THE EFFECT OF BRISTOL MYERS MJ 12880-1 AND

2-TETRADECYLGLYCIDIC ACID (McN-3802, TDGA)

ON FATTY ACID METABOLISM, TISSUE FFA AND

TG CONTENT IN DIABETIC (db/db) MICE

by

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To my family

ACKNOWLEDGMENTS

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ABSTRACT

The C57BL/KsJ-db/db mouse, a genetic model of experimental diabetes mellitus, was used to evaluate two drugs, Bristol Myers MJ 12880-1 and 2-tetradecylglycidate (TDGA or McN-3802) -- an inhibitor of long chain fatty acid oxidation. The studies in these diabetic mice were primarily concerned with lipid metabolism, and measurement of free fatty acids (FFAs) and triglycerides (TGs) in tissues and plasma.

Bristol Myers MJ 12880-1 was an ineffective hypo-glycemic agent and had no effect on plasma FFAs. MJ 12880-1 exacerbated the diabetic metabolism of the db/db mice aged 2-3 months and 5-6 months.

The hearts of db/db mice orally treated with TDGA at 5, 10 and 25 mg/kg/day for 12 weeks showed no significant differences in their TG content as compared to untreated db/db and non-diabetic mice. However, the cardiac FFA content of the 10 mg/kg/day treated db/db mice was significantly higher than the untreated db/db mice.

The tissues of the TDGA study were stored for 7-14 months at -70°C until analysis; the lipids may have hydrolysed. The study should be repeated using a method of quickly freezing the tissues at dry ice temperature (-78.5° C) and then rapidly extracting the lipids before hydrolysis can occur.

INTRODUCTION

Diabetes Mellitus

Diabetes mellitus is a common health problem in the United States. In 1979, diabetes mellitus was the attributed cause of death in 33,060 or 15/100,000 Americans according to the National Center for Health Statistics. Death usually results from the vascular complications arising from the disease.

Throughout the centuries, physicians have recognized that the clinical picture, course and severity of diabetes mellitus follows two general patterns: a juvenile onset, ketosis-prone type of diabetes and a less severe, maturity-onset, obesity-related, ketosis resistant diabetes (Skyler and Cahill, 1981). The National Diabetes Data Group (1979) classified diabetes mellitus and other categories of glucose intolerance based on contemporary knowledge of this heterogenous disorder. Juvenile onset diabetes and maturity onset diabetes have been labeled insulin-dependent diabetes mellitus (IDDM) and noninsulin-dependent diabetes mellitus (NIDDM), respectively. Other types have been recognized, and include secondary diabetes mellitus related to pancreatic disease (pancreatitis, surgical pancreatectomy), other hormonal disturbances (Cushing's disease, acromegaly, hyperthyroidism), insulin receptor abnormalities and certain

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rare genetic syndromes and other types [Lilly (Eli) Research Company, 1980; National Diabetes Data Group, 1979; Rotter and Rimoin, 1981].

Insulin dependent diabetes mellitus or Type I diabetes usually begins before 35 years of age, often in childhood or adolescence. The onset is rapid with almost complete failure of pancreatic islet function and insulin secretion leading to a state of insulin dependency. During the initial onset, islet cell antibodies are frequently present in the circulation. The oral hypoglycemic drugs are rarely effective for Type I diabetes, and exogenous insulin therapy is necessary to prevent death. Hyperlipidemia occurs frequently and ketoacidosis only occurs in insulin dependent diabetics.

Noninsulin dependent diabetes mellitus or Type II diabetes is divided into two subtypes: nonobese and obese. About 60-90% of the Type II diabetic individuals are obese (National Diabetes Data Group, 1979). Type II diabetes results from a defective insulin secretion to a given glucose signal and a state of relative insulin deficiency results. Histologically, the pancreatic islet cell appears essentially normal, and plasma insulin levels are normal or elevated. The elevated plasma insulin levels are associated to a decreased number of insulin receptors or a defect in insulin binding to cells, thus causing insulin resistance (Gepts and Lecompte, 1981; Rizza, Mandarino and Gerich, 1981). Type II

diabetes often occurs after 35 years of age (Kryston, 1981).
Diet and weight control can usually maintain metabolic
control adequately.   Oral hypoglycemic drugs including
sulphonylureas act by stimulating pancreatic islet cells to
secrete endogenous insulin, promoting growth of beta cells
and increasing receptor numbers [Skillman and Feldman, 1981;
Lilly (Eli) Research Company, 1980].   Insulin is sometimes
required to correct hyperglycemia which may develop under
conditions of stress and infection.   Cholesterol and tri-
glyceride levels are frequently elevated and related to
obesity.   Carbohydrate induced hypertriglyceridemia is
commonly observed [Lilly (Eli) Research Company, 1980].

Glucose is a potent stimulus for insulin secretion.
There are two separate phases of insulin secretion.   The
first phase involves the rapid release of small labile
stores of insulin from their granules into blood circula-
tion.   The second phase results in gradually increasing
levels of insulin secretion.   In Type II diabetes, the early
phase of insulin secretion following stimulation of the beta
cell by glucose appears to be impaired as suggested by the
characteristic delay of insulin secretion.   In Type I
diabetes, insulin secretion is often totally absent indicating
complete beta cell failure.

The uptake and utilization of glucose is impaired in
states of insulin deficiency.   In addition glucose enhanced

gluconeogenesis from glycogen, lactate, pyruvate, amino acids
and glycerol results from decreased insulin activity. Thus,
the hyperglycemia of insulin results from both a diminished
glucose utilization and an increased gluconeogenesis.

Insulin deficiency also causes alterations in protein
and fat metabolism. Proteins are catabolized to amino acids,
which in turn act as an additional substrate for glucose
production. In a situation when intracellular glucose is
unavailable for energy, the body increasingly relies on fat
as an energy source. The fatty acids are oxidized to two
carbon fragments and acetyl CoA which allows for ATP pro-
duction (Wakil, 1970). If lipolysis occurs at an increased
rate exceeding the capacity for free fatty acid utilization,
large amounts of ketone bodies, beta-hydroxybutyrate, acetone
and acetoacetate, are produced. Furthermore, the ketone
bodies impair glucose utilization and contribute excessive
amounts of metabolic acids to the circulation causing
metabolic acidosis.

Experimental Models of Diabetes Mellitus

Several animal models of diabetes mellitus have
received extensive study. The most common is that induced
in rats by streptozocin, which results in pancreatic beta
cell necrosis and insulinopenia (Mordes and Rossini, 1981).

In 1966, diabetes occurred as a mutation in an
inbred mouse strain C57BLKsJ (Hummel, Dickie and Coleman,

1966). The mutation which caused diabetes was inherited as
a single autosomal recessive gene with complete penetrance
on chromosome 4, linkage group VIII. Animals homozygous
for the diabetic gene (db/db) exhibited hyperphagia and
obesity with increased fat deposition in axillary and
inguinal regions at about 3-4 weeks of age, in association
with progressive hyperglycemia after 6-8 weeks.

The diabetic mice are infertile and result from the
breeding of animals heterozygous for the diabetic gene
(db/+). Breeding of the heterozygous BL/KsJ-db/+ mice
results in three types of littermates in the C57BL/KsJ
inbred mouse strain which can be identified by coat color
and body build (Herberg and Coleman, 1977; Coleman, 1978).
The normal heterozygotes (db/+) used for breeding are thin
and black. The normal homozygous misty (+/+) mice are thin
and gray. The diabetic (db/db) mice are black and fat.

Two stages of the diabetes syndrome are recognized in
the diabetic mice (Herberg and Coleman, 1977; Coleman, 1978).
Coleman and Hummel (1974) found the diabetic mice to be
hyperinsulinemic as early as 10 days of age. The diabetic
mice rapidly accumulates fat at 3-4 weeks of age (Hummel,
Dickie and Coleman, 1966). Subsequently, hyperglycemia,
polyuria and glycosuria follow. The diabetic mice remain
hyperinsulinemic until 3-4 months of age, while they gain
weight. At about 4 months of age the diabetic mice stop

gaining weight and insulin secretion decreases. Diabetic mice lose about one-third of their body weight between 4.5-9 months of age (Wyse and Dulin, 1970). Mice with genetic diabetes are more prone to succumb to the disease and often die before 10 months of age. The symptoms of hyperinsulinemia and obesity exhibited by the diabetic mice suggest a developing pattern similar to human Type II diabetes (Coleman, 1978).

Steinmetz, Lowry and Yen (1969) were the first ones to investigate FFA release from the adipose tissue of diabetic mice, 3-5 months of age. They found that the basal lipolysis rates of adipose tissue in vitro were similar in the diabetic and normal heterozygous mice. However, the plasma free fatty acid levels of the diabetic mice were about 1.5 times normal heterozygous mice.

The catecholamines, norepinephrine and epinephrine, and ACTH stimulate FFA mobilization. They act on adenyl cyclase, and thereby raise the intracellular level of cyclic adenosine-3',5'-monophosphate (c-AMP). The free fatty acid mobilization of the epididymal adipose tissue from the diabetic mice exposed to epinephrine, norepinephrine, isoproterenol and corticotropin were slightly decreased from controls (Steinmetz, Lowry and Yen, 1969). In comparison to the normal mice, the diabetic mice also showed a slightly lower lipolytic response to theophylline. Theophylline stimulates lipolysis by inhibiting phosphodiesterase from

catalyzing the conversion of c-AMP to 5'-AMP. However, the incubations of adipose tissue with dibutyryl c-AMP stimulated lipolysis in normal and diabetic mice to the same degree. Dibutyryl c-AMP is lipolytically active and more permeable to the cell membrane than c-AMP. From the above data, Steinmetz, Lowry and Yen (1969) concluded that the lipolysis system of the diabetic mice is defective. Furthermore, adenyl cyclase and lipase were implicated as causing the decreased fatty acid mobilization from the adipose tissue.

Allan and Yen (1976) studied the effects of iso-proterenol on lipolysis. At the peak response of about 6-7 minutes after subcutaneous administration of isoproterenol, the plasma free fatty acids (FFAs) were similar in diabetic and control mice. However, after peak response time, the plasma FFA levels were higher in the diabetic mice than the normal mice. The slow fall in plasma FFA was postulated to result from the following:

1) a prolonged response due to increased deposits of substrate triglyceride;

2) a longer response because of the slower degradation of isoproterenol in thelarge mass of adipose tissue;

3) a slower release of FFA from the larger adipose tissue mass; and/or

4) a slower clearance of FFA from the plasma.

The clearance of FFAs from the plasma depends on the FFA oxidation rates in various tissues as well as the FFA re-esterification rate in the liver and adipose tissue. Allan and Yen (1976) further observed that propranolol, a beta adrenergic blocker with anti-lipolytic properties, blocked most of the response by the db/db and normal mice to isoproterenol, when administered 30-40 minutes prior to isoproterenol.

Triglycerides account for about 50-56% of the diabetic mouse's body weight (Yen et al., 1976; Allan and Yen, 1976), but the blood volume of an obese mouse is not increased in proportion to its weight. Theoretically, if the diabetic mice responded normally to a lipolytic agent, the plasma FFA level of the diabetic mice would be approximately sixfold greater than normal. Allan and Yen (1976) assumed that the slower decay of plasma FFA in the diabetic mice was a result of the increased triglyceride deposit and was not caused by a decreased clearance rate of the FFA from the plasma. Furthermore, Allan and Yen (1976) estimated that the plasma FFA level in the db/db mice was only 1.5 times that of normal mice. From the in vivo lipolysis study by Allan and Yen (1976) and in vitro lipolysis studies by Steinmetz, Lowry and Yen (1969), Yen and Steinmetz (1972), and Kupiecki and Adams (1974), a quantitative deficiency in lipolysis was implicated in the diabetic mice.

Yen et al. (1976) investigated the triacylglycerol content and in vivo lipogenesis rates of the liver and carcass in diabetic and normal mice before and after the diabetics accumulated fat deposits. The carcass and liver triacylglycerol content was twofold greater at 14 weeks of age when compared to that of a diabetic mouse at 5 weeks of age. In young diabetic mice, the carcass exhibited high rates of lipogenesis which decreased to normal in older mice when their weight gain had stabilized. The liver of the diabetic mice had high lipogenesis rates at 5 and 14 weeks of age. The initial increase in the accumulation of liver triacylglycerols occurred at 7-8 weeks of age (Chan and Exton, 1977), and remained elevated in the diabetic mice. Hepatic triacylglycerols in the diabetic mice at 5 and 8 weeks of age exhibited a wide range (Chan and Exton, 1977; Chan et al., 1975).

Chan et al. (1975) found the free fatty acid levels in serum were about 3 times normal in db/db mice at 8 weeks of age. Steinmetz, Lowry and Yen (1969) found the plasma FFA levels of 14-20 week old diabetic mice to be 1.5-2 times that of normal mouse. The age of the mouse, metabolic condition at that age and the handling of the mouse in obtaining blood or tissue as well as the method used may play an important role in contributing to the variation of free fatty acid in plasma or serum and hepatic triacylglycerol levels.

Experimental Drugs

The two drugs investigated in the C57BL/KsJ-db/db mice were Bristol Myers MJ 12880-1 and 2-tetradecylglycidic acid (TDGA). These drugs or related agents might be of value in the treatment of diabetes mellitus.

Bristol Myers MJ 12880-1

The following is a summary of recent work with the drug, MJ 12880-1, performed on rats (communication from Bristol Myers Laboratories).

MJ 12880-1's effect on plasma FFA levels, lipolysis rates and oxidative metabolism were evaluated in rats. Three groups of rats were dosed with the drug at a concentration of 6.25, 12.5 and 25 mg/kg body weight/day in two equal doses for 15 days. A control group of rats was used to establish the baseline for the plasma FFA levels and lipolysis studies. The plasma FFA levels of all three groups of drug treated rats were significantly elevated from control rats.

The lipolysis studies involved a basal incubation of epididymal fat pads from control and drug treated rats and the addition of dimethylformimide, norepinephrine and theophylline to the incubation medium. Norepinephrine and theophylline are known to induce lipolysis in normal mice (Steinmetz, Lowry and Yen, 1969). The basal incubations of epididymal fat pads from the three dosed groups of rats

showed a significant increase in lipolysis rates from the control rats. The addition of dimethylformimide to the incubations of epididymal fat pads resulted in a slower lipolysis rate for the rats treated with drug at 12.5 and 25 mg/kg body weight/day. The addition of norepinephrine or theophylline to the incubation medium decreased the amount of FFA mobilized from the epididymal pads of 25 mg/kg/day drug treated rats.

Brain homogenates and liver slices from the three groups of dosed rats were used in the metabolic oxidation studies. Hepatic oxidation of acetoacetate was elevated in all three groups of dosed rats. However, glucose oxidation and palmitate oxidation by the liver decreased or remained the same. Acetoacetate oxidation by the brain decreased or stayed the same for rats treated with 12.5 and 25 mg/kg/day of drug. Palmitate oxidation was decreased or remained the same in brains from rats treated at 6.25 mg/kg/day with MJ 12880-1. Glucose oxidation by the brain decreased for all 3 dosed groups of rats. Further studies on MJ 12880-1 were required to conclude the drug's effect on oxidative metabolism.

2-Tetradecylglycidate (TDGA)

The common chemical name of the compound developed by McNeil Laboratories and identified as McN-3802 is 2-tetradecylglycidate (TDGA). The structure is shown in

Figure 1. This agent and its methyl ester (McN-3716) which
is rapidly converted to the free acid in the body are
inhibitors of long chain fatty acid oxidation that act by
inhibiting car-itine palmitoyl transferase (Tutwiler and
Dellevigne, 1979). TDGA and its methyl ester differ
structurally and mechanistically from the two oral hypo-
glycemic drug classes marketed, sulfonylureas and biguanides
(Tutwiler et al., 1978).

$$CH_3(CH_2)_{13}$$ COOH O
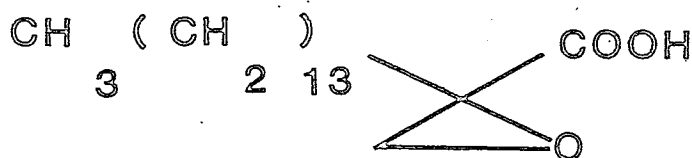
Figure 1. The Structure of 2-Tetradecylglycidate
(TDGA or McN-3802)--Specific Inhibitor of
Long Chain Fatty Acid Oxidation.

Randle et al. (1963,1966) has shown that in states
of increased lipid oxidation, the products of fatty acid
oxidation depress glucose utilization and stimulate hepatic
gluconeogenesis. Randle's glucose-fatty acid cycle
hypothesis states that glucose and fatty acid metabolism in
muscle and adipose tissue interact in a cycle. Therefore,
each controls the other's release into the blood and their
utilization. Randle et al. (1963,1966) further suggested
that excessive free fatty acid metabolism may be a key

factor underlying the decreased glucose tolerance in diabetes mellitus.

As predicted by the Randle hypothesis, drugs like TDGA and its methyl ester that decreased fatty acid oxidation resulted in lowered glucose concentrations, in situations where fatty acids are utilized as the major energy substrates, such as fasting, diabetes and high fat diets (Tutwiler, Mohrbacher and Ho, 1978). However, TDGA and its methyl ester did not lower glucose concentrations in conditions where carbohydrates were the main energy source. The study of specific inhibitors of fatty acid oxidation could increase our understanding of the role played by excessive fatty acid oxidation in diabetes and other diseases.

Free fatty acids are the major respiratory fuel for the fully aerobic mammalian heart. Free fatty acids are detrimental to a compromised heart. Pearce et al. (1979) summarized the proposed mechanisms of the FFA's effect on the heart in hypoxic conditions:

1) high intracellular levels of FFAs may bind intra-cellular calcium and limit the calcium required by the myofibrils;

2) increased fatty-acyl CoA levels may inhibit the adenine nucleotide translocator and thereby lower ATP availability to the muscle fibers;

3) ATP availability can be decreased by free fatty acid
    uncoupling oxidative phosphorylation; and

4) the fatty acid may increase the mass of anoxic
    tissue under hypoxic and ischemic conditions by
    increasing oxygen demands.

The oral hypoglycemic agent, 2-tetradecylglycidic
acid, specifically inhibits long chain fatty acid oxidation
but not short chain fatty acid oxidation in isolated rat
hepatocytes. Gluconeogenesis and ketogenesis are also
inhibited (Tutwiler and Dellevigne, 1979). TDGA also
inhibits long chain fatty acid oxidation in rat hemidiaphragm
(Tutwiler, Mohrbacher and Ho, 1979).

From the above data, Tutwiler and Dellevigne (1979)
suggested that TDGA inhibited an enzyme in the pathway of
fatty acid degradation prior to beta oxidation. Since the
concentrations of TDGA or its methyl ester used to inhibit
microsomal acyl-CoA synthetase activity were 100 times higher
than those required to inhibit palmitate oxidation,
ketogenesis and gluconeogenesis, the long chain acyl-CoA
was obviously not the site of inhibition. Tutwiler and
Dellevigne (1979) found that long chain acyl-CoA derivatives
accumulated after adding TDGA to hepatocytes. Increased
triglyceride levels were observed in livers of McN-3716
treated rats. In the presence of TDGA, only palmitate
oxidation was inhibited, while palmitoyl-L-carnitine and

octanoate were oxidized at normal rates. Therefore, Tutwiler and Dellevigne (1979) concluded the site of action of TDGA to be carnitine acyltransferase. Furthermore, Tutwiler and Ryzlak (1980) found evidence that TDGA or its coenzyme A ester inhibited long chain fatty acid oxidation by irreversibly inhibiting mitochondrial palmitoyl-CoA transferase.

TDGA inhibits long chain fatty acid oxidation, with resulting stimulation of glucose oxidation and lowering plasma glucose and tissue glycogen stores. Gluconeogenesis is diminished. For this reason, TDGA might be efficacious in treating diabetes mellitus, a condition in which fatty acids are utilized as a major energy substrate.

STATEMENT OF PROBLEM

The C57BL/KsJ-db/db mouse, a strain manifesting genetic diabetes mellitus, was employed in studies to test two potential new treatment approaches to diabetes mellitus. These agents, Bristol Myers MJ 12880-1 and 2-tetradecyl-glycidic acid (TDGA or McN-3802), were studied in terms of their effects on metabolism and pathologic changes in diabetic mice.

The literature values for the liver triglyceride levels of the diabetic mice have a wide variation. The literature lacks information on heart triglyceride levels and free fatty acid levels in hearts and livers from the C57BL/KsJ diabetic (db/db), heterozygous (db/+) and misty (+/+) mice.

The major purpose of this research project was to determine the free fatty acid pool in the hearts of the diabetic (db/db) mice. In addition, the goals of the project included the following:

1. To evaluate the effects of Bristol Myers MJ 12880-1 on diabetic mice in terms of:

   a. Changes in plasma long chain free fatty acids and blood glucose following a single dose of drug.

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b.   Changes in plasma long chain free fatty acids
     and blood glucose following 14 days of daily
     drug administration.

c.   The effects of 14 days of daily drug administra-
     tion on glucose oxidation by the heart, liver
     and brain homogenates of young diabetics (2-3
     months old) and old diabetics (5-6 months old).

d.   The effects of the 14 days of drug administered
     daily on palmitate oxidation by the heart, liver
     and brain homogenates from the two different age
     groups of diabetic mice;

2.   To examine the free fatty acid and triglyceride
     content of tissues kept at dry ice temperature
     $(-78.5^{\circ}C)$; and

3.   To quantitate the triglyceride and free fatty acid
     levels in heterozygous (db/+), and misty (+/+) mice
     as well as the diabetic (db/db) mice, which were
     treated or not treated with 2-tetradecylglycidate
     (TDGA or McN-3802).

MATERIALS AND METHODS

Chemicals and Supplies

Chemicals and supplies were obtained from the
following sources: high pressure liquid chromatography
grade methanol, chloroform, hexane and methylene chloride
from Burdick & Jackson, Muskegeon, Mich., Fisher Scientific
Co., Fair Lawn, New Jersey, Water Associate, Inc., Milford,
Mass. The following were purchased from Supelco Inc.,
Bellefonte, Pa.: glyceride kit, 3 N methanolic HCl, Gas
Chromosorb WHP 100-120 mesh and 100 microliter GC automatic
sampler vials. The following chemicals were obtained from
Sigma Co., St. Louis, Mo.: heptadecanoate (C17); methyl
esters of palmitate (C16), palmitoleate (C16:1), hepta-
decanoate (C17), stearate (C18), oleate (C18:1) and linoleate
(C18:2); 2',7'-dichlorofluorescein, bovine serum albumin
and Trizma base. EGTA (ethylene bis(oxyethylenenitrilo)
tetraacetic acid) was obtained from Eastman Kodak Co.,
Rochester, N.Y. The 1-^{14}C-palmitate, (U-^{14}C)glucose and
omnifluor premixed LSC powder was bought from New England
Nuclear, Boston, Mass. The ultra high purity (UHP) nitrogen,
hydrogen and air were obtained from Liquid Air Inc., San
Francisco, Ca.

Animals and Tissues

The plasma and tissue samples of heart, liver and brain from the diabetic (db/cb), misty (+/+) and hetero-zygous (db/+) mice were obtained from the breeding colony maintained at the Animal Resource Center of the University of Arizona Medical Center. The breeding colony was established with animals originally obtained from Jackson Laboratories, Bar Harbour, Maine.

Methodology

Palmitate and Glucose Oxidation

Fresh samples of heart, liver and brain from animals sacrificed by exsanguination were immediately placed in cold Tris sucrose buffer on ice. Each tissue was blotted dry, weighed, minced on ice and homogenized in Tris sucrose buffer (1:10; g:ml). These tissue homogenates were used for the glucose oxidation and palmitate oxidation experiments. Glucose and palmitate oxidation were performed utilizing the methods described by Brendel and Meezan (1974). The reagents for the experiment are listed in Table 1.

For the glucose oxidation experiment, each sample was analyzed in triplicate as follows, 5 mM (U-^{14}C)glucose (0.05 ml) and tris sucrose buffer (0.35 ml) were placed in a 2 ml plastic vial. After the addition of 0.1 ml of tissue homogenate (1:10; w/v), the vial was placed on an oxidation

Table 1. Methods and Materials Formulae.

A. Tris Sucrose Buffer
    2.422 g Tris
    54.77 g Sucrose
    0.292 g NaCl
    3.727 g KCl
    0.038 g EGTA
    950 ml distilled deionized
    H_2O to make 1 l
    pH to 7.2 with @ 2 ml of
    concentrated HCl
    add 5 mM Mg^{+2}

C. 1-^{14}C-Palmitate Substrate
    Palmitate-BSA (2:1, molar
    ratio)
    0.255 g BSA (0.75 mM)
    5 ml Tris Sucrose Buffer
    Mix BSA and buffer
    Warm up to 40-49°C
    Stir while adding 0.05 ml
    warmed 1-^{14}C-Palmitate
    Allow to stir 1-2 hours
    loosely capped to obtain
    minimal background.

B. $^{14}C_u$-Glucose (5 mM)
    50 mM Cold Glucose
    0.045 g Glucose
    5 ml Tris Sucrose buffer
    0.05 ml (0.05 mCi)

    $^{14}C_u$-Glucose
    Stir for 1-2 hours
    loosely capped to obtain
    minimal background

D. Scintillation Fluor
    2 l Toluene
    1 l Triton X-100
    .250 l distilled H_2O
    12 g Omnifluor
    Stir

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apparatus set on a 37^OC water bath. All CO_2 produced from glucose oxidation during a 30 minute period was collected in scintillation vials containing 1.5 ml of 0.3 N NaOH with a trace of Triton. The glucose blank consisted of 0.45 ml tris sucrose buffer and 0.05 ml (U-^{14}C)glucose.

The oxidation apparatus was capable of handling 28 vials at one time. To each vial, there were two small lengths of plastic tubing. One tube brought in the oxygen gas used to displace the labeled and unlabeled CO_2 produced from the glucose substrate by the homogenate. The other tube passed the CO_2 gas into the vial containing the NaOH.

The same procedure was used for the determination of the 1-^{14}C-palmitate oxidation. In these experiments, 0.05 ml of 1-1.5 mM 1-^{14}C-palmitate substrate was used instead of the radiolabeled glucose substrate. In control vials, tissue homogenate was omitted. The palmitate blank contained the same volume of 1-^{14}C-palmitate instead of the radiolabeled glucose substrate.

After 30 minutes, the scintillation vial was removed from the oxidation apparatus and 14 ml of scintillation fluor was added. The scintillation vial was capped, vortexed and counted for ^{14}C on a Beckman model LS-3133P scintillation counter for 5 minutes or a preset error of 0.2%.

In addition to the samples and blanks, triplicate sets of 0.01 ml 5 mM (U-^{14}C)glucose and 0.01 ml 1 mm 1-^{14}C-palmitate in 7 ml of fluor was counted. These two sets provided the specific activity information of the radiolabeled glucose and molarity of the substrate palmitate. The examples for calculating the specific activity of the radiolabeled glucose and the nmoles of glucose or palmitate oxidized to CO_2 appear in Appendix A.

Protein Assay

The amount of protein in tissue homogenates was determined by the method of Lowry et al. (1951). All reagents were freshly prepared on the day of the protein assay. 2% Na_2CO_3 in 0.1 N NaOH, 1% $CuSO_4$, 20% sodium tartrate and 10% (w/v) deoxycholic acid in water were combined in the ratios of 10:0.1:0.1:0.5 by volume, respectively. Into several empty test tubes labeled for each homogenate sample and standard, 2.5 ml of the mixture was added.

Prior to the assay, the tissue homogenates were sonicated to ensure a homogenous solution. Bovine serum albumin (BSA) was used as the protein standard. The standard stock solution of BSA was 1 mg/ml. Distilled, deionized water was added to approximately 0.5 mg of protein contained in the tissue homogenate or 0.02-0.20 ml of 1 mg/ml BSA to bring the total volume to 1 ml. The 0.5 mg of

protein in the homogenate was equivalent to 0.5 ml of a 1% (w/v) tissue homogenate.

The 1 ml of tissue homogenate or BSA standard was transferred to their respective tubes containing the 2.5 ml mixture. The test tubes were vortexed and allowed to sit at room temperature for 30 minutes. After 30 minutes the tubes were vortexed while adding in 0.20 ml of Folins phenol reagent. The test tubes were allowed to sit at room temperature for another 30 minutes. The absorbance of the samples and standards was read at 720 nm on a Beckman Acta CIII spectrophotometer. A standard protein curve is shown in Appendix B. The volumes used for the homogenates of brain, heart and liver gave concentrations between 1 and 22 mg/ml of protein.

Plasma Long Chain Free
Fatty Acid Analyses

A modification of the method of Grunert and Babler (1973) was used to assay plasma free fatty acids (FFAs). FFAs were converted to methyl ester (FAMES) derivatives and analyzed by gas liquid chromatography (GLC). Duplicates for each plasma sample were assayed provided that there was adequate plasma available. The plasma samples, frozen at $-20^{\circ}C$ for 1-8 months, were thawed at room temperature prior to extracting the FFAs.

Extraction. An aliquot of 0.003-0.010 ml of plasma was added to a test tube containing 0.05 μmoles heptadecanoate (C17) and 1.1 ml of chloroform-methanol (2:1, v/v). The tube was capped with a Teflon lined cap, vortexed, and 0.01 ml of 1 N H_2SO_4 and 1 ml of 0.05 M NaCl were added. The tubes were capped, vortexed and centrifuged in a VWR Scientific GT2 centrifuge at 898 x g for two minutes. After centrifuging, two distinct layers were observed. An aliquot of 0.50 ml $CHCl_3$, lower phase, was taken up with a Glenco 1 ml syringe. Then half of the chloroform was placed in a 0.3 ml esterification vial and evaporated to dryness under ultra high purity (UHP) nitrogen gas. The above step was repeated with the other half of the chloroform being transferred to the same esterification vial. The chloroform contained the plasma free fatty acids.

Esterification. The dried residue of plasma free fatty acids were esterified to their methyl esters with 0.1 ml of 3 N methanolic HCl (Metcalfe and Schmitz, 1961; Sheppard and Iverson, 1975). Nitrogen gas was passed over the vial's contents to prevent the polyunsaturated fatty acids from undergoing autoxidation (Christie, 1973). The vial was capped, vortexed and placed in a $90^{\circ}C$ water bath for one hour, then they were permitted to cool to room temperature. The esterification solvent was evaporated under UHP nitrogen. To each vial, 0.1 ml methylene chloride

was added and then dried under UHP nitrogen. The dried residue was dissolved in 0.05 ml methylene chloride. Aliquots of the methylene chloride were transferred to a 0.1 ml automatic sampler and evaporated under UHP nitrogen. The above steps involving the transfer of the fatty acid methyl esters from the esterification vial to the automatic sampler vials were repeated twice. Finally, the FAMES of the sample were dissolved in 0.025 ml of metbylene chloride for gas liquid chromatographic analysis.

Gas Liquid Chromatography Analysis. A Hewlett Packard (HP) 5711A dual flam ionization detector (FID) chromatograph with an HP7671A automatic sampler and a HP7123A chart recorder were used to analyze the plasma FAMES. The glass columns were 6 ft x 7 mm in diameter and packed with 5% DEGS (diethylene glycol succinate) on Gas Chromosorb WHP, 100-120 mesh. The flow rates of the gases were 30 ml/min for nitrogen, 60 ml/min for hydrogen and 240 ml/min for air. The temperatures for the FID and injection port were $250^{\circ}C$ and $200^{\circ}C$, respectively. The oven temperature was programmed at $155^{\circ}C$ for 8 minutes to $180^{\circ}C$ for 32 minutes at a rate of $8^{\circ}C$ per minute.

The fatty acid methyl esters were identified by comparison of their retention times with standard FAMES (Sigma Co., St. Louis, Missouri). The area of the fatty acids: palmitate (C16), palmitoleate (C16:1),

heptadecanoate (C17), stearate (C18), oleate (C18:1) and
linoleate (C18:2) was obtained by peak height x width at
one-half peak height. The amount of fatty acids in the
sample was calculated in terms of the internal standard,
heptadecanoate (C17). The amount of FFAs in the sample was
obtained using the formula below:

$$\text{Amount FFAs (micromoles)} = \frac{\text{Areas C16} + \text{C16:1} + \text{C18} + \text{C18:1} + \text{C18:2}}{\text{Area C17}} \times$$

$$\text{Amount C17 (micromoles)}$$

The concentration of plasma FFAs (µmoles/ml) was obtained by
dividing the amount FFAs by the volume of plasma used.

Tissue Content of Total
Fatty Acids

The procedure for analyzing tissue fatty acids was
similar to that for plasma FFA with three exceptions:
(1) The 1 N H_2SO_4 and heptadecanoate (C17), internal
standard, used in the extraction step were doubled; (2) the
volume of tissue homogenate extracted was 0.05-0.1 ml; and
(3) a step was added after the extraction step and prior to
esterification with 3 N methanolic-HCl.

To the dried residue obtained from the extraction
step with $CHCl_3$-CH_3OH (2:1, v/v), 0.25 ml of hexane was
added. The lipids which contain no noticeably polar
functional groups, for example triglycerides (TGs) or
cholesteryl esters, are highly soluble in hydrocarbon

solvents such as hexane, benzene, or cyclohexane and slightly more polar solvents such as chloroform and diethyl ether (Christie, 1973). However, they are insoluble in highly polar solvents such as methanol. The polar lipids such as phosphoglycerides may be sparingly soluble in hydrocarbon solvents unless solubilized by association with other lipids. The dissolved lipid residue of TGs and FFAs predominantly was transferred to a 0.3 ml esterification vial where the hexane was dried under UHP nitrogen gas. The esterification, actually transesterification of the lipids, and GLC analysis followed as described in the procedure for plasma long chain free fatty acids.

Tissue Free Fatty Acid and
Triglyceride Analysis

The assay of the tissue FFAs and triglycerides (TGs) differed from the long chain plasma FFA analysis on two major points. The first major difference was the sample treatment prior to extracting the lipids:

1. The tissues from the sacrificed animal were frozen at dry ice temperature ($-78.5^{\circ}C$) (Fernando-Warnakulasuriya et al., 1981) and then homogenized in ethanol using a polytron (Brinkman PCU-2 Kinematica).

2. The tissues were placed in cold tris sucrose buffer
    on ice and homogenized in the buffer, as was done
    in the assay of total lipids.

3. Some tissues had been placed in liquid nitrogen
    immediately after sacrificing the mice. The tissues
    were then stored in isopentane for 7-14 months at
    -70°C. The tissues were homogenized at room
    temperature in methanol using a polytron (Brinkman
    PCU-2 Kinematica).

The tissues were homogenized in ethanol, methanol or tris
sucrose buffer to make a 0.5-10% (w/v) solution. In one
experiment where tissue availability was limited, methanol
was the solvent for preparing homogenates. The other major
difference was the separation of lipid classes by thin
layer chromatography (TLC). TLC was done after extraction
of the lipids and subsequently esterification and GLC
analysis followed. The schematic of the tissue FFA and TG
analysis is shown on Figure 2.

Extraction. The extraction of 0.05-1.0 ml alcoholic
or tris sucrose buffer homogenates were similar to the
plasma long chain FFAs with two exceptions. The first was
the addition of 0.1 ml of 4.2% butylated hydroxy toluene
(BHT) to prevent autoxidation of the polyunsaturated lipids
such as palmitoleate (C16:1), oleate (C18:1) and linoleate
(C18:2) (Christie, 1973). The second minor change was the

I. LIPID EXTRACTION

Add 0.05-0.10 ml tissue homogenate

1.1 ml CHCl₃-OH (2:1, v/v)
0.05 micromoles C17 acid
0.1 ml 4.2 % BHT

vortex

Add 0.01 ml 1 N H₂SO₄

Add 0.05 ml 0.05 M NaCl

vortex

centrifuge 898 x G

Aspirate aqueous layer
Dry CHCl₃ under UHP nitrogen

Aqueous layer

CHCl₃ and lipids

Add 0.1 ml CH₂Cl₂ to the lipid residue
Vortex

lipid residue

II. THIN LAYER CHROMATOGRAPHY (TLC)

Spot sample and TLC lipid standard on 0.25 micrometer silica gel G TLC plate
Air dry

Develop plate in petroleum ether-diethyl ether-glacial acetic acid (80:20:2, by volume)
Air dry

Spray with 2',7'-dichlorofluorescein
Air dry
Place under the long wavelength of UV light to see lipids
Scrape the silica gel containing the FFAs and TGs and place the silica gel in their respective test tubes

Standard
TGs
FFAs
Start
Sample

FFAs : Extract the FFAs from the silica gel with CHCl₃-CH₃OH-glacial acetic acid (42:20:1, by volume)
Extract two more times with CHCl₃ alone
Combine all extractions

TGs : Add 0.05 or 0.1 micromoles C15 methyl ester
Extract the TGs from the silica gel with diethyl ether
Repeat two more times
Combine all extractions

Add 3 ml of 0.05 M NaCl to the FFA and TG extractions
Vortex and allow to separate into two phases

TGs
FFAs

FFAs: Aspirate aqueous layer
Add 2 g anhydrous Na₂SO₄
Decant CHCl₃ to a clean test tube
Dry under UHP nitrogen

TGs : Transfer ether layer to clean test tube
Dry under UHP nitrogen

Aqueous layer

ether layer and TGs

CHCl₃ layer and FFAs

III. ESTERIFICATION

FFAs   TGs

Esterify FFAs to their methyl esters in 0.8 ml of 3 N methanolic-HCl for 1 hour at 90°C

Transesterify the TGs to fatty acid methyl esters in 0.8 ml of 3 N methanolic-HCl and 0.5 ml of hexane for 1 hour at 90°C

FFAs   TGs

Cool tubes to room temperature
Dry esterification solvent under UHP nitrogen
Add CH₂Cl₂ and dry under UHP nitrogen
Dissolve FAMES in CH₂Cl₂ and transfer to automatic sampler vials

IV. GAS LIQUID CHROMATOGRAPHY (GLC) ANALYSIS

Injection port

Detector (FID)

Columns

Recorder

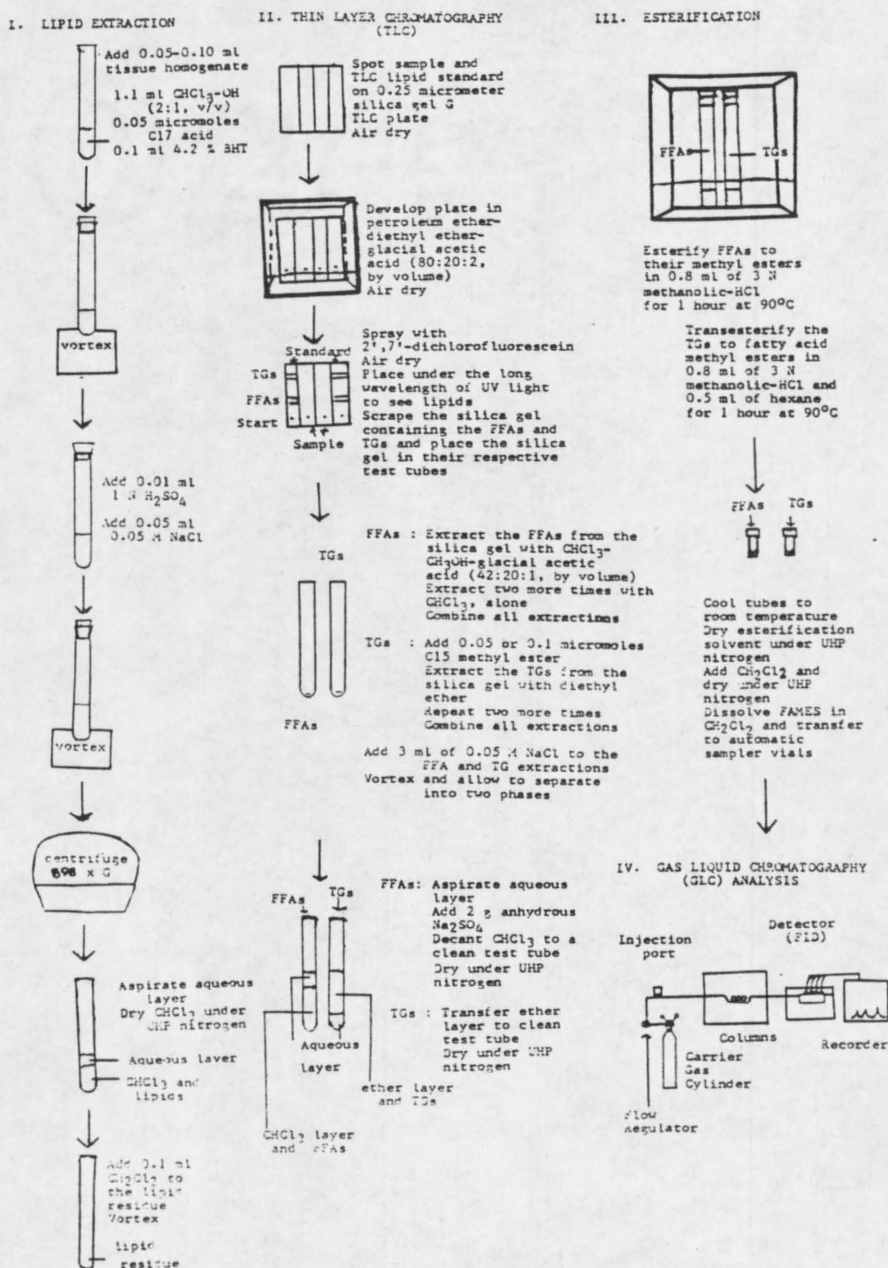Carrier Gas Cylinder

Flow Regulator

Figure 2.   Schematic of the Tissue Free Fatty Acid and TG Content Analysis--Extraction, TLC, Esterification and GLC Analysis.

use of 0.5 ml of NaCl to wash the $CHCl_3$ layer which contained the lipids. However, for the 0.5-1.0 ml alcoholic tissue homogenates, twice the volume of chloroform was added and all other reagents used in extracting the lipids were doubled. Subsequently, the lipids in the $CHCl_3$ layer were dried under UHP nitrogen. The lipids were dissolved in 0.1 ml of Burdick & Jackson methylene chloride (CH_2Cl_2). The lipids were either stored in a $-20^{\circ}C$ freezer or immediately spotted on a thin layer chromatography plate.

Thin Layer Chromatography. The lipids dissolved in methylene chloride were spotted onto a 0.250 micrometer silica gel G TLC plate (Christie, 1973; Christie, Noble and Moore, 1976; Fernando-Warnakulasuriya et al., 1981; Skipski et al., 1965). The plates were also spotted with a TLC mixture consisting of monoglyceride, diglyceride, free fatty acid (linoleate C18:2) and triglyceride. The plates were developed in petroleum ether (b.p. $40-60^{\circ}C$)-diethyl ether-glacial acetic acid (80:20:2, by volume) (Gloster and Fletcher, 1966; Skipski et al., 1965). The plates were sprayed with 2'7-dichlorofluorescein-CH_3OH-H_2O (0.05:75:25, w/v/v) and then air dried again (Myher, 1978). The standard lipids showed up under UV irradiation.

The lipids of interest were the FFAs and TSs, the FFAs and TGs were identified according to their positions with respect to the compounds in the standard TLC mixture.

The silica gel containing the FFAs or TGs was scraped off the TLC plate and placed in their respective labeled glass test tubes. The extraction from the silica gel and esterification differed for the FFAs and TGs.

Free Fatty Acids. The FFAs were extracted from the silica gel with 4 ml of $CHCl_3$-CH_3OH-glacial acetic acid (40:20:1, by volume) (Myher, 1978). The test tubes were centrifuged to allow the silica gel to settle down. The liquid was decanted into a 26 x 100 mm glass test tube. The above extraction steps were repeated twice using 4 ml of $CHCl_3$ each time. The $CHCl_3$ decantants were combined with the first decantant. The decantants were washed with 3 ml of 0.05 M NaCl and then centrifuged. The upper phase, aqueous layer, was aspirated off leaving the $CHCl_3$ layer which contained the FFAs. About 2 grams of anhydrous sodium sulfate was added to the chloroform layer. The chloroform was then decanted into a 13 x 28 mm glass test tube. The $CHCl_3$ was dried under UHP nitrogen. The FFAs were esterified in 0.8 ml of 3 N methanolic-HCl for one hour at $90^{\circ}C$. The subsequent steps that followed are the same as described for the plasma long chain FFA analysis.

Triglycerides. Prior to extracting the TGs from the silica gel, 0.05 or 1.0 micromoles of pentadecanoate methyl ester (C15) was added. The C15 methyl ester served

as the internal standard for quantitating the transesterified TGs as fatty acids. The C 17 acid, heptadecanoate, migrated with the FFAs of the sample, therefore another internal standard was necessary for the TGs. The TGs were extracted with 4 ml of diethyl ether. The test tube was centrifuged and then the ether was decanted into a 26 x 100 mm glass test tube. The above steps were repeated twice (Myher, 1978). The combined ether fractions were washed with 3 ml of 0.05 M NaCl. The test tube was capped with a Teflon lined screw cap and vortexed. When two distinct layers were visible, the upper phase, ether layer, was transferred with a disposable glass pipet to a 13 x 28 mm glass test tube. The ether layer containing the pentadecanoate methyl ester and the TGs were dried under UHP nitrogen. For one hour, the TGS were transesterified in 0.8 ml of 3 N methanolic-HCl and 0.5 ml hexane at $90^{O}C$. Hexane increased the solubility of the TGs in the polar esterification solvent. The subsequent steps are the same as described for the plasma long chain FFAs.

Calculations. The concentration of FFAs were obtained using the amounts of FFAs (see page 26 for formula) divided by the ml of homogenate used. The micromoles FFA/ml tissue homogenate can be readily converted to micromoles FFA/g wet weight tissue by dividing by the initial concentration of the tissue homogenate.

The calculations for the fatty acids produced from the transesterification of the TGs used pentadecanoate (C15) methyl ester instead of heptadecanoate (C17) as the standard. The amount of fatty acids can be divided by 3 to give the amount of TG (one micromole TG contains 3 micromoles of fatty acid). The concentration of tissue TGs was given in micromoles TGs/g wet weight of tissue.

Typical chromatograms of the FAMES from the GLC standard, tissue FFAs and transesterified TGs are shown in Appendix C.

EXPERIMENTAL PROTOCOL

Bristol Myers Laboratories and McNeil Laboratories provided the experimental drugs, Bristol Myers MJ 12880-1 and tetradecylglycidic acid (McN-3802), respectively.

Bristol Myers MJ 12880-1

The effect of MJ 12880-1 on several parameters in genetically diabetic mice at two different ages were studied. Young diabetic mice, 2-3 months old, and older diabetic mice, 5 months or older, were used in the study. At 2 months of age, the diabetic mice are overtly hyper-glycemic and weigh significantly more than age-matched non-diabetic littermates (Coleman and Hummel, 1967). The weight gain progresses until 4-5 months of age. Hyper-insulinemia, hyperphagia and blood glucose levels of 300-500 mg/100 ml are observed in the db/db mice during the period they accumulate fat. After 4-5 months of age, the diabetic mice's weight stabilizes and the insulin levels decrease in relation with the degenerative changes in the pancreatic islet cells.

The drug was administered in a dose of 50 mg/kg in 0.5% methyl cellulose solution. The drug was vortexed prior to dosing the mice. Fresh drug suspension was prepared

every four days. The drug in a volume of 0.25 ml was placed directly into the stomach via orogastric tube. The trauma to the animals was minimal due to the experience of laboratory personnel.

Control groups of diabetic mice at the two different ages were utilized. The control diabetic mice were dosed with 0.5% methyl cellulose (vehicle) alone using the same technique and a volume equivalent to the drug treated mice.

In the first experiment, the effects of a single dose of MJ 12880-1 on blood glucose of diabetic mice, aged 5-6 months, were evaluated. Blood samples for measurement of blood glucose were obtained by capillary puncture of the retro-orbital venous plexus. Animals were bled after a 14 hourfast (zero time) and then given drug or vehicle. Blood samples were obtained after dosing at tht time intervals of 0.5, 1, 2 and 4 hours. Each mouse served as its own control. Each of the four control groups treated with methyl cellulose alone contained 2 mice. The number of mice in the drug treated groups were as follows: 0.5 hour (n = 3), 1 hour (n = 4), 2 hours (n = 5) and 4 hours (n = 2).

In a second experiment, MJ 12880-1 was administered daily for 14 days to young (2-3 months old) and old (6 months old) diabetic mice to evaluate its effect on blood glucose. The treated db/db mice received 50 mg/kg drug while the control db/db mice received 0.5% methyl cellulose.

The diabetic mice were fasted overnight before obtaining blood by venipuncture for blood glucose measurements.

In a third experiment, the effects of a single dose of drug on plasma free fatty acid levels were studied in diabetic mice aged 5 months or older. After an overnight fast, the mice were bled from the retro-orbital venous plexus (zero time) followed by administration of 50 mg/kg drug or 0.5% methyl cellulose. Blood was obtained at the same time intervals specified in Experiment 1, the blood glucose study involving an acute single dose of drug or methyl cellulose, alone. Blood was obtained from 2 mice treated with drug at 0.5 hour. Each of the other 3 groups of drug treated mice from which blood was obtained at 1, 2 and 4 hours had 3 mice. Each of the four groups of control mice receiving methyl cellulose contained 2 mice. Each mouse served as its own control.

In a fourth experiment, the plasma FFA levels of young (2-3 months) and old (5-6 months) diabetic mice given 50 mg/kg drug for 14 days were evaluated. After 14 days of dosing, the mice were fasted overnight prior to obtaining plasma for the FFA analysis. The group of young diabetic mice included 4 given drug and 2 given vehicle. The group of old diabetic mice consisted of 3 given vehicle and 3 given drug.

The mice used in the preceding experiment were sacrificed by exsanguination after 14 days of drug administration and their tissues were analyzed for palmitate and glucose oxidation. Fresh samples of heart, liver and brain were immediately obtained and placed in cold tris sucrose buffer on ice. The tissues were used in the oxidative metabolism studies employing as substrates, palmitate and glucose.

2-Tetradecylglycidic Acid
(McN-3802 or TDGA)

The TDGA experiments were designed to evaluate the effects of long term administration of TDGA in groups of diabetic and non-diabetic mice. The mice entered the study at 5-6 weeks of age and were treated for 12 weeks. Diabetic mice of either sex was randomly selected to receive drug or vehicle or act as controls.

Three dosage schedules were chosen: (1) 25 mg/kg/da6 (n = 6), (2) 10 mg/kg/day (n = 4), and (3) 5 mg/kg/day (n = 4). The drug was given daily to the db/db mice by gavage in a volume of 0.4-0.7 ml. A single operator administered the drug. No morbidity resulted from the administration of the drug. The dosing of the mice with the drug took about 10 seconds per animal.

A group of vehicle treated diabetic mice (n = 6) received 0.5% tragacanth solution in a volume equivalent to

the drug treated diabetic mice. A group of control diabetic mice (n = 6) were not treated.

Non-diabetic controls included a group of hetero-zygotes (db/+) (n = 6) and a group of homozygotes (+/+) (n = 6). These two groups of mice were not treated.

At the end of the twelve weeks of study, the mice were sacrificed by decapitation. Sections of the heart and liver were analyzed for oxidative metabolism, carnitine assay and histological examination. The tissue portions designed for the carnitine assay were also used for free fatty acid (FFA) and triglyceride (TG) analysis. The tissues were immediately placed in liquid nitrogen and stored in isopentane at -70°C for 7-14 months, until analysis.

RESULTS

The two potential agents for treating diabetes,
MJ 12880-1 and McN-3802, were investigated according to
study protocols provided by Bristol Myers Laboratories and
McNeil Laboratories, respectively. In addition, the
triglyceride and free fatty acid levels from 6 months old
diabetic, misty and heterozygous mice heart and liver were
assayed.

Bristol Myers MJ 12880-1

Effect on Blood Glucose and Plasma FFAs

The blood glucose and plasma FFA levels were
similar in the control and experimental diabetic mice (5-6
months of age) following a single dose of drug or vehicle.
The results for the plasma FFA levels of the control and
experimental diabetic mice prior to dosing and after dosing
with drug or vehicle are shown in Table 2.

The repeated dose effects of MJ 12880-1 produced no
significant differences in the plasma FFA or blood glucose
levels of the two different ages (2-3 months and 5-6 months
or older) of db/db mice and their respective age-matched
controls. Table 3 shows the plasma FFAs of the 2-3 months
and 5-6 months or older diabetic mice treated for 14 days

Table 2. Plasma Free Fatty Acid Levels (Micromoles FFA/ml
          Plasma) of Each Control and Bristol Myers MJ
          12880-1 Treated Diabetic Mouse Aged 5 Months or
          Older at Time 0 hr and at 0.5, 1, 2, and 4 Hours
          after Dosing.

Mouse No.	Control		Experimental	
	Time = 0 hr	Time = 0.5 hr	Time = 0 hr	Time = 0.5 hr
1	5.72	4.05	3.41	2.59
2	3.39	4.4	3.93	4.86
	Time = 0 hr	Time = 1.0 hr	Time = 0 hr	Time = 1.0 hr
1	3.12	3.06	3.05	3.10
2	4.51	3.24	3.86	2.94
3	-	-	1.79	1.34
	Time = 0 hr	Time = 2.0 hr	Time = 0 hr	Time = 2.0 hr
1	4.01	3.31	3.01	2.87
2	7.92	3.70	7.95	6.47
3	-	-	3.13	3.27
	Time = 0 hr	Time = 4.0 hr	Time = 0 hr	Time = 4.0 hr
1	5.78	3.74	5.33	3.35
2	5.3	2.53	2.14	2.91
3	-	-	1.83	2.74

Table 3. The Plasma Free Fatty Acid Levels (Micromoles FFA/ml Plasma) of Bristol Myers MJ 12880-1, 14 Days Daily Treated and Untreated Diabetic Mice.

Mouse No.	Age	Control	Experimental
1	5-6 months	2.92	3.11
2		4.46	3.38
3		3.42	3.10
1	2-3 months	4.46	4.57
2		4.25	4.64
3		–	3.34
4		–	2.58

with 50 mg/kg drug, and their age-matched controls who received vehicle methyl cellulose.

Effect on Palmitate and
Glucose Oxidation

The results of oxidative metabolism studies on the 14 day drug treated 2-3 months and 5-6 months old diabetic mice and their age-matched controls are shown in Figures 3 and 4. The 2-3 months old diabetic mice treated with 50 mg/kg MJ 12880-1 for 14 days showed a 35% decrease in palmitate oxidation by the liver and a 17.8% increase in hepatic glucose oxidation. In the 5-6 months old diabetic mice, there was a 65% increase in palmitate oxidation by the liver and a 11% increase in glucose oxidation by the brain.

The total lipids of the liver and heart were measured only in the 2-3 months old diabetic mice. This information was needed to evaluate the effect of MJ 12880-1 on palmitate metabolism by the heart and liver. The total lipid content of the liver was 2.02 ± 0.24 and 2.57 ± 0.46 micromoles/g wet weight of liver for the control and experimental 2-3 months old diabetic mice, respectively. The total lipids in the heart were 0.88 ± 0.01 and 1.48 ± 0.44 micromoles/g wet weight of heart, respectively. There was no significant difference between the vehicle and drug treated diabetic mice in hepatic and cardiac lipid levels. The total lipids in the heart and liver of 5-6 months old control and experimental diabetic mice were not measured.
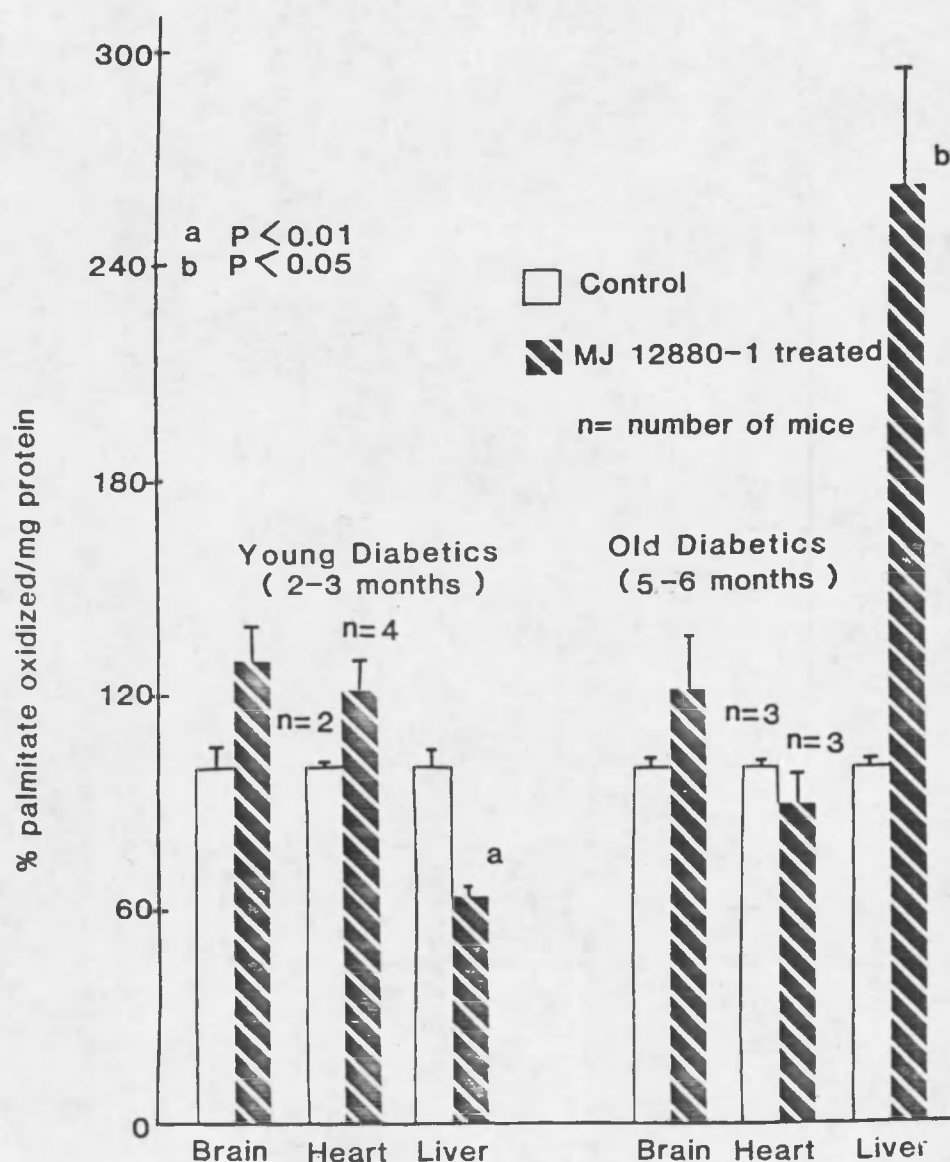
Figure 3.   The Effect of MJ 12880-1 on Palmitate Oxidation
by the Brain, Heart and Liver Homogenates of
Young and Old Diabetic Mice Based on Their Age-
Matched Controls. -- The percent palmitate
oxidized/mg protein (Means ± S.E.) was not
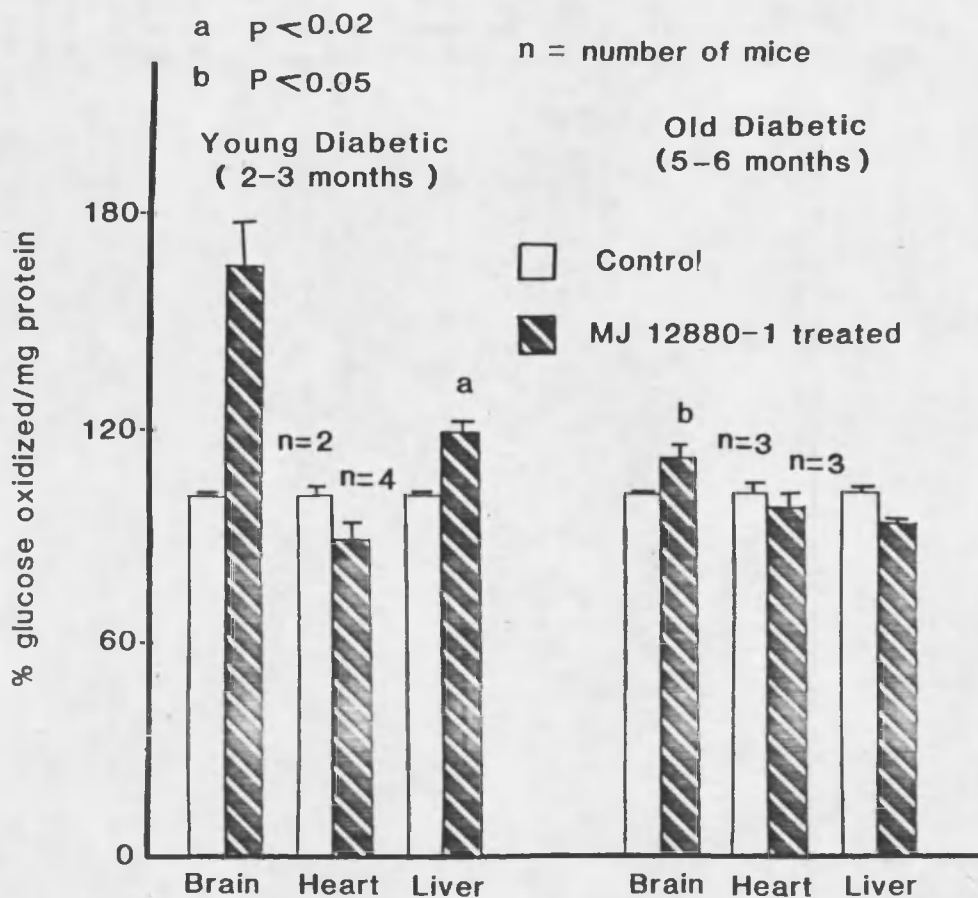adjusted for tissue lipid content.

Figure 4. The Effect of MJ 12880-1 on Glucose Oxidation by the Brain, Heart and Liver Homogenates of Young and Old Diabetic Mice Based on Their Age-Matched Controls. -- The percent glucose oxidized/mg protein (Means ± S.E.) of the tissue homogenates from age-matched control mice were set at 100%.

Therefore, the percent palmitate oxidized/mg protein based on their age-matched controls (Figure 4) was not adjusted to account for the tissue lipids.

2-Tetradecylglycidic Acid (McN-3802)

Mice entered into this study at the age of 5-6 weeks, and received drug or vehicle for a period of 12 weeks. Untreated diabetics, heterozygous and misty mice served to establish the baseline of the cardiac FFA and TG levels. Groups of diabetic mice received McN3802 at 5, 10, and 25 mg/kg/day. Control diabetics received the vehicle tragacanth.

The total lipids of the heart and liver from 5 and 10 mg/kg McN-3802 treated diabetics were assayed within two weeks after obtaining tissue homogenates. The results are shown in Table 4. There were no significant differences in hepatic and cardiac lipids between the two groups received McN3802 at 5 and 10 mg/kg/day using tissues homogenized in Tris sucrose buffer.

The results of the cardiac FFA and TG levels of the drug and/or vehicle treated diabetic and untreated diabetic, misty and heterozygous mice are shown in Figures 5 and 6. There were no significant differences between the cardiac TG levels using the analysis of variance (ANOVA) test. However, using the Student's test on the diabetics only, the

Table 4. Cardiac and Hepatic Lipid Levels (Micromoles
Total FAs/g Wet Weight Tissue) of 5 and 10 mg/kg
McN-3802 Treated Diabetic (db/db) Mice.

Heart

$\dfrac{\mu\text{moles total FAs}}{\text{g wet weight}}$	$\dfrac{\mu\text{moles total FAs}}{\text{g wet weight}}$
71.07	62.40
80.69	49.24
57.62	79.07
75.13	63.16
67.56	55.70
$\bar{X}=$ 70.41	$\bar{X}=$ 61.91
S.E.= ±3.87	S.E.= ±4.98

- -

Liver

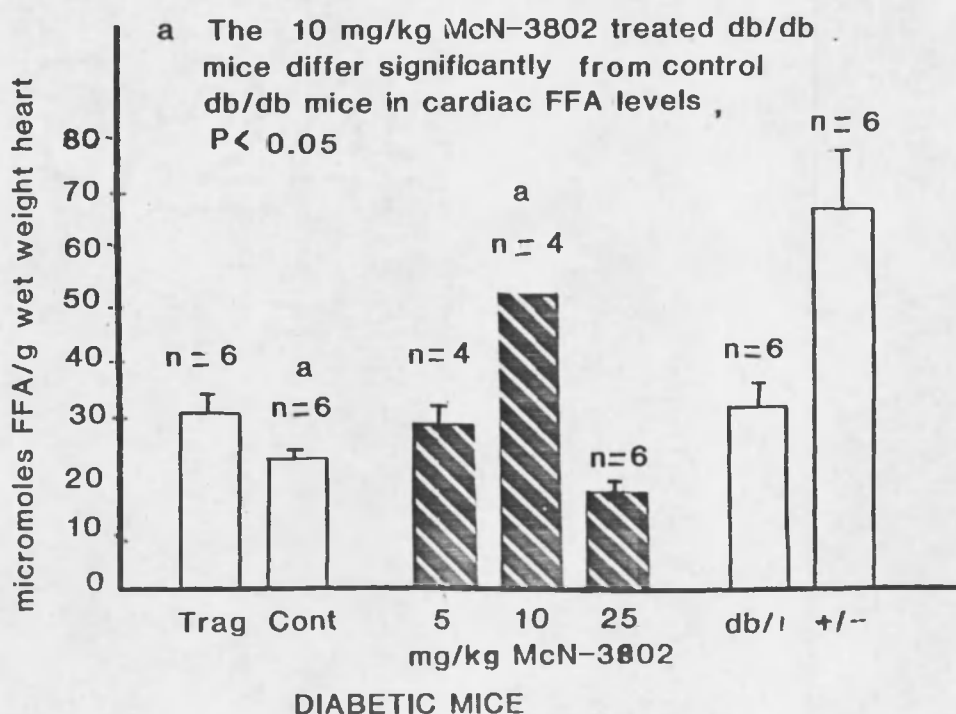| $\dfrac{\mu\text{moles total FAs}}{\text{g wet weight}}$	$\dfrac{\mu\text{moles total FAs}}{\text{g wet weight}}$
54.92	97.88
94.62	54.82
98.08	126.62
146.08	147.70
145.05	200.90
159.11	----
$\bar{X}=$ 116.31	$\bar{X}=$ 125.58
S.E.= ±16.45	S.E.= ±24.43

Figure 5. The Effect of McN-3802 on Cardiac FFAs
(Means ± S.E.) of Treated Diabetic Mice as
Compared to Untreated Diabetics and Vehicle
Treated Diabetics and Untreated Non-Diabetic
Mice. -- The diabetic mice were treated with
vehicle (Trag), not treated (Cont) or drug
treated at 5, 10 and 25 mg/kg. The non-
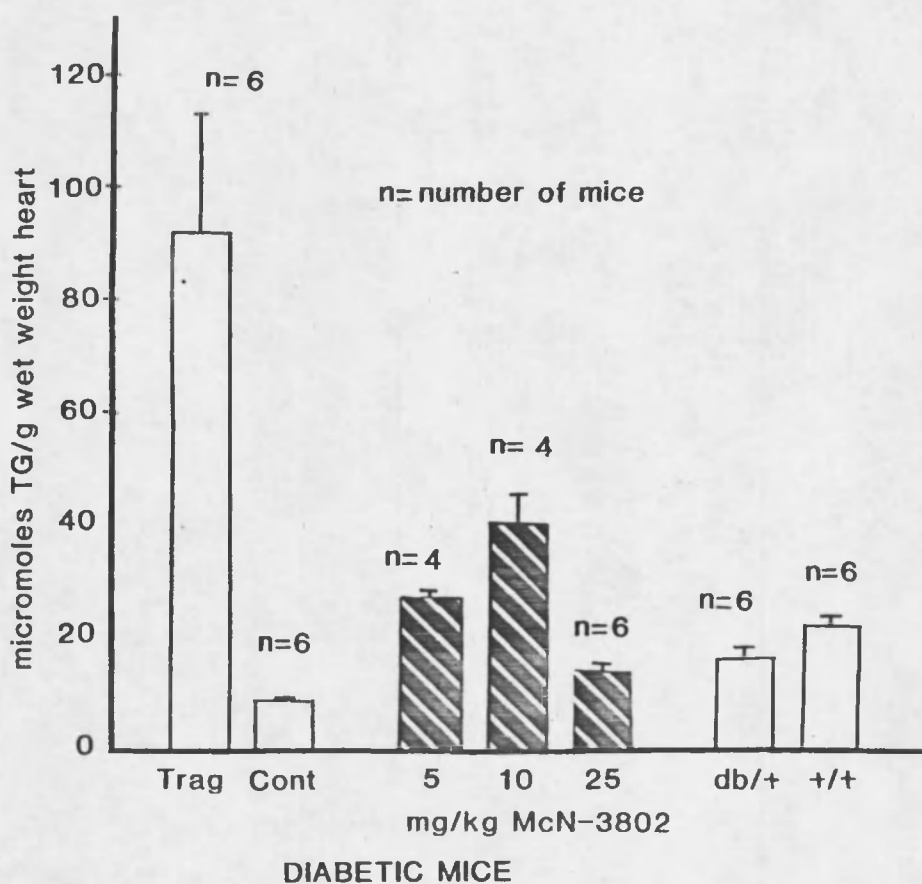diabetic mice were heterozygotes (db/+) and
misty mice (+/+).

Figure 6. The Effect of McN-3802 at 5, 10 and 25 mg/kg on Cardiac TG Content of Diabetic Mice as Compared to Vehicle Treated (Trag) Diabetics, Untreated (Cont) Diabetics and Untreated Non-Diabetic Mice. -- The untreated non-diabetic mice were heterozygous (db/+) and misty (+/+) mice. The cardiac TG content were given as Means ± S.E.

FFA level of the 10 mg/kg McN-3802 treated diabetics was significantly higher than the untreated diabetic mice (p <0.05).

Frozen Hearts and Livers

The hearts and livers from 6 months old littermates of the C57BL/KsJ-db inbred strain of mice, misty, diabetic and heterozygous, were obtained for FFA and TG analyses. The results are shown on Fig. 7 and 8. No significant differences were found using an analysis and variance test on the FFAs and TGs. The mice had not been treated with any drugs.
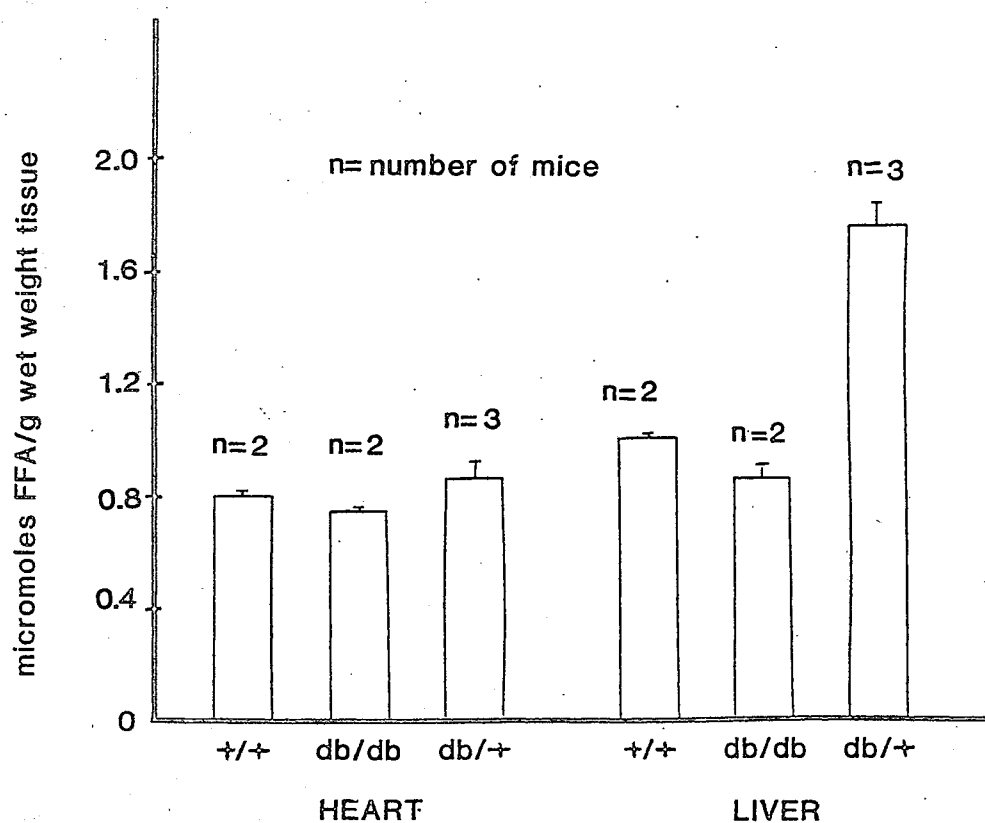
Figure 7. The FFA Levels (Means ± S.E.) in Heart and Liver Tissues of 6 Months Old Untreated Diabetic (db/db), Misty (+/+) and Heterozygous (db/+) Mice. -- After sacrificing the mice, the tissues were immediately frozen at dry ice temperature, homogenized and extracted for lipids.
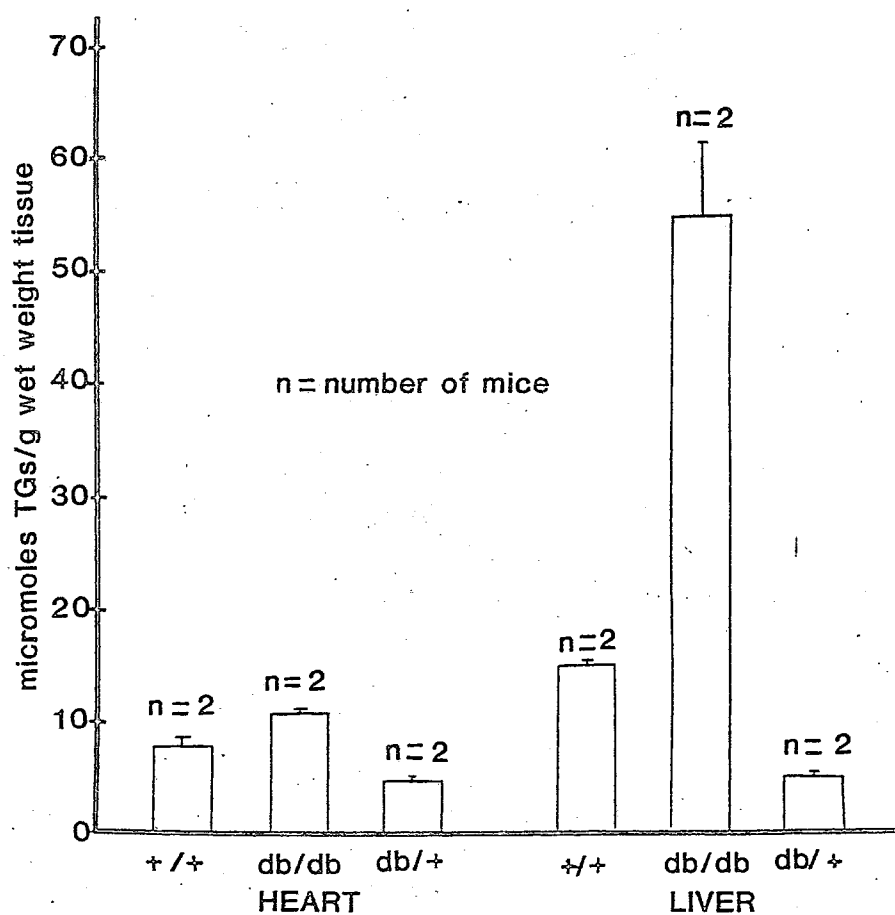
Figure 8. The TG Content (Means ± S.E.) in Heart and Liver in Tissues of 6 Months Old Untreated Diabetic (db/db), Misty (+/+) and Heterozygous (db/+) Mice.

DISCUSSION

Bristol Myers MJ 12880-1

The study of Bristol Myers MJ 12880-1 on blood glucose, plasma free fatty acids and oxidative metabolism suggested that the drug did not influence those parameters significantly. The drug, MJ 12880-1, had no effect on blood glucose levels or plasma long chain FFAs in young or old diabetic mice. This was observed in animals given a single dose of drug or a repeated daily dose of drug for 14 days. The results are similar to previous studies utilizing rats which showed no change in blood glucose and plasma FFAs with the exception of higher plasma FFAs in rats treated with drug for 14 days.

The plasma FFA levels of young and old diabetics receiving drug for 14 days were similar to their age-matched controls. The plasma FFA level of the db/db mice were not affected by the drug, possibly because of their hugh deposits of substrate triglyceride, and a slower release of free fatty acids from the adipose tissue mass (Allan and Yen, 1976).

Previously reported values for FFAs in serum and plasma have shown some variability. Steinmetz, Lowry and Yen (1969) found the plasma levels of diabetic mice aged

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3-5 months to be 1.86 ± 0.27 micromoles FFA/ml plasma (1.5-2 times normal heterozygous mice, 0.81 ± 0.04 micromoles FFA/ml plasma). Chan et al. (1975) found the free fatty acid levels in serum to be about 3 times greater in diabetic mice as compared to normal (2.69 ± 0.80 as compared to 0.91 ± 0.26 microequivalents/ml serum in normal mice). The units micromoles and microequivalents are equal in this case since the fatty acids analyzed contain only one ionizable hydrogen (H^+). The serum FFA level (Chan et al., 1975) and the plasma FFA level (Steinmetz, Lowry and Yen, 1969) of normal mice are very similar. However, in the diabetic mice, the serum FFA level is about 1.5 times the plasma levels (Chan et al., 1975; Steinmetz, Lowry and Yen, 1969). A possible explanation is that the FFA level of the db/db mice is actually higher at 8 weeks than at 12 weeks of age. At 7-8 weeks of age, the levels of triacylglycerols in the carcass and liver increase dramatically (Yen et al., 1976). The FFAs are precursors of triacylglycerols and are transported to the liver and adipose tissue by the blood. Additionally the bleeding procedure and FFA analysis could be influenced by technical variation.

In this study, the plasma levels of FFA in both drug treated and control mice aged 2-3 months and 5-6 months, were higher than the previous literature reported values (Steinmetz, Lowry and Yen, 1969). However the plasma FFA levels obtained in the study were closer to the literature

value given for the serum FFAs (Chan et al., 1975). In a few cases the plasma FFA level was about 2-3 times the serum FFA levels of 8 weeks old db/db mice (Chan et al., 1975).

The plasma samples had been stored for over 9 months at -20°C prior to analysis. Plasma is a good antioxidant and provides protection to its component lipid. Furthermore, at -20°C, lipids stored in plasma do not undergo degradation for several years (Nelson, 1972). Therefore the long storage period of the plasma samples would not produce the high FFA levels found in this study. However, accidental overnight thawing of the plasma samples occurred twice during that 9 month period. The accidental thawing of the samples resulted in autoxidation of polyunsaturated acids and degradation of lipids (Nelson, 1972). No antioxidant had been added to the samples. The polyunsaturated acids like palmitoleate (C16:1), oleate (C18:1) and linoleate (C18:2) autoxidize rapidly in plasma at room temperature exposed to oxygen (Christie, 1973).

MJ 12880-1 caused a decrease in palmitate oxidation and an increase in glucose oxidation by the liver of 2-3 months old diabetic mice. These correspond with Chan and Exton's (1977) conclusions that 8 week old diabetic mice have decreased fatty acid oxidation and increased fatty acid esterification to monoglycerols, diglycerols, triglycerols and phospholipids. Additionally, lipogenesis activity in the db/db mice at 8 weeks of age was 4 times normal. Chan

and Exton (1977) found that hepatic oxidation of non-esterifiable octanoate to CO_2 and ketone bodies was not reduced as much as the oxidation of esterifiable oleate. The changes in fatty acid oxidation appeared to be secondary to glyceride synthesis.

The total hepatic lipid content of control and MJ 12880-1 treated diabetic mice were found to be similar. MJ 12880-1 may have enhanced fatty acid esterification thereby decreasing hepatic palmitate oxidation in the 2-3 months old db/db mice. However, the triglyceride (TG) and FFA levels of mice were not measured at the time of the oxidation study. The FFAs and TGs were measured one year later and did not provide useful information since extensive autolysis of lipids, especially phospholipids may have occurred (Fairbairn, 1945).

The increased hepatic glucose oxidation was enhanced in 2 months old mice treated with MJ 12880-1. The gluconeogeic enzymes and glycolytic enzymes were found to be higher in the 2 months old db/db mice (Chang and Schneider, 1970; Wyse and Dulin, 1979). Chan et al. (1975) found that 8 week old diabetic mice had almost 3 times the liver glycogen of normal mice (64 ± 3 and 22 ± 3 mg/kg liver in db/db and normal mice, respectively). Additionally, the 8 week old diabetic mice were found to have higher glycogen synthetase I and total synthetase activities than normal mice.

Paradoxically, livers from 8 week old diabetic mice were found to have a glycogen phosphorylase activity of 177% of normal mice. The studies of Chan et al. (1975) and Chan and Exton (1977) suggested that the glycogen turnover is very rapid in the db/db mice. The glycolytic enzymes, pyruvate kinase, glycokinase and hexokinase, are twofold higher for 8 weeks old db/db mice compared with normal mice (Chan et al., 1975). Therefore glucose oxidation in the 2 months old diabetic mice is higher and MJ 12880-1 appears to enhance glucose oxidation by the liver.

The increase in palmitate oxidation by the liver and glucose oxidation by the brain showed that MJ 12880-1 merely enhanced the existing metabolic conditions in the 6 months old diabetic mice. At 4.5 months of age, gluconeogenic enzymes increased further while glycolytic enzymes decreased (Chang and Schneider, 1970; Wyse and Dulin, 1970). The older db/db mice are characterized as having normal or decreased plasma insulin, and retarded glucose oxidation accompanied by the depressed activities of pyruvate kinase and phosphogluconate dehydrogenase (Chang and Schneider, 1970; Wyse and Dulin, 1970). Additionally, the insulinopenic diabetic mice may lose weight at 4.5 months of age and about 50-56% of their body weight is composed of triacylglycerols (Yen et al., 1976; Chan et al., 1977). The diabetic mice 4.5-9 months of age, catabolize more fat to compensate for decreased glucose

oxidation which results from insulin deficiency or tissue resistance to insulin. However, the brain requires glucose or beta-hydroxybutyrate for its energy substrates in fasting and diabetic states. Increased glucose oxidation by the brain and increased palmitate oxidation by the liver in older diabetics was observed in untreated mice. Thus, MJ 12880-1 appeared to increase the oxidation of substrates already utilized by the mice at 2-3 months and 5-6 months of age.

2-Tetradecylglycidate (TDGA or McN-3802)

The cardiac triglyceride and free fatty acid levels of misty (+/+), diabetic (db/db) and heterozygous (db/+) mice have not been investigated previously.

In this study, the cardiac triglyceride levels of TDGA treated diabetic (db/db) mice were not significantly different from the untreated diabetic mice and their non-diabetic littermates (misty [+/+] mice and heterozygotes [db/+]). However, the cardiac FFA levels of the 10 mg/kg/day TDGA treated diabetic mice were significantly higher than the untreated diabetic mice. The 25 mg/kg/day TDGA treated diabetic mice had similar values for cardiac FFA and TG content as the untreated db/db mice.

Tutwiler and Dellevigne (1979) found that TDGA inhibited palmitate oxidation in isolated rat liver cells in vitro. Additionally, livers of rats treated with the methyl

ester of TDGA had increased triglyceride content. Similarly, Pearch et al. (1979(found that TDGA inhibited fatty acid oxidation in vitro by 80% when rat hearts were perfused with the drug 10 minutes prior to the addition of fatty acids. However, complete long chain fatty acid oxidation by the heart was produced when TDGA was added 30 minutes earlier than oleate and (^{3}H)-palmitate. No in vivo studies of the effect of TDGA on rat hearts have been done previously. The in vitro studies of TDGA inhibiting long chain fatty acid oxidation suggest that a study on FFA and TG content of hearts and livers from rats treated with TDGA daily for 14 days or more may provide interesting data.

The total lipid levels of the heart and liver from diabetic mice treated both 5 and 10 mg/kg 2-tetradecylglycidic acid were similar. However, the FFA and TG levels of the heart from the 10 mg/kg treated mice were about 25% and 50% higher than the 5 mg/kg treated diabetic mice, respectively. The measurement of the total lipids in the heart and liver of the drug treated diabetic mice did not provide useful information. The TG levels in liver have been found to increase in rats treated with the methyl ester of McN-3802 (Tutwiler and Dellevigne, 1979). It would be particularly interesting to find out the hepatic TG levels in the McN-3802 treated db/db mice. The hepatic TG levels should be higher in the TDGA treated db/db mice than untreated db/db mice.

The study of 2-tetradecylglycidic acid on the db/db
mice was not conclusive and should be repeated. However, the
study showed the need to extract the lipids from tissues as
soon as possible. The effect of tetradecylglycidate on the
cardiac FFA and TG levels should be further investigated
before conclusions can be made on the drug's effect on
db/db mice.

Frozen Hearts and Livers

There are no literature values available on the FFA
and TG levels of the 6 months old diabetic, misty and
heterozygous mice. The TG level of the db/db mice was not
significantly different from the TG levels of non-diabetic
littermates, the misty and heterzygous mice. The TG level
of the db/db mice may be statistically significant from the
TG levels of its non-diabetic littermates if the sample
size was larger than 2 mice.

Stearns and Benzo (1981) found diabetic (db/db) mice
of 21 weeks of age to have 1.77 ± 0.23 microM FFAs/g liver
and 38.6 ± 10.1 mg TG/g liver. The micromoles TG/g wet
weight of liver of the 6 months old db/db mice studied was
equivalent to 44.77 mg TG/g wet weight of liver. Both the
FFA and TG content in the dry ice frozen livers of the 6
months old db/db mice agreed with the values obtained by
Stearns and Benzo (1981).

The hepatic TG content (4.3 ± 1.9 mg TG/g liver) of control 21 weeks old mice studied by Stearns and Benzo (1981) agreed very well with the TG content of the heterozygous mice livers (3.96 mg TG/g wet weight liver) but not the misty mice livers (12.26 mg TG/g wet weight liver). The misty mice analyzed in this study had threefold the TG level in liver found by Stearns and Benzo (1981). The control mice used by Stearns and Benzo (1981) were the lean litter-mates of the db/db mice.

The FFA content of the 21 weeks of age normal mice were found to have 2.48 ± 0.27 microM FFA/g liver by Stearns and Benzo (1981). The FFA content in the liver of the 21 weeks of age mice was twofold of the misty mice and about threefold of the heterozygous mice that were 6 months of age. Autolysis of lipids may have occurred, thereby accounting for the high FFA content of the liver from the 21 weeks of age normal mice studied by Stearns and Benzo (1981).

The FFA and TG levels in the heart of 6 month old diabetic, misty and heterozygous mice appear to be closer to the expected range of values than the heart FFA and TG levels found in the 4.5 months old untreated McN-3802 misty, heterozygous and diabetic mice. However, the db/db mice lose weight at about 4.5-9 months of age and about 50% of their body weight is triacylglycerols (Wyse and Dulin, 1970; Yen et al., 1976). So the amount of FFA and TG may be

slightly higher in the 4.5 months old diabetic mice since
that is the initial age weight loss starts. Additionally,
Fairbarin (1945) stated that the technique of quick freezing,
pulverization of tissue at dry ice temperature and extrac-
tion of the lipids should be taken as the maximum values for
the tissue analyzed.

CONCLUSIONS

Bristol Myers MJ 12880-1 had no effect on the metabolic abnormalities in the diabetic mouse. Bristol Myers MJ 12880-1 appeared to exacerbate the diabetic metabolism of the 2-3 months and 5-6 months old diabetic mice.

2-Tetradecylglycidate (McN-3802) treated diabetic mice dosed at 10 mg/kg had significantly higher cardiac FFA levels compared to the untreated db/db mice. However, the 25 mg/kg McN-3802 treated db/db mice did not have apparently higher cardiac FFA or TG levels than the untreated db/db mice. Further studies are needed to evaluate McN-3802 on the heart and particularly the liver in terms of the FFA and TG levels.

The cardiac FFA and TG levels from the animals used in the McN-3802 study showed the need for extracting lipids from the tissue as soon as possible. The TG and FFA levels obtained from the frozen heart and liver of 6 months old db/db mice were lower than the values obtained from the tissues of the McN-3802 study which were kept at -70°C for 7-14 months.

The lack of data on cardiac and hepatic FFA and TG levels show the need for more research in quantitating the lipid levels in the C57BL/KsJ inbred strain of misty (+/+),

diabetic (db/db) and heterozygous (db/+) mice at various ages. The preferred method would be to freeze the tissue at dry ice temperature, pulverize the tissue at dry ice temperature, homogenize the tissue in an alcoholic solvent and quickly extract the lipids. Lower values for the FFAs, TGs and other classes of lipids may be obtained. Additionally, the variation of the TG and FFA levels in the livers and hearts of the diabetic (db/db), heterozygous (db/+) and misty mice should be further investigated.

APPENDIX A

CALCULATIONS USED IN THE PALMITATE AND
GLUCOSE OXIDATION EXPERIMENT

Glucose

Specific Activity = K (micromole/microCi)

$$K_{glucose} = \frac{5 \text{ mM x micromole/ml x } 1/\text{mM x } 2.2 \times 10^6 \text{ dpm/microCi}}{\text{dpm/100 } \mu l \text{ sample x } 1000 \text{ } \mu l/\text{ml}}$$

$$= \frac{1.1 \times 10^6 \text{ micromole/microCi x } 1/\text{dpm}}{\text{dpm sample}}$$

where dpm sample $= \dfrac{cpm_{10 \text{ } \mu l \text{ hot glucose}} - cpm_{blank}}{C^{14} \text{ efficiency}}$

$$= \text{dpm/100 } \mu l \text{ sample}$$

nmoles Glucose oxidized by tissue homogenate $= \text{dpm x } K_{glucose} \text{ } (\mu moles/\mu Ci)$

$$= \frac{\text{dpm x } K_{glucose} \text{ } (\mu moles/\mu Ci) \times 10^3 \text{ nmoles/}\mu mole}{2.2 \times 10^6 \text{ dpm/}\mu Ci}$$

$$= \frac{\text{dpm x } K_{glucose} \text{ nmoles}}{2.2 \times 10^3 \text{ dpm}}$$

where dpm $= \dfrac{cpm_{homogenate} - cpm_{blank}}{C^{14} \text{ eff}}$

Palmitate

Specific Activity 1-C^{14} Palmitate = Y moles/μCi is stated
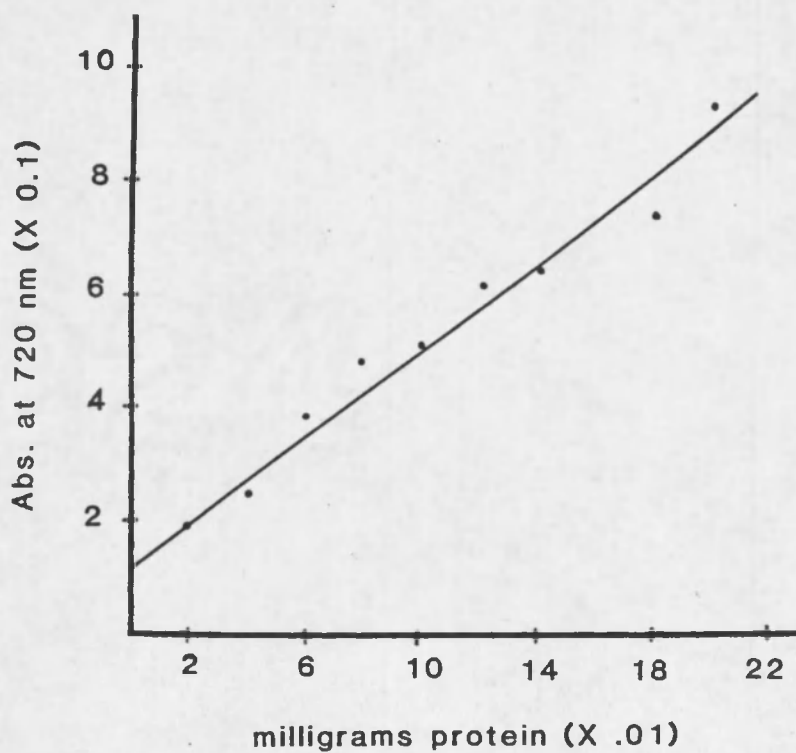 on the container of the radiolabeled substance.

$$\text{nmoles Palmitate oxidized by tissue homogenate} = \frac{Y\ \text{moles}/\mu Ci}{2.2 \times 10^6\ \text{dpm}/\mu Ci}$$

$$\times\ \frac{(cpm_{homog} - cpm_{blank})}{C^{14}_{eff}}$$

$$\times\ \frac{dpm \times C^{14}_{eff}}{CPM}\ \times\ \frac{10^3\ \text{nmoles}}{\mu\text{mole}}$$
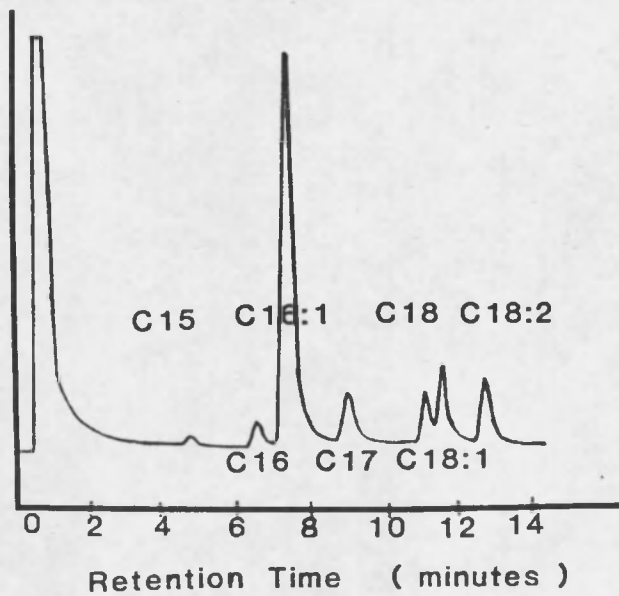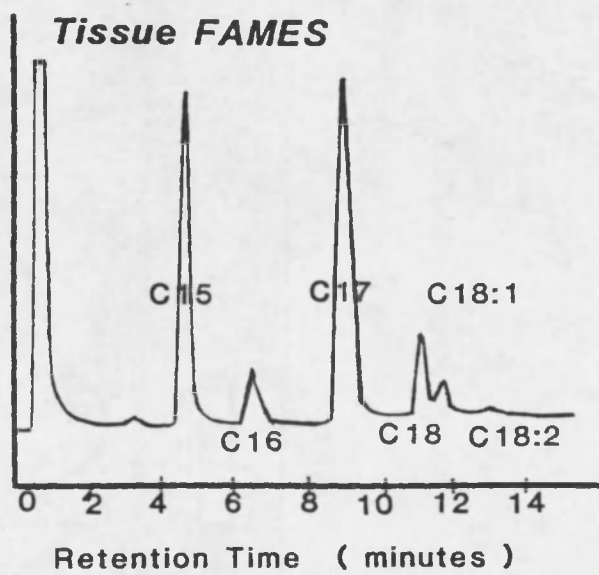
APPENDIX B

STANDARD CURVE FOR THE HOMOGENATE PROTEIN
BY THE LOWRY PROCEDURE
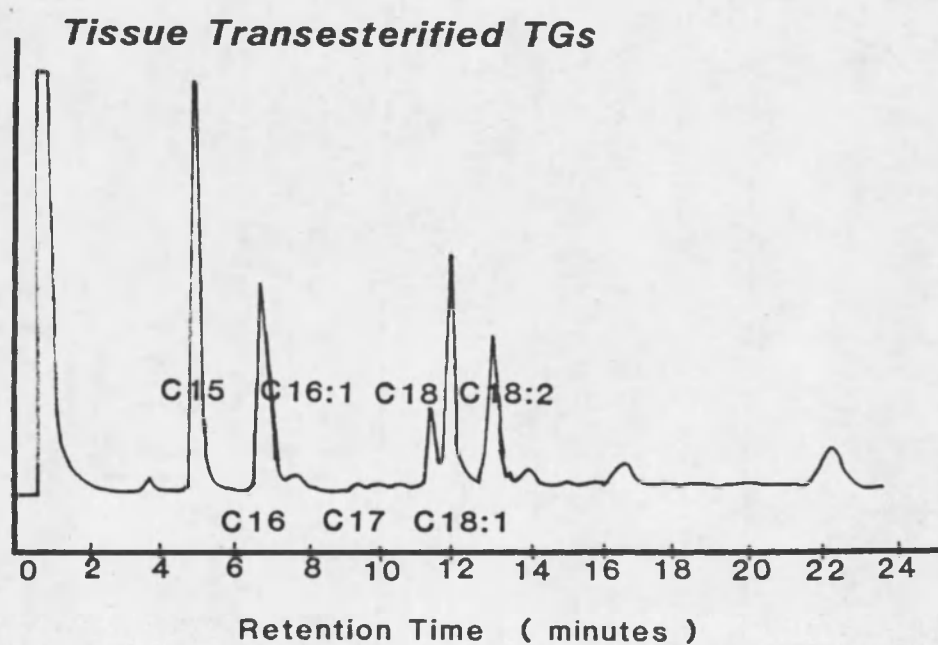
APPENDIX C

TYPICAL CHROMATOGRAMS OF THE FAMES FROM THE GLC STANDARD, TISSUE FFAS AND TRANSESTERIFIED TGS

GLC FAMES STANDARD

Retention Time (minutes)

Tissue Transesterified TGs

Retention Time (minutes)

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