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DISSESTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by
Kenneth Jon Kellar, B.A.

*** ***
The Ohio State University
1974

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Finally, to my wife, Beth, whose contagious happiness has at times kept me going, I dedicate my life's future work.
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The central nervous system controls energy balance through the adjustment of several important variables among which, the most important appear to be: food intake, stored energy, and energy requirements (1). The mechanisms which control these variables appear to operate to maintain constancy of milieu intérieur. To achieve such regulation, the brain must monitor signals from the periphery which supply information about the state of the organism. These signals appear to be routed in two ways -- first, via afferent nerves from peripheral systems that take part in energy exchange such as the gut and the skin and second, via the changes that take place in the internal environment and that are conveyed by the blood. The second signal, that conveyed by the blood, is largely a result of the exchange of energy across the gastrointestinal tract after the intake of food. This energy that is carried by the blood to all cells of the body is in the form of the basic foodstuffs required by cells to carry out their activities. However, the signal that appears to be monitored by the brain most closely, at least for short term regulation in most species, is the concentration of glucose carried in the blood (2) and the rate of utilization of that glucose (3).

The area of the brain that is responsible for monitoring the glucose signal is thought to be the hypothalamus -- specifically, the
ventromedial hypothalamic nucleus (VMN) which is a nuclear group that forms a cellular column on either side of the third ventricle beginning just caudal to the optic chiasm and ending just rostral to the premammillary nucleus. The VMN is characterized by small to medium-sized cells forming an ovoid cell group which stains deeply in Nissl preparations. The nucleus is surrounded by a cell-poor zone and is therefore easily delimited from neighboring cell groups: the dorsomedial nucleus dorsally, the arcuate nucleus ventrally, the lateral hypothalamic region laterally.

The ventral medial hypothalamus (VMH) containing the VMN has been the subject of much experimental work ever since Hetherington and Ranson demonstrated in the early 1940's that, in the rat, obesity regularly follows bilateral electrolytic destruction of the VMN (4). Brobeck and his colleagues soon extended these observations and found that the obesity could be attributed almost entirely to increased food intake. He termed the syndrome "hypothalamic hyperphagia" (5). In 1951, Anand and Brobeck found that bilateral lesions placed in the more lateral hypothalamic area caused animals to become aphagic (6); in addition, they demonstrated that animals made hyperphagic by VMN lesions become aphagic following destruction of the lateral hypothalamic areas (6). Anand and his colleagues postulated dual
hypothalamic control systems for feeding behavior involving the VMN, the ventrolateral area (LH), and the fiber connections between these two centers (7). In this system, the LH represented a chronically active "feeding center" responsible for the initiation of feeding, and the VMN represented a "satiety center" responsible for cessation of feeding behavior by exerting inhibitory control over the lateral area.

Since the enunciation of this model, a variety of behavioral and physiological evidence has confirmed the existence of such a system and more than a hundred papers have been published dealing with the phenomena of hypothalamic hyperphagia and hypothalamic aphagia [for reviews, see references 8 and 9].

In 1955, Jean Mayer postulated the presence of chemoreceptors in the VMN and possibly other neural areas. These receptors were postulated to have a high affinity for and to be activated by glucose (2). Mayer recognized that the level of glucose alone was not sufficient to regulate the activity of the VMN, and he predicted that more important than the level of glucose was the rate at which glucose was utilized by the VMN. A corollary to this hypothesis was that the glucose receptor cells in the VMN would be sensitive to insulin -- a concept at odds with the generally accepted idea that insulin does not play a direct role in brain metabolism (10).
In high doses, insulin reduces blood glucose levels by promoting the uptake of glucose into adipose tissue and muscle and preventing the release of glucose from the liver. When hypoglycemia becomes severe (blood levels of less than 40mg percent) a well known sequence of central nervous system events ensues: hyperirritability, disorientation, lethargy and somnolence, convulsions and finally coma and death (11). At almost any stage of this sequence, an intraperitoneal (I.P.) or intravenous (I.V.) injection of glucose immediately reverses the sequence. It seems clear that in this case insulin is exerting an indirect effect on the brain by lowering blood glucose levels and thus depriving the brain of its primary substrate for energy production.

High energy phosphate (ATP and phosphocreatine) decreases have been demonstrated in brains of mice undergoing electogenic (12) and audiogenic (13) seizures. Paradoxically, no significant decreases in high energy reserves were observed in cerebral cortex of mice (14) or whole brain of rat (15) before, during or after insulin induced hypoglycemic convulsions.

Since cells within the VMH appear to monitor blood glucose levels and thereby regulate the short term feeding behavior of animals, it should be of interest to know what role the VMH plays under conditions of severe hypoglycemic stress. Curiously, there have been
very few reports dealing with this subject. Several reports (16, 17) have shown that the frequency of spikes recorded from the VMN increased and those recorded from the LH decreased after I.V. glucose administration. The infusion of insulin caused a reverse pattern of activity (16, 18). A further investigation of the role of the VMH during severe hypoglycemic stress is the subject of the first part of this dissertation.

To study the relationship of the VMH to hypoglycemic stress, goldthioglucose (GTG) was used to lesion the VMH in mice. This use of GTG led to further investigation of the conditions under which GTG lesions the hypothalamus and the early neurochemical changes associated with its use.

During a toxicological study of gold compounds, in mice, Brecher and Waxler found that about a third of the survivors of a midlethal dose (LD50) of goldthioglucose became obese (19). The obesity resembled that seen after electrolytic destruction of the VMN of mice or rats and could be attributed to increased food intake. Other organic gold compounds tested such as goldthiomalate (GTM), goldthio-caproate, goldthiosorbitol, or goldthiogalactose were without effect in producing obesity. Upon histological examination, however, Brecher and Waxler could detect no lesion in the brains of obese mice.
Subsequent studies by Marshall, et al. (20) established that GTG does produce lesions in the ventral medial hypothalamus very close to the center of the area described by the coordinates used for stereotaxically producing lesions in the ventral medial nucleus of the hypothalamus. The lesion, however, becomes increasingly more difficult to detect with time. Presumably, the disappearance of the lesion is due to the collapse of the broad area of necrosis into a narrow scar on either side of the third ventricle; so that, after approximately 14 days, only the residual scar can be seen. After two months, even this scar is difficult to detect (21). The optimal time to see the lesion is from 24 to 72 hours after injection.

Goldthioglucose has been used to produce lesions in mice by many groups; however, its use in rats and other mammals has been less successful. Several groups have reported lesions in rats injected with GTG (22, 23); however, other workers have not been able to reliably produce lesions in this species (24, 25).

Several factors have been reported to influence the ability of GTG to lesion the VMH of mice. Among these are strain differences, sex of the mice and the concentration and rate of utilization of glucose at the time of injection. Brecher and his associates (26) found that the degree of lesioning of the VMH of normal mice was
directly correlated with the blood glucose concentration at the time of injection of GTG. That is, mice that were made hyperglycemic by the administration of glucose prior to the GTG injection had larger lesions than control mice that were normo-glycemic at the time of GTG injection. Conversely, as the blood glucose concentration at the time of injection of GTG was lowered, the lesion became less extensive. Thus, they found that mice that were fasted for 24 hours prior to GTG injection had smaller lesions than non-fasted mice.

Debons, et al. (27) showed that diabetes in mice, whether produced by alloxan or guinea pig anti-insulin serum prevented GTG lesions in the VMH. Furthermore, the administration of insulin either intravenously (28) or intrahypothalamically (29) restored the sensitivity of the VMH to lesion by GTG.

The above observations led us to examine whether GTG lesioned the VMH of rats and to define the conditions under which such lesions might be produced. The rat was chosen because, being approximately seven times larger than the mouse brain, the rat brain would offer obvious advantages for neurochemical studies. Also, if these studies showed that the rat hypothalamus could be successfully lesioned by GTG, then the previously stated disparity in reports by other groups concerning this point would be resolved. The
histological examination of the brains of normal and diabetic rats after GTG injection is the subject of the second part of this dissertation.

The pharmacology and biochemical effects of GTG are not well known, but certain aspects of its absorption, distribution and excretion have been studied in the mouse [for review of earlier literature, see reference 30].

After an intraperitoneal injection of GTG peak blood levels are reached between 30 and 60 minutes in the mouse (31). Chemical analysis of tissues (31) and radioactive tracer studies (32) of mice injected with GTG have suggested that the compound has access to most tissues of the body, but tends to concentrate in certain organs. In the early post-injection period, the highest concentrations of gold are found in the kidney and adrenal gland (31, 32). Three to four hours after injection, the concentrations of gold in the kidney and adrenal have fallen and those of the liver and spleen have risen to their highest levels (31, 32). A uniform distribution of gold has been reported throughout the brain one hour after injection of GTG (21). After six hours, however, the highest concentration in the brain is found in the medial hypothalamus (21, 33, 34). Twenty-four hours
after the injection of GTG, the gold levels in the ventral medial hypothalamus were more than three times those of temporal lobe areas (26).

The high concentration of gold in the VMH of mice following GTG injection is consistent with the observations of many groups that, the brain lesion, if present, is always found in that area. Only occasionally have studies revealed anatomical lesions of other areas -- notably, the hippocampal commissure and the medulla oblongata (35).

It has been suggested that the localization of lesions in the hypothalamus and the hippocampal commissure indicates area of a deficient blood-brain barrier (35), but the uniform distribution of gold throughout the brain one hour after GTG injection (21) does not support this concept.

The uniform distribution of gold in the brain shortly after injection of GTG and the failure of goldthiomalate and other goldthio compounds not containing a glucose moiety to produce lesions in the hypothalamus support the concept of a distinct metabolism of glucose, possibly involving glucoreceptors, in the VMH. Such a concept had been postulated by Mayer (2) to explain the regulation of feeding by
the VMH. However, to date, no such distinct metabolism of glucose by the VMH has been reported.

Very little about the biochemical changes induced by GTG has been reported. Several studies have examined the effects of GTG obesity on carbohydrate and lipid metabolism of adipose tissue (36, 37) and liver (38, 39), but no studies have been reported in which biochemical changes preceding the anatomical lesions in brain or other organs were investigated.

In previous studies, a possible role of the VMH in regulating the response of mice to hypoglycemia was examined, and the ability of GTG to cause VMH lesions in rats was confirmed. Therefore, it was decided to examine the early biochemical changes in the hypothalamus induced by GTG.

Since glucose appears to be an obligatory substrate for production of energy and the maintenance of physiologic function in the brain (40), and because the glucose moiety of GTG had been shown to be essential for the production of lesions (19), these biochemical studies focused on the uptake and metabolism of glucose by the hypothalamus and the effect GTG has on these biochemical processes.
METHODS AND MATERIALS

Convulsion Experiments

Preparation of animals. Young adult female CBA/J mice (Jackson Laboratories) weighing 18-22 grams were used for the convulsion experiments. These mice were housed ten to a cage in a temperature and humidity controlled vivarium with a 6:00a.m. to 6:00p.m. light cycle. They were fed standard laboratory chow (Ralston Purina Co.) and water ad libitum. The mice were assigned randomly to one of three groups: goldthioglucose (GTG), experimental group; goldthiomalate (GTM) control group; or saline control group. The mice in each cage were numbered by notching their ears. After acclimating to the animal quarters for 14 days, the mice were fasted for 24 hours before being given a single intraperitoneal injection of either goldthioglucose* (0.4mg/g), goldthiomalate (0.4mg/g) or saline, according to the group to which each had been assigned. All injections were given in saline in a volume equal to 0.01cc/gram body weight. The fast was continued for an additional 24 hours following injection, and then the mice were allowed free

*goldthioglucose was a gift of Dr. Merl Steinberg of the Schering Corporation, Bloomfield, New Jersey.
access to food. The purpose of fasting the mice before and after the injections was to decrease the mortality due to GTG. Weights of the mice were recorded periodically.

**Insulin injections.** Insulin injections were begun 73 days after initial treatment. The mice were fasted for 18 hours prior to each late morning injection of insulin (beef and pork regular insulin, Lilly Iletin). The insulin was diluted in saline and injected intraperitoneally in a volume equal to 0.012cc/gram body weight. The dose range of insulin used in these experiments was from 2 to 50mU/g (milliunits/gram).* After each insulin injection, the mice were observed for 70 minutes in their home cages with no food available. A convulsion was scored when a mouse displayed tonic or clonic spasms or nose to tail axial torsion spasms. Each mouse that convulsed was immediately injected intraperitoneally with 25 milligrams of glucose dissolved in water. At the end of the observation period, all mice were given free access to food and water and allowed five days to recover prior to the next insulin injection.

Another group of saline control and another group of GTG experimental mice were tested with insulin 7 and 12 days after initial treatment.

*A unit of insulin is defined as 1/24mg of a recrystallized sample (U.S.P.). A milliunit (mU) = 0.001 unit.
In order to determine if insulin resistance had been acquired by the mice after having been injected with increasing doses of insulin on successive test days, the saline and GTM groups were re-tested with a dose of insulin which had previously been shown to produce convulsions. Their response to the second injection was compared to their response to the first exposure at this dose.

To compare the insulin induced convulsions of these mice with another convulsant, each mouse was fasted for 18 hours and then injected intraperitoneally with 35mg/kg of pentylenetetrazol (Knoll Pharmaceutical Company, Orange, New Jersey).

**Blood glucose measurements.** The blood glucose of each mouse was measured after an 18 hour fast and again 60 minutes after the injection of insulin (14mU/gram body weight) or during convulsions, whichever occurred first. A small scalpel cut on the tail was made and 50 microliters (μl) of blood was withdrawn with a micropipette. The blood was deproteinized by placing it in a small tube containing 100μl of 0.45 molar perchloric acid and mixing on a Vortex-Genie (Scientific Industries, Inc., Springfield, Massachusetts). The deproteinated blood was spun for 10 minutes at 2500 rpm in a GLC-1 table-top centrifuge (Ivan Sorvall, Inc., Newtown, Connecticut). The acid-deproteinated plasma was then neutralized
with a mixture of potassium hydroxide-ethylenedinitrilo tetraacetic acid (KOH-EDTA) in 0.1 molar tris (hydroxymethyl) aminomethane (tris buffer) adjusted to pH 7.4. Aliquots were then taken for glucose measurement by the fluorometric method of Lowry, et al. (41).

**Histology.** Each mouse was anesthetized with sodium pentobarbital (30mg/mouse, I.P.). The chest wall was opened and the right ventricle of the heart was cut. Through the left ventricle, the mouse was perfused with isotonic saline for approximately one minute and then with sodium phosphate buffered 10 percent formalin for approximately four minutes. The brain was removed, the hypothalamus blocked out, and the entire brain placed in screw cap vials containing buffered 10 percent formalin. After being in the formalin for seven to ten days, the brains were placed in an aqueous 30 percent sucrose solution for one to two days. The brain block containing the hypothalamus was then placed on a microtome (Sartorius-Werke, Göttinger, Germany) cooled by a tissue freezer (Erickson Tool Co., Solon, Ohio) and frozen sections were cut at a thickness of 40 micrometers (μ). Each cut section was placed serially in a compartment of an ice tray filled with water. The cut sections were transferred, one at a time, to a shallow glass baking dish containing 200ml of a 1 percent gelatin solution (Purified gelatin,
Allied Chemical). The individual sections were floated onto a glass slide and positioned with a camel hair brush. Every second or third section was mounted. The mounted sections were placed in an oven at 50° overnight. The dried, mounted sections were then dehydrated, stained with cresyl violet and differentiated according to the following procedure:

Dehydration --

- 95% ethyl alcohol (ETOH): chloroform (1:1 solution) 15 minutes
- 100% ETOH 5 minutes
- 80% ETOH 5 minutes
- 70% ETOH 5 minutes
- Distilled water (to remove ETOH) 5 minutes
Stain -- 1gm cresyl violet acetate (Matheson, Coleman and Bell, Cincinnati, Ohio)

2000ml distilled water
3ml glacial acetic acid
0.025gm sodium acetate

Stain at approximately 50°C approximately 5 minutes
Distilled water rinse 5 minutes
Distilled water rinse 3 dips
95% ETOH 3 dips
95% ETOH + 5 drops glacial acetic acid; 3 changes of this, or until background is clear.

Differentiation --
100% ETOH 3 dips
100% ETOH 3 dips
100% ETOH (fresh) 3 dips
Xylene 5 minutes
Xylene 5 minutes
Xylene (fresh) 5 minutes

Coverslip
Statistical methods. The Student's t test (42) was applied to the measurements of the weights of the mice and to the measurements of blood glucose levels. The percentage of mice which convulsed in each group at each dose level was transformed using an arc sin $\sqrt{x}$ transformation (43). This was done to impart homogeneity to the variances, so that a two way analysis of variance (42) could be used to determine whether differences existed across the different groups of mice and/or across the different dose levels of insulin used.

The level of significance between groups was determined using the Tukey Multiple Comparison Test (42, 43) and the Biometrik Tables for Studentized Range (44).

Histological Experiments

Preparation of animals. Since strain differences exist in mice with regard to the production of VMH lesions by GTG, we tested two different strains of rats for the production of VMH lesions by GTG. Young adult female Wistar and Sprague Dawley rats weighing 150-200 grams were used for histology experiments. All rats were housed, four or five per cage, in a temperature and humidity controlled vivarium with a light cycle of 6:00a.m. to 6:00p.m. All
rats were given at least four days to acclimate to their environment before being prepared for experiments. Rats that were to be made diabetic were fasted for 48 hours and then given a single intraperitoneal injection of alloxan monohydrate (Nutritional Biochemicals Corporation, Cleveland, Ohio) in dose of 160mg/kg. The fast was continued for six hours following the administration of alloxan. The urine of these animals was tested for sugar daily using Tes-Tape urine sugar analysis paper (Eli Lilly and Co., Indianapolis, Indiana). Only those rats whose urine registered four-plus were considered diabetic. The others were not used.

Four non-alloxanized Sprague-Dawley rats were fasted for 24 hours and given a single intraperitoneal injection of GTG in a dose of 0.5mg/g. The fast was continued for another 24 hours, and 48 hours after the injection the rats were perfused with 10 percent buffered formalin through a heart puncture and prepared for histological examination of the brains exactly as described previously for mice.

Six non-diabetic and 18 alloxan-diabetic Wistar rats were divided into five groups which received the following intraperitoneal injections:
Group a -- non-diabetic -- saline and
30 minutes later GTG (0.5mg/g).

Group b -- alloxan-diabetic -- saline and
30 minutes later GTG (0.5mg/g).

Group c -- alloxan-diabetic -- saline,
10 minutes later glucose (50mg),
and 20 minutes later GTG (0.5mg/g).

Group d -- alloxan-diabetic -- insulin (5mU/g),
and 30 minutes later GTG (0.5mg/g).

Group e -- alloxan-diabetic -- insulin (5mU/g),
10 minutes later glucose (50mg),
and 20 minutes later GTG (0.5mg/g).

Forty-eight hours after the GTG injections, the rats were prepared
for histological examination of the brains exactly as described
previously for mice.
Biochemical Experiments

Preparation of animals. Young female Wistar rats were used for all biochemical experiments. Rats that were to be made diabetic were fasted for 48 hours and given a single injection of alloxan monohydrate (160mg/kg, I.P.). The fast was continued for six hours following the injection. The urine of these animals was tested for sugar daily for three days using Tes-Tape urine sugar analysis paper. All rats, diabetic and non-diabetic, were fasted for 18 hours (overnight) before each experiment.

Dissection. Each rat was decapitated by small animal guillotine and the head allowed to fall into a large beaker containing ice water (4°C). The skull was quickly opened with heavy scissors and the brain removed and rinsed three times in ice cold tris-saline buffer (see incubation medium). The brain was then placed, on filter paper in a large petri dish containing cold buffer. With the brain resting on its dorsal cortex surface and the ventral side of the brain up, the hypothalamus was dissected out using a stainless steel razor blade. A transverse cut was made just caudal to the optic chiasm and just rostral to the mammillary bodies (Fig. 1-1). The temporal cortex on both sides was trimmed away by making two oblique cuts dorso-laterally through the hypothalamic indentation just medial to
Fig. 1

Dissection Procedure: I. Ventral surface of rat brain. Isolation of hypothalamus and temporal cortex. 1A -- transverse cut rostral to mammillary bodies. 1B -- transverse cut caudal to optic chiasm.

II. Frontal section of rat brain at the level of ventromedial nucleus of the hypothalamus. Isolation of ventral hypothalamus. 2A and 2B oblique cuts through hypothalamic indentation just medial to optic tracts.

3 -- transverse cut at level of fornix. Structures: DMN, dorsomedial nucleus; VMN, ventromedial nucleus; LHA, lateral hypothalamic area; ARN, arcuate nucleus; Fx, fornix; OT, optic tract. III. Dissection of the ventral hypothalamus. 4 -- mid-parallel cut through third ventricle, 5A and 5B cuts parallel to ventricle and 1mm on either side of cut 4. Structures: LH, lateral hypothalamus; VMH, ventral medial hypothalamus; III third ventricle.
the optic tracts (Fig. 1-II). The ventral hypothalamus was isolated by a single transverse cut just ventral to the fornix or about one-third down the third ventricle (Fig. 1-II). The lateral hypothalamus from each side was dissected using a number 3 scalpel with a number 11 blade, and the ventral medial area was divided in half by a mid-sagittal cut through the third ventricle (Fig. 1-III). Each half of the ventral medial area dissected contained the ventromedial nucleus and the arcuate nucleus. Sections of temporal cortex were dissected with a scalpel. Dissections were completed in less than three minutes. Each section was gently touched three times to a glass plate to remove excess water and weighed on a Precision Balance (Roller-Smith Co., Bethlehem, Pennsylvania). The "apparent" wet weights (45) of the sections prepared in this manner were between 4 and 10 milligrams.

Incubation medium. The incubation medium was a standard tris-saline buffer containing the following salts: sodium chloride (139mM), potassium chloride (5.5mM), potassium phosphate dibasic (1.4mM), magnesium sulfate (1.4mM), calcium chloride (2.9mM), tris buffer adjusted to pH 7.35 (38mM) and glucose (9mM). The mixture was kept cold and gassed for 15 minutes with 100 percent oxygen. During the gassing, the calcium chloride was added slowly to prevent the precipitation of insoluble calcium phosphates.
Incubation conditions. Incubations were carried out in 10ml reaction flasks (Kontes Glass Company, Vineland, New Jersey) stoppered with rubber caps (Kontes Glass Company) which contained plastic center wells (Kontes Glass Company) descending from the center of the cap into the flask. The center wells contained accordion-folded squares of Whatman #1 filter paper and 0.2ml of 13 percent sodium hydroxide (NaOH) for the trapping of carbon dioxide given off by the tissues. Drugs and radiolabeled substrates were added directly to the incubation medium in each flask.

After the tissues were dissected and weighed, they were minced with a razor blade so that no dimension was greater than 0.4mm. This allowed oxygen to diffuse throughout the tissue and prevented the centers of the tissues from becoming anoxic (46). With the tissues in the flask and the rubber caps tightly sealed, the flasks were gassed with 100 percent oxygen for ten seconds to ensure a well oxygenated atmosphere during incubation. The tissues were incubated at 37°C while shaking at 100rpm in a metabolic shaker (model 2156, Research Specialties Co., Richmond, California). Incubation periods were 60, 120, or 180 minutes.
At the end of the incubation period, the flasks were placed in ice. This effectively lowered the temperature of the medium to 6°C in less than 30 seconds.

**Drug and radioisotope additions.** All flasks contained 0.5 microcuries (µci) of D-glucose-\(^{14}\)C (U.L.) (4.9mci/mm, New England Nuclear, Boston, Massachusetts), and 0.1µci of 2-Deoxy-D-glucose-1-\(^{14}\)C (54mci/mm, New England Nuclear).

Experimental tissues were incubated in the presence of varying concentrations of GTG and/or 100 microunits of crystalline porcine insulin (glucagon free)*. The insulin additions were taken from a stock insulin solution of 1mg of crystalline insulin per ml of dilute hydrochloric acid (pH 2.6).

**\(^{14}\)CO\(_2\) estimations.** The flasks were unstoppered and the alkali saturated filter papers and NaOH in the center wells were transferred to scintillation counting vials containing 15ml of scintillation solution. The center wells were rinsed into the vials with three volumes of scintillation solution. The scintillation

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*Gift of Dr. I.H. Slater, Eli Lilly and Company, Indianapolis, Indiana.*
solution consisted of 0.3gm of 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP), 12gm of 2,5-diphenyl-oxazole (PPO), and 76.8gm of naphthalene dissolved in 960ml of dioxane (spectral grade, peroxide-free).

Samples were counted four times at ten minutes each time in a model 3320 Packard Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Company, Inc., Downers Grove, Illinois).

\(^{14}C\) uptake estimations. In order to estimate the uptake of radiolabeled substrates during the incubation periods, the ice chilled tissues and media were transferred to 10 X 75mm culture tubes. The flasks were rinsed into the tubes with 1.5ml of buffer. The culture tubes were spun for one minute in a Sero-Fuge II centrifuge (Clay Adams, Inc.), and the media plus the rinse were removed from the packed tissue with a pasteur pipette, transferred to clean culture tubes and stored at -20°C. The packed tissue was washed three times with 0.5ml of buffer, and the washes discarded. The tissue was then taken up in 2.0ml double distilled water, transferred to homogenization tubes and homogenized for approximately one minute in a jacketed bath maintained at ice temperature. The homogenized tissue was then transferred back into the culture tubes and centrifuged at 2000g for 15 minutes at 4°C in a model RC-2B refrigerated centrifuge
equipped with a SS-34 head (Ivan Sorvall, Inc., Norwalk, Connecticut). After centrifugation, an exact aliquot of the supernatants was transferred to scintillation solution (the scintillation solution was of the same composition as that used for $^{14}$CO$_2$ estimates) and counted in the Packard Tri-Carb Scintillation Counter.

**Fluorometry.** Measurements of glycolytic intermediates and high energy phosphates were carried out on extracts of brain homogenates or incubation media employing the microfluorometric enzymatic methods established by Lowry and Passonneau and their co-workers (47). Fluorescence of reduced pyridine nucleotides (NADH, NADPH) coupled to specific enzymatic reactions were followed in a Farrand Ratio Fluorometer (Farrand Optical Co., Inc., New York, New York). Reaction kinetics were routinely determined prior to each assay to establish the time for 50 percent substrate conversion (half-time). The conduct of the assay was usually completed in ten half-times. Standard solutions and enzyme blanks were assayed along with unknowns.

**Ion-exchange chromatography.** A Dowex 1-X8 formate resin column was employed to separate major intermediates of glucose-$^{14}$C (U.L.) metabolism. A 1 X 30cm Pyrex Column fitted with a
fine porosity fritted disk and Teflon stopcock was filled to approximately one-third of its total volume with distilled water, and an aqueous slurry of Dowex 1-X8 formate resin (200-400 mesh) was slowly poured into the column and permitted to settle to a height of 14cm by gravity. During the settling, the stopcock was opened to hasten packing of the resin bed. The column was washed free of fines by reverse flushing (this also prevented layering of the packed column). The resin was again permitted to settle (14cm height) and was washed with at least five hold-up volumes of water. At no time was the resin permitted to run dry.

Loading of the columns was accomplished by diluting 1ml aliquots of tissue extracts to 100ml with double distilled water in a separatory funnel. Ten micromoles of the following cold carrier solutions of the following major intermediates were added to the diluted samples: L-glutamic acid, D,L-aspartic acid, pyruvate, citrate, isocitrate, lactate, 3 phosphoglyceric acid, D L glycerophosphate, fructose 1,6 diphosphate, glucose 6-phosphate, and 6 phosphogluconate. After thorough mixing, the separatory funnel was secured above the formate resin column and the stopcocks were opened. The diluted samples and carriers passed over the resin at a flow rate of approximately 1ml per minute. The non-
adsorbed effluent was collected in an Erlenmeyer flask. When the entire volume contained in the separatory funnel had passed over the column, the funnel was rinsed with 10ml of double distilled water which also was allowed to pass over the column and was collected in the Erlenmeyer flask. The non-adsorbing effluent in the Erlenmeyer flask was stoed at 4°C. The resin was then washed with two hold-up volumes of distilled water to remove any non-adsorbing 14C-labeled contaminants. The column was then refilled with water and arranged for linear gradient elution with 0-0.5M formic acid and 0-0.5M ammonium formate buffer (pH 3.6). All column elutions were carried out at room temperature (20°C).

The apparatus for continuous elution chromatography consisted of: (a) two cylindrical Pyrex bottles (500ml capacity) of equal cross sectional area and connected by regulator siphon; one bottle designated as reservoir bottle and the other, with an outflow, as the mixing bottle; (b) a magnetic stirrer (Dyla Dual, Will Scientific Co.); (c) Teflon-coated stirring bar (Ohmite, A.H. Thomas Co.); (d) 1/16 inch bore rubber tubing connected to the outflow of the mixing bottle at one end and to a standard tapered insert (10/30) at the other. The tapered insert was fitted into the chromatography column [for further details, see reference 48].
Fractions were collected using a 200 tube capacity Gilson Automatic Fraction Collector (Gilson Medical Electronics, Middleton, Wisconsin) equipped to deliver 4ml fractions.

The mixing bottle was filled with water and the reservoir bottle with the concentrated elutant (either 1M formic acid or 1M ammonium formate buffer). The contents of the mixing bottle were continuously stirred and delivered by gravity directly to the column containing the resin. This allowed a linearly increasing concentration of the elutant to be passed over the column.

Four milliliter fractions were collected at the rate of approximately 1ml/minute. Routinely, an elution volume of 950ml was collected for each complete chromatographic pattern. Collections were capped and stored at 4°C until assayed for radioactivity.

The column was washed with approximately four hold-up volumes of water between the formic acid elution and the ammonium formate buffer elution.

\[ ^{14} \text{C}-\text{intermediate estimations of column fractions.} \] Radioisotopic counting of elution fractions was conducted on 1ml sample aliquots taken from individual collecting tubes. Aliquots were pipetted into ringed aluminum planchets (32mm diameter x 2.4mm
high; Sigma Chemical Company, St. Louis, Missouri), a drop of detergent solution was added and the planchets were dried on stainless steel pans in a small oven at 85°C. The planchets were transferred to an automatic sample changer of 100 planchet capacity (model 1042, Nuclear Chicago Corp., Des Plaines, Illinois) equipped with a gas-flow detector for thin-window Geiger counting. The sample changer was coupled to a Decade Scaler and Lister (model 8703, Nuclear Chicago). Counting efficiency with this method was calculated to be approximately 25 percent.

**Statistical methods.** Student's paired t test was employed to test statistical significance between groups.
RESULTS

Convulsion Experiments

Weights of mice. The increases in weights of the mice in each group in which insulin testing was begun at 73 days were used as an indication of a ventral medial hypothalamic lesion. In Table 1 the weights of the mice in these groups are presented at day 0, the treatment day (GTG, GTM, or saline); at day 73, the beginning of the insulin test period; and at day 118 the conclusion of the insulin test period. At day 73, 76 percent of the GTG group had attained a weight two standard deviations greater than controls. Also, the weight of the GTG group was 21 percent greater than controls and had stabilized, as shown by the fact that their weights did not increase significantly between day 73 and day 118.

The weights of the GTG and saline groups in which insulin testing was begun at seven days were not significantly different from each other.

Response of mice to insulin hypoglycemia. In Fig. 2 the response of the three groups of mice in which insulin testing was begun at 73 days is plotted over a dose range of insulin of 2-30mU/g.
**TABLE 1**

Weights of Mice in Convulsion Experiments

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>(n)</th>
<th>Average Fasting Weight (grams ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>(9)</td>
<td>19.5 23.3 (0.4) 24.2 (0.5)</td>
</tr>
<tr>
<td>GTM (0.4mg/g)</td>
<td>(9)</td>
<td>19.5 23.2 (0.4) 23.6 (0.8)</td>
</tr>
<tr>
<td>GTG (0.4mg/g)</td>
<td>(27)</td>
<td>20.2 28.2 (0.5)* 29.4 (0.5)*</td>
</tr>
</tbody>
</table>

Weight ratios
GTG/controls    | 1.04 | 1.21 | 1.23 |

*P<0.01 compared to controls
Fig. 2

Log dose-response curves for saline (n=8) controls, GTM controls (n=7) and GTG treated group (n=25). Insulin testing begun 73 days after initial treatment. See text for details.
% CONVULSIONS

- SALINE
- GTM
- GTG

* p<.01

GTG vs. CONTROLS

LOG DOSE INSULIN mU/g
The dose of insulin required to produce convulsions in 50 percent of the mice in each group (CD₅₀) was 4.7mU/g and 5.8mU/g for the saline and GTM groups respectively, and 11mU/g for the GTG group. The dose of insulin required to produce convulsions in 90 percent of the mice in each group (CD₉₀) was 7mU/g and 9mU/g for the saline and GTM groups respectively; however, no dose of insulin up to 50mU/g elicited convulsions in more than 60 percent of the GTG group. Thus, the GTG group appeared to be protected against insulin-induced hypoglycemic convulsions.

To determine whether these mice which had been subjected to repeated exposures to increasing doses of insulin on successive test days had developed a resistance to insulin, the saline and GTM control groups were challenged with a second dose of 11mU/g at the end of the experiment. All of the saline group and all but one of the GTM group convulsed. This indicated that insulin resistance had not developed sufficiently over this period of time (seven weeks) to impart protection against insulin-induced hypoglycemia.

This conclusion was supported by blood glucose measurements determined after an 18 hour fast and again 60 minutes after injection of insulin (14mU/g) or during convulsions, whichever occurred first. In all animals the blood glucose fell from a fasting level of
approximately 65mg percent (3.6mM) to approximately 23mg percent (1.2mM). There were no significant differences in blood glucose levels among the groups (Fig. 3).

To determine whether the apparent protection conferred by the GTG was specific for hypoglycemic convulsions or was a more general protection against central nervous system convulsions, the three groups of mice were treated with pentylentetrazol (35mg/kg, I.P.) after an 18 hour fast. There were no significant differences in response to this central nervous system stimulant among the groups -- 88 percent of the GTG group, 100 percent of the GTM group and 88 percent of the saline group convulsed (Fig. 4). Therefore, the protection conferred by GTG appeared to be specific for hypoglycemic convulsions.

The GTG and saline groups in which insulin testing was begun seven days after initial treatment received the following doses of insulin: 5mU/g on day 7 and 11mU/g on day 12. There were no significant differences in response between the groups at either dose level. Fifty percent of each group convulsed after receiving 5mU/g; and all of the mice in each group convulsed after receiving 11mU/g.
Fig. 3

Blood glucose levels of GTG, GTM, and saline groups. Left, fasting blood glucose levels following 18 hour fast. Right, blood glucose levels 60 minutes after insulin (14mU/g, I.P.) or during convulsion, whichever occurred first.
FASTING BLOOD GLUCOSE

GTG  GTM  SALINE

BLOOD GLUCOSE
60 mins after INSULIN
14 mU/g (I.P.)

(25)  (7)  (8)
Fig. 4

Convulsion response of GTG, GTM and saline groups to pentylenetetrazol (35mg/kg, I.P.) following 2 hour fast.
% CONVULSIONS

GTG  GTM  SALINE
Histology. The hypothalamic lesion resulting from the administration of GTG extends as far rostral as the posterior edge of the optic chiasm and as far caudal as the medial mammillary nucleus. The lesion is most consistently seen at the level of the median eminence. The lesion is bilateral rostrally but often becomes confluent in the midline at its caudal extent.

Until approximately day seven, the lesion can be seen as a sharply circumscribed area of necrosis nearly devoid of Nissl staining materials. After about seven days, the lesion begins to be transformed into a narrowing band on either side of the ventricle. This shrinkage of the lesion is believed to be due to loss of fluid and diffusible products of autolysis (21). By the fourteenth day after injection, the lesion is transformed into a narrow scar which becomes progressively more difficult to visualize with time (21).

The CBA mice used in the convulsion studies were not fixed for histology until more than 130 days after initial treatment. Upon examination of the stained sections, no lesions were apparent. In sections from a few mice from the GTG group, residual scars and distortion of the third ventricle were noted. No scars or distortions were seen in mice from the GTM or saline groups.
However, in groups of CBA mice treated identically to those in the convulsion studies but fixed for histology 48 hours after treatment, lesions of the ventral medial hypothalamus were clearly evident in all GTG treated mice; while no mouse treated with GTM or saline showed evidence of lesions. Representative sections of normal and lesioned hypothalamus are shown in Plates I and II (appendix).

Histology Experiments

Although the ability of GTG to produce hypothalamic lesions leading to obesity in mice has been well documented (49), similar attempts to produce hypothalamic lesions or obesity in the rat or other laboratory animals have been less successful. Goldthioglucose has been reported by several groups to cause lesions in albino rats (22, 23), but others have failed to demonstrate lesions (24, 25).

This study of the ability of GTG to lesion the VMH of rats was based on our high rate of success in lesioning mice with GTG, and the need for a rat model of the lesion to be used in biochemical studies.
Sprague-Dawley rats. Four Sprague-Dawley female rats treated with GTG all had discrete lesions through the ventral medial hypothalamus. Representative sections of normal and lesioned hypothalamus from Sprague-Dawley rats are shown in Plates III and IV (appendix).

Wistar rats. Table 2 summarizes the incidence of VMH lesions in the five groups of Wistar rats studies. All of the non-diabetic rats (group a) which received saline and GTG had lesions in the VMH. Representative sections of non-lesioned (control) and lesioned hypothalamus from Wistar rats are shown in Plates V and VI (appendix).

None of the alloxan-diabetic rats treated with saline instead of insulin had lesions of the VMH, regardless of whether they received saline alone (group b) or saline plus glucose (group c) prior to GTG injection. None of the diabetic rats that received insulin alone prior to the GTG injection (group d) had lesions in the VMH, and only one rat treated with both insulin and glucose prior to GTG injection (group e) had a lesion in the VMH. Thus, it appears that parenteral insulin injection does not immediately restore the sensitivity of the VMH to damage by GTG injection, regardless of the level of blood glucose at the time of injection.
<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>Pre-Treatment</th>
<th>GTG</th>
<th>Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.) non-diabetic</td>
<td>(6)</td>
<td>saline</td>
<td>0.5mg/g</td>
<td>6/6</td>
</tr>
<tr>
<td>b.) alloxan-diabetic</td>
<td>(4)</td>
<td>saline</td>
<td>0.5mg/g</td>
<td>0/4</td>
</tr>
<tr>
<td>c.) alloxan-diabetic</td>
<td>(2)</td>
<td>saline + glucose</td>
<td>0.5mg/g</td>
<td>0/2</td>
</tr>
<tr>
<td>d.) alloxan-diabetic</td>
<td>(6)</td>
<td>insulin</td>
<td>0.5mg/g</td>
<td>0/6</td>
</tr>
<tr>
<td>e.) alloxan-diabetic</td>
<td>(6)</td>
<td>insulin + glucose</td>
<td>0.5mg/g</td>
<td>1/6</td>
</tr>
</tbody>
</table>
Biochemical Experiments

After having demonstrated that GTG can produce lesions in the VMH of rats, in vitro experiments were begun in an attempt to elucidate the early neurochemical changes in the VMH brought about by GTG and what role, if any, insulin may play during the course of these changes.

**Effects of insulin on $^{14}$C-uptake and $^{14}$CO$_2$ generation.** The effects of insulin (100μU/incubation) on $^{14}$C-uptake and $^{14}$CO$_2$ generation in the VMH, LH and cortex of alloxan-diabetic rats were studied over a time range of 60-180 minutes. Insulin was found to make no significant difference on the amount of $^{14}$C labeled substrate or its metabolites in the tissue (Fig. 5), or on the amount of $^{14}$CO$_2$ generated (Fig. 6).

**Effects of GTG on $^{14}$C-uptake and $^{14}$CO$_2$ generation.** Incubations of VMH, LH, and cortex for 120 minutes with increasing concentration of GTG resulted in a steady decrease of $^{14}$C-glucose given off as $^{14}$CO$_2$ in the hypothalamic areas, but much less of a decline in cortex (Fig. 7). Maximum inhibition of approximately
Fig. 5

Uptake of $^{14}$carbon label into the VMH, LH and temporal cortex in the presence and absence of insulin (100μU/incubation) at times shown.
I$^4$C UPTAKE CPM/mg (±SEM)

CORTEX

500-1

VMH LH

-INSULIN

+INSULIN

INCUBATION TIME (MINUTES)

INCUBATION TIME (MINUTES)
Fig. 6

Generation of $^{14}$CO$_2$ from $^{14}$C-glucose (U.L.) by the VMH, LH and temporal cortex in the presence and absence of insulin (100µU/incubation) at the times shown.
Inhibition of $^{14}\text{CO}_2$ production from $^{14}\text{C}$-glucose (U.L.) by increasing concentrations of GTG in the VMH, LH and cortex after 120 minutes of incubation.
$^{14}\text{CO}_2$ CPM/mg

PERCENT OF CONTROL

GOLDTHIOGLUCOSE CONCENTRATION (mM)

* $P < .05$
50 percent was produced in the VMH and approximately 30 percent in the LH by 10mM GTG. Subsequent incubations were carried out using this concentration of GTG.

Goldthioglucose (10mM) caused decreased uptake of $^{14}$C-labeled substrate (Fig. 8) and an even larger decrease in $^{14}$CO$_2$ generation (Fig. 9) in the hypothalamic tissue at 60, 120 and 180 minutes. Maximal effects were seen at 120 minutes. The VMH was notably more sensitive to GTG than was the LH. At 120 minutes, the VMH $^{14}$C-uptake was depressed by approximately 33 percent by GTG, while the LH $^{14}$C-uptake was depressed by approximately 18 percent. During the same time period, the $^{14}$CO$_2$ generated by the VMH was decreased by 57 percent by GTG, while the $^{14}$CO$_2$ generated by the LH was decreased by 42 percent (Figs. 8 and 9).

**Effects of insulin on GTG inhibition of $^{14}$C-uptake and $^{14}$CO$_2$ generation.** Insulin did not appear to have a significant influence on $^{14}$C-uptake in the VMH or the LH in the presence of GTG after 120 minutes incubation (Fig. 10). However, in the presence of insulin, the inhibition by GTG of $^{14}$CO$_2$ generated by the VMH and LH was not as pronounced (Fig. 11). This was especially apparent in the VMH where the presence of insulin plus GTG increased the $^{14}$CO$_2$ generated
Fig. 8

Uptake of $^{14}$C label into the VMH and LH in the presence and absence of GTG (10mM) after incubation for 60, 120 and 180 minutes.
Fig. 9

Generation of $^{14}\text{CO}_2$ from $^{14}\text{C}$-glucose (U.L.)

by the VMH and LH in the presence and absence of

GTG (10mM) after incubation for 60, 120 and

180 minutes.
Fig. 10

Effect of insulin (100μU/incubation) on the inhibition by GTG (10mM) of uptake of $^{14}$C label into the VMH and LH. Control levels (No GTG):
VMH, 342cpm/mg; LH, 250cpm/mg.
Fig. 11

Effect of insulin (100μU/incubation) on the inhibition by GTG (10mM) of $^{14}$CO$_2$ production from $^{14}$C-glucose (U.L.) by the VMH and LH. Control levels (No GTG): VMH, 136cpm/mg; LH, 107cpm/mg.
to approximately 75 percent of control levels (no GTG) compared to
43 percent of control levels in the presence of GTG alone.

**Effect of GTG on lactate production.** Lactate is a major
product of glycolysis. Most of the lactate produced by the tissues is
lost to its surrounding medium. If glycolysis is inhibited to a
significant extent, the lactate production will be decreased as well as
the production of acetyl CoA, which feeds directly into the tri-
carboxylic acid cycle leading to the generation of CO₂. If, on the
other hand, inhibition occurs at a step beyond glycolysis, the
generation of CO₂ will be decreased without necessarily decreasing
lactate production.

In these experiments lactate production in the presence of
GTG was inhibited less than 20 percent in the VMH and less than
10 percent in the LH (Fig. 12). This suggests that the earliest
block in ¹⁴CO₂ production occurred at a step (or steps) beyond the
glycolytic sequence.

**Effect of GTG on high energy phosphates.** Brain is uniquely
dependent on glucose as a substrate for energy production. Under
most conditions, no other substrate serves to allow adequate
Fig. 12

Effect of GTG (10mM) on rate of lactate production by the VMH and LH after incubation for 120 minutes.
production of ATP and phosphocreatine for the myriad energy requiring processes in brain tissue (50).

In these studies, only phosphocreatine (PC) was measured in the brain tissues because the more labile ATP had fallen below measurable levels during cold storage of the tissues. In Fig. 13 the PC levels in the VMH and the LH are shown after incubation with and without GTG. Note that GTG reduced PC levels to approximately 60 percent of the control values in both the VMH and LH.

Effect of GTG on $^{14}$C-glycolytic and $^{14}$C-tricarboxylic acid cycle intermediates. The chromatographic elution profiles following ion-exchange chromatography indicates that GTG caused a decrease in the formation of $^{14}$C-hexosemonophosphates in both the VMH and LH (Figs. 14 and 15). This was especially pronounced in the VMH. Goldthioglucose also caused a decrease in citrate and isocitrate formation (Figs. 14 and 15). This was evident in the VMH as well as the LH; again, the decrease was more pronounced in the VMH. The formation of 2-phosphoglyceric acid and 3-phosphoglyceric acid as well as fructose 1,6-diphosphate, all intermediates of glycolysis, appeared to be depressed in the VMH and LH in the presence of GTG.
Fig. 13

Phosphocreatine content of VMH and LH after incubation for 120 minutes in the presence and absence of GTG (10mM).
Fig. 14

Chromatographic profile of pooled VMH tissue extracts after incubation in the presence or absence of GTG. Formic acid elution. Labels: A, lactate; B, hexosemonophosphate; C, citrate and isocitrate. See text for details.
LITERS OF FORMIC ACID

DPM/TUBE

VMH - GTG

LITERS OF FORMIC ACID

VMH + GTG
Fig. 15

Chromatographic profile of pooled LH tissue extracts after incubation in the presence or absence of GTG. Formic acid elution. Labels: A, lactate; B, hexosemonophosphate; C, citrate and isocitrate. See text for details.
LH - GTG

DPM/TUBE

LH + GTG

DPM/TUBE

LITERS OF FORMIC ACID

0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

LITERS OF FORMIC ACID

0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

LITERS OF FORMIC ACID

0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

LITERS OF FORMIC ACID
(Figs. 16 and 17). The product of the first reaction of the pentose phosphate pathway, 6-phosphogluconate, was decreased in both the VMH and LH in the presence of GTG (Figs. 16 and 17).

In general, the results of the ion-exchange chromatography suggest that all of the intermediates of uniformly labeled glucose $^{14}$C metabolism are decreased by GTG in both the VMH and LH.
Fig. 16

Chromatographic profile of pooled VMH tissue extracts after incubation in the presence or absence of GTG. Ammonium formate buffer elution.

Labels: D, 6-phosphogluconate; E, 2-phosphoglyceric acid and 3-phosphoglyceric acid; F, fructose 1,6-diphosphate.
VMH - GTG

VMH + GTG

LITERS OF AMMONIUM FORMATE BUFFER

DPM/TUBE

LITERS OF AMMONIUM FORMATE BUFFER

DPM/TUBE
Fig. 17

Chromatographic profile of pooled LH tissue extracts after incubation in the presence or absence of GTG. Ammonium formate buffer elution. Labels: D, 6-phosphogluconate; E, 2-phosphoglyceric acid and 3-phosphoglyceric acid; F, fructose 1,6-diphosphate.
LH - GTG

LH + GTG

DPM/TUBE

LITERS OF AMMONIUM FORMATE BUFFER

1000

500

100

50

10

5

1

LITERS OF AMMONIUM FORMATE BUFFER

1000

500

100

50

10

5

1
DISCUSSION

Convulsion Experiments

The ventral medial hypothalamus exerts a modulating effect on feeding behavior by inhibiting the tonically active lateral hypothalamus (7). Through this dual control system, energy balance is regulated and maintained (51).

It is clear from ablation experiments that neither the VMH nor the LH is essential for the maintenance of life. Indeed, rats with bilateral lesions in both the VMH and LH have survived for long periods of time. The electrolytic or chemical destruction of the VMH results in an animal that fails to maintain his food intake at the previous level. The threshold for satiation is increased (52) and the "set point" for maintaining a relatively constant body weight is raised. These changes in regulatory mechanisms within the brain are manifested by increased food intake (per meal as well as daily), and increased body weight to new levels which are then maintained fairly constantly (53, 54). The electrolytic bilateral destruction of the LH results in an animal that is completely aphagic and which, if not force fed, will die of starvation. The period of complete aphagia,
however, is transient, and after about three days of forced feeding, the LH lesioned animal will begin to eat wet, palatable foods on its own (55). Gradually, the animal will recover its appetite and regain its ability to regulate its body weight under most conditions; that is, it adjusts its food intake to compensate for changes in nutrient density of the food, eats less when the ambient temperature is raised and more when the ambient temperature is lowered (56).

The point being made here with regard to VMH and LH lesioned animals is that the deficit incurred, however apparently severe, results in only a temporary inability of the animal to regulate its food intake and maintain a relatively constant body weight -- albeit, grossly higher in the case of the VMH lesioned animal. Other areas of the brain are apparently involved in the function of regulating food intake and are capable of taking over this function after destruction of the primary regulatory mechanisms in the hypothalamus. One such area may be the septal region, destruction of which leads to hyperphagic eating patterns as well as increased water intake (57).

One interesting and important exception to the recovery of regulated feeding behavior of LH lesioned animals has been reported by Epstein and Teitelbaum (56). This exception is the failure of LH
recovered rats to increase their food intake in response to insulin-induced hypoglycemia. Both normal and VMH lesioned rats will increase their food intake and prevent the onset of severe hypoglycemia in response to insulin injections. The recovered lateral rat, however, does not increase its food intake to compensate for the hypoglycemia induced by insulin (56). The deficit appears to be permanent, having been demonstrated more than a year after damage to the LH. A specific impairment of the hypoglycemic control of food intake appears to be a permanent sign of the lateral hypothalamic syndrome. It is noteworthy that the VMH lesioned rats in Epstein and Teitelbaum's study did respond to insulin-induced hypoglycemia, since the opposite might have been predicted from Mayer's "glucostatic hypothesis" (2), which postulated the presence of chemoreceptors in the VMH which have a special affinity for glucose and which are activated by increased glucose utilization rate. This hypothesis postulated that the VMH, unlike the rest of the brain, was sensitive to insulin and that insulin increases the glucose utilization rate of the VMH. Obviously, the response of VMH lesioned rats to insulin-induced hypoglycemia does not support Mayer's hypothesis.

The convulsion experiments reported in this dissertation were an attempt to study the regulatory mechanisms of feeding behavior
from a different aspect. Since hypoglycemia is apparently involved as a regulatory signal in the hypothalamus for feeding behavior, and since severe hypoglycemia results in a dramatic sequence of behavior culminating in convulsions and coma, the question was asked: can the severe hypoglycemia behavioral sequence be interrupted or altered by lesioning the VMH? The answer is that apparently the sequence can be changed. Lesioning the VMH of mice by GTG injections led to a reduction in the incidence of hypoglycemic convulsions when 73 days were allowed to pass between the time of lesion and the beginning of the insulin test periods.

The behavioral sequence of the lesioned mice prior to the convulsion stage remained essentially unchanged from non-lesioned mice. That is, the VMH lesioned mice became irritable, lethargic and somnolent just as did the controls. It is presumed that the lesioned animals would have entered the coma stage eventually, but these experiments were usually terminated before that stage was reached. Even so, blood glucose levels fell below 22mg percent without causing more than half the VMH lesioned mice to convulse. Up to this point, these experiments support the idea of a glucose sensitive VMH which under conditions of severe hypoglycemia discharges to other areas of the brain in a dyssynchronous manner leading to
convulsion. However, it was a consistent observation of this study that as soon as the experiments were terminated by placing food in the cage, the mice would begin to eat -- ravenously, if they were able or more slowly if they were too weak. This is consistent with the observations of Epstein and Teitelbaum, who reported that VMH lesioned animals respond to insulin-induced hypoglycemia by immediately increasing their food intake (56).

A parsimonious explanation for the apparent protection against insulin-induced hypoglycemic convulsions seen in mice with VMH lesions is not possible at this time. However, certain possibilities exist which are consistent with the observations of others and which should be investigated. Among these is the possibility that the VMH acts as a "trigger zone" for the induction of hypoglycemic convulsions. As stated previously, no changes in high energy phosphate reserves have been noted in cerebral cortex of mice (14) or whole brain of rat (15) during hypoglycemic convulsions. Tarr, et al., (15) suggested that a decrease in high energy reserves might occur only in small but critical areas of the brain and that these changes might go undetected when the critical area is assayed as part of a much larger area. The VMH may be such a critical area, and lesioning the VMH may, in effect, prevent the signal for severe hypoglycemic
stress from being activated, thus decreasing the probability of a convolution. However, if this were the case then the mice tested 7 and 12 days after lesioning should have shown some degree of protection also. Since they did not, it is difficult to invoke such an explanation.

Another possibility is suggested from studies of the response of fasted animals to insulin-induced hypoglycemia. In one study (58), mice fasted for 48 hours displayed significant protection from insulin-induced hypoglycemic convulsions when compared to non-fasted controls. The increased resistance to convulsions appeared to be specific for hypoglycemic convulsions since fasting did not confer protection against pentylenetetrazol or strychnine induced convulsions. In another study (59), increased cerebral uptake of ketoacids and increased β-hydroxy-butyrate dehydrogenase activity were found in the brains of fasted rats. Thus, the brain may become less dependent upon glucose as a fuel and, instead, utilize ketoacids which are available in increased quantities during fasting. Ketoacids are also increased during period of severe hypoglycemia. Fat must be oxidized to maintain body functions during periods of low blood glucose and glycogen reserves, and if incomplete oxidation of fat occurs due to the low carbohydrate reserves, ketosis prevails (60).
It is possible that lesions in the VMH induce changes in the metabolic requirements of certain areas of the brain or of the entire brain. If so, one of the long term effects of VMH lesions may be to induce resistance to hypoglycemic convulsions by increasing the utilization of ketoacids by the brain.

A third possibility is that changes in peripheral organ systems induced by GTG, or increased body fat following long term hyperphagia, contribute to the protection from insulin-induced hypoglycemic convulsions. For example, the liver may inactivate insulin more rapidly, thus effectively lowering the circulating levels of exogenous insulin. This is not likely the case, however, since blood glucose concentrations fell as quickly and to the same levels in lesioned mice as in non-lesioned controls. Somatotrophin levels are reduced in rats (61) and mice (62) with VMH lesions. This is probably due to loss of cells in the VMH responsible for synthesizing somatotrophin releasing factor. It would be tempting to implicate reduced somatotrophin levels as a factor in the increased thresholds to hypoglycemic convulsions seen in the VMH lesioned mice. For example, if somatotrophin were necessary to maintain electrical excitability of brain tissue, then reduced somatotrophin levels might raise the threshold for spread of convulsive foci in the brain.
No evidence for such a role of somatotrophin exists, however. Furthermore, if electrical excitability were decreased due to lower somatotrophin levels, then the threshold for convulsions induced by other agents would be expected to be raised also.

Obviously, much work remains to be done in defining the role of the hypothalamus in the response to severe hypoglycemic stress.

Histological Experiments

The histological examination of the rat brain after GTG treatment revealed that pretreatment with alloxan prevents the GTG lesion of the hypothalamus. These results are in agreement with the findings of Debons, et al. (29) in mice, and support the contentions of others that insulin increases the glucose utilization rate of cells in the VMH (2) and that increased glucose utilization of cells in the VMH makes the VMH more vulnerable to lesion by GTG (26). However, in this study, treatment of alloxan diabetic rats with insulin alone did not restore the sensitivity of the hypothalamus to lesion by GTG. Even when glucose was administered along with insulin in order to maintain high levels of blood glucose, only one rat had a lesion in the VMH.
It appears, therefore, that the diabetic rat differs from the diabetic mouse with regard to restoration by insulin of sensitivity to GTG lesions. One possible explanation for the discrepancy between the two species may lie in the time required for insulin to activate the VMH. Debons, et al. (28) found that insulin was effective in restoring the sensitivity of the VMH to GTG lesion when administered five minutes or more before GTG. In the rat studies, I allowed 30 minutes between the injection of insulin and GTG, but found no lesions. The possibility remains that chronic treatment with insulin over a number of days would restore the sensitivity of the VMH to lesion by GTG. This possibility is currently being explored. One other point should be made with regard to the differences obtained in the mouse and the rat. In the mouse, Debons, et al., used doses between 0.8 and 1.2mg/g body weight, while in the rat, I used 0.5mg/g body weight. It is possible that GTG would produce lesions in diabetic rats if administered in higher doses.

Biochemical Experiments

The most notable effect of GTG on carbohydrate metabolism observed in the biochemical studies reported here was on the generation of CO$_2$ by the hypothalamus. While having little effect
on the temporal cortex, GTG inhibited $^{14}\text{CO}_2$ production by
approximately 40 percent in the LH and more than 50 percent in the
VMH after 120 minutes of incubation. The inhibition of $^{14}\text{CO}_2$
production in the hypothalamus was not strictly a function of
decreased uptake of $^{14}\text{C}$-glucose, since in nearly every case $^{14}\text{CO}_2$
production was inhibited to a greater extent than was $^{14}\text{C}$-uptake.
The data suggest that GTG acts somewhere along the metabolic
route of glucose to severely inhibit its use as an energy producing
substrate in the hypothalamus. This suggestion is supported by the
fact that GTG caused an approximate 40 percent decrease in
phosphocreatine levels in the VMH and LH.

Studies designed to localize the major site of inhibition by
GTG were not entirely successful. However, the fact that lactate
production in the VMH and LH was inhibited to a much lesser degree
than was the $\text{CO}_2$ production suggests that the earliest factor con-
tributing to the decreased $\text{CO}_2$ production was beyond the glycolytic
sequence. This may have been at either the pyruvate dehydrogenase
step in which the product of aerobic glycolysis, pyruvate, is prepared
for entrance into the tricarboxylic acid cycle as acetyl CoA, or in
the tricarboxylic acid cycle directly. The inhibition of high energy
phosphate production in the tricarboxylic acid cycle is probably a
factor in the decreased levels of phosphorylated intermediates seen in the chromatographic profiles of VMH and LH tissues incubated in the presence of GTG. High energy phosphate, in the form of ATP, which is generated in the tricarboxylic cycle, is necessary for the enzymatic synthesis of glucose 6-phosphate from glucose. The formation of glucose 6-phosphate is a necessary priming step for the subsequent steps in glycolysis, as well as those of the pentose phosphate pathway. The decreased levels of citrate and isocitrate in tissues incubated in the presence of GTG are probably a direct reflection of lower metabolic activity of the tricarboxylic acid cycle.

In these studies, insulin had no effect on the uptake of $^{14}$C-glucose, nor on the generation of $^{14}$CO$_2$ in the VMH or the LH. These studies, however, do not rule out the suggestion of Mayer (2) that there exists a small number of cells within the VMH that are responsive to insulin. The possibility remains that the response of a small number of cells to insulin would have been obscured by the surrounding tissue. Another possibility is that insulin was exerting an action on the VMH which was not measured in these studies. For example, insulin is known to increase protein and RNA synthesis in many peripheral tissues, and the VMH and LH are sites of active protein and RNA synthesis.
Finally, in these studies insulin did not increase the effects of GTG on the hypothalamic tissue. In fact, there was some indication that insulin actually reduced the degree of inhibition of $^{14}$CO$_2$ production caused by GTG. These results are not consistent with what was found in vivo. That is, normal levels of circulating insulin are apparently necessary to produce a lesion in the VMH of rats.
SUMMARY AND CONCLUSIONS

These studies attempted to look at the ventral medial hypothalamus at three different levels: first, its putative role in the regulation of blood glucose levels and its possible involvement in the development of hypoglycemic convulsions in mice; second, the histology of the VMH of rats after goldthioglucose administration and the consequence of insulin or the lack of it for the necrotizing effects of GTG in the VMH; and third, the early biochemical effects of GTG on the VMH, especially its effect on glucose metabolism.

Lesioning the VMH of mice with GTG resulted in substantial protection against insulin-induced hypoglycemic convulsions. This effect was seen in mice in which insulin testing was begun 73 days after lesioning the VMH but not in mice tested 5 or 12 days after lesioning.

These observations suggest that the lesion or the resultant hyperphagia and consequent obesity produce a change in the absolute metabolic requirements of the brains of these mice. A similar, though somewhat less substantial, resistance to insulin-induced
hypoglycemic convulsions has been reported in mice fasted for 48 hours (58). Also, increased cerebral uptake and utilization of ketoacids have been found in fasted mice (59).

It is not known by what mechanism a VMH lesion may produce such changes in the metabolic requirements of brain at this time.

The results of the histology experiments in the rat support, in part, the contentions of other investigators (26-28) that the presence of insulin and the rate of glucose utilization is a critical factor in determining the extent of the VMH lesion caused by GTG. In my investigations on the rat, prior treatment with alloxan prevented the occurrence of GTG lesions of the VMH. However, unlike previous studies in the mouse (28), the administration of insulin to the diabetic rat 30 minutes before GTG injection did not restore the sensitivity of the VMH to lesion by GTG. The possibility remains that the rat differs from the mouse in the length of time required by insulin to restore the sensitivity of the VMH to lesion by GTG. Currently, studies are planned in which diabetic rats will be treated with insulin for two to five days prior to GTG injection.
One point stands out in these studies which appears to be in agreement with the conclusion of others (26, 27); that is, in the absence of insulin, GTG is completely ineffective in producing lesions in the VMH.

It is not possible, at this time, to extrapolate the role of insulin in the development of the GTG lesion of the VMH to its possible role of increasing glucose utilization in the same area. However, it is tempting to draw the analogy in the continued search for the answer to the question of whether insulin has a direct effect on glucose metabolism in the VMH.

The biochemical studies in which the early effects of GTG on glucose metabolism of brain were studied indicate that in vitro GTG has an effect on glucose uptake and utilization in both ventral and lateral hypothalamic tissue, but not on cortical tissue. The major effect seen in these experiments was a decreased production of $^{14}$CO$_2$ from $^{14}$C-glucose (U.L.). Lactate production by these tissues was diminished much less than the CO$_2$ production. This suggests that GTG was acting at a site or sites beyond the glycolytic sequence. This suggestion is supported by the fact that phosphocreatine
levels were reduced to about 60 percent of control levels by GTG, a finding which is in concert with inhibition at the level of the tricarboxylic cycle.

However, the observation that inhibition of the metabolic processes studied occurred in both ventral and lateral hypothalamus while in vivo the lesion is seen only in the VMH, raises the possibility that the effects seen are not directly related to the formation of the anatomical lesion. Furthermore, adequate insulin levels appear to be an absolute requirement for production of the lesion in vivo; whereas, in vitro insulin diminished the effect of GTG. Nevertheless, the ability of GTG to disrupt glucose metabolism in the hypothalamus may provide clues to how it causes the anatomical lesion in the VMH and what distinguishing characteristics of metabolism in this area result in it being sensitive to destruction by GTG.
APPENDIX
Plate I

Transverse section through the hypothalamus of CBA/J mouse. Structures: VMN, ventromedial nucleus; ar, arcuate nucleus; III, third ventricle.

Thickness, 40μ.
Plate II

Transverse section through the hypothalamus of CBA/J mouse 48 hours following GTG injection.

Scale bar equals 100μ.
Plate III

Section through the hypothalamus of female Sprague-Dawley rat.
Plate IV

Section through the hypothalamus of female Sprague-Dawley rat 48 hours following GTG injection.
Plate V

Section through the hypothalamus of

female Wistar rat.
Plate VI

Section through the hypothalamus of female

Wistar rat 48 hours following GTG injection.
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