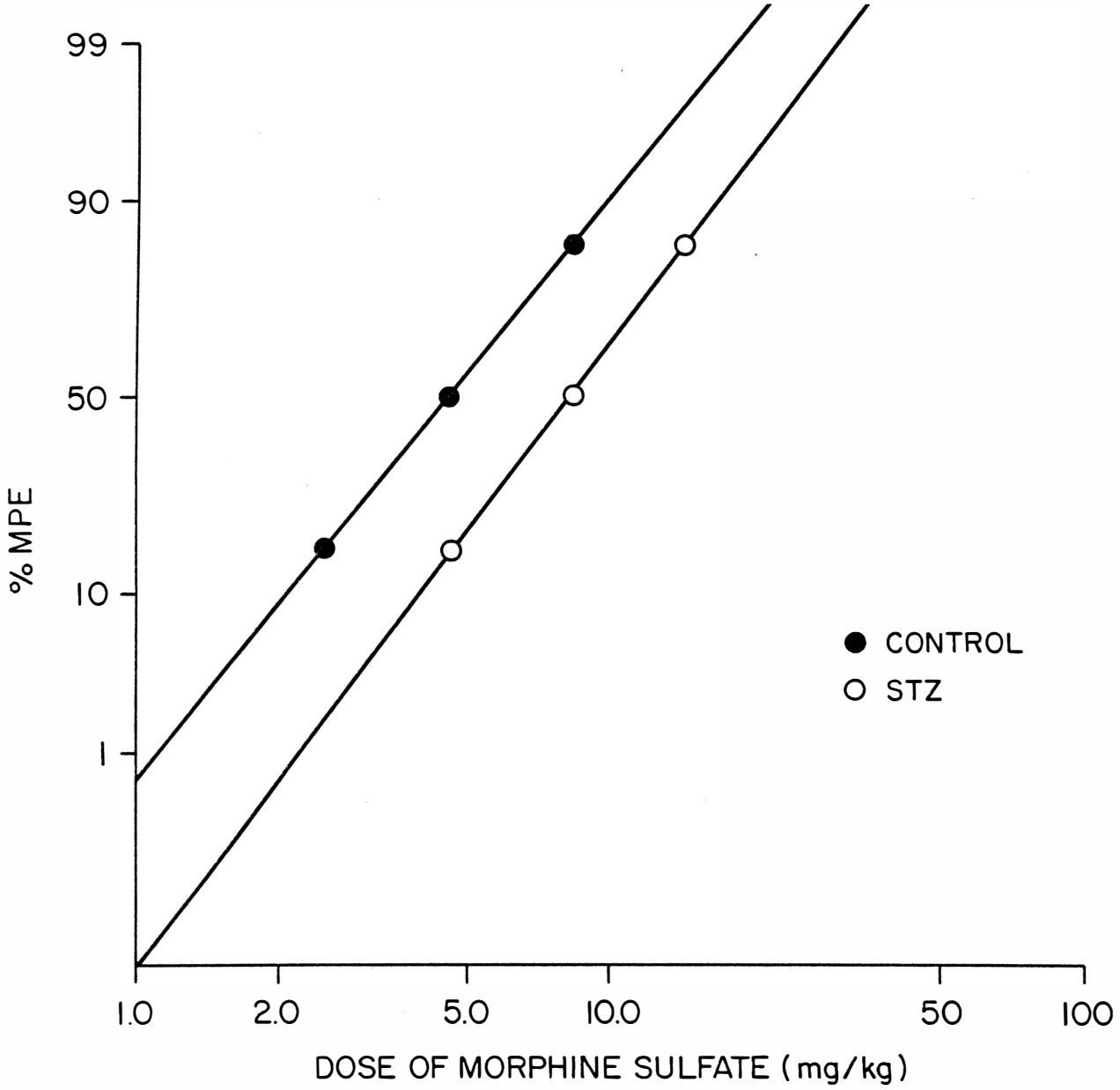
[d] 0.028 mol/kg i.p. 25 min before meperidine

[e] 18 hr fast before meperidine

[f] 18 hr fast + 1 U/kg insulin s.c. 20 min before meperidine

Figure 2

The effects of Streptozotocin (STZ)-induced diabetes in rats on the anti-nociceptive potency of morphine as quantitated in the tail-flick test. STZ was administered 6 days earlier at a dose of 60 mg/kg i.v. The equations for both lines were generated by analysis of covariance (Barr et al., 1976).


99
90
50
10
1
% MPE
CONTROL
STZ
1.0
2.0
5.0
10.0
50
100
DOSE OF MORPHINE SULFATE (mg/kg)

very similar in magnitude to the results in STZ-induced diabetic Swiss mice.

3. C57Bl/KsJ mice. The next attempt was to determine whether the decrease in morphine's potency seen in STZ-induced diabetic mice and rats would also be seen using still another animal model of human diabetes mellitus. In Table 13, it can be seen that mice homozygous for the gene "dbm" (diabetes) are significantly less sensitive to the antinociceptive effects of morphine sulfate in the tail-flick test, when compared with mice who are merely heterozygous. The potency ratio between these groups of mice (0.09) is of even greater magnitude than that between STZ-induced diabetic mice and their respective control. This relationship is shown in Figure 3. The C57Bl/KsJ dbm/++ mice as a group appear to be more sensitive to morphine than the Swiss mice while the spontaneously diabetic mice (dbm/dbm) are even less sensitive to the antinociceptive effects of morphine than are the STZ-induced diabetic mice.

In summary, STZ-induced diabetes and other pretreatments which affect blood glucose levels can affect the antinociceptive potency of morphine, phenazocine and levorphanol, but not methadone, propoxyphene or meperidine. The decreased sensitivity to morphine seen in STZ-induced diabetic mice was also capable of being produced in mice with spontaneous diabetes, STZ-induced diabetic rats, and mice pretreated with dextrose or fructose while 3-O-methyl glucose pretreatment had no significant effect on morphine potency. The potency of morphine in STZ-induced diabetic and dextrose-pretreated mice was returned to normal by the administration of insulin. Additionally, severe hypoglycemia rendered mice supersensitive to the antinociceptive effects of morphine. Pretreatment of mice by fasting showed only a trend towards significance.

Table 13

The ED_{50} of Morphine in Spontaneously Diabetic Mice and Their Nondiabetic Littermates in the Tail-Flick Test

Genotype	ED_{50}[a]	Potency Ratio[b]	95% Confidence Limits of Potency Ratio
dbm/++ (nondiabetic)	1.5	1.00	---
dbm/dbm (diabetic)	17.2	0.09[c]	0.04 - 0.20

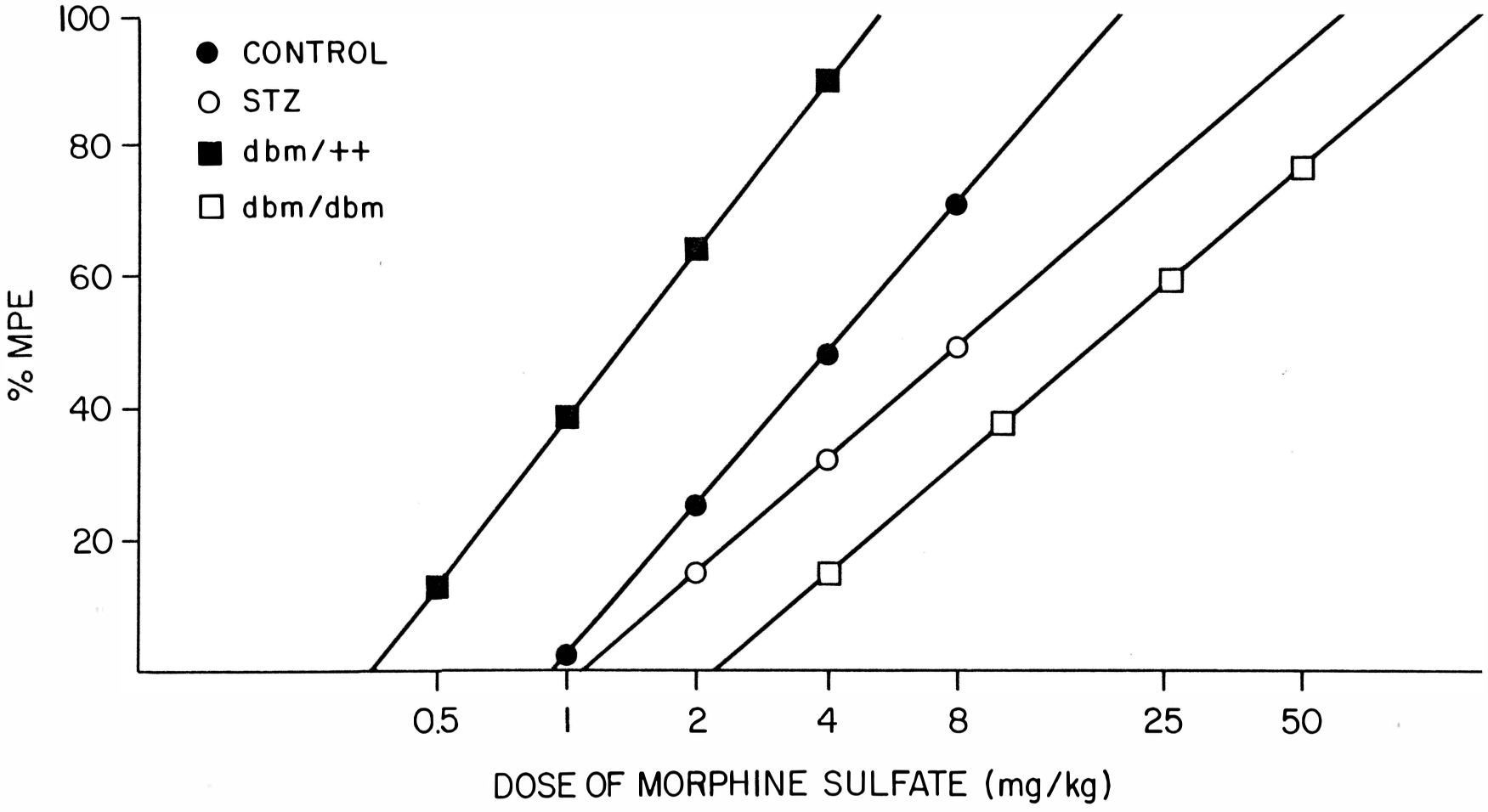
[a] mg/kg s.c., n = 6/dose of morphine

[b] vs control

[c] $p < 0.05$ vs control (Barr et al., 1976)

Figure 3

Comparison of the sensitivity to morphine shown by mice with different models of human diabetes mellitus and their respective controls using the tail-flick test. STZ = Streptozotocin 200 mg/kg i.v. 6 days earlier.


100
80
60
40
20
% MPE
CONTROL
STZ
dbm/++
dbm/dbm
0.5
1
2
4
8
25
50
DOSE OF MORPHINE SULFATE (mg/kg)

B. Phenylquinone-Induced Writhing

To verify that the effects seen in the tail-flick test comparing STZ-induced diabetic mice with control mice in their response to morphine was not peculiar to just the tail-flick reflex response, the phenylquinone (PPQ)-induced writhing test was performed. The ability of morphine to inhibit the PPQ-induced writhing response was evaluated in STZ-induced diabetic, and fasted + insulin pretreated mice vs control. In Table 14, it can be seen that the STZ-induced diabetic mice were significantly less sensitive to the effects of morphine in this assay. The hypoglycemic mice were somewhat, but not significantly more sensitive to the effects of morphine than were control mice. Neither of the experimental pretreatments had any significant effect on the writhing response elicited by PPQ alone. None of the log-dose response curves deviated significantly from parallelism.

The next series of experiments were designed to further elucidate the mechanism(s) responsible for differences in the antinociceptive potency of morphine seen in STZ-induced diabetic mice.

C. Duration of Action of Morphine

If, as a result of STZ-induced diabetes, the absorption, biotransformation or excretion of morphine were altered, this could account for the drug's decreased potency in the diabetic mice. A change in one of these three parameters might be indicated if the duration of antinociception following a single dose of morphine differed for STZ-diabetic mice vs control mice, unless two or more parameters were altered in opposite directions and in equal amounts. A graph showing the % MPE at several times after morphine administration in control and STZ-induced diabetic mice is presented in Figure 4. In both groups of mice, peak antinocicep-

Table 14

The ED_{50} of Morphine in Control, Streptozotocin (STZ)-Induced Diabetic and Fasted + Insulin Pretreated Mice in the Phenylquinone-Induced Writhing Test

Pretreatment (n/dose)	ED_{50}[a]	95% Confidence Limits of ED_{50}	Potency Ratio[b]
Control (6)	0.74	0.39 - 1.39	1.00
STZ[d] (6)	2.16	1.17 - 3.99	0.34[e]
Fasting + Insulin[d] (6)	0.50	0.30 - 0.82	1.48

[a] mg morphine/kg

[b] vs control

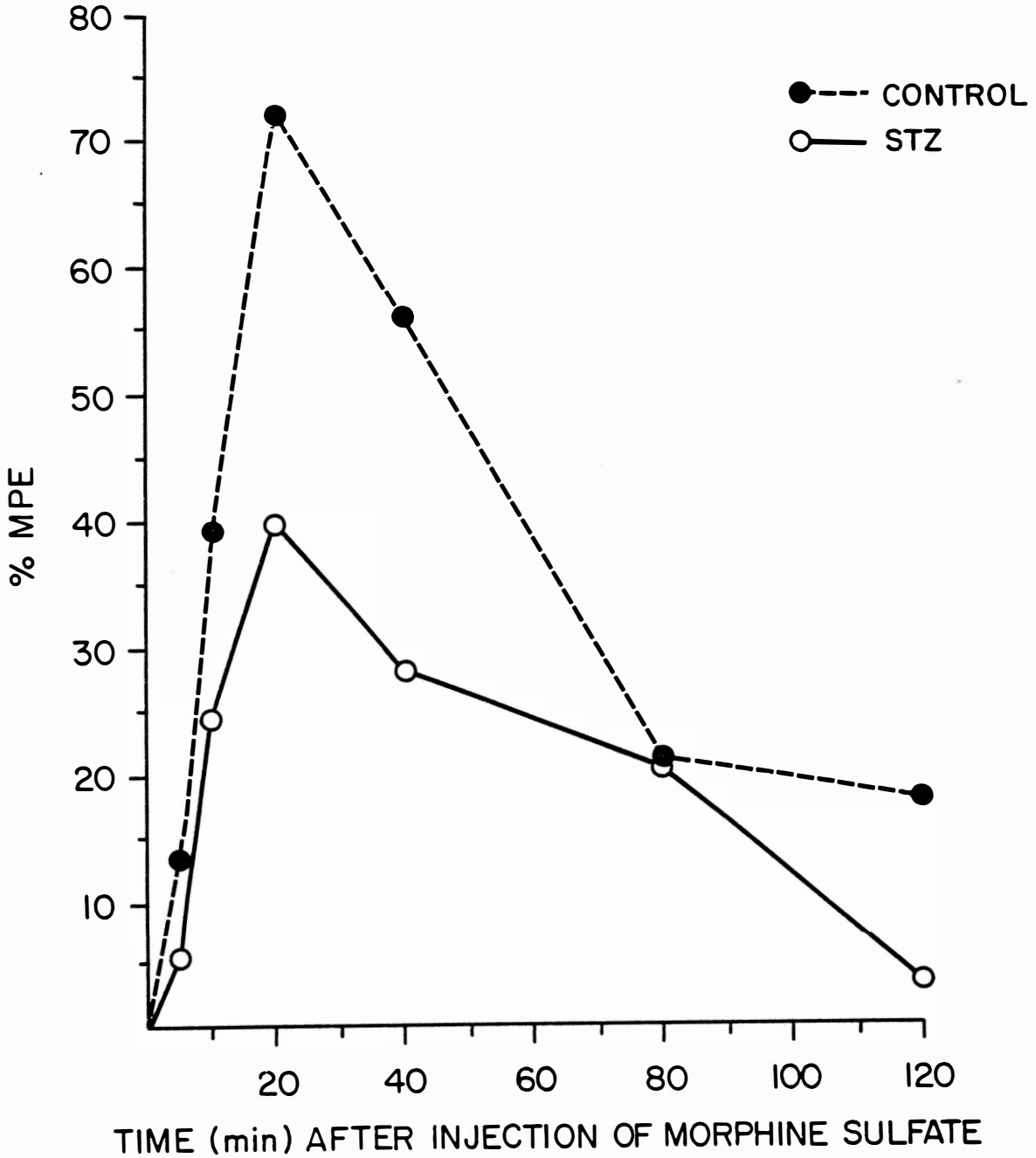
[c] 200 mg/kg STZ i.v. 6 days earlier

[d] 18 hr fast + 1 U/kg insulin s.c. 20 min before morphine

[e] $p < 0.05$ vs control (Litchfield and Wilcoxon, 1949)

Figure 4

Duration of action of morphine (4 mg/kg s.c.) in control and Streptozotocin (STZ)-induced diabetic mice as quantitated in the tail-flick test. Six different mice were used at each time point. STZ-induced diabetic mice were injected with 200 mg/kg STZ i.v. 6 days earlier.


80
70
60
50
40
30
20
10
% MPE
CONTROL
STZ
20
40
60
80
100
120
TIME (min) AFTER INJECTION OF MORPHINE SULFATE

tion was seen at 20 min (the time routinely used in tail-flick experiments). In this laboratory, 25% MPE is considered to be pharmacologically significant: In both groups of mice the % MPE reached 25% at 10 min, and dropped to that mark at 80 min. Thus, there appears to be no difference in the duration of action, although the response in the STZ-induced diabetic mice was lower than control mice at all time points.

D. Naloxone Antagonism of Morphine-Induced Antinociception

The ability of naloxone, a pure narcotic antagonist, to inhibit the effects of morphine in the tail-flick test was determined in control and STZ-induced diabetic mice (Table 15). A significant difference in the pA_2 or slope of the log dose-response curves (% MPE vs log-dose naloxone) using a constant dose of morphine would suggest that morphine and naloxone are not interacting at the same receptor in STZ-induced diabetic and control mice. Using a modification of the method of Takemori et al. (1969), no significant difference in the pA_2 values were observed. The slopes of the log dose-response curves also did not differ significantly from parallelism (Litchfield and Wilcoxon, 1949; Barr et al., 1976). There was, however, a decreased potency of morphine in STZ-induced diabetic mice compared with control mice at all doses of naloxone.

E. Radiotracer Studies

One possible reason for the different sensitivities to morphine shown by several pretreatment groups is that the level of morphine in the brains of these animals could be altered as a result of the pretreatment. In a series of three experiments, summarized in Table 16, it was found that levels of ^{3}H-morphine equivalents in the brains of control, STZ-induced diabetic and insulin-treated STZ-induced diabetic mice was almost identical, although the antinociceptive potency of the morphine was sig-

Table 15

Naloxone[a] Antagonism of Morphine[b]-Induced Antinociception in Streptozotocin (STZ)-Induced Diabetic and Control Mice in the Tail-Flick Test

	Control	STZ[c]
pA_2[d]	1.23	1.46
95% Confidence Limits of pA_2	0.82 - 1.64	0.74 - 2.18
Slope[e]	3.25	7.96
Potency Ratio[f]	1.00	3.92[g]
95% Confidence Limits of Potency Ratio	----	1.44 -10.67

[a] 0.03, 0.1 or 0.3 mg/kg s.c., n = 6/dose of naloxone

[b] 6 mg/kg s.c.

[c] 200 mg/kg STZ i.v. 6 days earlier

[d] Takemori et al. (1969)

[e] Litchfield and Wilcoxon (1949)

[f] vs control (Barr et al., 1976)

[g] $p < 0.05$ vs control (Barr et al., 1976)

Table 16

Levels of ^{3}H-Morphine Equivalents and Antinociception in Control, Streptozotocin (STZ)-Induced Diabetic, and Insulin-Pretreated STZ-Induced Diabetic Mice Administered ^{3}H-Morphine[a]

Pretreatment	% MPE[b]	(n)	Brain ^{3}H[b,c]	(n)	Serum ^{3}H[b,d]	(n)
Control	55.6 ± 5.7	(41)	126.1 ± 5.0	(33)	2711.5 ± 147.1	(29)
STZ[e]	30.2 ± 5.3[g]	(20)	119.1 ± 8.1	(19)	2909.8 ± 237.1	(20)
STZ + Insulin[f]	69.2 ± 7.1[g,h]	(20)	119.1 ± 8.9	(20)	3888.6 ± 292.0[g]	(20)

[a] Morphine sulfate 4 mg/kg 20 min earlier

[b] $\bar{X}$ ± S.E.

[c] ngEq of morphine/g brain tissue

[d] ngEq of morphine/ml serum

[e] 200 mg/kg STZ i.v. 6 days earlier

[f] 200 mg/kg STZ i.v. 6 days earlier + 3 U/kg insulin s.c. 20 min before morphine

[g] $p < 0.05$ vs control using Student's t-test

[h] $p < 0.05$ vs STZ using Student's t-test

nificantly less. No significant difference was found in the serum-^{3}H-morphine equivalent levels of control and STZ-induced diabetic mice. The STZ-induced diabetic mice also administered insulin, however, had a significantly higher serum-^{3}H level than either of the other two groups. The reason for this is unknown. It can be concluded from these data that the decreased response of STZ-induced diabetic mice to morphine is probably not due to a change in the distribution of morphine in the brain or blood.

III. Other Pharmacologic Studies

The next series of experiments were designed to determine whether the decreased potency of morphine in STZ-induced diabetic mice would occur for lethality as well as antinociception. Also under investigation was whether altered sensitivity to non-narcotic centrally acting drugs would occur.

A. LD_{50} Determinations

1. Morphine. STZ-induced diabetes caused a significant increase in the acute lethality of morphine sulfate (Table 17). The slopes of the dose-response curves did not differ significantly from parallelism (1.7 vs 1.5). The LD_{50} and 95% confidence limits in control mice were 503 mg/kg and 409-618 mg/kg, respectively. This is similar to the LD_{50} of morphine reported in the literature, 531 mg/kg (Eddy, 1948). In STZ-induced diabetic mice, the LD_{50} and 95% confidence limits were 337 mg/kg and 247-459 mg/kg, respectively. This results in a potency ratio (LD_{50} control/ LD_{50} STZ) of 1.5, which is significant at $p < 0.05$. All animals showed increased activity and Straub tail within 5 min following the morphine administration. Deaths occurred following convulsions in control mice

Table 17

The Acute LD_{50} of Subcutaneously Administered Morphine Sulfate in Control and Streptozotocin (STZ)-Induced Diabetic Mice

Pretreatment	Dose (s.c.)	n	Number Dead (%)
Control	300 mg/kg	8	1 (11)
	500	9	4 (44)
	600	9	5 (56)
	700	9	8 (89)
LD_{50}[c] = 503 mg/kg; 95% Confidence Limits = 409-618 mg/kg; Slope = 1.5; 95% Confidence Range = 1.1-1.9			
STZ[a]	300	9	4 (44)
	500	16	11 (69)
	600	9	9 (100)
	700	9	9 (100)
LD_{50} = 337 mg/kg; 95% Confidence Limits = 247-459 mg/kg; Slope = 1.7; 95% Confidence Limits = 0.9-3.4			

[a] 200 mg/kg STZ i.v. 8 days earlier

[b] 24 hr following morphine injection

[c] Litchfield and Wilcoxon (1949)

[d] $p < 0.05$ vs control

generally 60-120 min after morphine administration. Diabetic mice appeared to die of respiratory depression, without convulsions, although excitation was observed initially. No signs of gross pathology were observed. So, STZ-diabetic mice are less sensitive to morphine's antinociceptive effects but more sensitive to its lethality.

2. Methadone. Administration of toxic doses of methadone to STZ-diabetic and control mice caused Straub tail initially, but was followed by behavioral and respiratory depression, and death 30-90 min following administration in both groups of animals. No signs of gross pathology were observed. The acute s.c. LD_{50} of methadone was not significantly different in STZ-induced diabetic mice vs control (Table 18). The slopes of both dose-response curves (control vs STZ) were nearly identical (1.56 vs 1.49) as were the LD_{50}'s (36.7 vs 35.1). The reported acute s.c. LD_{50} of methadone is 33 mg/kg (Chen, 1948). When considered with the tail flick data for methadone and morphine this suggests that although morphine and methadone are classified pharmacologically as narcotic analgesics, their mechanisms and/or sites of action in the CNS may be different.

3. Nicotine. STZ-induced diabetes did not have a significant effect on the acute, oral LD_{50} of nicotine, a central cholinergic stimulant (Table 19). Convulsions occurred 5-15 sec following intubation, followed immediately by death. No signs of gross pathology were observed. The LD_{50} of nicotine in control mice was 65.5 mg/kg with 95% confidence limits of 31.6 to 135.9 mg/kg. In STZ-induced diabetic mice, the LD_{50} was 77.3 mg/kg with 95% confidence limits of 34.4 to 174.1 mg/kg. The slopes of the dose-response curves were not significantly different. Oral LD_{50} values for nicotine alkaloid reported in the literature vary greatly (Larsen *et al.*, 1961) although 24 mg/kg is generally accepted as the

Table 18

The Acute LD_{50} of Subcutaneously Administered Methadone Hydrochloride in Control and Streptozotocin (STZ)-Induced Diabetic Mice

Pretreatment	Dose (s.c.)	n	Number Dead (%)[b]
Control	18	10	0 (0)
	24	10	1 (10)
	30	10	5 (50)
	56	10	9 (80)
	100	10	10 (100)
LD_{50}[c] = 36.7 mg/kg; 95% Confidence Limits = 29.3-46.0 mg/kg; Slope = 1.6; 95% Confidence Limits = 1.1-2.1			
STZ[a]	24	10	1 (10)
	30	9	4 (44)
	56	10	9 (90)
LD_{50} = 35.1 mg/kg; Confidence Limits = 25.3-48.7 mg/kg; Slope = 1.4; 95% Confidence Limits = 1.0-2.1			

[a] 200 mg/kg STZ i.v. 8 days earlier

[b] 24 hr following methadone injection

[c] Litchfield and Wilcoxon (1949)

Table 19

The Acute Oral LD_{50} of Nicotine in Control and Streptozotocin (STZ)-Induced Diabetic Mice

Pretreatment	Dose	n	Number Dead (%)[b]
Control	7.6 mg/kg	8	0 (0)
	13.5	8	0 (0)
	24.0	8	0 (0)
	42.7	8	3 (37.5)
	76.0	8	4 (50.0)
	202.0	8	7 (87.5)
	240.0	10	10 (100)
LD_{50}[c] = 65.5 mg/kg; 95% Confidence Limits = 31.6-135.9 mg/kg; Slope = 2.8; 95% Confidence Limits = 0.8-10.6			
STZ[a]	13.5	6	0 (0)
	24.0	8	2 (25)
	42.67	8	3 (37.5)
	76.00	7	3 (42.9)
	202.0	8	6 (75.0)
LD_{50} = 77.3 mg/kg; 95% Confidence Limits = 34.4-174.1 mg/kg; Slope = 1.4; 95% Confidence Limits = 0.8-32.5			

[a] 200 mg/kg STZ i.v. 10 days earlier

[b] 24 hr after nicotine administration

[c] Litchfield and Wilcoxon (1949)

minimum lethal dose. In Table 18 it can be seen that 24 mg/kg was not lethal, but at 42.7 mg/kg, 37.5% lethality occurred.

B. Duration of Hexobarbital-Induced Anesthesia

Pretreatment of female Swiss mice with either STZ or dextrose did not have a significant effect upon the duration of hexobarbital-induced anesthesia. The mean duration of anesthesia ranged from 44.1 min (STZ) to 51.0 min (dextrose) with control mice having a duration of anesthesia of 49.1 min (Table 20).

Following injections of phenobarbital sodium for five days, the duration of hexobarbital-induced anesthesia in STZ and control mice decreased to 13.7 and 13.5 min, respectively (Table 21). These durations were significantly shorter than each group's pre-phenobarbital durations of anesthesia (13.7 vs 49.1 min in control mice; 13.5 vs 44.1 min in STZ-induced diabetic mice), but the two post-phenobarbital durations were not significantly different from each other. These data are in agreement with those of Lamson et al. (1951).

It thus appears that the hyperglycemic aspect of experimental diabetes selectively alters the antinociceptive potency of certain narcotic analgesics.

IV. Analyses of Biochemical Changes Induced by Pretreatments

A. Serum Glucose Levels (SGL)

1. Swiss mice. Since light etherization was required to enable rapid blood sampling, the effect of ether inhalation on SGL was determined. Blood was collected initially by cutting off approximately 5 mm of the mouse's tail. Thereafter, each mouse was etherized and blood collected from the retroorbital sinus. Because serial blood samples were occassion-

Table 20

Duration of Anesthesia in Control, Streptozotocin (STZ)-Diabetic and Dextrose-Pretreated Mice following Injection of Hexobarbital (100 mg/kg i.v.)

Pretreatment	n	Duration (min)[a]
Control	9	49.1 ± 4.1
STZ[b]	7	44.1 ± 5.0
Dextrose[c]	5	51.0 ± 8.8

[a] $\bar{X}$ ± S.E.

[b] 200 mg/kg STZ i.v. 6 days earlier

[c] 0.028 mol/kg i.p. 25 min before hexobarbital

Table 21

Effect of Injections of Phenobarbital Sodium for 5 Days in Control and Streptozotocin (STZ)-Diabetic Mice on the Duration of Hexobarbital-Induced Anesthesia (100 mg/kg i.v.)

Pretreatment	n	Duration (min)[a]
Control	9	13.7 ± 0.7[c]
STZ[b]	6	13.5 ± 1.0[c]

[a] $\bar{X} \pm$ S.E.

[b] 200 mg/kg STZ i.v. 13 days earlier

[c] $p < 0.001$ vs non-induced duration in Table 20 for same pretreatment, using Student's t-test

ally required, the effects of frequent etherization were also investigated. These data appear in Table 22. At time 0, blood samples from the sinus had a significantly higher SGL than samples of blood from the tail. At 45, 90 and 180 min later, sinus blood samples did not have significantly different SGL than time 0 tail or sinus samples. The significantly higher SGL of sinus blood at time 0 could be due to a stress-induced release of epinephrine which would temporarily increase SGL as a result of the tail-cutting at first and be detected in the sinus blood sample taken immediately afterwards (Himms-Hagen, 1967). The SGL from the time 0 sinus puncture was much closer to the time 0 tail values (181 vs 142 mg/dl) than to the level required for an animal to be considered hyperglycemic under the criteria established (> 250 mg/dl), and in subsequent experiments, where tail-cutting was not initially performed, control SGL were never significantly different from the aforementioned time 0 tail-value of the first experiment (Table 22). On the basis of these data, it was concluded that light etherization did not significantly affect the animals' SGL sufficiently to cause a possible misinterpretation of the data.

For both dextrose and insulin pretreatments, the alteration in SGL (hyper- and hypoglycemia, respectively) were observed by 30 min following injection and persisted through 60 min (Table 23). The effect peaked for both pretreatments at 45 min, so 40-45 min was chosen as the pretreatment time.

The effects of the various pretreatments on SGL of mice are shown in Table 24. Blood samples were taken at the time corresponding to the point where the effect of morphine would have been determined. Significant increases above control levels were seen in STZ-induced diabetic and dextrose pretreated mice, whereas fasting or fasting + insulin caused

Table 22

Effect of Sampling Method on Serum Glucose Levels (SGL) in Control Mice

Time	SGL[b]
0 (tail)	142.0 ± 11.0
0[a]	181.0 ± 9.0[c]
45[a]	151.8 ± 13.1
90[a]	164.8 = 7.0
180[a]	153.0 ± 10.9

[a] blood collected from retroorbital sinus

[b] mg/dl, $\bar{X}$ ± S.E., n = 5

[c] $p < 0.05$ vs "0" time (tail) using Student's t-test

Table 23

Duration of Effects of Dextrose- or Fasting + Insulin-Pretreatment on Serum Glucose Levels in Mice

Time[b]	Pretreatment		
	Control	Dextrose[c]	Fasting + Insulin[d]
	Serum Glucose Levels[a]		
0	129.7 ± 6.8	118.3 ± 3.9	91.5 ± 2.7[f]
30	127.8 ± 5.2	307.7 ± 26.1[e,f]	67.3 ± 2.4[e,f]
45	129.2 ± 6.7	327.0 ± 29.2[e,f]	37.3 ± 2.4[e,f]
60	126.8 ± 5.1	302.5 ± 24.0[e,f]	40.5 ± 2.1[e,f]
90	129.3 ± 6.0	239.8 ± 9.4[e,f]	48.2 ± 2.0[e,f]
120	126.8 ± 5.4	184.3 ± 10.7[e,f]	52.0 ± 2.3[e,f]

[a] mg/dl, $\bar{X}$ ± S.E., n = 6

[b] minutes after injection of dextrose or insulin

[c] 0.028 mol/kg i.p.

[d] 18 hr fast + 1 U/kg insulin s.c.

[e] $p < 0.05$ vs time "0" using Student's t-test

[f] $p < 0.05$ vs control at same time using Student's t-test

Table 24

Serum Glucose Levels (SGL) in Swiss Mice
Receiving Various Pretreatments

Pretreatment	n	SGL[g]
Control	6	129.7 ± 6.8
Morphine Sulfate[a]	8	138.6 ± 5.8
STZ[b]	8	472.6 ± 34.2[h]
STZ + Insulin[c]	22	75.4 ± 5.4[h]
Dextrose[d]	6	327.0 ± 29.2[h]
Fructose[d]	6	120.2 ± 4.7
3-O-methyl glucose[d]	6	116.2 ± 4.2
Fasting[e]	6	95.5 ± 2.4[h]
Fasting + Insulin[f]	6	48.2 ± 2.0[h]

[a] 8 mg/kg s.c. 20 min before sampling

[b] 200 mg/kg Streptozotocin (STZ) i.v. 6 days earlier

[c] 200 mg/kg STZ i.v. 6 days earlier + 3 U/kg insulin s.c. 40 min before sampling

[d] 0.028 mol/kg i.p. 45 min before sampling

[e] 18 hr fast before sampling

[f] 18 hr fast + 1 U/kg insulin s.c. 40 min before sampling

[g] mg/dl, $\bar{X}$ ± S.E.

[h] $p < 0.05$ vs control using Student's t-test

significant decreases from control levels. STZ-diabetic animals administered insulin also had significantly lower SGL than control. The variability in the severity of the animals' diabetes makes it impossible to predict what dose of insulin is sufficient to reverse the hyperglycemia without causing mild hypoglycemia in some mice. It should be noted that the difference in the SGL of STZ-induced diabetic and dextrose-pretreated mice vs control is four times the difference between fasting and insulin vs control. Mice that have died from convulsions following injections of higher doses of insulin have had SGL < 30 mg/dl (data not shown). Pretreatment of mice with fructose or 3-O-methyl glucose, unlike dextrose pretreatment, had no significant effect on SGL, yet both dextrose and fructose pretreatments caused significant decreases in morphine potency. Morphine sulfate (8 mg/kg s.c.) had no significant effects on SGL.

2. Rats. The effect of STZ-induced diabetes on SGL in rats is shown in Table 25. The significant increase in SGL induced by STZ-diabetes is similar for both Swiss mice and rats.

3. C57Bl/KsJ mice. The mutation "diabetes" in these mice follow classical Mendelian genetics (Hummel et al., 1966). Thus only those mice homozygous for the gene "dbm" should be diabetic and hyperglycemic. The heterozygote (dbm/++) was considered a better control genotype than the pure wild type (++/++). The diabetic mice had significantly higher SGL than the heterozygotic controls (Table 26).

B. Brain Glucose Levels in Swiss Mice

The effects of various pretreatments on levels of glucose in mouse brains is shown in Table 27. The level of glucose in the brains of the control mice were similar to that reported by King et al. (1977) for male mice. With the exception of STZ-induced diabetic mice administered

Table 25

Serum Glucose Levels (SGL) in Streptozotocin (STZ)-Induced Diabetic Rats Compared with Vehicle-Injected Control Rats

Pretreatment	n	SGL[b]
Control	6	104.2 ± 4.7
STZ[a]	6	390.0 ± 35.8[c]

[a] 60 mg/kg STZ i.v. 6 days earlier

[b] mg/dl, $\bar{X}$ ± S.E.

[c] $p < 0.05$ vs control using Student's t-test

Table 26

Serum Glucose Levels (SGL) in Spontaneously Diabetic Mice Compared with Their Nondiabetic Littermates

Genotype	n	SGL[a]
dbm/++ (nondiabetic)	8	153.4 ± 5.5
dbm/dbm (diabetic)	8	354.8 ± 21.8[b]

[a] mg/dl, $\bar{X}$ ± S.E.

[b] $p < 0.05$ vs dbm/++ using Student's t-test

Table 27

Brain Glucose Levels in Swiss Mice Receiving Various Pretreatments

Pretreatment	Brain Glucose Level[f]
Control	2.20 ± 0.11
STZ[a]	5.17 ± 0.20[g]
STZ + Insulin[b]	3.13 ± 0.32[g,h]
Dextrose[c]	5.31 ± 0.42[g]
Fasting[d]	1.76 ± 0.13[g]
Fasting + Insulin[e]	0.41 ± 0.04[g]

[a] 200 mg/kg Streptozotocin (STZ) i.v. 6 days earlier

[b] 200 mg/kg STZ i.v. 6 days earlier + 3 U/kg insulin s.c. 40 min before sacrifice

[c] 0.028 mol/kg i.p. 45 min before sacrifice

[d] 18 hr fast before sacrifice

[e] 18 hr fast + 1 U/kg insulin s.c. 40 min before sacrifice

[f] mMoles/kg wet weight, $\bar{X}$ ± S.E., n = 6

[g] $p < 0.05$ vs control using Student's t-test

[h] $p < 0.05$ vs STZ using Student's t-test

insulin, changes in SGL (Table 24) were similar to changes in brain glucose levels. That is to say, pretreatments that caused increased SGL produced correspondingly increased brain glucose levels, and pretreatment that caused decreased SGL also caused decreased brain glucose levels. However, while injection of 3 U insulin/kg to STZ-diabetic mice completely reversed their hyperglycemia and, in fact, caused some hypoglycemia, this dose of insulin was not sufficient to return brain glucose levels to normal. These levels were still significantly greater than control levels, but also significantly lower than those of STZ-diabetic mice without insulin injection.

C. Serum Insulin Levels in Swiss Mice

The effects of various pretreatments on serum insulin levels are shown in Table 28. When sugars or insulin were administered, serum insulin levels were determined at the time when morphine's effects would have been determined. Morphine sulfate (6 mg/kg s.c.) did not alter serum insulin-levels 20 minutes after injection. It can be seen that fasting alone did not have a significant effect on serum insulin levels. Subcutaneously administered porcine insulin in fasted and STZ-diabetic mice (1 and 3 U/kg, respectively) significantly increased serum insulin levels from a mean of 8.86 uU/ml in control mice to 38.00 and 77.83 uU/ml respectively. Thus, both serum insulin levels and SGL (Table 24) in insulin-injected STZ-diabetic mice were significantly higher than in insulin-injected fasted animals.

Injection of dextrose, fructose or 3-O-methyl glucose significantly increased serum insulin levels vs control 45 min after injection. Dextrose had the greatest effect on serum insulin levels, while fructose and 3-O-methyl glucose had an equal effect.

Table 28

Serum Insulin Levels in Swiss Mice Receiving Various Pretreatments

Pretreatment	n	Serum Insulin Level[a]
Control	7	8.86 ± 3.30
Morphine Sulfate[b]	7	7.57 ± 1.34
STZ[c]	6	0.67 ± 0.33[h]
STZ + Insulin[d]	6	77.83 ± 9.41[h]
Dextrose-45[e]	7	42.43 ± 5.47[h]
Fructose-45[e]	7	22.57 ± 5.42[h]
3-OMG-45[e]	5	27.40 ± 6.48[h]
Fasting[f]	7	6.86 ± 1.37
Fasting + Insulin[g]	6	38.00 ± 4.47[h]

[a] uU/ml, $\bar{X}$ ± S.E.

[b] 6 mg/kg s.c. 20 min before sampling

[c] 200 mg/kg Streptozotocin (STZ) i.v. 6 days before sampling

[d] 200 mg/kg STZ i.v. 6 days earlier + 3 U/kg s.c. 60 min before sampling

[e] 0.028 mol/kg i.p. 45 min before sampling

[f] 18 hr fast before sampling

[g] 18 hr fast + 1 U/kg insulin s.c. 40 min before sampling

[h] $p < 0.05$ vs control using Student's t-test

The only pretreatment group whose serum insulin level was significantly lower than control was the STZ-induced diabetic mice. It should be noted that the STZ-diabetic group had serum insulin levels significantly lower (about 1/10) than the control group, while the dextrose-pretreatment group had significantly higher (almost 10X) serum insulin levels than the control group. There is a hundred fold difference in serum insulin levels between the STZ- and dextrose-pretreatment groups, while serum glucose levels (Table 22) and morphine potency (Table 4) in these two groups are similar. These results indicate that the altered potency of morphine is not due to a change in serum insulin levels.

D. Brain Water Content and Serum Osmolarity in Swiss Mice

STZ-induced diabetes had no significant effect upon brain water content or serum osmolarity, suggesting that if the diabetic hyperglycemia did tend to cause central dehydration due to an increase in serum osmolarity, other perturbations compensated for this change (Table 29).

Table 29

Brain-Water Content and Serum Osmolarity in
Streptozotocin (STZ)-Induced Diabetic and Control Mice

Pretreatment	Brain-Water Content[a,b]	Serum Osmolarity[a,c]
Control	73.0 ± 0.5	323 ± 1
STZ[d]	71.9 ± 0.3	332 ± 6

[a] $\bar{X}$ ± S.E.

[b] $\frac{\text{Wet weight - dry weight}}{\text{Wet weight}} \times 100 = \%$ water; n = 10

[c] m Osmolar Units/kg; n = 6

[d] 200 mg/kg STZ i.v. 6 days earlier

DISCUSSION

The data presented in this dissertation indicate that STZ-induced diabetes can affect the potency of some centrally-acting drugs (most notably morphine) while not affecting the potency of other drugs, even some that are considered to be in the same pharmacologic class (e.g. methadone).

The results of a number of experiments suggest the hypothesis that it is the hyperglycemic aspect of the diabetic state which is initially responsible for the observed decrease in the antinociceptive potency of morphine. STZ-induced diabetic mice and rats, and spontaneously diabetic mice were all significantly less sensitive to the antinociceptive effects of morphine than their respective nondiabetic controls. When insulin was administered to STZ-induced mice to lower their BGL's, their sensitivity to the antinociceptive effects of morphine returned to control values.

In contrast to the hypoinsulinemia which occurs with hyperglycemia in STZ-induced diabetes, pretreatment of mice with dextrose caused hyperglycemia and hyperinsulinemia, yet the sensitivity of these two groups of mice to the antinociceptive effects of morphine were both significantly lower than mice with normal BGLs. When insulin was administered to dextrose-pretreated mice to prevent the dextrose-induced hyperglycemia from occurring, sensitivity to the antinociceptive effects of morphine was not significantly different than in control mice.

The fasted plus insulin (hypoglycemic) pretreated mice were more, but not significantly more sensitive to the antinociceptive effects of levorphanol in the tail-flick test and the effects of morphine in the phenylquinone-induced writhing test. These were the only two instances

where a pretreatment did not follow the pattern generally seen in these experiments. This could be explained by the fact that the difference in BGL's between the fasted plus insulin pretreatment group and control mice was not as great as the difference between the pretreatment-induced hyperglycemic mice and controls. Making their hypoglycemia any more severe caused convulsions and death, however (data not presented).

Spontaneously-diabetic mice are hyperglycemic and hyperinsulinemic (Coleman and Hummel, 1967), and have a decreased sensitivity to morphine's antinociceptive effects. This supports the hypothesis that hyperglycemia is the condition responsible for the decreased sensitivity to the antinociceptive effects of morphine seen with diabetes-like syndromes.

Insulin administration to STZ-induced diabetic mice, which restored BGL's and the sensitivity to morphine's antinociceptive effects to control values, decreased brain glucose levels significantly lower than before insulin, but still significantly greater than control levels. This suggests that brain glucose levels are not as important as BGL's, in altering the sensitivity of animals to morphine. It has previously been reported that BGL's may not always accurately reflect brain glucose levels (Thurston, 1976).

An attempt was made to correlate the BGL's of individual mice with their sensitivity to morphine's antinociceptive effects. A statistically significant correlation was not found, suggesting that a threshold must be achieved for a significant change in sensitivity to morphine to occur.

Several experiments were performed in an attempt to elucidate the mechanism whereby increasing BGL cause a decrease in the antinociceptive potency of morphine. Pretreatment of mice with fructose, like dextrose, caused a significant decrease in morphine's antinociceptive potency, while

pretreatment with 3-O-methylglucose had no effect. This suggests that changes in morphine's potency can be affected only by certain sugars.

The results of the experiment which demonstrated that there was no difference in the duration of action of morphine in STZ-induced diabetic and control mice suggests that the absorption, metabolism and excretion of morphine are not affected by STZ-induced diabetes. However, this can not be ruled out solely on the basis of there being no difference in the duration of action. For example, two or more of these parameters could be altered in opposite directions yielding no net effect on the duration of action of morphine.

Patrick et al. (1975) have reported that the level of morphine in the brains of mice can be correlated with both the dose of morphine administered and the degree of antinociception observed. Control, STZ-induced diabetic and insulin-injected STZ-induced diabetic mice did not have levels of morphine in their brains significantly different from each other, despite the fact that the degrees of antinociception were significantly lower and higher, than control, respectively, in these groups of mice. This suggests that under certain conditions the level of morphine in the brain may not always reflect the level of antinociception, and also suggests that hyperglycemia ultimately exerts a central effect on the action of morphine. This central effect is apparently selective for only certain narcotic analgesics.

The experiments with STZ-induced diabetic mice demonstrate that the antinociceptive potency of morphine can be selectively altered, without affecting the potency of all narcotics (e.g. methadone). Other investigators have been able to selectively affect either morphine's or methadone's effects. Sprague and Takemori (1978) have reported that pretreatment of

mice with methamphetamine significantly increased both the antinociceptive potency of morphine and brain morphine levels. There was a slight increase in methadone potency and no increase in brain methadone levels. Rosecrans and his colleagues have reported that various treatments will alter the potency of morphine while not affecting the potency of methadone. Rosecrans et al. (1977) reported that depletion of brain dopamine significantly attenuated morphine- but not methadone-induced antinociception. Chance et al. (1978) have reported that lesions of the medial raphe nucleus of rats (effectively lowering serotonin levels in ascending systems) antagonized the analgesic effects of morphine but not methadone, whereas lesions of the descending systems blocked the effects of both drugs, suggesting different sites of action for the two drugs. Also suggestive of a difference in the actions of morphine and methadone is the observation that apomorphine- (a dopamine receptor stimulant) induced stereotypic behavior is potentiated by methadone, but not morphine. Conversely, d-amphetamine- (which stimulates release of and blocks reuptake of norepinephrine and dopamine) induced stereotypic behavior is markedly potentiated by morphine but only slightly so by methadone (John A. Rosecrans, personal communication).

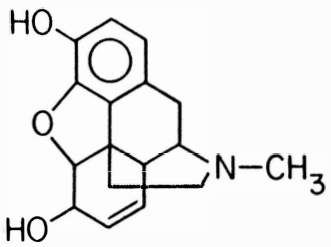
An alternative explanation for differences between morphine and methadone has been offered by May (1972). In his article reviewing attempts to convert narcotic agonists into antagonists, he remarks that certain classes of agonists have not been successfully converted to antagonists, and makes the observation that those drugs which have been successfully converted all share a particular structural similarity; the molecular structure of the drug is quite rigid. In these narcotics, twisting of the groups attached to their quaternary carbon cannot occur. Those drugs which have

not been successfully converted to antagonists do not share this same rigid structure. The groups attached to their quaternary carbons can twist to varying degrees depending upon the bulk of the substitutions.

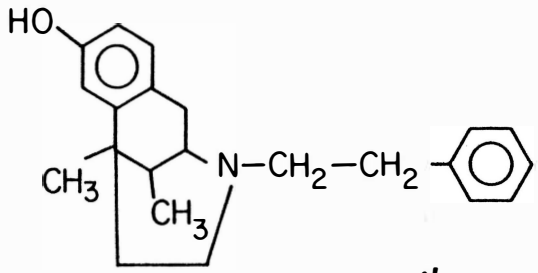
In this dissertation, those analgesics whose potencies were altered by STZ-induced diabetes all have rigid molecular structures (morphine, levorphanol, phenazocine), while the potencies of methadone, propoxyphene and meperidine, which do not have rigid structures (Figure 5), were not altered by STZ-induced diabetes. There is other evidence to support the view that the structural rigidity of a narcotic can affect its actions. Since the discovery of stereospecific opiate-receptor binding (Pert and Snyder, 1973a, b) many narcotic agonists and antagonists have been evaluated with respect to their relative affinity for the receptor. Drugs that show high affinity binding all have rigid molecular structures (Pert and Snyder, 1973a, b). In contrast, drugs with low receptor affinity have non-rigid structures. Narcotics with rigid structures are not always more potent than those with non-rigid structures. For example, morphine and methadone are equipotent in producing antinociception. Binding affinity may thus be more important for rigid than non-rigid narcotics to produce their effects. Another factor (e.g. intrinsic activity) could be more important than binding affinity for non-rigid narcotics. Diabetes-induced hyperglycemia may then yield another peripheral effect that could result in a change in the opiate receptor. This might affect the binding affinity of all narcotics, and therefore have a greater effect on the antinociceptive potency of those narcotics with rigid than non-rigid structures. STZ-induced diabetic rats have significantly elevated serum levels of ACTH and corticosterone (DeNicola et al., 1977). ACTH can attenuate the antinociceptive potency of morphine (Takemori, 1976) reportedly by inhibiting its affinity for binding to the opiate receptor (Terenius, 1975). This could

Figure 5

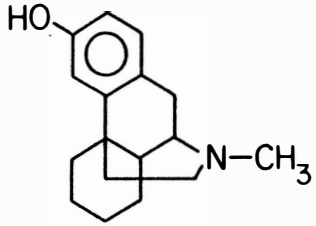
Structures of the narcotic analgesics and antagonist tested in mice with altered blood glucose levels. Those drugs whose antinociceptive potencies were altered in mice with STZ-induced diabetes or other pretreatments are marked with an asterisk (*).



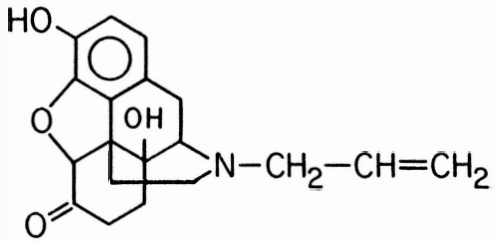
MORPHINE*



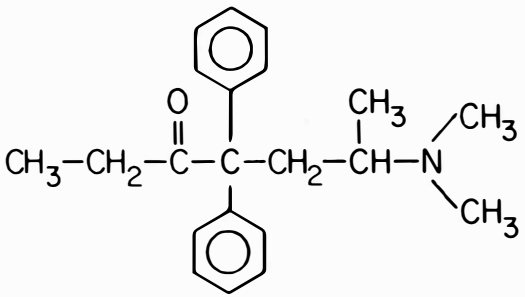
PHENAZOCINE*



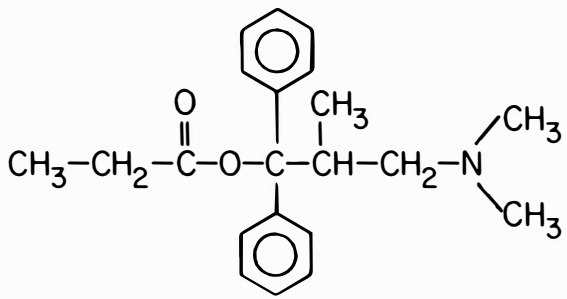
LEVORPHANOL*



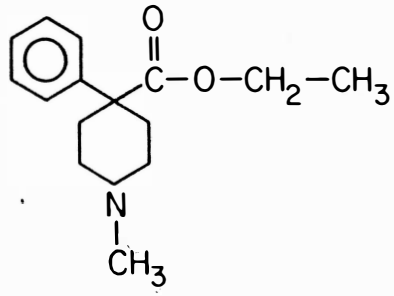
NALOXONE



METHADONE



PROPOXYPHENE



MEPERIDINE

be the mechanism by which hyperglycemia affects the antinociceptive potency of morphine.

When naloxone was used to antagonize the antinociceptive effects of morphine, the STZ-induced diabetic mice demonstrated a decreased sensitivity to morphine compared with control mice, regardless of the dose of naloxone. One can speculate that there may be a decrease in the number or an alteration in the conformation of receptors available to bind to morphine. The antinociceptive potencies of methadone, propoxyphene and meperidine were not altered by STZ-induced diabetes perhaps because the change in the receptors that may be speculated to have occurred affected the binding of certain drug molecules (e.g. morphine) which are too rigid in structure to fit into the altered receptor. It is widely accepted, since having been proposed by Martin et al. (1976), that there are at least three different classes of opiate receptors and that two of these are involved in antinociception. Perhaps the less rigid structures (e.g. methadone) have affinity and intrinsic activity for different receptors than morphine including the possibly altered receptors in STZ-induced diabetic mice. The speculated change in conformation is apparently not due to changes in brain glucose levels.

In addition to the attenuated antinociceptive potency of morphine seen in STZ-induced diabetic mice was the significantly potentiated lethal effect of morphine demonstrated in the LD_{50} experiment. The physiologic alteration in the STZ-induced diabetic mice responsible for morphine's decreased antinociceptive effects and increased lethality could be different. Neither the antinociceptive potency nor the lethality of methadone were altered by STZ-induced diabetes, providing further support for the position that there is a difference in the actions of morphine and methadone and a selective effect of diabetes on drug potency. Different receptors may also

be involved for morphine- vs. methadone-induced lethality.

There are reports that morphine exerts a direct effect on blood glucose levels. Intravenous and intraventricular administration (at 1/50 the i.v. dosage) of morphine produce marked hyperglycemia within 15 min which lasted for 60 min (Borison et al., 1962 and 1964). The hyperglycemia they observed following morphine administration was not as much as was achieved in this dissertation by STZ-induced diabetes or dextrose pretreatment. The highest dosage of morphine utilized in this dissertation to produce antinociception (8 mg/kg s.c.) was not sufficient to cause hyperglycemia in control Swiss mice (Table 24). The hyperglycemic effect of intraventricularly administered morphine occurs concommitantly with a temporary partial depletion of hypothalamic catecholamines (Moore et al., 1965). The interaction between certain narcotics and glucose metabolism is further suggested by reports that heroin addicts have a delayed and attenuated hyperglycemic response after a glucose tolerance test, high resting insulin levels, and a delayed hyperinsulinemic response following the test (Reed and Ghodse, 1973; Brambilla et al., 1976; Ghodse, 1977). Whether the reported "tolerance" to the effects of glucose ingestion in addicts has any direct relationship to the more familiar tolerance development to heroin (or to the decreased antinociceptive potency of morphine reported in this dissertation) is uncertain, but intriguing to consider. Leslie et al. (1979) have even hypothesized that non-insulin-dependent diabetes may be due to an inherent super-sensitivity to enkephalin. However, the data to support their hypothesis is purely circumstantial. Levorphanol, like morphine, has been reported to cause marked hyperglycemia (Keith et al., 1955; Pittinger et al., 1955). In contrast, methadone (Isbell et al., 1947), propoxyphene (Wiederholt et al., 1967) and meperidine (Dey and Feldberg,

1975) whose antinociceptive potencies were not altered by STZ-induced diabetes are either very weakly hyperglycemic, hypoglycemic, or have no effect on BGL's.

Levine and Goldstein (1955) and Battaglia and Randle (1959) have reported that insulin does not have an effect on the uptake or utilization of fructose by body tissues, in contrast to insulin's facilitation of the uptake and utilization of glucose and certain other sugars. A number of tissues in the body (peripheral nerves, erythrocytes, lens) do not require insulin for the uptake of glucose (Bhagavan, 1978), and can freely absorb fructose from the blood (Levine and Goldstein, 1955). When hyperglycemia occurs (as in diabetes) these tissues will convert the excess glucose to fructose and sorbitol in quantities much greater than normally occurs. The decreased sensitivity to morphine seen in diabetic mice is not likely to be due to a buildup of fructose or sorbitol in a key insulin-insensitive peripheral tissue or brain. This is because the tail-flick test detects central effects, and without a narcotic present, tail-flick latency was not affected by hyperglycemia.

King et al. (1977 and 1978) have reported that morphine has dose-dependent effects on brain energy metabolism, most notably decreasing brain malate levels as morphine-induced antinociception increases. Levorphanol, too, has a significant dose-dependent effect on malate levels, while methadone and meperidine, whose antinociceptive potencies were not altered by STZ-induced diabetes, had significant effects on brain malate levels only at intermediate and not high doses (L.J. King, personal communication). Since malate is a component of the citric acid cycle, one could speculate that the hyperglycemia could affect the cycle, increasing its activity and preventing morphine's effects. Experimentally-induced

diabetes has already been shown to have a myriad of effects on carbohydrate metabolism in the brain (Prasannan and Subramanvan, 1968; Flock et al., 1969). Ruderman et al. (1974) have reported that STZ-induced diabetic rats have significantly increased concentrations of fructose-6-phosphate, acetyl CoA, glycogen and citric acid in their brains compaired to nondiabetic rats. They did not determine the level of malate in these brains. If a decrease in malate levels of the brain is necessary for morphine-induced antinociception, one could speculate this to be a possible site of interaction between diabetes and morphine's effects.

Formal reviews of many pharmacologic agents that can either increase or decrease morphine potency have been prepared by Hansten (1971) and Takemori (1976). It is noteworthy that many of the pharmacologic agents that can alter the potency of morphine are drugs that affect neurotransmitter systems in the brain (Harris et al., 1969; Dewey et al., 1969; Harris et al., 1969; Howes et al., 1969; Dewey et al., 1970; Calcutt et al., 1971; Major and Pleuvry, 1971; Sparkes and Spencer, 1971; Ayhan, 1972; Clouet, 1975; Govoni et al., 1975; Bloom et al., 1976; Racagni et al., 1977; Rosecrans et al., 1977; Phebus and Lytle, 1978; Strombom and Svensson, 1978; Elchisak and Rosecrans, 1979; Rosecrans et al., 1979). There is thus reason to speculate that if the CNS neurochemical systems are altered in diabetics, a change in morphine potency may be observed.

Several reports have appeared suggesting that several of the consequences of diabetes affect the function of both the dopaminergic and serotonergic systems of diabetics. Blood vessels in kidneys of diabetic humans do not vasodilate after dopamine infusion (Schacht and Baldwin, 1977). An unusually high percentage of patients in mental institutions have diabetes (Waitzkin, 1966a and b; Koranyi, 1979). Many of these patients, once diagnosed and treated for diabetes, have had spontaneous

remissions of the signs and symptoms of their mental illnesses (Koranyi, 1979). Affective disorders were most common in these patients, suggesting that these patients' central biogenic amine systems were not functioning properly.

STZ-induced diabetic rats have lower brain tryptophan levels than nondiabetics (MacKenzie and Trulson, 1978c). Insulin administration to STZ-induced diabetic rats returned their brain tryptophan levels to normal (MacKenzie and Trulson, 1978a, b, c and e; Crandall and Fernstrom, 1979). Serotonin turnover rates were normal even in severely diabetic rats (MacKenzie and Trulson, 1978c and e). These same authors (1978d) reported that significantly fewer STZ-induced diabetic rats displayed a behavioral syndrome (Jacobs, 1976) induced by p-chloroamphetamine and tryptophan plus pargyline than did nondiabetic rats. There have been other reports that the central serotonergic (Fernando and Curzon, 1978) and dopaminergic (Marshall et al., 1976; Marshall, 1978a and b; Marshall et al., 1978) systems of diabetic rats are resistant to pharmacologic manipulation. Thus there is circumstantial evidence to support the speculation that alterations of the serotonergic and/or dopaminergic systems in the brains of STZ-induced diabetic mice could be partly responsible for morphine's decreased antinociceptive potency.

It should be noted that species differences in basal and intermediary metabolism and differences between human diabetes mellitus and the animal models used in this dissertation make it impossible to assume that human diabetics are less sensitive to the analgesic effects of morphine. There is no such evidence in the literature that human diabetics are less sensitive to the analgesic effects of any narcotic analgesic, perhaps due to the rigid control of BGL's in diabetics exerted by physicians during

hospitalization (Giddings, 1974).

In summary, any one or a combination of the following may be intermediary steps in the alteration of the antinociceptive potency of morphine by STZ-induced diabetes: alterations in the conformation of certain opiate receptors; changes in the responsiveness to morphine of the neurotransmitter systems in the brain; and alterations in brain energy metabolism as indicated by changes in brain malate levels. The data do not allow more than speculation on these ideas. However, there is sufficient evidence to hypothesize that the principal factor of the diabetic state responsible for the altered antinociceptive potency of morphine is probably the presence of hyperglycemia. This is supported by the experiments demonstrating that reversal of the STZ- and dextrose-induced hyperglycemia in mice by insulin returned sensitivity to morphine's antinociceptive effects to normal. Blood glucose levels but not serum insulin levels or brain glucose levels correlated with changes in the antinociceptive potency of morphine.

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