CARBOHYDRATE METABOLISM IN ADRENALECTOMIZED MICE GIVEN ENDOTOXIN AND TRYPTOPHAN

Thesis for the Degree of M. S.
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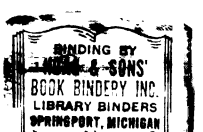
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ABSTRACT

CARBOHYDRATE METABOLISM IN ADRENALECTOMIZED MICE
GIVEN ENDOTOXIN AND TRYPTOPHAN

By

Patricia Enid Gardner

A study was undertaken to observe the effects of endotoxin or endotoxin plus tryptophan on carbohydrate metabolism in adrenalectomized mice. Mice were injected with 1 LD_{50} endotoxin alone, and in combination with 20 mg of L-tryptophan. In some instances adrenalectomized mice were pretreated with 0.5 mg of cyproheptadine, an antiserotonin drug, 2 hours prior to injections. Sensitivity to endotoxin and tryptophan was estimated by significantly increased numbers of deaths after 8, 24 and 48 hours when compared to appropriate controls and by significantly decreased blood glucose and liver glycogen.

Endotoxin significantly depleted blood glucose and liver glycogen during an 8 hour experimental period although the decrease in liver glycogen was not as striking due to rapid depletion of liver glycogen even in control mice given saline. A concurrent injection of endotoxin and tryptophan resulted in hyperreactivity as evidenced by depletion of carbohydrates and increased deaths after 8 hours. Cyproheptadine did little to protect adrenalectomized mice against the effects of endotoxin and tryptophan, and in some instances toxic manifestations of this drug were seen.

Patricia Enid Gardner

Endotoxin-poisoned adrenalectomized mice were hyperreactive to an injection of tryptophan given 10 hours after endotoxin and had increased deaths after 24 hours. Although adrenalectomized mice are more susceptible to situations of stress than normal mice, the former did not display as dramatic a response as observed in intact mice. Epinephrine was given to observe whether the lack of hyperreactivity might be due to lack of epinephrine. Tryptophan had no effect on adrenalectomized mice when given concurrent with endotoxin and epinephrine. When a delayed injection of epinephrine and tryptophan was given 10 hours after endotoxin, adrenalectomized mice behaved more like normal mice in that there were more deaths with a delayed injection of epinephrine and tryptophan after endotoxin than when a concurrent injection of all three were given.

CARBOHYDRATE METABOLISM IN ADRENALECTOMIZED MICE GIVEN ENDOTOXIN AND TRYPTOPHAN

By

Patricia Enid Gardner

A THESIS

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in partial fulfillment of the requirements
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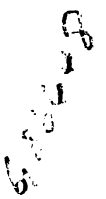

DEDICATION

To my husband, Trevor George Gardner

to my parents, Mr. & Mrs. F. A. Sebro, and

to my research advisor, Dr. Robert J. Moon

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I wish to express my deepest appreciation to Dr. Robert J. Moon, my research advisor, for his guidance, patience and personal interest throughout the course of my investigation.

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INTRODUCTION

The adrenal hormones play important roles in host response to both acute and chronic stress (6,11). Epinephrine and nor-epinephrine are important in the acute stress response, particularly by activating liver phosphorylase which results in rapid glycogenolysis. Of the many corticosteroids secreted by the adrenal cortex, the glucocorticoids can be singularized as the group concerned with the control of metabolism and the body's defense against stress. Cortisol is the major glucocorticoid hormone and its secretion is indirectly controlled by the hypothalamus. The glucocorticoids affect carbohydrate metabolism by stimulating gluconeogenesis with the probable result of an increase in levels of glycogen in the liver cells and the induction of certain liver enzymes (7,21). Glucocorticoids also can decrease glucose utilization by enhancing fat utilization. Their further effects on protein and fat metabolism, all of which can be related to carbohydrate metabolism, demonstrate the significance of these hormones when energy is in demand. Under conditions of internal stress, the body needs increased energy supplies. Lacking the adrenal hormones which could aid by stimulating rapid glycogenolysis and gluconeogenesis, the adrenalectomized animal is severely compromized in its ability to respond to the metabolic assaults such as endotoxin poisoning.

Endotoxin poisoning significantly decreases blood glucose and liver glycogen in intact animals (5,11,15,30,35,47). This response is attributed mainly to failure of gluconeogenesis, i.e., a diminished capacity to synthesize glucose from non-carbohydrate precursors, and the inability to convert pyruvate into blood glucose (glucogenesis) (14,30,33,47). The major metabolic lesion occurs at the conversion of pyruvate to phosphoenolpyruvate (PEP) and involves the enzymes phosphoenolpyruvate carboxykinase and pyruvate carboxylase.

Adrenalectomized mice are at least 1000-fold more susceptible to endotoxin than normal mice (12). Further, adrenalectomized rats and intact endotoxin-poisoned mice are hyperreactive to the amino acid L-tryptophan, an effect possibly related to depression of the adaptive liver enzyme tryptophan oxygenase (6,35). When tryptophan is given to intact endotoxin-poisoned mice at a time when tryptophan oxygenase activity is depressed, mice frequently die in convulsions within 4-8 hours and evidence suggests that tryptophan cannot be metabolized through the kynurenine pathway and hence is funneled into the serotonin pathway (26,35,36,37). Overproduction of serotonin could be at the basis of the hyperreactivity in such animals (28).

Previous results in this laboratory have shown a significant decrease in blood glucose and liver glycogen levels in intact mice at 4 and 8 hours after endotoxin, a phenomenon which is significantly enhanced by tryptophan. Previous experiments have also shown that endotoxin-poisoned adrenalectomized mice are sensitive to tryptophan when the amino-acid is given concurrent with or delayed 4 or 10 hours

(3) whereas normal mice are sensitive only to delayed injections of tryptophan.

Our prior rationale for this study is that the adrenalectomized animal, because of its generally enhanced susceptibility to stress, should be even more hyperreactive to tryptophan than the intact animal. By contrast to this rationale Beine (3) has shown that while endotoxin-poisoned adrenalectomized mice die sooner and in greater numbers after a delayed or concurrent injection of tryptophan, for some reason the toxic manifestations are not nearly as dramatic as in intact mice.

Since alterations in carbohydrate reserves are quite dramatic in endotoxin-poisoned intact mice given tryptophan and since cyproheptadine, an anti-serotonin drug can protect intact mice against the severe depletion of carbohydrate reserves, the primary objective of this study became to describe and compare the effects of endotoxin and tryptophan singly or in combination, on carbohydrate metabolism in adrenalectomized mice and to determine the effects of cyproheptadine on this response. We expect such a study will clarify the nature of the responses of adrenalectomized endotoxin-poisoned mice to tryptophan and aid in interpreting Beine's data suggesting that such animals are, in fact, less and not more hyperreactive to tryptophan than normal mice.

LITERATURE REVIEW

Primary determinants of host resistance to endotoxin are the reticuloendothelial system and the adrenal glands. Deprivation of glucocorticoids by adrenalectomy not only unarms animal species to endotoxic shock but endotoxin detoxification is also affected (23). Adrenalectomized animals are 100 to 1000 times more sensitive to endotoxin than intact animals. This alone is ample proof that the adrenals contribute in a major way to an animal's response to endotoxin (12).

Endotoxin poisoning in intact animals causes a significant decrease in blood glucose and liver glycogen levels (5,11,15,35,47) with the first deaths occurring after about 18-20 hours and completed by 48 hours (9,15,48). During the first 12 hours liver glycogen is reduced to less than 1% (5.5% is normal) and muscle glycogen to 2/3 of normal values (14). Experiments with hyperreactive BCG mice showed that the mechanism of the hypoglycemic activity of endotoxin involves the inhibition of the synthesis of glucose from non-carbohydrate sources or failure of gluconeogenesis and the inability to convert pyruvate into blood glucose (glucogenesis) (14,30,31,33,36,47,48). Endotoxin also causes an uncoupling of oxidative phosphorylation (31,48) and an inhibition of pyruvate oxidation (15). BCG infected mice died in less than 4 hours after endotoxin, with convulsions and severe hypoglycemia

(13,47,48). A probable explanation is that due to the uncoupling of oxidative phosphorylation caused by endotoxin, hyperreactive mice depleted their glucose more rapidly due to a general hypermetabolic state (48). Associating profound hypoglycemia with convulsions and death may suggest that hypoglycemic activity and lethality of endotoxin might be causally related (47).

Adrenalectomized mice did not show as severe a hypoglycemia nor did it occur as rapidly as in BCG infected mice, despite the LD_{50} being almost the same. Adrenalectomized mice died later (> 6 hours) with only an occasional seizure and a behavior more typical to normal mice (13,47).

The liver is the chief site of glucogenesis. Rat livers infected with endotoxin show a significant impairment of this function (30). In an assay of three important glucogenic enzymes, glucose-6-phosphatase, fructose 1,6 diphosphatase, and phosphoenolpyruvate carboxykinase, glucose-6-phosphatase was markedly decreased in endotoxin rat livers as compared with normal control rat livers. This is consistent with the assumption that possibly the key metabolic lesion in glucogenesis is at a lower level in the glycolytic pathway than glucose-6-phosphatase, perhaps involving a cofactor in the series of metabolic steps leading from pyruvate to phosphoenolpyruvate (30). Pseudomonas infected animals were unable to synthesize glucose from the glycolytic pathway precursors at a normal rate. Enzyme assays showed that endotoxin lowered the activity of glucose-6-phosphatase in the liver (30, 31). The resulting hypoglycemia produced by this enzyme deficit may not be significant in the pathogenesis of infection per se but the

underlying cause may be an aid in understanding events that lead towards the animal's death in infection.

Cortisone acetate is effective in protecting mice against the lethal dose of endotoxin (5,9,15,28,35) when the correct ratio between the two is ascertained. Animals given endotoxin and cortisone had more carbohydrate reserves and a decreased number of deaths than those given endotoxin alone. While poisoned animals were virtually depleted of all carbohydrate, mice given cortisone alone had concentrations of carbohydrate 3 to 4 times that of normal mice (13). The probable action of cortisone is in synthesizing liver enzymes that are inhibited by the action of endotoxin. When cortisone is administered concurrently or before endotoxin, protection is achieved. If given more than 1 hour after endotoxin, the action of cortisone is negated (9).

Endotoxin can suppress urinary nitrogen as a result of an injection of adrenocorticotrophic hormone (ACTH) (10,12). The increase in protein degradation and carbohydrate synthesis in normal mice after ACTH did not occur in endotoxin-treated mice. A balance between the quantity of catabolized protein (estimated by increase in urinary nitrogen excretion) and the total storage of carbohydrates as a result of cortisone administration was found in fasted and fed mice but not in endotoxin poisoned mice (11). Failure of a rise in urinary nitrogen in endotoxin-treated mice possibly involves a reduction in renal excretion of accumulated nitrogenous wastes alone with suppressed responses of the adrenal cortex to ACTH (12).

Tryptophan oxygenase is an adaptive liver enzyme responsible for oxidatively converting tryptophan to formylkynurenine in the pathway leading to the biosynthesis of nicotinamide adenine dinucleotide (NAD) (1). Endotoxin-poisoned mice are sensitive to tryptophan as evidenced by severe hypoglycemia when mice were injected with tryptophan 4 hours after endotoxin poisoning (35,36,37,39). The time of sensitivity differed with Carworth Farm (CF-1) mice which were sensitive 10 hours after poisoning as compared to Rockland Farm mice showing sensitivity as early as 4 hours after endotoxin (36). The sensitivity of endotoxin-poisoned mice to tryptophan is possibly related to the time of tryptophan oxygenase depression after endotoxin poisoning (36).

An increase in tryptophan oxygenase activity (8-10 units) was observed within 2 hours of an injection of 1 LD_{50} endotoxin. In 5 hours the enzyme activity was normal and by the 10th hour it was decreased by approximately 50% of its normal activity (37). Concurrent administration of endotoxin and tryptophan did not alter significantly the survival of endotoxin-poisoned mice. This could be attributed to the activity of tryptophan oxygenase not being significantly depressed before tryptophan administration. It appears that a critical amount of time must elapse before endotoxin-poisoned mice become sensitive to tryptophan (37). The administration of tryptophan at a time when tryptophan oxygenase activity is depressed results in death in 6-8 hours in experimental animals (36).

Allopurinol rapidly decreases tryptophan oxygenase activity and in the absence of endotoxin was given to test the sensitivity of mice to

tryptophan. The depression of tryptophan oxygenase activity and substrate induction in greater amounts than in endotoxin-poisoned mice, without deaths after tryptophan suggests that tryptophan oxygenase activity, though necessary for increased sensitivity does not account for the hyperreactivity that occurs. Raising and lowering the level of this enzyme at the time of challenge did not alter mortality and further supports this (28,35,37). Depression of tryptophan oxygenase activity with 5-hydroxytryptophan did not increase susceptibility of mice to endotoxin (35). This again is consistent with the concept that tryptophan oxygenase per se is not directly related to the survival of endotoxin-poisoned mice, and assumes that tryptophan and endotoxin may be exerting other effects on the host which alters its response to endotoxin.

Endotoxin's action on tryptophan oxygenase is possibly by inhibiting its synthesis or by accelerating its denaturation (8). Although depression of this enzyme's activity is necessary for endotoxin-poisoned mice to show sensitivity to tryptophan, its depression does not increase the toxicity of tryptophan in normal mice (36,37). The inhibition of tryptophan oxygenase suggests that tryptophan cannot be metabolized through the kynurenine pathway and hence is funnelled into the serotonin pathway. The ability of an anti-serotonin drug, cyproheptadine, to protect against the number of deaths occurring when tryptophan is injected 4 hours after endotoxin suggests that the amino-acid is being converted to serotonin, a toxic product (28,35,36,37,39). Cyproheptadine had no effect on increasing

mortality when endotoxin and tryptophan were given concurrently. It also counteracts the sensitivity that occurs but does nothing to alter the lethality of the bacterial poison. Serotonin injection 4 hours after endotoxin resulted in severe hypoglycemia similar to that caused by the injection of tryptophan (39), and increased deaths in animals (35). Further implications of funnelling of tryptophan into serotonin synthesis comes from protection by cyproheptadine against the hypothermia produced by tryptophan in endotoxin-poisoned mice (39). Another factor involved in the increased sensitivity in endotoxin-poisoned mice given tryptophan may result from the extreme hypoglycemia experienced by mice after endotoxin and tryptophan as compared to mice given endotoxin alone. In endotoxin-poisoned mice tryptophan and its metabolites were found in greater quantities and for longer periods of time in the tissues. The accumulation of toxic products as serotonin persisting in the tissues of endotoxin-poisoned mice is perhaps significant in determining the basis of the hyperreactivity of these animals to tryptophan (38).

Blood glucose depression after tryptophan administration is a result of inhibition of gluconeogenesis in the liver (25,33). This was characterized by a decrease in phosphoenolpyruvate (PEP) and a rise in oxalacetate and its precursors in the liver thus indicating blockage of PEP carboxykinase. The administration of L-tryptophan to intact or adrenalectomized rats greatly elevated phosphoenolpyruvate carboxykinase activity, primarily by an activation process, but blocked the <u>in vivo</u> catalysis of the enzyme (25). Inhibition of glucogenesis from

precursors beyond PEP carboxykinase in the glyconeogenic pathway was also a result of tryptophan administration. The apparent glycogenolytic effect of tryptophan is due presumably to mobilization of hepatic glycogen reserves in order to meet requirements for glucose normally met by gluconeogenesis. The inhibitory effect of tryptophan on gluconeogenesis results in the blocking of the catalytic functions of PEP carboxykinase in vivo (25). Metabolites of tryptophan in the nicotinic acid pathway through quinolinic acid were capable of blocking the formation of glucose from lactate, pyruvate and alanine by the liver, suggesting that the inhibition was due to quinolinic acid (33).

Studies done on the role of both synthesis and degradation in controlling the enzyme level of tryptophan oxygenase suggests that the enzyme is inducible by substrate and adrenocortical hormones. The administration of hydrocortisone increases the rate of synthesis (43) and also maintains the level of total oxidized pyridine nucleotides in contrast to decreased levels seen in endotoxin poisoning (5). Cortisone markedly stimulates RNA turnover and the accumulation of RNA and protein accounts for part of the general liver hypertrophy following cortisone administration (21). Tryptophan acts to decrease the rate of degradation of tryptophan oxygenase (8,28,35,45).

Increased synthesis and decreased degradation are responsible for increased activity of tryptophan oxygenase. In endotoxin-poisoned mice cortisone maintained the normal level of tryptophan oxygenase activity and prevented death in 100% of cases (28). Cortisone does not act on the enzyme by preventing its destruction (8).

Glucocorticoids function as inducers of certain liver enzymes by stimulating the production of new RNA, presumably messenger RNA (7,21). A relationship exists between maintaining the activity of liver enzymes inducible by adrenocortical hormones and survival against endotoxin lethality in animals (1). Cortisone acetate caused a four-fold increase in liver tryptophan oxygenase (1) while endotoxin reduced the enzyme to one-third of its activity in a few hours and prevented an increase when cortisone administration was delayed for 2-4 hours. Concurrent injection of endotoxin and cortisone resulted in normal enzyme activity (1). It is suggested that the maintenance of tryptophan oxygenase is necessary for continued survival of endotoxin-poisoned mice. Endotoxin suppresses the synthesis of tryptophan oxygenase and prevents its induction by cortisone for 16-20 hours when both are given concurrently.

The effect of Actinomycin D and ethionine on tryptophan oxygenase is similar to that of endotoxin in inhibiting protein synthesis although Actinomycin D inhibits enzyme induction by cortisone for a longer period of time. Carbon tetrachloride (CCL_4) also prevents induction of certain liver enzymes (48). These compounds prevented cortisone elevation of tryptophan oxygenase activity in intact mice and prevented cortisone maintenance of the enzyme's activity at control levels in endotoxin-poisoned mice when endotoxin, cortisone and inhibitors were given concurrently. Endotoxin significantly depresses but does not prevent substrate induction. Fasted animals had a higher tryptophan oxygenase activity than fed, apparently due to the release of endogenous adrenocortical hormones as part of the stress response to

fasting (8). The increase in liver glycogen under stress situations is also attributed to the release of these hormones (7).

Substrates or inhibitors of microbial enzymes are not the only enzyme inducers. Analogues of tryptophan such as D-tryptophan, α methyl-DL-tryptophan and N-acetyl-L-tryptophan showed substrate type induction of tryptophan oxygenase in adrenalectomized mice, although they were less effective than L-tryptophan. The inducing activity of some of these is attributed to their conversion in the animal to L-tryptophan (17).

MATERIALS AND METHODS

Mice: Eighteen to 20 gram, female CF-1 mice (Carworth Farms, Portage, Michigan) were used in all experiments. They were housed, six per cage, with pine wood chips as bedding. Food (Purina mouse chow, Ralston Purina Co., St. Louis, Missouri) and water were available ad libitum, unless otherwise noted. Adrenalectomized mice received 0.9% saline as drinking water.

Adrenalectomy procedures: Hair was removed from the backs of mice using electric hair clippers. Mice were anesthesized with 0.25 mg Nembutal (sodium pentobarbarbital injected subcutaneously in 0.2 ml of 0.85% NaCl, followed 10-15 minutes later by light etherization. After etherization the mice were restrained on a board with tape. The back of the mouse was disinfected with 0.2% iodine (Rexall). A 2-cm incision was made through the skin over the back bone, using small surgical scissors. The skin was pulled to the right side and a 1-cm incision made through the muscle layer, beginning near the rib cage and extending down at a slight angle. The right adrenal gland was removed using two pairs of straight point surgical forceps. The skin was then pulled to the left side and the procedure repeated, removing the left adrenal gland. No stitches were used to close the muscle layer incisions. The medial dorsal incision was closed with two stitches of silk surgical thread, gauge 000, using a 4-0 suture needle.

Completeness of adrenalectomy was determined 24-48 hours prior to experimentation utilizing the water retention test of Beatty (2). Mice were used for experimentation 3-12 days after surgery.

Blood glucose: Blood glucose was determined by the Glucostat method. Mice were bled (0.1 ml) from the retro-orbital plexus. The blood cells were lysed by diluting the blood with 1.9 ml of distilled water. The solution was deproteinized by the addition of 1.0 ml $Ba(OH)_2$ followed by 1.0 ml of $ZnSO_4$. ($Ba(OH)_2$ and $ZnSO_4$ were made up to concentrations of 1.8% and 2.0%, respectively. Solutions were adjusted so that 4.8 ml of $Ba(OH)_2$ exactly neutralized 5.0 ml of $ZnSO_4$. Phenolphthalein was used as an end point indicator.) After centrifugation, 2 ml of the supernate was used for each test. Distilled water served as the reagent blank. Two ml of Glucostat reagent was added at timed intervals to each sample and after 10 minutes the reaction was stopped by adding one drop of 4 N HCl. Five minutes later the absorbance of the solutions were read spectrophotometrically at 420 nm. Concentrations of unknowns were determined directly from a standard curve.

A standard curve for blood glucose was prepared using concentrations ranging from 0-200 mg % and is shown in Appendix A. Appropriate dilutions of glucose were made with distilled water and the tests were performed as described above.

Liver glycogen: Tissue glycogen was assayed by the procedure of Kemp and Van Heijninger (29). After obtaining blood for blood glucose assay, mice were killed by cervical dislocation and a slice

of liver (50-100 mg) was removed. The tissue was homogenized in 5 ml of deproteinizing solution (trichloroacetic acid 5 g A.R. and Ag_2SO_4, 100 mg A.R. dissolved in water and made up to 100 ml) using a glass tube and teflon pestle. The samples were placed in a boiling water bath for 15 minutes. After cooling, the volume of fluid lost by evaporation was replaced by deproteinizing solution. The samples were centrifuged at 3000 rev/min for 5 minutes and 1 ml of clear supernatant added to 3 ml of concentrated H_2SO_4 and mixed by vigorous shaking. These were placed in a boiling water bath for exactly 6.5 minutes, subsequently cooled in running tap water and the resultant color intensity produced measured spectrophotometrically at 520 nm. Data were expressed as % glycogen/gram wet weight of liver. The glycogen concentrations were determined from a standard curve (Appendix B) prepared from appropriate dilutions of a 16 mg/ml solution of dextrose. Dilutions ranged from the undiluted stock to 0.25 mg/ml and were measured as above.

Endotoxin: Heat killed cells of Salmonella typhimurium, strain SR-11, suspended in isotonic saline served as the source of endotoxin. Cells were prepared from organisms grown overnight in 500 ml brain heart infusion broth to a concentration of approximately 1×10^9 cells/ml and harvested by continuous flow centrifugation using a Sorvall continuous flow system. The cells were washed twice with non-pyrogenic isotonic saline (Baxter Laboratory, Morton Grove, Illinois) and resuspended in saline to approximately 10 times the original concentration.

The pooled cells were heat killed by autoclaving at 6 lb pressure and 222°F for 6 minutes. The preparation was judged sterile by lack of growth in 48 hours on subculture in brain heart infusion broth. The LD_{50} of this preparation for both normal and adrenalectomized mice was determined according to the method of Reed and Munch (42). The LD_{50} dose for normal mice was a 1:4 dilution of the final preparation and that for adrenalectomized mice a 1:1000 dilution.

Chemicals: Nembutal (sodium pentobarbital), 50 mg/ml was purchased from a local supply house. L-tryptophan and epinephrine were purchased from Nutritional Biochemical Corporation, Cleveland, Ohio. Epinephrine was suspended in Freund's incomplete adjuvant prior to injection. Cyproheptadine was purchased from Merck, Sharp and Dohme, West Point, Pa.

RESULTS

Blood glucose, liver glycogen and survival of adrenalectomized mice given endotoxin or endotoxin plus tryptophan: Blood glucose levels were maintained in control animals (given saline injection) over the 8 hour experimental period despite the fact that all food was removed (Figure 1). Blood glucose was significantly depressed ($P < 0.05$) at 2, 4 and 8 hours after 1 LD_{50} endotoxin (Figure 1). In contrast to blood glucose levels, liver glycogen was rapidly depleted in control, fasted mice. Endotoxin poisoning did not result in significantly different liver glycogen levels compared to controls until 8 hours after endotoxin ($P < 0.05$). Although there was a decrease in liver glycogen 4 hours after endotoxin, the decrease was not statistically significant.

Concurrent injection of endotoxin and tryptophan significantly depleted blood glucose levels (Figure 2) and increased deaths (Table 1) by 8 hours after injection. This phenomenon has been previously referred to in the literature as "hyperreactivity" of endotoxin-poisoned mice to tryptophan (36,39). Liver glycogen was significantly depressed below control values ($P < 0.05$) at 8 hours but not at earlier times. Tryptophan alone did not produce severe hypoglycemia after 8 hours though an early depletion of liver glycogen and blood glucose at 2 hours was observed ($P < 0.05$).

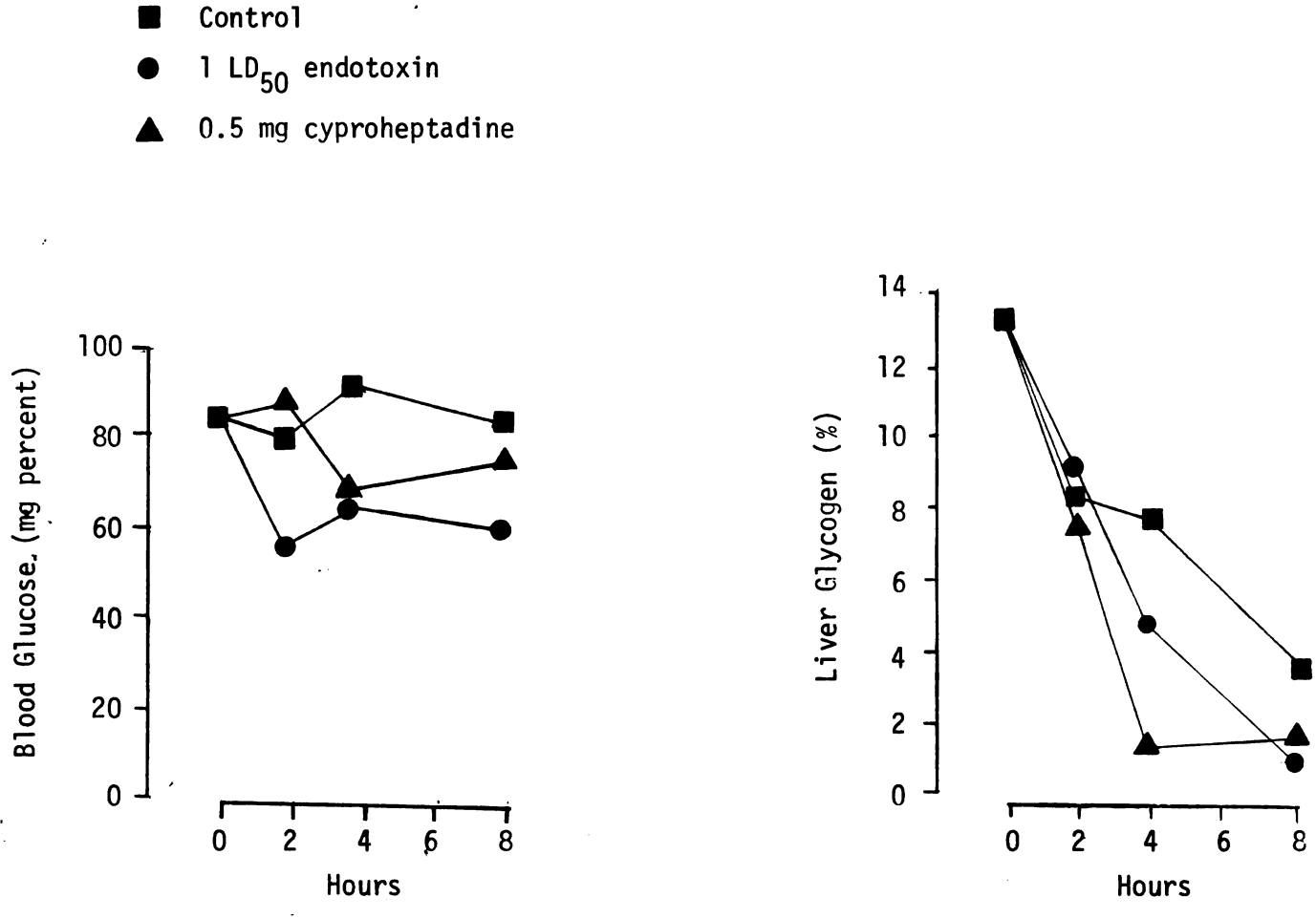


Figure 1. Blood glucose and liver glycogen of adrenalectomized mice given 1 LD_{50} endotoxin and 0.5 mg cyproheptadine.

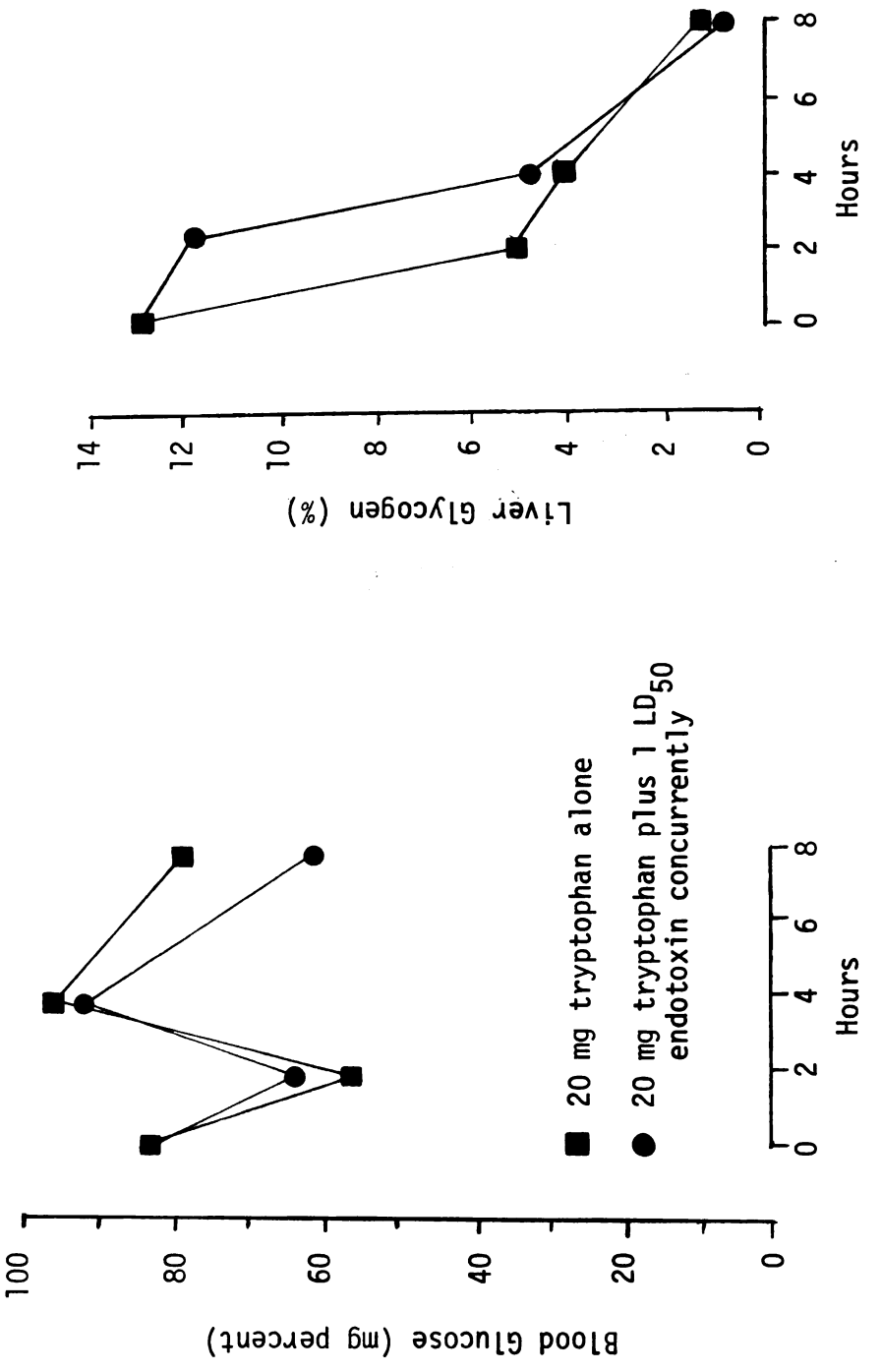


Figure 2. Blood glucose and liver glycogen of adrenalectomized mice given endotoxin plus tryptophan or tryptophan alone.

Table 1. Survival of adrenalectomized mice given endotoxin or endotoxin plus tryptophan concurrently

Experimental treatment	# survivors/total injected		
	Hours		
	8	24	48
1.5 ml saline (control)	11/11[a]	10/11[c]	9/11[g]
1 LD_{50} endotoxin	9/9	8/9	6/9[f]
1 LD_{50} endotoxin plus 20 mg tryptophan concurrently	6/9[b]	5/9[d]	4/9[e]

b vs a $P < 0.05$.

d vs c $P < 0.05$.

e vs g $P < 0.05$.

f vs g Not statistically significant.

Table 1 shows survival of adrenalectomized mice given endotoxin or endotoxin plus tryptophan. All data were compared to control mice given saline alone. In this latter group 1 of 11 mice had died by 24 hours and 2 of 11 by 48 hours. This fact made the number of mice given endotoxin alone not statistically different from controls although a greater number died. Tryptophan given concurrent with endotoxin statistically enhanced the number of deaths when compared to appropriate controls ($P < 0.05$) at 8, 24 and 48 hours.

Blood glucose, liver glycogen and survival of cyproheptadine treated adrenalectomized mice given endotoxin or endotoxin plus tryptophan concurrently: Administration of cyprophetadine, an antiserotonin drug (0.5 mg/mouse), resulted in a slight depression of blood glucose when compared to control mice given saline but the depression was not as great as that seen in adrenalectomized mice given either endotoxin or endotoxin plus tryptophan (cf., Figure 1).

Pretreatment with cyproheptadine 2 hours prior to endotoxin resulted only in very minor and seldom statistically significant changes in either blood glucose or liver glycogen in animals given endotoxin alone (Figure 3). Endotoxin-poisoned adrenalectomized mice had increased blood glucose 2 hours after 1 LD_{50} endotoxin and increased liver glycogen ($P < 0.05$) after 8 hours (Figure 3).

When cyproheptadine was given 2 hours before endotoxin plus tryptophan, the antiserotonin drug did not protect and in fact seemed to enhance at later hours, the hypoglycemic effects of the bacterial poison given either alone or with tryptophan (Figure 3). Cyproheptadine enhanced liver glycogen breakdown (cf., Figure 1) when given alone to control animals and did not significantly alter the rate of glycogen breakdown when given 2 hours before endotoxin plus tryptophan (Figure 3).

Adrenalectomized mice given cyproheptadine 2 hours before 1 LD_{50} endotoxin showed a significant decrease in deaths at 24 hours ($P < 0.02$) when compared to appropriate controls. Pretreatment with cyproheptadine before 1 LD_{50} endotoxin plus 20 mg tryptophan resulted in significantly decreased deaths ($P < 0.02$) at 8 hours but not at 24 and 48 hours (Table 2).

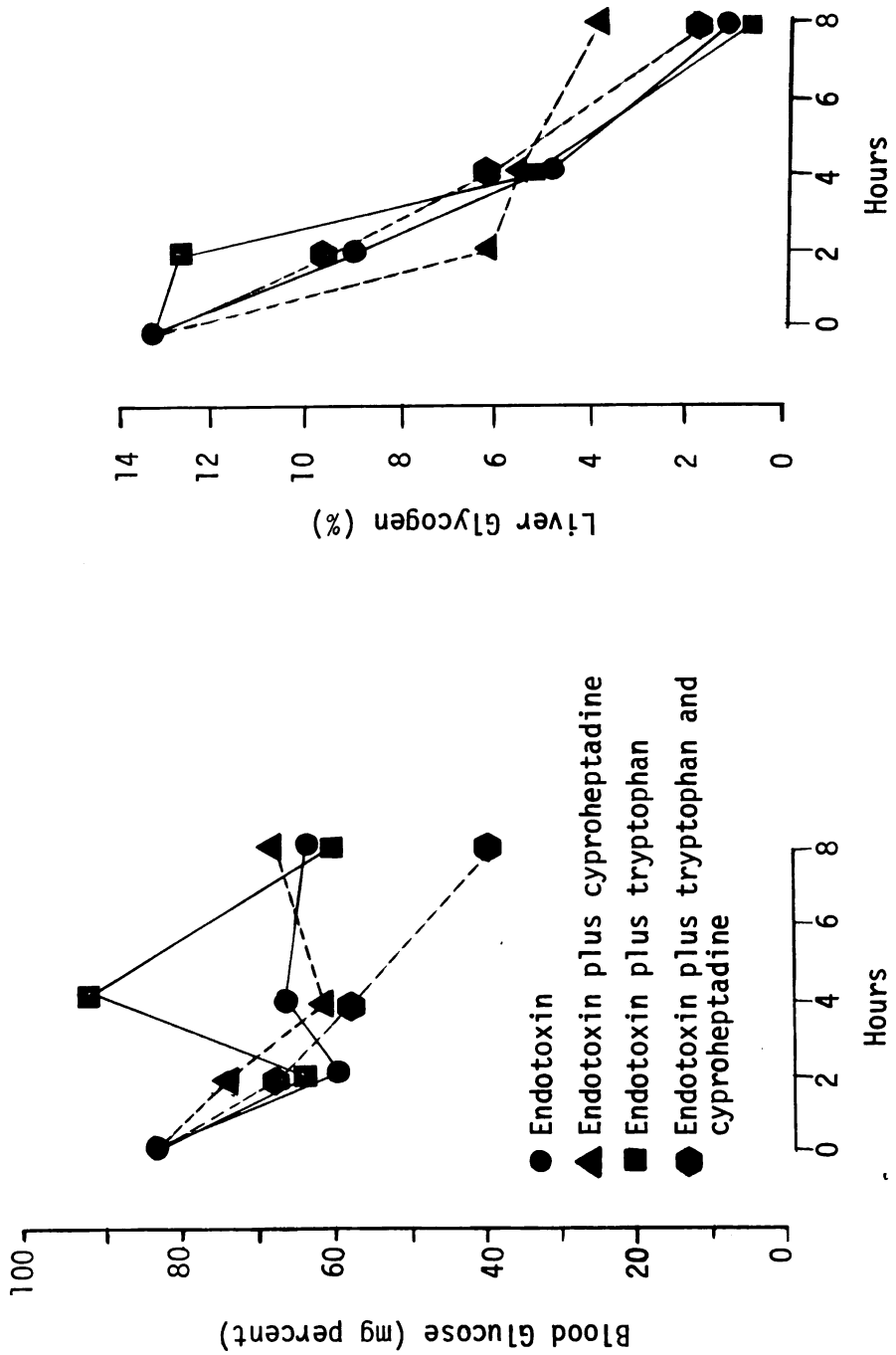


Figure 3. Effect of cyproheptadine on blood glucose and liver glycogen of adrenalectomized mice given endotoxin plus tryptophan.

Table 2. Effect of cyproheptadine on survival of adrenalectomized mice pretreated 2 hours before 1 LD_{50} endotoxin and endotoxin plus 20 mg tryptophan concurrently

Experimental treatment	# survivors/total injected					
	Hours					
	8		24		48	
	Without CH*	With CH	Without CH	With CH	Without CH	With CH
1 LD_{50} endotoxin	5/7[a]	7/7[b]	2/7[c]	6/7[d]	1/7	4/7
1 LD_{50} endotoxin plus 20 mg tryptophan	6/9[g]	10/10[h]	5/9[i]	8/10[j]	4/9	6/10

*CH = Cyproheptadine.

b vs a Not statistically significant.

d vs c $P < 0.02$.

h vs g $P < 0.02$.

j vs i Not statistically significant.

Table 3. Effect of cyproheptadine on survival of adrenalectomized mice given greater doses of endotoxin plus tryptophan

Experimental treatment	# survivors/total injected		
	Hours		
	8	24	48
0.5 ml saline plus 10 LD_{50} endotoxin plus 20 mg tryptophan 2 hours later	8/12[a]	2/12[c]	2/12
0.5 mg. cyproheptadine plus 10 LD_{50} endotoxin plus 20 mg tryptophan 2 hours later	12/12[b]	9/12[d]	4/12
0.5 ml saline plus 25 LD_{50} endotoxin plus tryptophan 2 hours later	7/12[e]	1/12[g]	0/12
0.5 mg cyproheptadine plus 25 LD_{50} endotoxin plus tryptophan 2 hours later	10/12[f]	4/12[h]	1/6

b vs a $P < 0.02$.

d vs c $P < 0.01$.

f vs e Not statistically significant.

h vs g Not statistically significant.

Survival experiments were then done on mice pretreated with cyproheptadine before tryptophan plus greater doses of endotoxin (10 LD_{50} and 25 LD_{50}) to observe whether cyproheptadine protects against death in animals where blood glucose and liver glycogen would be expected to be severely depleted. Cyproheptadine protected against death in 2 instances when adrenalectomized mice were given cyproheptadine before tyrptophan and greater than 1 LD_{50} endotoxin. Deaths in mice pretreated with cyproheptadine 2 hours before 10 LD_{50} endotoxin plus tryptophan concurrently, were significantly decreased at 8 and 24 hours. No significant differences were noted at 48 hours. Pretreatment with cyproheptadine did not protect against death in mice given 25 LD_{50} of endotoxin and tryptophan concurrently (Table 3).

Blood glucose, liver glycogen and survival of endotoxin-poisoned adrenalectomized mice given a delayed injection of tryptophan: When tryptophan was given to adrenalectomized mice 10 hours after endotoxin, statistically greater numbers of animals died after 24 hours (hyperreactivity to tryptophan) but the tryptophan did not change the ultimate LD_{50} of the lipopolysacchardie after 48 hours (Table 4).

Figure 4 shows that the depressed blood glucose levels 10 hours after endotoxin were not further decreased by an injection of tryptophan in adrenalectomized mice. Control values at 2 and 8 hours were lower than values after tryptophan. An injection of tryptophan 10 hours after endotoxin did not decrease liver glycogen levels below that of controls. Liver glycogen at 2, 4 and 8 hours were increased above controls, indicating no effect of tryptophan at any of these times.

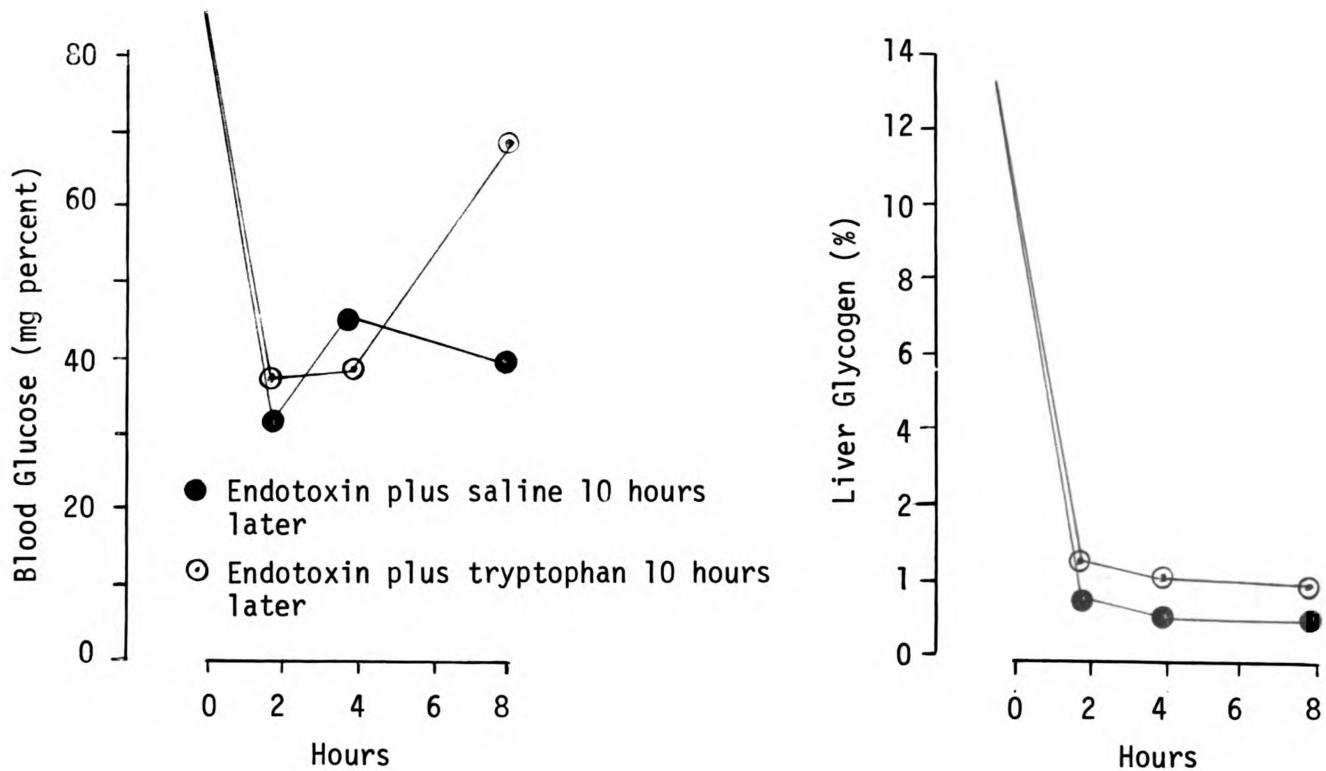


Figure 4. Blood glucose and liver glycogen of endotoxin-poisoned adrenalectomized mice given a delayed injection of tryptophan (saline or tryptophan injected at zero hour).

Table 4. Survival of endotoxin poisoned adrenalectomized mice given tryptophan 10 hours later

	# survivors/total injected		
	Hours		
Experimental treatment	8	24	48
1 LD_{50} endotoxin plus 1 ml saline 10 hours later	11/13[a]	7/9[c]	5/9[e]
1 LD_{50} endotoxin plus 20 mg tryptophan 10 hours later	10/17[b]	4/11[d]	3/11[f]

b vs a Not statistically significant. d vs c $P < 0.05$.

f vs e Not statistically significant.

Effect of epinephrine on survival of endotoxin-poisoned adrenalectomized mice given concurrent and delayed injections of tryptophan: Table 5 shows survival of adrenalectomized mice given epinephrine and endotoxin or endotoxin plus tryptophan. An injection of 0.25 mg of epinephrine alone to adrenalectomized mice resulted in deaths after 8, 24 and 48 hours. Four of 10 mice had died in 24 hours and 6 of 10 in 48 hours. When 1 LD_{50} endotoxin was given concurrent with epinephrine there were increased deaths when compared to mice given epinephrine alone, although the increase was not statistically different. Endotoxin and tryptophan concurrent with epinephrine did not further increase deaths than when epinephrine and endotoxin were given alone.

Table 5. Effect of epinephrine on survival of endotoxin-poisoned adrenalectomized mice given tryptophan

Experimental treatment	# survivors/total injected		
	Hours		
	8	24	48
0.25 mg epinephrine	9/10	6/10	4/10
Epinephrine plus 1 LD_{50} endotoxin	8/10[a]	4/10[c]	4/10
Epinephrine plus endotoxin and 20 mg tryptophan	8/10	4/10	3/10
Epinephrine plus tryptophan 10 hours after endotoxin	4/9[b]	1/9[d]	1/9
Endotoxin plus tryptophan and epinephrine repeated after 1/2 hour	2/10	0/10	0/10

b vs a Not statistically significant.

d vs c Not statistically significant.

Endotoxin-poisoned adrenalectomized mice did not show increased deaths when tryptophan was given concurrent with epinephrine. Previous results show a significant increase in deaths after 8, 24 and 48 hours when endotoxin-poisoned adrenalectomized mice were given a concurrent injection of tryptophan (Table 1). Adrenalectomized mice reacted more like intact mice and showed hyperreactivity to a delayed injection of epinephrine and tryptophan 10 hours after endotoxin. Although no significant differences were observed, endotoxin-poisoned adrenalectomized mice given a delayed injection of tryptophan and epinephrine had twice

as many deaths after 8 hours as mice given a concurrent injection of epinephrine, endotoxin and tryptophan. Deaths at 24 and 48 hours were also more increased than with concurrent injections. When 2 doses of epinephrine were given within half-hour along with endotoxin and tryptophan, there were significantly increased deaths after 8, 24 and 48 hours. Eight of 10 mice died within 8 hours and by 24 and 48 hours, there were no survivors. Deaths were far more increased with a delayed injection of tryptophan 10 hours after endotoxin and with a repeated dose of epinephrine than at other times.

DISCUSSION

Fasted adrenalectomized control animals (given saline) maintained blood glucose levels during the 8 hour experimental period. In contrast, liver glycogen levels of control mice were rapidly depleted during this period. This suggests that liver glycogen may be the main source of blood glucose in the adrenalectomized animal. Since endotoxin-poisoned mice do not eat, control mice were denied food during the experiments in an effort to establish similar conditions. Without the stimulation of gluconeogenesis by the glucocorticoid hormones, these animals appear to use glycogen rapidly and this may account for the continued depletion of liver glycogen even in control mice. Liver glycogen was also rapidly depleted in adrenalectomized mice given endotoxin but the rate of the depletion was greater only at later periods than control mice. In contrast to liver glycogen, blood glucose was significantly decreased in poisoned mice when compared to controls at both early and late times (cf., Figure 1).

Previous work done by Dr. Moon (36) and compared with present results (Figure 1) shows that intact mice had approximately 3 1/2 times more blood glucose than adrenalectomized mice 2 hours after endotoxin and 2 times as much after 4 hours. This evidence, along with limitations due to lack of glucocorticoids in these animals could suggest that adrenalectomized animals are less capable of coping with situations that

might induce stress, and may be more hyperreactive than the intact animal.

Severe hypoglycemia, depletion of liver and muscle glycogen and increased deaths occur in endotoxin-poisoned intact mice within 3-6 hours after a tryptophan load (36). Our experiments show similar data in that a concurrent injection of endotoxin and tryptophan load into adrenalectomized mice also resulted in reactivity of the poisoned mice given tryptophan (Table 1 and Figure 2), as evidenced by increased deaths and depletion of carbohydrates. The significant decrease in blood glucose and liver glycogen occurring 8 hours after concurrent endotoxin and tryptophan suggests but does not prove that these responses may be a significant factor in the increased deaths occurring at this time.

Survival experiments in adrenalectomized mice are particularly difficult because the animals are usually varied in their biological responses. This fact is clearly pointed up in Table 1 where 2 of 11 saline control animals succumbed, an effect which not only would never be expected in intact mice but also made the statistical evaluation of the endotoxin survival difficult to interpret even though the evidence suggests that approximately 50% of the mice did die following endotoxin. Such variability among the animals necessitates utilization of much larger groups in survival studies in adrenalectomized mice since variability also seems to be correspondingly enhanced. Literature data on adrenalectomized mice must be carefully scrutinized for appropriate control values.

In experiments with intact mice, the antiserotonin drug cyproheptadine prevented the hyperreactivity seen in endotoxin-poisoned mice given tryptophan, presumably by acting against the build-up of serotonin. Cyproheptadine significantly decreased the hypoglycemia as well as the number of deaths in endotoxin-poisoned intact mice given a delayed injection of tryptophan (36). The effect of cyproheptadine on adrenalectomized mice does not appear to be consistent with that seen in intact mice. Cyproheptadine alone seems to display toxic manifestations. Blood glucose and liver glycogen levels after cyproheptadine were lower than those of saline controls. Cyproheptadine caused only minor enhancement of blood glucose and liver glycogen in adrenalectomized mice. In only two instances did cyproheptadine protect against the hypoglycemia seen with endotoxin alone or a concurrent injection of endotoxin and tryptophan. On a few occasions cyproheptadine further enhanced the hypoglycemia and decreased liver glycogen (Figure 3). The percentage of liver glycogen in intact mice 4 hours after cyproheptadine was 8.6% (36) as compared to 1.6% seen in adrenalectomized mice at this same time (Figure 1). Liver glycogen values in intact mice 16 hours after cyproheptadine were almost twice as much as that seen in adrenalectomized mice 8 hours after cyproheptadine. These results suggest that (1) adrenalectomized mice given cyproheptadine behave differently from intact mice (cyproheptadine might be acting as an inhibitor of some important mechanism concerned with maintaining liver glycogen) and (2) cyproheptadine itself seems to be causing toxicity in the adrenalectomized animal.

In a few instances pretreatment with cyproheptadine before endotoxin or endotoxin plus tryptophan protected against death in adrenalectomized mice (Tables 2 and 3). No consistent pattern of cyproheptadine protection was observed. The variability in the data makes any interpretation so inconclusive as to be almost meaningless except to say that in some instances cyproheptadine seemed to protect against death in the adrenalectomized animal.

Endotoxin-poisoned adrenalectomized mice were hyperreactive to a delayed injection of tryptophan as evidenced by a greater number of deaths after 24 hours in control mice (Table 4). Evidence of hyperreactivity was not seen in depressed levels of blood glucose and liver glycogen. An injection of tryptophan 10 hours after endotoxin did not further decrease blood glucose while liver glycogen was increased above controls. Adrenalectomized mice showed sensitivity to a delayed injection of tryptophan, not by decreased blood glucose and liver glycogen but by increased deaths in 24 hours after tryptophan.

Because adrenalectomized mice are more susceptible to situations of stress than normal mice, these animals were expected to be more hyperreactive to tryptophan than the intact animal. Results from these experiments suggest that this is not really so. It seems clear that these animals do not display as dramatic a response as that seen in intact mice. Previous reports by Shands (47) in his comparison of adrenalectomized mice with BCG infected mice shows that adrenalectomized mice were neither as severely hypoglycemic nor was it as rapid in development as that seen in BCG infected mice. Adrenalectomized mice also

died later (> 6 hours) and only occasionally had seizures. Beine (3) showed that while endotoxin-poisoned adrenalectomized mice died sooner and in greater numbers after a delayed or concurrent injection of tryptophan, the toxic manifestations were not nearly as dramatic as in intact mice.

These observations, along with the results from present data, were the basis for the use of epinephrine in the last part of this experiment. Since our initial results showed that adrenalectomized mice were not as hyperreactive to tryptophan as intact mice, it was thought that the lack of epinephrine in these animals might be in part responsible for lack of hyperreactivity. To test this hypothesis, a dose of 0.25 mg of epinephrine was given subcutaneously in an oil emulsion. Epinephrine was suspended in oil so that it would be gradually absorbed into the system and not become detoxified as rapidly as if it were injected intraperitoneally. Due to limited time and numbers of animals, only 1 dose of epinephrine was used throughout the experiment. Again the survival data was difficult to interpret due to the numbers of deaths occurring in control animals given epinephrine alone. The latter group of animals had 60% survivors in 24 hours and 40% in 48 hours. Epinephrine did cause toxicity in adrenalectomized animals as evidenced by increased deaths after injection. Although deaths were observed with epinephrine alone, the number of deaths with subsequent injections were greater than with epinephrine alone. One significant aspect of this data was observed with a delayed injection of epinephrine and tryptophan 10 hours after endotoxin. Mice receiving this injection

behaved more like intact animals than adrenalectomized mice in that they had more deaths when a delayed injection of epinephrine and tryptophan were given to endotoxin-poisoned adrenalectomized mice than when a concurrent injection of all three were given (Table 5). A concurrent injection of endotoxin, epinephrine, and tryptophan resulted in the same numbers of deaths as an injection of epinephrine and endotoxin alone. This indicates no effect of tryptophan on adrenalectomized mice when given concurrently with endotoxin and epinephrine. This is contrary to previous data which indicates increased sensitivity of adrenalectomized mice to a concurrent injection of endotoxin and tryptophan alone (Table 1). It also suggests that epinephrine does cause adrenalectomized mice to react more like intact mice.

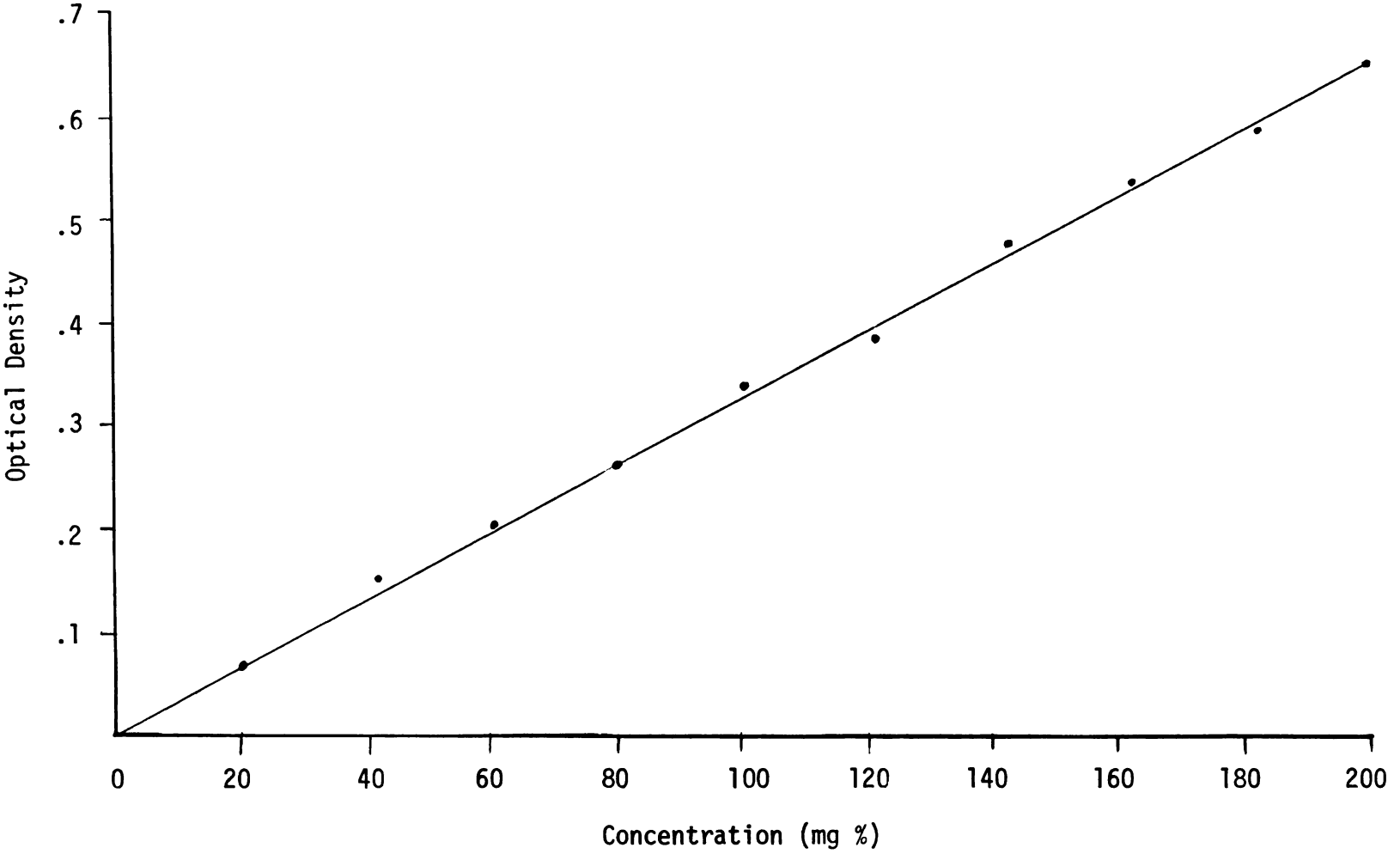
Further work on this particular aspect of the data needs to be done to clarify and expand on the results obtained. Additional experiments should be done utilizing different doses of endotoxin in order to judge as accurately as possible the dosage that would be least toxic to the animal. Different routes of administration should be tried to observe and compare results and to decide which is the most effective. Varied timings and modes of administration are also facets which could be dealt with later on.

SUMMARY AND CONCLUSIONS

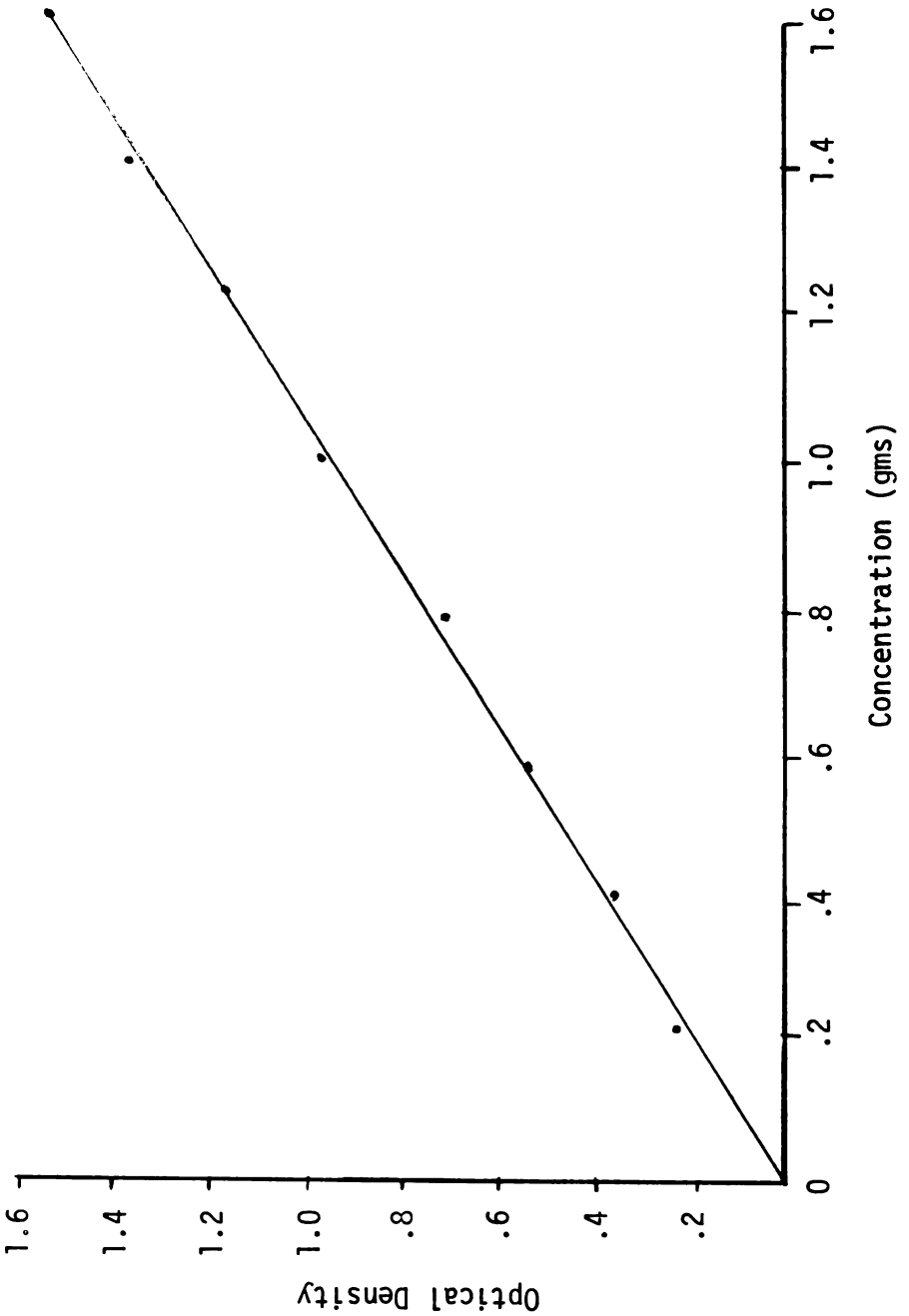
Although adrenalectomized mice are more sensitive to situations of stress than normal mice, they do not display responses as dramatic as that seen in intact mice. One LD_{50} endotoxin decreased blood glucose and liver glycogen in adrenalectomized mice and caused more deaths than in control mice although dramatic manifestations were not observed. Concurrent endotoxin and tryptophan resulted in hyperreactivity of endotoxin-poisoned mice given tryptophan. There was depletion of carbohydrate and increased deaths after 8 hours. Because of varied biological responses, survival data using adrenalectomized mice were difficult to interpret. Control adrenalectomized animals (given saline) died in greater numbers than intact mice given saline. Adrenalectomized mice were hyperreactive to both concurrent and delayed injections of tryptophan as evidenced by increased deaths 8 hours after a concurrent injection and 24 hours after a delayed injection. Adrenalectomized mice behaved more like intact mice when epinephrine was injected. There were more deaths when a delayed injection of epinephrine and tryptophan were given to endotoxin-poisoned adrenalectomized mice than when a concurrent injection of all three were given. A concurrent injection of endotoxin, epinephrine and tryptophan resulted in the same number of deaths as an injection of epinephrine and endotoxin alone. This suggests that tryptophan has no effect on adrenalectomized mice when given concurrently

with endotoxin and epinephrine. This also contradicts previous data showing increased sensitivity of adrenalectomized mice to a concurrent injection of endotoxin and tryptophan alone.

APPENDICES



APPENDIX A. STANDARD CURVE FOR BLOOD GLUCOSE



APPENDIX B. STANDARD CURVE FOR LIVER GLYCOGEN

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VITA

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