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RESEARCH TITLE: __Glucocorticoid Antagonism by Endotoxin: Biological Effects During__

__Stress and Basis for Inhibition of Phosphoenolpyruvate Carboxykinase__

AUTHOR: ___William B. Huff___

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STATEMENT(s):

GLUCOCORTICOID ANTAGONISM BY ENDOTOXIN: BIOLOGICAL

EFFECTS DURING STRESS AND BASIS FOR

INHIBITION OF PHOSPHOENOLPYRUVATE

CARBOXYKINASE

by

William B. Huff,Capt,USAF,BSC

THESIS

129 Pages

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF ARTS

THE UNIVERSITY OF TEXAS AT AUSTIN

August, 1985

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ABSTRACT

Bacterial endotoxins produce a variety of patho-
physiologic effects by inducing the release of humoral
factors from cells of the reticuloendothelial system,
primarily macrophages. This study focusses on one particular
factor, glucocorticoid antagonizing factor (GAF). Antagonism
was augmented when mice were subjected to conditions of
stress where adrenoglucocorticoids are necessary for survival.
Mice were sensitized to both toxic endotoxin and irradiated
detoxified endotoxin during the stress of cold, heat, and
tourniquet shock. Endotoxin, as well as detoxified endotoxin,
induced the release of GAF from cells of the RES. Treatment
of mice with this GAF-rich serum also resulted in their sensi-
tization to stress.

Survival of LPS-poisoned animals subjected to stress
could be improved when exogenous hydrocortisone was given
prior to the administration of exogenous GAF-rich serum or
prior to the release of endogenous GAF in response to endo-
toxin. Naloxone, a B-endorphin antagonist, failed to increase
the survival rate of animals under the experimental con-
ditions investigated.

Endotoxin given 1 hour prior to hydrocortisone or
GAF-rich serum given concurrent with the hormone inhibited
the induction of the gluconeogenic rate-limiting enzyme
phosphoenolpyruvate carboxykinase (PEPCK). It was shown that

uninducible catalytic activity was not a result of suppressed or modified catalysis as the amount of enzyme, measured by ELISA, correlated directly with catalytic activity as measured by carboxylation with radiolabeled bicarbonace.

The data reported in this thesis suggest that GAF produces a pharmacological antagonism of the animals ability to manifest an adrenal cortical response. This antagonism may be the determining factor in the animals demise during conditions of stress and it is characterized by impairment of carbohydrate homeostasis due to noninduction of PEPCK.

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INTRODUCTION

Almost a century ago Boise noted that infection pre-
ceded a state of septic shock (26). Another half-century
passed before the hemodynamic alterations associated with
gram-negative septic shock were directly attributed to a cell
wall constituent referred to as endotoxin or lipopolysac-
charide (LPS) (103,166). Since that time bacterial LPS has
been under extensive investigation. Although much has been
learned about the pathophysiology of this toxin, today it
still stimulates the curiosity of many researchers due to the
undefined manner by which LPS precipitates death during gram-
negative sepsis and the associated endotoxin (septic) shock.

Septic shock can be induced by a wide variety of
microorganisms. These include gram-negative bacteria (56,
163), gram-positive bacteria (4), spirochetes (87), ricket-
tsia (91), and fungi (32). Endotoxin shock caused by
gram-negative bacteria is of special interest because of its
continued prominence despite antimicrobial therapy (122,172).
Sepsis associated with gram-positive bacteria is complicated
by the clinical incidence of shock in less than 5 percent of
the cases (143,144). This is contrasted to gram-negative
bacterial sepsis where approximately 30 percent of the
bacteremic patients develop septic shock and the average
mortality rate of these patients is greater than 50 percent.
This is compared to a mortality rate of less than 20 percent

when gram-negative sepsis is uncomplicated by shock (70,105, 122,168). Thus, the importance of resolving the nature of how LPS exerts its metabolic effects is clearly indicated.

The events of endotoxin shock can be divided into early and late phases based upon the pathophysiologic effects on the circulatory system (17,122). Immediate hemodynamic alterations include an initial vasoconstriction followed by vasodilation with an initial increase followed by a decrease in systemic arterial blood pressure (79,167). Leukocytosis is followed by leukopenia (153) and a hypercoagulable state precedes a hypocoagulable state due to disseminated intra-vascular coagulation (DIC) with depletion of clotting factors (45,46). These early events lead to arteriovenous shunting, microvascular insufficiency and anoxia due to inadequate perfusion of tissues (122). Ultimately, progression to irre-versible shock characterized by terminal hypotension, severe organ dysfunction and severe myocardial depression are believed to contribute to the animals demise (17. 62,122).

The exact nature of an initial mechanism(s) leading to irreversible shock and death has not been defined and several mediator substances released by endotoxin-mediated shock may be involved. No intention is made to slight any authors contributions in this important field of investi-gation but time constraints will limit this discussion to only a brief overview of those mediators which have been implicated as initiators of irreversible shock. Endotoxinemia

elicits the release of endogenous histamine from intra-
vascular as well as extravascular storage sites (76,77). In
addition, elevated histidine decarboxylase activity occurs
during challenge with LPS or stress (141). In an effort to
correlate histamine production with endotoxin shock it was
shown that exogenous histamine, LPS, or histamine releasor
agent, known as 48/80, produce increased portal venous
pressure with subsequent pooling of blood in the hepato-
splanchnic area, decreased venous return, and decreased
systemic arterial pressure in dogs (78). These data implicate
histamine as a causitive factor of hypotension seen during
endotoxin shock. Further support for similar pathophysiologic
effects of histamine and LPS was shown when phenoxybenzamine,
an anti-histaminic agent, prevented increased portal
venous pressure and pooling of blood upon exposure of dogs
to either LPS or histamine (31). However, decreased systemic
arterial pressure was not blocked by phenoxybenzamine in LPS-
poisoned dogs even though blockage did occur in dogs treated
with histamine (31). It was also shown that phenoxybenzamine
could not afford protection against systemic hypotension or
death of endotoxin shocked dogs (31,106). In addition, others
have reported that the LPS-induced acute increase in portal
venous pressure and decrease in systemic arterial pressure
observed in dogs was not an acute episode in cats, rabbits,
or monkeys (79,93). Therefore, it appears that histamine
does not play a major role in the initiation of irreversible

endotoxir shock and consequently other factor(s) are
implicated.

A proposed model for the pathophysiology of endo-
toxin shock suggests that LPS interacts with antibody and
complement. The subsequent generation of C3a and C5a in turn
activates leukocyte aggregation and adherence to vascular
endothelium where the release of lysosomal enzymes and very
potent toxins, superoxide and hydrogen peroxide occurs. These
can cause endothelial damage leading to endogenous volume
loss and hypotension (88,97). The basis for this model is an
initial reaction between LPS and anti-LPS antibody. This is
quite reasonable since most mammals contain antibodies to
LPS (63,95). it is well known that LPS activates the comple-
ment cascade (42,68,152). Fearon et al (55) showed that
complement activation correlates with LPS-induced hypotension
and death. Furhermore, increased vascular permeability can
be aborted by protecting the vascular endothelium from the
insult of complement activation (156). This was shown to be
true in neutropenic animals, ie, the lack of response that
occurs as a result of the elimination of C5a responder and
of oxygen radical producing cells and by the protection
afforded these animals by treatment with superoxide dismu-
tase (156). Also, dogs undergoing exchange transfusion with
plasma that had been made hypocomplementary by heating at
56°C were refractory to the lethal effects of LPS (151).
However, others have shown that during hypocomplementemia

that was induced by treatment with cobra-venom factor (CVF) or by congenital C6 deficiency, the initial (ie, within the first few minutes after challenge with LPS) hypotension is not manifested but that overall, hypotension and mortality is unaltered (60,161). Therefore, complement may be involved in an initial LPS-induced hypotension, but the terminal hypotension must be explained by another mechanism.

Catecholamine production has also been shown to occur during endotoxinemia (25,135). It is believed that in an effort to maintain homeostasis, elevated histamine levels cause the release of these adrenergic amines from the adrenals (31,35,78). This concept is supported by showing hypersensitivity of rats and mice to histamine following adrenalectomy (71). This hypersensitivity could be counter-acted by administering exogenous epinephrine and it thus appears that catechols and amines exert opposite and compens-atory effects on one another (71,150). In addition, increased urinary excretion of epinephrine and norepinephrine was observed in man following subcutaneous injections of hista-mine (51). However, catecholamine production during endotoxin shock does not appear to be a major factor in the irrever-sible phase of shock as adrenalectomized animals exhibited no significant change in portal hypertension or systemic hypo-tension relative to intact animals when given LPS (30,80). Also catecholamine antagonists, such as reserpine, do not abolish LPS-induced hypotension (89). These results indicate

that catecholamines are not responsible for irreversible endotoxin shock.

Endogenous opiates (endorphins) have drawn recent interest as mediators of endotoxinemic shock. Adrenocortico-tropin hormone (ACTH) and B-endorphin , the most potent endorphin, are stored in a common pituitary site (69,137) and are synthesized from the same precursor glycoprotein (69,104). Both ACTH and B-endorphin are elevated in a number of stressed states, including LPS-poisoning (69,81,110,137). Exogenous endorphins have been shown to produce significant hypotension in rats and dogs (81,98). Therefore, these endogennus opiates may play a critical role in the classical LPS-induced hypotension. This is substantiated by studies with naloxone, a B-endorphin antagonist, which can reverse the hypotension seen with LPS challenge (52,53,81,98). This is a selective reversal of pathophysiologic effects and not a direct pressor mechanism as naloxone given to nonendotoxi-nemic rats or dogs does not alter systemic blood pressure (52,81). Although naloxone reverses hypotension in endo-toxinemic rats it has no effect on their survival rate (53). Thus, in the rat system at least, there appear to be factors other than hypotension and cardiac collapse leading to death. This is different from LPS-poisoned dogs, where naloxone not only attenuates hypotension but also increases the survival rate (52). This work has also been extended to primates: in Cynomolgus monkeys naloxone has been shown to improve

hypotension and survival following endotoxinemia (83). In
addition to reversing hypotension, naloxone also prevents
other common features of septic shock, eg, the initial hyper-
glycemia in mice due to depletion of hepatic glycogen stores
(73). The depletion of hepatic glycogen and other carbohy-
drate anomalies may be critical in the demise of LPS-poisoned
animals as hypoglycemia is a hallmark of terminal endotoxin
shock (10,58,94).

Total depletion of hepatic glycogen during LPS-
challenge results in an initial, but transient hypergly-
cemia, followed by a profound and terminal hypoglycemic state
(19,58,94). Adrenalectomy, with subsequent corticosteroid
replenishment, abolished this LPS-induced glycogen depletion
(139,153). Therefore, enhanced glycogenolysis during endo-
toxinemia is likely due to the release of catecholamines
from the adrenal glands. Elevated glycogenolysis by itself
however, should not account for terminal hypoglycemia. An
increase in carbohydrate consumption and/or hepatic gluco-
neogenesis should compensate for any glycogenolytic derange-
ment. Unfortunately, both of these parameters are blocked by
LPS (58,94,159).

Using a phenol red solution as a tracer, Turner and
Berry (159) showed that gastric emptying is prevented within
5-10 minutes after exposure to LPS. Prior to this, it had
been shown that LPS suppresses gastric motility (117). Since
gastric contractions assist in the elimination of chyme from

the stomach it appears that the inhibition of gastric
emptying is due to decreased gastric motility. the overall
effect is essentially a condition of starvation due to de-
creased food and water uptake during endotoxinemia. Since
LPS blocks the consumption of exogenous carbohydrates the
animal becomes dependent on hepatic gluconeogenesis to
neutralize terminal hypoglycemia.

Gluconeogenesis, or reverse glycolysis, depends on
the reversal of three highly exergonic reactions (146):
glucose-6-phosphate to glucose, fructose-1,6-diphosphate
to fructose-6-phosphate, and pyruvate to phosphoenolpyruvate.
The former two reactions are catalyzed by hydrolysis of
ester phosphate bonds by the enzymes glucose-6-phosphatase
and fructose-1,6-diphosphatase, respectively, while the
latter reaction involves the coupling of two nucleoside
triphosphate cleavage reactions, as shown below.

$$\begin{array}{c}
\text{Pyruvate} \\
|\ \ \ \ \ \\
|\ \ \ \ \ \\
|\ CO_2\ +\ ATP \\
\text{Pyruvate carboxylase}\quad |(\\
\text{(PC)}\quad\quad |\ ADP\ +\ P_i \\
\Downarrow \\
\\
\text{Oxaloacetic acid} \\
|\ \\
\text{Phosphoenolpyruvate}\quad |\ \\
\text{Carboxykinase}\quad\quad |\ GTP(ITP) \\
\text{(PEPCK)}\quad\quad\quad |(\\
|\ GDP(IDP)\ +\ CO_2 \\
\Downarrow \\
\\
\text{Phosphoenol pyruvate}
\end{array}$$
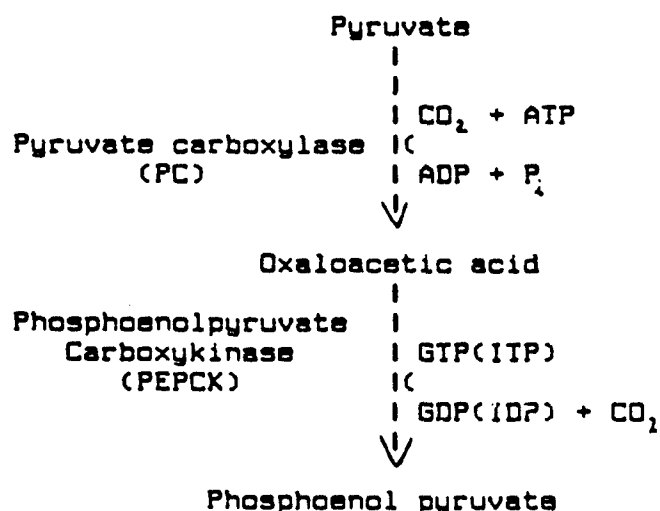
Endotoxin inhibits the induction, by glucocorticoids or fasting, of each of these enzymes: glucose-6-phosphatase (101), fructose-1,6-diphosphatase (101), and PEPCK (16). Although it may be unusual for a single enzyme to control the expression of an entire metabolic pathway, there is strong evidence to suggest that PEPCK is the rate-limiting enzyme in gluconeogenesis; therefore, the interaction of LPS with PEPCK will be emphasized. Seubert et al (146) measured gluconeogenesis by the formation of glucose in hepatic or renal cortex slices from animals receiving cortisol 6 hours ealier. The amount of glucose formed was directly proportional to the activity levels of pyruvate carboxylase and PEPCK, but not to glucose-6-phosphatase or fructose-1,6-diphosphatase. They concluded that the conversion of pyruvate to phosphoenolpyruvate is the rate-limiting step in gluconeogenesis. Rongstad (134) showed PEPCK to be the rate-limiting enzyme by using 3-mercaptopicolinate, a strong inhibitor of PEPCK. Inhibition of PEPCK correlated directly with the reciprocal rate of synthesis of glucose in rat hepatocytes. Since PEPCK appears to be the regulatory enzyme in gluconeogenesis, further details of its role and regulation are warranted.

PEPCK was first observed in chicken liver mitochondria in 1954 (162). It is found primarily in the cytosol of hamsters, rats, and mice and in the mitochondria of rabbits and chickens (72), and equally proportioned in the cytosol

and mitochondria in man (155). Ballard and Hanson showed the cytosolic and mitochondrial isoenzymes to be immunologically distinct proteins. While some investigators have shown that only the cytosolic form responds to diet and hormones (118,148), others have implicated a role for mitochondrial PEPCK (3,72). Although details of the physiologic interaction between cytosolic and mitochondrial PEPCK has yet to be resolved, they both appear to be regulators of gluconeogenesis (155).

Hepatic PEPCK is readily induced by glucagon (a cAMP stimulator) (127,169), cAMP (127,169,170), norepinephrine (127), glucocorticoids (118, 126,148,170), fasting (59,148), alloxan or mannoheptulose induced diabetes (148), and thyroxir. (28,116). As a negative control, insulin inhibits the induction of hepatic PEPCK (67,149,169,170). This appears to be a direct suppression as insulin, in the absence of glucose (a glucagon, hence, cAMP modulator), inhibits the dibutyryl cAMP induction of PEPCK activity in Reuber H-35 cells (67,154). It should be noted that adrenalectomy or induced diabetes does not interfere with the circadian cycle of PEPCK (123). Thus, multiregulation of this enzyme is apparent and when one regulatory mechanism becomes compromised the other(s) may compensate.

Recent attention has been given to investigating the level at which hepatic PEPCK is regulated. Since actinomycin D blocks the induction of PEPCK by cAMP (169) or glucocorti-

coids (130,131), regulation at the level of transcription
is probable. This is substantiated by measuring the increase
in amount of immunoprecipitable PEPCK from in vitro trans-
lation of hepatic RNA following treatment with cAMP (84,85)
or glucocorticoids (85,107). In addition, a direct measure
of glucocorticoid or cAMP induced levels of mRNA for PEPCK
using cDNA probes has also confirmed a transcriptional level
of regulation (2,8,38,39,164). With this background of PEPCK
and its regulation, a discussion of how LPS interacts with
this crucial gluconeogenic enzyme will ensue.

Kun (94) was the first to observe an inhibition of
gluconeogenesis by LPS when he noted severe hypoglycemia in
LPS-poisoned rats. Other studies have confirmed this early
report. Shands et al (147) showed that LPS neither altered
the rate of glucose clearance from the blood nor altered
oxygen consumption, measured by metabolism and oxidation of
various substrates. They concluded that LPS-induced hypogly-
cemia was due to inhibition of gluconeogenesis and not due
to insulin or increased metabolism. McCallum and Berry (102)
saw an almost 50 percent inhibition of incorportaion of
[U-^{14}C]alanine into blood glucose after LPS-poisoning in
mice. Additional studies have also shown impaired gluconeo-
genesis upon endotoxinemia (58,113,171). The question re-
mained however, as to how LPS inhibits gluconeogenesis.
Rippe and Berry (18,130,131) reported the inhibition of in-
duction of hepatic PEPCK following either LPS-poisoning or

actinomycin D. Although LPS inhibits the induction of other
hepatic enzymes, glucose-6-phosphatase (101), fructose-1,6-
diphosphatase (101), and tryptophan oxygenase (16,132), this
is not due to gross hepatic tissue damage since it does not
inhibit the induction of hepatic tryosine amino transaminase
(16). The above data clearly show that LPS inhibits gluco-
neogenesis at the level of PEPCK. Several studies have
provided insight as to how this antagonism occurs.

Using a compilation of reports, Berry (17) suggested
LPS antagonizes the glucocorticoid induction of PEPCK by a
a humoral factor. First, the large size of endotoxin made it
an unlikely intracellular toxin (121). After endotoxinemia,
Noyes (121) detected LPS in phagocytic Kuppfer cells in the
liver but not in nonphagocytic hepatic parenchymal cells.
Second, aimals with a hyperactive reticulcendothelial system
(RES) are sensitized to the lethal effects of LPS (9,44).
Thus, the basis for an RES mediating substance was noted and
subsequent studies supported this.

Moore et al (111) showed serum from LPS-poisoned
mice inhibited the glucocorticoid induction of PEPCK in mice
made tolerant to LPS. Tolerant mice are refractory to the
effects of LPS. In addition, supernatant from peritoneal
exudate cells (PEC's), primarily macrophages, also inhibited
the glucocorticoid induction of PEPCK in mice made tolerant
to LPS (111). Furthermore, normal mice pretreated with rabbit
anti-macrophge serum did not exhibit LPS inhibited PEPCK

activity (111). Pretreatment with anti-thymocyte serum did not abolish the inhibition of PEPCK activity by LPS (111). This investigation clearly establishes an LPS-induced humoral substance produced by macrophages of the RES which antagonizes the glucocorticoid induction of PEPCK and was thus called 'glucocorticoid antagonizing factor (GAF)' (111). Other studies have since confirmed GAF as the LPS-induced mediator which inhibits the glucocorticoid induction of hepatic PEPCK.

Congenitally athymic nude mice (nu/nu) exhibit hydrocortisone induced PEPCK activity regardless of LPS treatment (112). Their PEPCK activity is, however, inhibited by serum rich in GAF (ie, serum collected 2 hours after administration of LPS to mice pretreated with zymosan, an RES activator) (112). In addition, C3H/HeJ mice, which do not respond to LPS and exhibit a fully inducible PEPCK activity even in the presence of LPS, resist hydrocortisone induced enzyme levels in the presence of GAF-rich serum (114). Also, Reuber H-35 hepatoma cells which are refractive to the LPS inhibition of hydrocortisone induced PEPCK activity, displayed total inhibition of PEPCK activity when treated with GAF-rich serum (65,66). Finally, Couch et al (43) showed that mice made tolerant to LPS and subsequently placed in the cold were sensitized to the lethal effects of GAF-rich serum but not LPS.

It is the purpose of this paper to evaluate the

glucocorticoid antagonism of LPS, to further support the role of GAF as an LPS-induced mediator, and to assess the dysfunction of carbohydrate metabolism at the level of PEPCK by either LPS or GAF.

MATERIALS AND METHODS

CHEMICALS:

All chemicals, reagents, and enzymes used in these experiments were obtained from Sigma, unless otherwise noted.

ENDOTOXIN (LPS):

Commercially prepared and lyophilized LPS from Salmonella typhimurium was purchased from Difco, Detroit, MI, cat # 3125-25. It was stored dessicated at 4 C and reconstituted in sterile, nonpyrogenic 0.9% isotonic saline. The reconstituted solution was stored at -20 C. Freeze thawing did not alter, in a detectable way, the activity of LPS.

RADIATION DETOXIFIED ENDOTOXIN (RD-LPS):

This preparation was graciously provided by Dr. Lorand Bértók of the National Institute of Radiobiology and Radiohygiene in Budapest, Hungary. It is a phenol-water endotoxin preparation from Escherichia coli 089, human strain, which was detoxified by exposure to 150 kGy (kiloGrays) of 60-Co gamma irradiation. Reconstitution and storage was the same as that for toxic LPS.

ANIMALS:

CFW mice initially purchased from Charles River breeding Laboratories Inc. (Wilmington, MA) and subsequently

bred at this facility were employed in all experiments. Both
male and female mice were used, ranging in weight from 20 to
25 grams. Mice were housed no more than 12 per large cage or
5 per small cage with Beta Chip bedding (Northeastern Pro-
ducts Corp., Warrenburg, N.Y.). Food pellets from Wayne Lab-
Blox, Allied Mills, Chicago, Il. and tap water were available
at all times except during controlled starvation.

TOLERANCE:

Mice were made tolerant to LPS by a modification of
the procedure described by Goodrum and Berry (66). A series
of intraperitoneal injections were given at increasing dose-
levels of 10 ug, 15 ug, and 25 ug LPS in volumes of 100,
150 and 250 ul prepared from a 100 ug/ml solution. Mice were
injected on days 1,2 and 4, respectively, prior to challenge
48 hours after the last injection. Mice rendered tolerant do
not respond to the lethal effects of LPS.

STIMULATION OF THE RETICULOENDOTHELIAL SYSTEM (RES):

Hyperactivation of the RES sensitizes mice to LPS
and it also increases the yield of induced humoral factors
known to be produced by macrophages. In order to stimulate
the RES, mice were given an intravenous injection of 700 ug
of heat-killed Corynebacterium parvum (Burroughs Wellcome,
cat. # CN 6134) in a total volume of 100 ul. Mice were then
challenged 6 days later.

COLLECTION OF GAF-RICH SERUM (CpES):

GAF (glucocorticoid antagonizing factor) rich serum, also called Corynebacterium parvum - endotoxin serum (CpES), was collected by decapitating mice 2 hours after an intravenous injection of 25 ug LPS in mice whose RES had been stimulated with C. parvum 6 days earlier. Blood was allowed to clot approximately 3 hours at 4°C then centrifuged at 10,000 rpm for 10 minutes. The serum was filtered through a 0.45 um millipore filter and stored at -20°C.

COLD AND HEAT STRESS MODELS:

Cold stress was induced according to Couch and Berry (43). Endotoxin tolerant mice were placed individually, without food, water, or bedding on a wire mesh screen, about 1.5 cm above the table top in a walk-in refrigerator at 5°C. Each mouse was covered with a 250 ml beaker. A brick, or other weight, was placed on top of the beaker to prevent the mouse from escaping. Temperature was regulated between 4°C and 5°C. Heat stress was induced by housing endotoxin tolerant mice in a 37°C walk-in incubator. There were 5 mice per cage and bedding, but no food or water was provided. No method for controlling humidity in either the cold or heat stress environment was available. Rectal temperatures were measured with a small animal thermistor, inserted about 1 cm into the rectum and read on a telethermometer (Yellow Springs Instrument Co.) after 3 minutes. Temperatures were

recorded every hour.

TOURNIQUET SHOCK STRESS MODEL:

Tourniquet shock was induced by a modified version
of that of Rosenthal (136). To a 1500 ml glass beaker was
added about 50 ml chloroform. A wire mesh stand was placed
into the beaker and the top of the stand was approximately
10 cm above the liquid chloroform surface. A single mouse
was placed onto the wire mesh and aluminum foil was then
placed over the beaker. Mice were anesthetized for 15-30
seconds. It is better to re-anesthetize than to over-anes-
thetize. The subdued mouse was then placed in a hollow brass
cylinder about 4 cm in diameter. A rubber band (size 30,
measuring 2 x 1/32 x 1/8 in) was then wrapped in 6 equal
turns around a tubercullin syringe case, 1 cm in diameter
and 3 cm long. This hollow case was coated lightly with
glycerol for easy removal of the rubber band. To the hind
leg of the mouse was tied, by a slip knot, fishing line
attached to an 11 gm lead weight. The weight was passed
through the TB syringe case pulling the hind leg into the
case. The rubber band was then slipped off the case so that
it fit snugly against the body and around the proximal hind
leg. The fishing line was then removed and the process was
repeated for the other leg. Tourniquets were left on for
1 hour before removal with sharp scissors.

PHOSPHOPOENOLPYRUVATE CARBOXYKINASE PURIFICATION:

This procedure is a modification of that of Colombo (41) and Iynedjian (86). Induced levels of PEPCK were obtained by starving mice 24 hours prior to challenge. After this time, mice were decapitated, their livers were perfused with ice cold sterile, nonpyrogenic 0.9% isotonic saline via the portal vein. The gall bladder was excised and discarded. The livers were excised and immediately frozen in liquid nitrogen. Livers were maintained at $-20^{\circ}C$ for a maximum of 48 hours until purification was begun. All purification procedures were performed at $4^{\circ}C$. Each liver was homogenized in 5 volumes (w/v) of buffer 1 (50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.25 M sucrose, pH 7.4) by a motor driven teflon Potter-Elvehjam homogenizer at one-quarter speed. The homogenate was then centrifuged at 100,000 x g for 1 hour at $4^{\circ}C$ in a Beckman Ti50 rotor using a Beckman model L ultracentrifuge. Solid ammonium sulfate, 45% (w/v), was added to the supernatant and stirred overnight at $4^{\circ}C$ and the precipitate was removed by centrifugation at 10,000 x g for 15 minutes. The supernatant was then made 65% (w/v) in ammonium sulfate. This was stirred for 4 hours at $4^{\circ}C$. The precipitate was collected after centrifugation at 10,000 x g for 15 minutes and dissolved in buffer 2 (50 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM DTT, pH 7.5). It was desalted by overnight dialysis against buffer 2. Further desalting was accomplished by passing it through a 2.3 cm x 26 cm BioGel

P-6 column equilibrated with buffer 2. Eluate fractions with OD 280 greater than 0.15 were pooled and applied to a 2.7 x 9 cm DEAE-cellulose column equilibrated with buffer 2. This column was washed with buffer 2 until the OD 280 was zero and the PEPCK fraction was then eluted with a 300 ml gradient of 0 to 300 mM NaCl in buffer 2. The PEPCK fraction eluted between 70 and 150 mM NaCl. Fractions containing PEPCK activity, assayed by the method of Colombo (41), were pooled and concentrated to 5 ml by pressure filtration using a 43 mm Amicon PM 30 membrane at 15 psi nitrogen. This solution was diluted to 40 ml with buffer 3 (10 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM DTT, pH 7.0) then dialyzed 24 hours against 2 changes of buffer 3. The dialysate was then applied to a 2.6 x 4 cm hydroxylapatite column equilibrated with buffer 3. This column was flushed with buffer 3 until the OD 280 read zero. The fraction containing PEPCK was then eluted with a 200 ml linear gradient of 0 to 200 mM potassium phosphate, pH 7.0. Fractions containing PEPCK activity were pooled and concentrated to 25-35 ml using the Amicon described above. This solution was then dialyzed for 2 days against 3 changes of buffer 4 (10mM tris-HCl, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol). The dialysate was then made 0.6 mM with manganese chloride and applied to a 1.2 x 2.4 cm agarose-hexane-GTP affinity column. this column was washed with 10 ml of buffer 4 (without manganese chloride). PEPCK was then eluted with buffer 4 containing 1 mM GTP in a

volume of 1-2 ml. Fractions containing PEPCK activity were
stored at -20°C in a 50 percent glycerol solution.

SDS-PAGE:

Sodium dodecyl sulfate polyacrylamide gel electro-
phoresis (SDS-PAGE) was by the method of Laemmli (95).
Thoroughly cleaned glass plates were sealed on three sides
using spacers and 1% hot agarose. These plates allowed the
formation of a gel 1.5 mm in thickness. The separating gel
was made in a 125 ml side-armed flask containing 10% acryl-
amide (Eastman), 0.26% bisacrylamide (Eastman), 0.375 M Tris-
HCl, pH 8.8, 0.1% SDS, 0.05% N,N,N',N'-tetramethylethylene-
diamine (TEMED). Following degassing of the solution, poly-
merization was initiated with freshly made 10% ammonium
persulfate (BioRad) to 0.015%. This solution was gently
poured between the sealed plates and allowed to polymerize
for 25 minutes at 25°C. To ensure a flat surface the solution
was overlaid with a 1:4 dilution of lower buffer (1.5 M Tris,
0.4% SDS) during polymerization of the acrylamide. The
stacking gel was prepared in essentially the same manner as
the separating gel except the acrylamide and bisacrylamide
concentrations were halved and the buffer was changed to
0.125 M Tris-Cl, pH 6.8. The overlay buffer was removed and
after initiating polymerization with ammonium persulfate the
stacking gel was placed on top of the separating gel. A
teflon well-forming comb was inserted into the stacking gel

and polymerization occurred at 25°C in 20 minutes. The teflon comb was gently removed and the plates were placed in an electrophoretic apparatus containing electrode buffer (25 mM Tris, 0.192 M glycine, 0.1% SDS, pH 8.6). Samples were prepared by boiling for 5 minutes in an equal volume of sample buffer (62.5 mM Tris-Cl, pH 6.8, 4% SDS, 18% glycerol, 10% B-mercaptoethanol, 0.002% bomophenol blue). The samples were loaded (5-50 ul) using a Hamilton syringe into the sample well chambers. Molecular weight markers were run with each gel. Gels were electrophoresed for 4 hours at 25 mA, constant current, or until the bromophenol blue tracking dye was 1 cm from the bottom of the gel. Gels were fixed for 30-60 minutes in 10% acetic acid, 30% methanol, 10% trichloroacetic acid (TCA), stained for 30-60 minutes in 0.1% Coomassie brilliant blue R, 10% acetic acid, 30% methanol, and then destained overnight in 15% acetic acid.

ANTI-PEPCK IgG FRACTION ISOLATION:

On day 1, a New Zealand white rabbit was injected with a total volume of 2.0 ml containing 3 mg/ml purified PEPCK in an equal volume of complete Freunds adjuvant (Difco, Detroit, MI, cat. # 0638-600). This antigen-adjuvant mix was given as a 1.0 ml intraperitoneal and 0.25 ml subcutaneous injection in all four haunches. On days 24 and 26, 0.5 mg/ml purified PEPCK in a total volume of 1.0 ml sterile, nonpyrogenic 0.9% isotonic saline was injected intravenously. On

day 35, and twice a week thereafter for 4 weeks, the rabbit was bled by cardiac puncture using a 30 cc syringe and an 18 gauge needle. Approximately 15 ml of serum was collected from each bleed. The IgG fraction was isolated from this immune serum according to the method described by Nowotny (119). The serum was centrifuged at 10,000 x g for 15 minutes to remove any cellular debris. To this was added saturated ammonium sulfate dropwise to 40% saturation at 25°C while stirring. After 30 minutes the solution was centrifuged at 1000 x g for 30 minutes. The pellet was washed once with phosphate buffered saline (PBS, 0.02 M phosphate, 0.9% NaCl, pH 7.0) containing 40% ammonium sulfate and the washed pellet was redissolved in PBS to 10% of the starting volume. This solution was dialyzed every 8-12 hours against 2-3 buffer changes of 0.0175 M phosphate buffer (PB), pH 6.3, until no sulfate could be detected in the dialysis buffer when mixed with solid barium chloride. The dialyzed solution was clarified by centrifugation at 10,000 x g for 15 minutes and the clear supernatant was diluted to approximately 15 mg/ml with PB, pH 6.3. This solution was then applied to a 2.3 x 25 cm DEAE-Sephadex A-50 column equilibrated with PB, pH 6.3. The first fractions, which contain IgG, with an OD 280 were pooled and concentrated to approximately 3 mg/ml by pressure filtration using a PM 10 Amicon filter under 15 psi nitrogen. This solution concentrate was divided into 1.0 ml aliquotes and stored at -20°C.

TITRATING ANTI-PEPCK IgG:

Rabbit anti-mouse PEPCK IgG fraction was titrated by its ability to bind and precipitate out PEPCK from high-speed mouse liver supernatants. This procedure is a modified version of that of Ballard and Hanson (5). In 1.5 ml Eppendorf tubes, anti-PEPCK was diluted in 0.0175 M phosphate buffered saline, pH 6.3, to yield a total volume of 50 ul of various concentrations (0-250 ug) of the antibody. To this was added 10 ul of 25-30% (w/v) high-speed (100,000 x g) mouse liver supernatant. After mixing, the solution was incubated for 1 hour at 0°C. Preimmune IgG fractions were in-included as controls. Following the first incubation, 0.2 ml of a 10% suspension of heat-killed, formalin-fixed Staphylo-coccus aureus, Cowan I strain (Sigma cat. # P-7155, binding capacity of 1.56 mg human IgG per ml) was added and after gentle mixing was incubated for 15 minutes at 0°C. After centrifugation for 1 minute, 0.4 ml of the supernatant was assayed for PEPCK by the radiometric assay described later.

IMMUNODIFFUSION:

This was performed according to Clausen (40). A solution of 1% purified agar (Difco cat. # 0560-01) in veronal buffer (15 mM diethyl barbituric acid, 75 mM sodium barbital, pH 8.6) was prepared. Then 2.8-3.0 ml of molten agar was pipetted onto a glass slide cleansed with 70% ethanol. This was allowed to harden for about 5 minutes in

a moist humidifier. After punching an appropriate pattern with a Gelman punch, 3 mm bore, the wells were removed by vacuum. To the appropriate wells was added 10 ul of antigen (high-speed, 100,000 x g, mouse liver supernatant) and antibody (rabbit anti-mouse PEPCK). Diffusion occurred overnight and the immune precipitin line was read using a back-lighting apparatus (Kallestad Labs, Inc., Minneapolis, MN).

IMMUNOELECTROPHORESIS:

Modification of the procedure described by Nowotny (120) was used. 1% Purified agar, as in the immunodiffusion method above, was employed. Wells were punched with a Gelman gel punch (a 1.5 mm well for the antigen and a 1 mm trough for the antibody). The antigen plug was removed by vacuum and 2 ul of antigen (high-speed mouse liver supernatant) was added. Electrophoresis was carried out for 1 hour (3-6 mA per slide) using a Pharmacia EPS 500/400 power source. After electrophoresis, the antibody trough was removed by vacuum and 0.1 ml of rabbit anti-mouse PEPCK serum was added. The slide was incubated overnight at 25 C and read as in the immunodiffusion method above.

WESTERN BLOT:

This was performed according to the procedure of Burnette (34) which was adapted from that of Towbin (157). Gels were prepared as in the SDS-PAGE section above, except they were electrophoresed overnight at 4 mA and left

unstained. Half of the SDS-PAGE contained samples and the other half contained molecular weight markers to monitor the transfer of proteins onto the nitrocellulose. Transblot materials were assembled in a plastic container filled with degassed electrophoretic buffer (20mM Tris-base, 150 mM glycine, 20% (w/v) methanol, pH 8.3), in the following sequence. Bottom plastic holder, filter pad, two pieces of filter paper, nitrocellulose sheet (Schleicher & Schuell, cat. # BA85), SDS-PAGE gel, two pieces of filter paper, filter pad, and top plastic holder. Care was taken to avoid entrapment of air bubbles in any layer of assembly. The assembled material was quickly transferred to an electrophoretic chamber (Hoeffer TE 50 Transfer Electrophoretic Cell) which had been previously filled with electrophoretic buffer. The nitrocellulose sheet was positioned between the SDS-PAGE gel and the anode. Electrophoresis was run at 4°C for 2 hours at 50 V. The transblot apparatus was dismantled and the nitrocellulose was cut to separate samples from molecular weight markers. The nitrocellulose containing samples was heat-sealed in plastic (Sears counter craft, seal N-save) and stored at 4°C. The nitrocellulose containing molecular weight markers was stained in India ink as follows. Nitrocellulose was washed 4 times for 10 minutes each time in 250 ml wash buffer (PBS-TW: 0.15 M NaCl in 0.01 M Na_2HPO_4 / NaH_2PO_4, pH 7.2, containing 0.3% Tween 20) at 37°C. After each wash the nitrocellulose was thoroughly rinsed in water.

After the final wash, the nitrocellulose was stained with India ink (1 ul ink per ml PBS-TW) for 2 hours. Following confirmation of the transfer of proteins from the SDS-PAGE gel to the nitrocellulose, the stored nitrocellulose containing samples was saturated by incubation in 15 ml of a 10% milk-Tris-saline (MTS) solution (10% Carnation instant nonfat dry milk, 10 mM Tris-Hcl, 0.9% NaCl, pH 7.4) at 40°C for 30 minutes under constant rocking. After protein saturation, the nitrocellulose was incubated in 15 ml 10% MTS containing 200 ul rabbit anti-mouse PEPCK serum for 90 min. at 25°C. Occassional handling and turning of the bag afforded maximum contact of the antibody with the antigen. Following this incubation, the nitrocellulose was washed in 200 ml Tris-saline without milk for 10 minutes at 25°C, then in 200 ml Tris-saline containing 0.05% NP-40 for 10 minutes at 25°C, and finally in 200 ml Tris-saline for 10 minutes at 25°C. Following this wash, the nitrocellulose was incubated in 15 ml MTS containing 3 ul of ^{125}I-protein A, 3.7×10^6 cpm, (kindly provided by Dr. H. Bose, Dept. of Micro., U.T. Austin). Incubation proceeded at 25°C for 1 hour after which the nitrocellulose was blot dried and then air dried for 1-2 hours. The nitrocellulose was sealed in Saran wrap and auto-radiographed in an intensifying screen exposed at -70°C to Kodak XR film.

PHOSPHOENOLPYRUVATE CARBOXYKINASE ASSAY:

Method 1: Essentially that of Colombo et al (41).
This method was used to monitor the presence of PEPCK during
its purification. In the reaction, PEPCK catalyzes the car-
boxylation of phosphoenolpyruvate (PEP) to oxaloacetic acid
(OAA) which is subsequently converted to malic acid by malic
dehydrogenase (MDH) with concomitant oxidation of NADH. The
disappearance of NADH is followed at 340 nm as a decrease in
absorbance over time. Purification of PEPCK was completed
through the desalting step before assaying for activity due
to the presence of NADH oxidizing activities in crude liver
supernatants. The reaction mix contained:

$$56 \text{ mM HEPES buffer, pH } 7.0-7.2$$

$$1 \text{ mM dithiothreitol (DTT)}$$

$$2 \text{ mM MnCl}_2 \cdot 4H_2O$$

$$0.2 \text{ mM NADH}$$

$$22 \text{ units malate dehydrogenase (MDH)}$$

$$2.3 \text{ mM inosine } 5'\text{-diphosphate (IDP)}$$

$$2.5 \text{ mM phosphoenolpyruvate (PEP)}$$

The reaction was run at 25°C by adding 0.7 ml of the reaction
mix, less IDP and PEP, to a cuvette. Then 0.1 ml of sample
containing PEPCK was added and the small decrease in absorb-
ance was recorded. The reaction was started by the addition
of 0.2 ml IDP/PEP and the decrease in absorbance was again
recorded. The enzyme activity was calculated as follows:

$$U/ml = \frac{[(\Delta A_{+IDP} - \Delta A_{-IDP})/min] (rxn\ vol\ in\ ml)}{(sample\ vol\ in\ ml)[6.23(absorption\ coefficient)]}$$

One unit is defined as that amount of enzyme that catalyzes the formation of one micromole product per minute at $25^{\circ}C$.

Method 2: This is a modification of the procedure of Ballard and Hanson (5) and Chang and Lane (36). This assay was used to measure the activity of PEPCK in crude liver supernatants. Livers were prepared according to the 'purification of PEPCK' method discussed earlier. The reaction is essentially that of the Colombo assay discussed above, except the carboxylation of PEP involved assaying the incorporation of radiolabeled $^{14}CO_2$ and the reaction mix contained 50 mM $KHCO_3$ containing 2 uCi $NaH^{14}CO_3$ (ICN # 17441-H), 100 mM imidazole buffer, pH 6.9, and 2.5 mM NADH. The reaction mixture was kept on ice in appropriately labeled 13 × 100 mm test tubes until the reaction was initiated. To begin the reaction, the tubes were prewarmed to $37^{\circ}C$ for 3 minutes. At timed intervals, 50 ul of a 15% (w/v) high-speed liver supernatant was added. The reaction was terminated 15 minutes later by the addition of 25 ul of 15% (w/v) ice cold TCA. Each tube was immediately placed on ice. A control reaction less PEP and IDP was run with each assay and the result was subtracted from the sample result. Free $^{14}CO_2$ was removed from the reaction tube by bubbling unlabeled CO_2 through the

reaction solution for 30 minutes. After centrifugation for 2 minutes at 2,000 rpm, 0.4 ml of the reaction solution was counted in a total volume of 6.0 ml complete counting cock-tail (RPI, Research Products International Inc., cat. # 11156), for 5 minutes on a scintillation counter. One unit of activity is defined as that amount of enzyme that cata-lyzes the fixation of one micromole of $NaH^{14}CO_3$ per minute at 37°C and was calculated as follows:

$$U/ml = \frac{\text{incorporated cpm as stable } ^{14}\text{C-malate}}{(\text{umole } NaH^{14}CO_3)(\text{rxn time, 15 min})(\text{ml sample, 0.05 ml})}$$

Specific activity was calculated as mU/mg protein.

Method 3: Enzyme linked immunosorbant assay (ELISA) was used to measure the amount of PEPCK in high-speed mouse liver supernatants. In this way a comparison between the amount of enzyme and activity of enzyme (method-2) can be made. The ELISA procedure used is an adapted version of that of Tsang et al (158). Plastic, 96 well, microtiter plates (Dynatech Laboratories cat. # 011-010-3650) were prewashed in 95% ethanol and rinsed in distilled water. After drying they were wrapped in plastic wrap and stored at 25°C until use . Upon use the wells were coated with 200 ul of 5 ug/ml rabbit anti-mouse PEPCK IgG in sensitizing buffer (0.05 M Tris, 2.0 mM EDTA, 0.3 .: KCl, ph 8.0). Coating of antibody occurred by incubating at 37°C for 1 hour and then overnight

at $4°C$. After coating the plates were washed 3 times for 3 minutes each with wash buffer (PBS-TW, 0.01 M Na_2HPO_4/NaH_2PO_4, 0.15 M NaCl, pH 7.2, with 0.3% Tween 20). Between each wash the plates were shaken dry and then blot dried on a layer of paper towells. To ensure complete saturation of all binding sites in the wells, the plates were then incubated overnight at $4°C$ with 200 ul of 0.5% Carnation nonfat dry milk in sensitizing buffer. Plates were washed as above. After the final wash, 200 ul of a 1:40 mouse liver homogenate in PBS-TW was added to the appropriate wells and the plates were incubated for 1 hour at 37 C. Plates were then washed as above. The enzyme was then sandwiched by the addition of 200 ul rabbit anti mouse PEPCK IgG:horseradish peroxidase (HRP) conjugate in PBS-TW made 0.01% with respect to thimerosal. Plates were incubated for 1 hour at $37°C$ then washed as before. During the final wash, fresh substrate was prepared as follows. To a scrupulously clean 100 ml graduated cylinder was added 10 ug o-phenylenediamine (OPD), dissolved in 1 ml of absolute methanol. This was taken to a volume of 100 ml to which 0.1 ml of a 3% hydrogen peroxide solution was added. After the final wash, 200 ul of this OPD substrate was added to each well. The conjugated HRP reduces H_2O_2 to 1/2 O_2 resulting in the subsequent oxidation of OPD and formation of an orange-yellow chromagen. The reaction was allowed to proceed at room temperature for 30 minutes, after which time it was terminated with 25 ul of 8 N H_2SO_4. The absorbance of the

plate wells were then read at 490 nm using an automatic microplate reader (Dynatech MR 600). A control with 5 ug/ml preimmune serum coated to the bottom of the wells was run with each assay and its absorbance was subtracted from that of the samples.

PREPARATION OF RABBIT ANTI-MOUSE PEPCK IgG:HORSERADISH PEROXIDASE CONJUGATE:

The procedure of Boorsma and Streefkerk (27) was employed. Horseradish peroxidase (HRP), 8 mg, was dissolved in 2 ml distilled, deionized water and 0.4 ml of freshly made 0.1 M $NaIO_4$ (periodate). This solution was stirred at 25 C for 20 minutes, during which time the periodate oxidized the aldehyde groups of HRP. After 20 minutes the oxidation was terminated by the addition of 3 drops of ethylene glycol. This solution was stirred at 25°C for 5 minutes. The solution was then desalted through a 2 x 12 cm Sepahadex G-25 fine column equilibrated with 0.001 M sodium acetate buffer, pH 4.2. The brown colored fractions were collected and pooled. To this was added 10 mg IgG (purified rabbit anti-mouse PEPCK antibody). The primary amines of the antibody reacted with the aldehyde groups of HRP. This conjugate solution was brought to pH 9.0-9.5 by adding 1 M Na_2HCO_3 / NaH_2CO_3, pH 9.5, in a dropwise fashion. After stirring for 2 hours at 25°C, 0.2 ml of freshly made NaBH (0.4 mg/ml) was added and the solution was incubated at 4°C for 2 hours.

This resulted in the reduction of any unreacted aldehyde groups. the conjugate solution was then dialyzed overnight against phosphate buffered saline (PBS), pH 7.0-7.2. The dialysate was stored at -20°C in aliquotes containing 0.01% thimerosal until use.

PROTEIN ASSAY:

The method of Bradford (29) was used. The absorbance of acidic Coomassie brilliant blue G-250 shifts from 465 to 595 nm when bound by protein and the amount of binding is directly proportional to color intensity. Concentrated dye reagent (BioRad cat. # 500-006) was diluted 1:4 in distilled water, filtered through Whatman no. 1, and stored in glass at 25°C. To 5.0 ml of diluted dye reagent was added 0.1 ml appropriate diluted protein (eg, a 1:10 dilution of a 15% (w/v) high speed mouse liver supernatant). This was mixed by gentle inversion and allowed to set at least 15 minutes, but no longer than 1 hour, before reading on a Beckman uv/vis spectrophotometer at 595 nm against a diluted dye:distilled water blank. Standard proteins were run with each assay to establish a standard curve from which protein concentrations (mg/ml) were read.

RESULTS

EFFECT OF STIMULATING THE RETICULOENDOTHELIAL SYSTEM
(RES) ON SENSITIZATION OF MICE TO ENDOTOXIN: Benarcerraf (9)
showed that mice were sensitized to the lethal effects of LPS
upon prior stimulation of their reticuloendothelial system
(RES) with zymosan. Since the biological effects of LPS are
mediated by leukocytes, primarily macrophages (111), it seems
likely that a proliferation of these cells in the spleen and
other RES tissue could result in this acute sensitization to
LPS. We primed the RES of CFW mice with a heat-killed, for-
malin-fixed preparation of Corynebacterium parvum prior to
challenge with toxin endotoxin (LPS) or radiodetoxified endo-
toxin (RD-LPS). These results are shown in Table 1. With a
sufficient dose-rate of LPS, C. parvum primed mice began to
die as early as 2 hours post-challenge. These were convulsive
deaths characterized by bloody diarrhea, hematuria, protein-
uria and splenomegaly. Eight hours after the administration
of LPS the LD_{50} for mice whose RES had been stimulated was
approximately 5 ug for LPS and 20 ug for RD-LPS. No deaths
were observed during this same time when unprimed mice were
challenged with as much as 500 ug of LPS. These data show
the lethal activity of detoxified LPS (RD-LPS) during bio-
logical conditions which optimize the lethal effects of endo-
toxin. However, even under such conditions RD-LPS remains
less toxic than LPS. The exact chemical reason for a decrease

Table 1: Sensitization of mice primed with Corynebacterium parvum to LPS or RD-LPS.

		% Mortality at time (hours)		
		Cumulative deaths/total		
Treatment	Dose level	4	6	8
Unprimed + LPS [a]	500 ug	0 0/13	0 0/13	0 0/13
Primed + LPS [b]	20 ug	47 7/15	80 12/15	100 15/15
	5 ug	27 3/11	54 6/11	54 6/11
	0.8 ug	0 0/12	25 3/12	42 5/12
Primed + RD-LPS [c]	20 ug	15 3/20	45 9/20	50 10/20
	5 ug	0 0/12	33 4/12	42 5/12
	0.8 ug	0 0/12	8 1/12	8 1/12

[a]
Normal mice given 500 ug LPS (iv) derived from S.typhimurium

[b]
Mice injected with 700 ug of heat-killed C. parvum (iv) and given various doses of LPS (iv) 6 days later.

[c]
Mice injected with 700 ug of heat-killed C. parvum (iv) and given various doses of RD-LPS (iv) 6 days later.

in the toxicity of RD-LPS is unknown, although irradiation
does result in a decrease in the keto-deoxyoctonate (KDO) and
glucosamine groups (23). Loss of these would render the
molecule more hydrophobic and potentially less reactive with
those cells of the RES which produce mediators involved in
the pathophysiologic effects of LPS. These biological dif-
ferences are even more speculative than the chemical ones
and further studies are needed to resolve the animals re-
sponse to LPS versus RD-LPS.

EFFECT OF HEAT AND COLD STRESS ON SENSITIZATION OF
MICE TO ENDOTOXIN: It is well known that mice subjected to
temperature stress survive poorly when treated with LPS (13,
124,125). We observed the effect of RD-LPS in mice which
were housed at $5°C$ and $37°C$ and compared this toxicity with
that of toxic LPS. In mice exposed to $5°C$ (Figure 1), LPS was
more lethal than RD-LPS. At 10 hours the LD_{50} for LPS was 0.5
ug. This is a 700-fold increase in toxicity over animals
poisoned at $25°C$ where the LD_{50} is 345 ug at 24 hours after
challenge with LPS. The LD_{50} for RD-LPS was approximately
20 ug at 10 hours of cold stress. This is a 50-fold increase
in toxicity over animals poisoned at $25 C°$ where the LD_{50} is
about 1000 ug at 24 hours (23). Though RD-LPS is less toxic
than LPS in mice exposed to cold, even its diminished toxi-
city is significant when compared to cold-stressed mice
receiving saline as a placebo. None of the unpoisoned mice

Figure 1: Effect of cold stress on sensitization of mice to
an intravenous injection of LPS (clear bars) or radiodetoxi-
fied LPS (shaded bars). Percent mortality was calculated and
plotted along the ordinate against various times (hours)
after challenge along the abscissa. Fasted mice were housed
at 5°C, singly, without bedding, in a walk-in refrigerator.
Mortality rates are calculated from at least 3 separate
experiments, each with at least 5 mice per group.
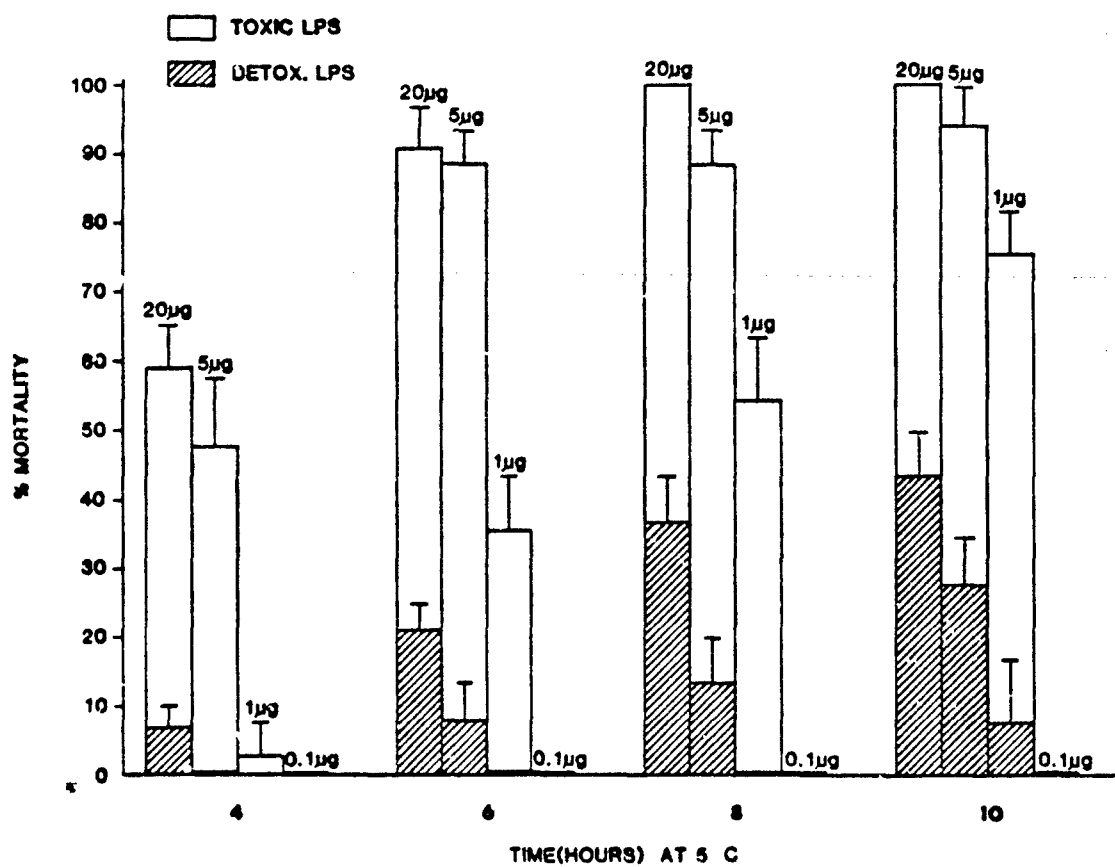
died when exposed to 5°C for 10 hours. The LD_{50} was calcu-
lated at 10 hours in all temperature-stress experiments
because there were significant mortality rates in mice
treated with LPS while those given only saline were rela-
tively unaffected within this time span (see legend to
Figure 3).

During heat stress the decrease in the LD_{50} for
RD-LPS and LPS is even more dramatic than during cold stress.
Figure 2 shows that at 10 hours of incubation at 37°C the
LD_{50} for LPS is 0.04 ug, or a 10,000-fold increase in toxi-
city over mice poisoned at 25°C. As in cold stress, RD-LPS
is less lethal than LPS during heat stress. At 10 hours of
incubation at 37°C the LD_{50} for RD-LPS is 0.5 ug. This is a
2,000-fold increase in toxicity relative to the LD_{50} for mice
housed at 25°C. These data show that the potency of both LPS
and RD-LPS are intensified during temperature stress and this
is more dramatic at 37°C than at 5°C. The reason for in-
creased sensitization at 37°C is unknown, but the animals
response to LPS may differ in heat versus cold stress. It
has been reported that B-endorphins, the endogenous opiates
released during endotoxinemia, may regulate hyperthermia [82]
but not hypothermia [137]. Since naloxone antagonizes the
effects of B-endorphins [52,64,81,82] and glucocorticoids
afford protection against the lethal effects of LPS [12,14,
61,99], we tried to protect our temperature-stressed mice
against the toxic effects of LPS with these two agents.

40

Figure 2: Effect of heat stress on sensitization of mice to
an intravenous injection of LPS (clear bars) or radiodetoxi-
fied LPS (shaded bars). Percent mortality was calculated and
plotted along the ordinate against various times (hours)
after challenge along the abscissa. Fasted mice were housed
at 37°C, 5 per cage, in a walk-in incubator. Mortality rates
were calculated from at least 3 separate experiments, each
with at least 5 mice per group.
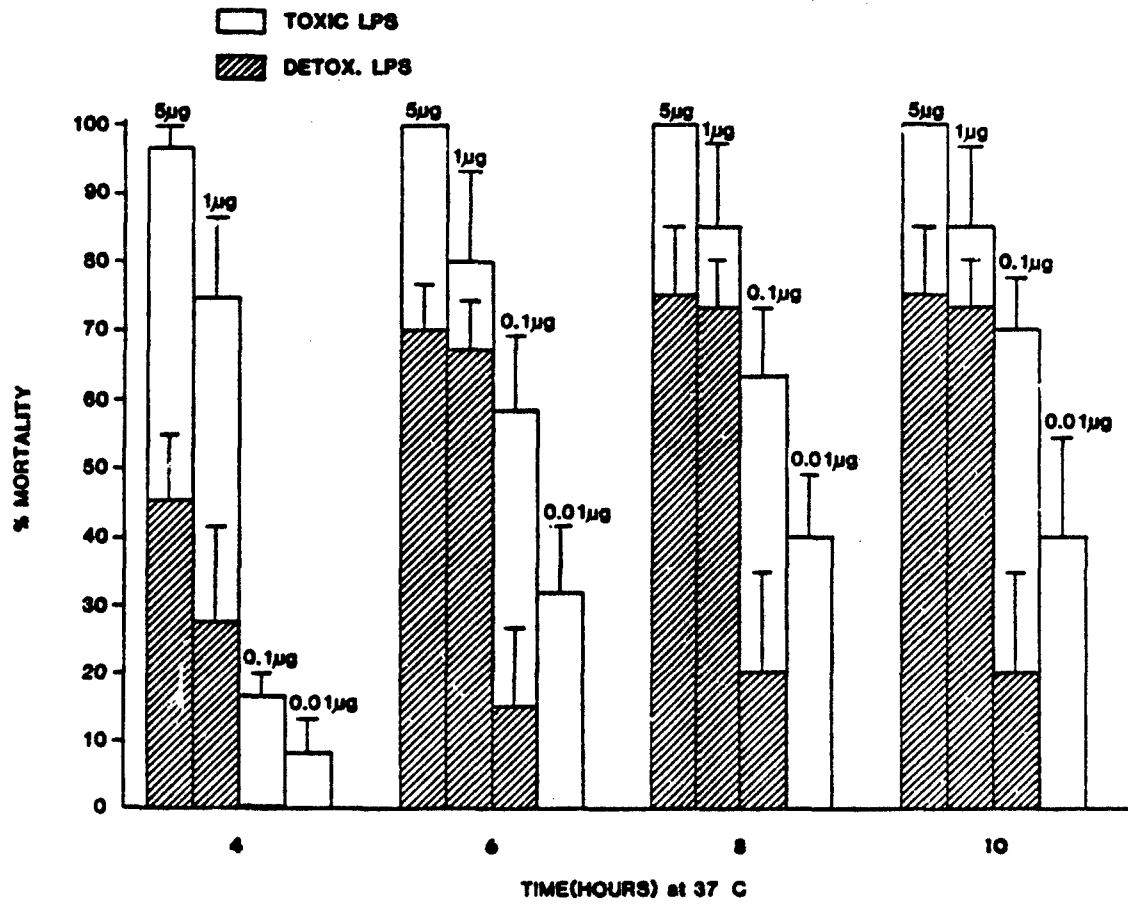
EFFECT OF HYDROCORTISONE OR NALOXONE IN PROTECTING MICE AGAINST LETHALITY DUE TO ENDOTOXIN DURING COLD AND HEAT STRESS: Hydrocortisone, given as a 50 mg/kg subcutaneous injection prior to LPS increased the survival rate of mice during cold or heat stress (Figures 3 and 4). Using an approximate LD_{70} dose of LPS (1 ug at 5°C and 0.1 ug at 37°C), 47% (16/34) and 33% (10/30) of the mice were dead when housed at 5°C or 37°C (Figures 3 and 4), respectively, at 10 hours after challenge with LPS plus hydrocortisone. At both temperatures this is about one-half the mortality rate seen in mice given LPS alone where 79% (26/33) of the mice died in the cold (Figure 3) and 73% (19/26) of the mice died in the heat (Figure 4) at 10 hours. In addition to increasing survival, hydrocortisone enabled mice to maintain a more normal body temperature (Figures 3 and 4, clear bars). At 10 hours LPS-poisoned mice had an average body temperature of 21°C in the cold and 40.2°C in the heat. LPS-poisoned mice given hydrocortisone maintained an average body temperature of 26°C and 39.6°C at 5°C and 37°C, respectively, at 10 hours. Regardless of the time of death after challenge with LPS, mice which died during cold stress exhibited a body temperature of about 20°C. This is almost 20°C cooler than normothermia. This wide fluctuation is not observed at 37°C. LPS-poisoned animals exposed to heat present with only a 2-3° C hyperthermic response at the time of death. This may indicate that regulation of hyperthermia is more critical

Figure 3: Ability of hydrocortisone or naloxone to protect
mice housed at 5°C against lethality from 1 ug of LPS.
Et - LPS (1 ug, iv). NX - naloxone (5 mg/kg, sc) plus LPS.
HC - hydrocortisone (50 mg/kg, sc) plus LPS. C - normal mice.
Percent mortality (dark bars) was calculated and colonic
temperatures (light bars) were measured and plotted along
the ordinate against various times (hours) after challenge
plotted along the abscissa. Fasted mice were housed singly,
without bedding, in a walk-in refrigerator. Mortality rates
and colonic temperatures were calculated from at least 3
separate experiments, each with at least 5 mice per group.
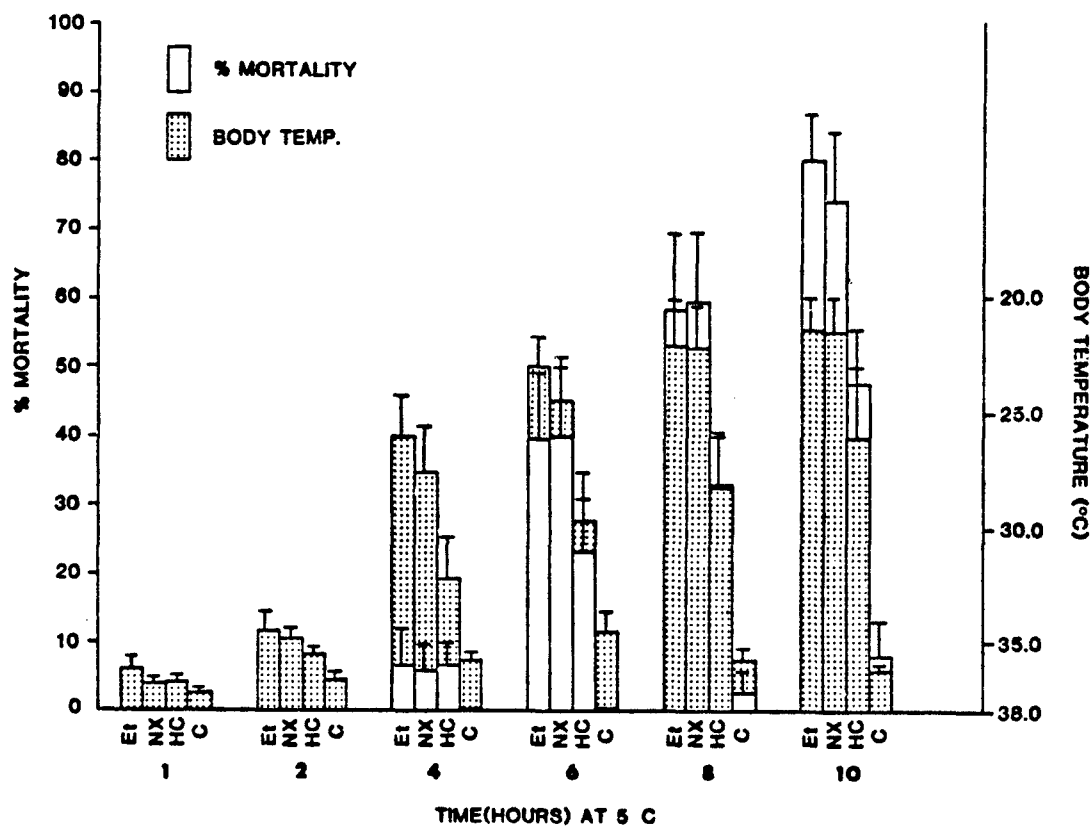
45

Figure 4: Ability of hydrocortisone or naloxone to protect
mice housed at 37°C against lethality from 0.1 ug of LPS.
Et = LPS (0.1 ug, iv). NX = naloxone (5 mg/kg, sc) plus LPS.
HC = hydrocortisone (50 mg/kg, sc) plus LPS. C = normal mice.
Percent mortality (dark bars) was calculated and colonic
temperatures (light bars) were measured and plotted along
the ordinate against various times (hours) after challenge
plotted along the abscissa. Fasted mice were housed 5 per
cage in a walk-in incubator. Mortality rates were calculated
and colonic temperatures were measured from at least 3 sepa-
rate experiments, each with at least 5 mice per group.
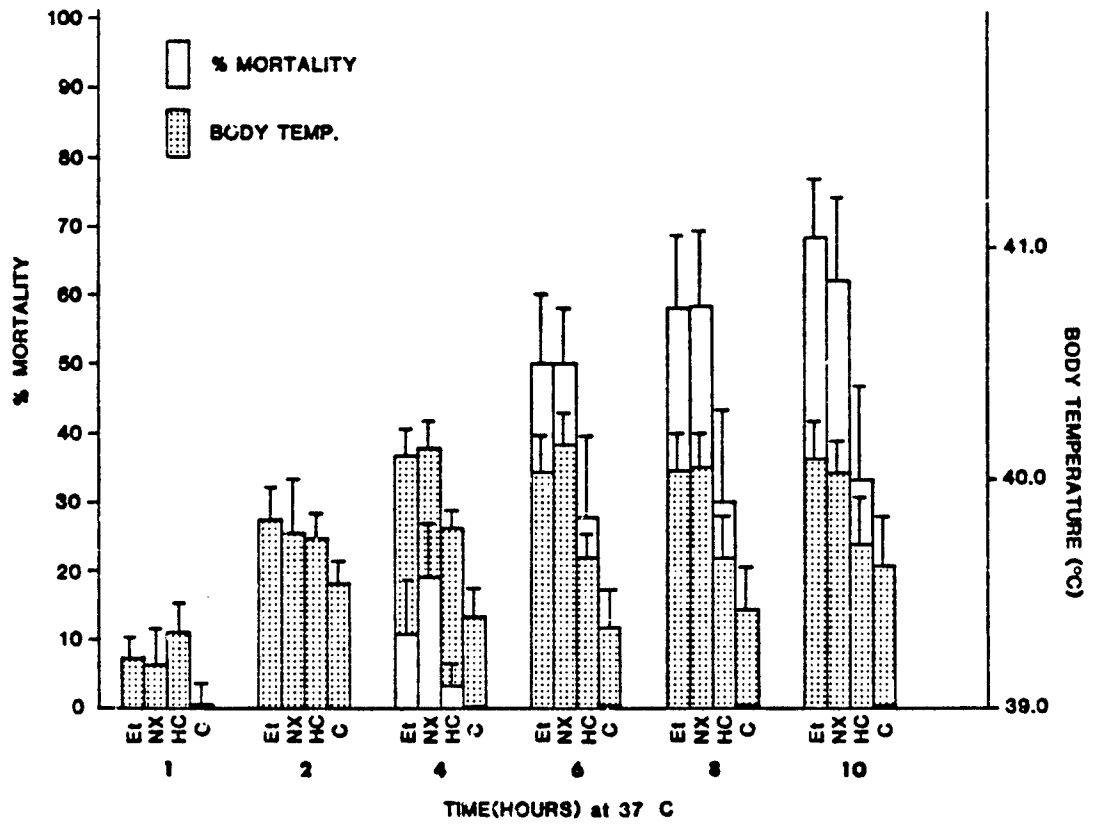
to the animals survival. It has been shown that B-endorphins regulate hyperthermia (82) but not hypothermia (64). It seemed reasonable then to study the effect of blocking endogenous B-endorphins with the opiate antagonist, naloxone, during temperature stress.

Naloxone, given as a 5 mg/kg subcutaneous injection 5 minutes prior to LPS, neither attenuated mortality nor cold-stress induced hypothermia or heat-stress induced hyperthermia. At 5°C mice given naloxone prior to LPS exhibited a mortality rate of 73% (27/37) and their average body temperature was 21°C at 10 hours (Figure 3). This is essentially the same as in mice treated with LPS alone where a mortality rate of 79% (26/33) and average body temperature of 21°C was observed at 10 hours. Similar parity was noted at 37°C (Figure 4). At 10 hours the mortality rate of mice treated with LPS alone or LPS plus naloxone were similar, 68% (19/26) and 62% (16/26), respectively. Mice treated with naloxone prior to challenge with LPS had an average body temperature of 40.1°C at 10 hours during heat stress and this is about the same as the average reading of 40.2°C taken in mice which received LPS alone.

From these studies it must be concluded that hydrocortisone increases the survival rate of endotoxinemic mice during both heat and cold stress. This is characterized by the animals ability to maintain a more normal body temperature. On the other hand, naloxone did not afford protection

or alter the animals hypothermic response in the cold or hyperthermic response in the heat. This suggests that B-endorphins do not contribute to the demise of LPS-poisoned animals during temperature stress and they have no clear role in long-term regulation of hyperthermia or hypothermia. However, during the early stages of heat stress, naloxone may have antagonized the ability of B-endorphins to regulate hyperthermia. At 4 hours, mice treated with LPS plus naloxone had a core temperature which was $0.3^{\circ}C$ higher than mice treated with LPS alone (Figure 4). This increase in body temperature may either be biologically insignificant or else deregulation of hyperthermia may merely be a symptom of the animals response to LPS as the mortality rates for both groups of mice is the same at this time. Since very small dose-levels of LPS and RD-LPS are fatal for mice during stress to heat or cold and for mice whose RES has been stimulated, the ability of RD-LPS to elicit an RES derived serum factor which could sensitize mice to the conditions of temperature stress was tested.

EFFECT OF HEAT AND COLD STRESS ON SENSITIZATION OF MICE TO GLUCOCORTICOID ANTAGONIZING FACTOR: Couch et al (43) showed that mice were sensitized to the cold with serum collected 2 hours after LPS was given to mice whose RES was primed with zymosan. This same format was used to collect serum from mice whose RES had been activated 6 days earlier

with 700 ug of heat-killed _Corynebacterium parvum_. The serum
was collected 2 hours after challenge with 25 ug of LPS or
RD-LPS. It was abbreviated CpES for _C. parvum_ endotoxin serum
and CpdES for _C. parvum_ detoxified endotoxin serum. Mice
which were made tolerant to LPS were used in this experiment
in order to test for the presence of a humoral factor and to
negate the effects of any residual endotoxin remaining in
the serum. At 5°C, CpES and CpdES are both very potent as
early as 4 hours after administration (Figure 5). At 4 hours
93% (25/27) of the mice challenged with CpES and 90% (18/20)
of the mice challenged with CpdES were dead. Control sera
collected from _C. parvum_ primed mice without LPS (CpS), from
unprimed mice given LPS (ES) and from unprimed mice given
RD-LPS (dES) were significantly less lethal. A control group
of mice (column C in Figure 5) was also included to show the
unresponsiveness of tolerant mice to a 10 x LD_{50} of LPS.
Similar results were obtained during heat stress. At 37 C,
CpES and CpdES killed 100% (25/25) and 88% (14/16), re-
spectively, of the endotoxin tolerant mice 4 hours after
challenge (Figure 6). The mortality rates for mice given
control sera (CpS, ES, and dES) were dramatically less even
at 8 hours after challenge.

These data indicate the presence of an LPS-induced
serum mediator produced by cells of the RES which sensitizes
endotoxin tolerant mice to cold and heat stress. Also, cells
of the RES produce this mediator in response to either LPS or

Figure 5: Effects of cold stress on sensitization of mice, made tolerant to endotoxin, to glucocorticoid antagonizing factor (GAF). Percent mortality was calculated and plotted along the ordinate against various times (hours) after challenge plotted along the abscissa. Fasted mice were housed singly, without bedding in a 5°C walk-in refrigerator. All mice were rendered tolerant to endotoxin. C = mice given a 10x LD_{50} of LPS. CpS = sera collected 6 days after challenge with C. parvum. ES = sera collected 2 hours after challenge with 25 ug LPS. dES = sera collected 2 hours after challenge with 25 ug RD-LPS. CpES = sera collected 2 hours after challenge with 25 ug LPS in mice primed 6 days earlier with C. parvum. CpdES = sera collected 2 hours after challenge with 25 ug RD-LPS in mice primed 6 days earlier with C. parvum.
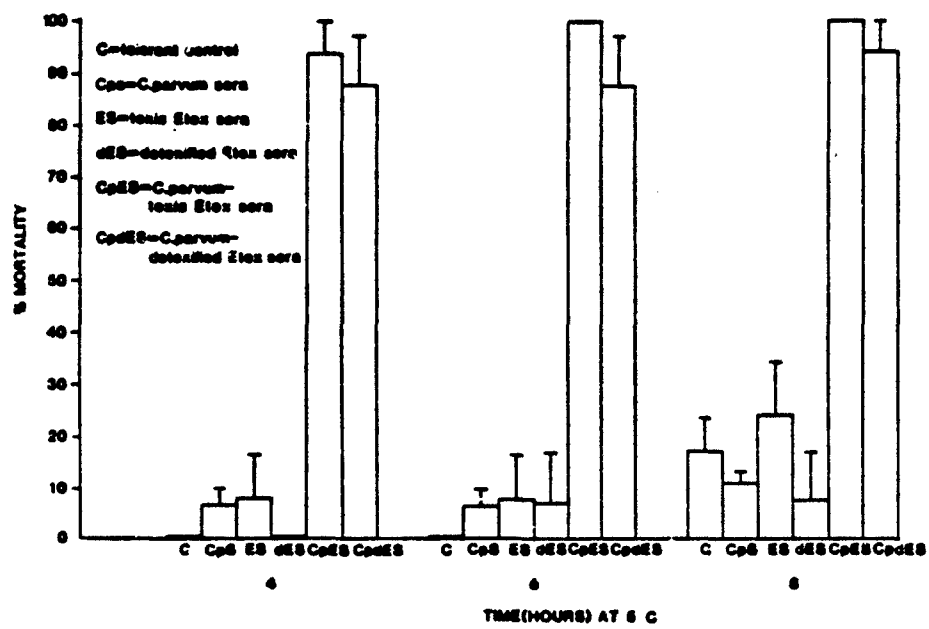
Figure 6: Effects of heat stress on sensitization of mice, made tolerant to endotoxin, to glucocorticoid antagonizing factor (GAF). Percent mortality was calculated and plotted along the ordinate against various times (hours) after challenge plotted along the abscissa. Fasted mice were housed in a 37°C walk-in refrigerator. All mice were rendered tolerant to endotoxin. C = mice given a 10x LD_{50} of LPS. CpS = sera collected 6 days after challenge with C. parvum. ES = sera collected 2 hours after challenge with 25 ug of LPS. dES = sera collected 2 hours after challenge with 25 ug of RD-LPS. CpES = sera collected 2 hours after challenge with 25 ug of LPS in mice primed 6 days earlier with C. parvum. CpdES = sera collected 2 hours after challenge with 25 ug of RD-LPS in mice primed 6 days earlier with C. parvum.
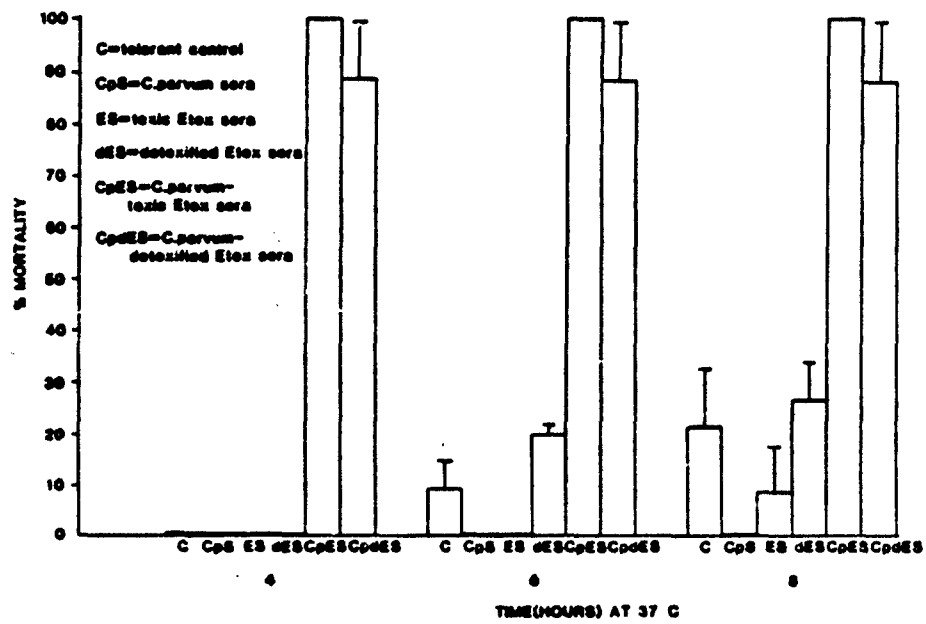
C=tolerant control

CpS=C.parvum sera

ES=toxic Etox sera

dES=detoxified Etox sera

CpES=C.parvum-
      toxic Etox sera

CpdES=C.parvum-
       detoxified Etox sera

% MORTALITY

TIME(HOURS) AT 37 C

RO-LPS. This is shown in figures 5 and 6 where lethality due to CpdES essentially parallels that of CpES from 4 to 8 hours of cold or heat stress. Because this serum is known to antagonize the effects of exogenous hydrocortisone in vivo (111, 112,114) and in rat hepatoma cells in vitro (65,66), the mediator was called 'glucocorticoid antagonizing factor (GAF)'. Inhibition of the adrenal cortical response during heat and cold stress is the most probable explanation for lethality due to GAF as adrenalectomized animals survive poorly during temperature stress (74) and this can be reversed by exogenous corticosteroids (74).

EFFECT OF HYDROCORTISONE IN PROTECTING MICE AGAINST LETHALITY DUE TO GLUCOCORTICOID ANTAGONIZING FACTOR (GAF) DURING COLD AND HEAT STRESS: GAF-rich serum (CpES and CpdES) is lethal for mice placed in the cold or heat (Figures 5 and 6); furthermore, exogenous hydrocortisone protects mice poisoned with LPS against lethality due to LPS during temperature stress (Figures 3 and 4). Therefore, it seemed necessary to try and protect temperature-stressed mice against lethality due to GAF by giving them exogenous hydrocortisone. At 5°C (Figure 7) hydrocortisone afforded partial protection to mice poisoned with CpES (GAF-rich serum). At 4 hours, 80% (8/10) of the mice given CpES were dead while only 23% (3/13) of the mice given 50 mg/kg of hydrocortisone plus CpES had died. The mortality rate of the hydrocortisone treated group

Figure 7: Protective effect of hydrocortisone on survival of tolerant mice subjected to GAF-rich serum during stress to cold. Percent mortality was calculated and plotted along the ordinate against various times (hours) after challenge plotted along the abscissa. HC = hydrocortisone given sc, 50 mg/kg (6 mice per group). CpES = C. parvum endotoxin serum given iv, 0.4 ml (10 mice per group). HC + CpES = hydrocortisone given sc, 50 mg/kg, 2 hours prior to CpES given iv, 0.4 ml (13 mice per group). Fasted mice were housed, singly, without bedding in a walk-in refrigerator at 5°C.

% Mortality vs Time (hours) at 5°C
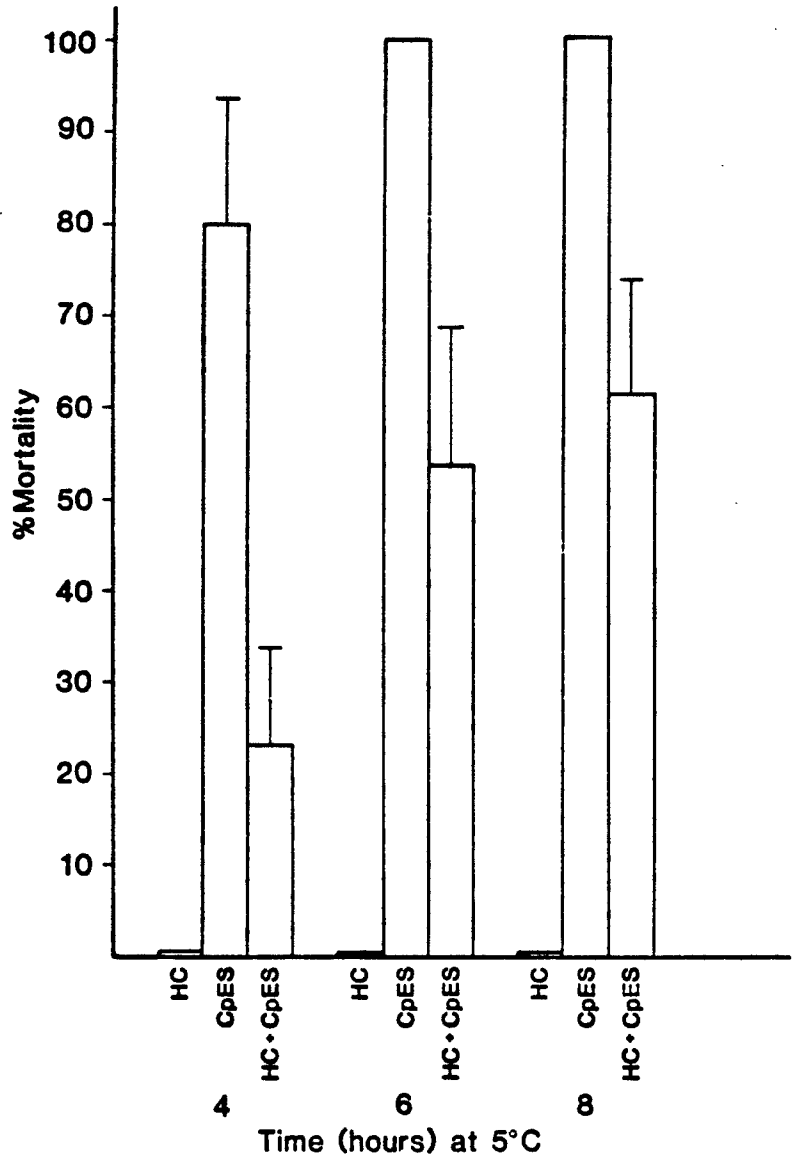
rose to 61% (8/13) at 8 hours, but this is still signifi-
cantly less than the number of deaths seen in mice which were
not pretreated with the hormone. At 37°C (Figure 8), similar
results were observed. All of the mice receiving CpES were
dead at 4 hours (25/25) compared to a 26% (7/27) mortality
rate for the group pretreated with hydrocortisone. As with
exposure to 5°C, stress for 8 hours at 37°C showed similar
results. The mice without hormone treatment had a mortality
rate about twice that of mice which did receive hydrocorti-
sone, 100% (25/25) and 52% (17/27), respectively. Thus
hydrocortisone, given 2 hours prior to serum (CpES) con-
taining enhanced levels of GAF notably increased the survival
rate of mice stressed by cold and by heat. This is signifi-
cant as it indicates that not only can GAF antagonize the
effects of hydrocortisone, but the hormone can also antago-
nize the effects of GAF.

EFFECT OF SENSITIZING MICE TO THE LETHAL EFFECTS OF
LPS WITH A PRIOR DOSE OF LPS OR RD-LPS: This experiment shows
that mice which are stressed with a small dose of LPS or de-
toxified LPS are sensitized to a second sublethal dose of LPS
6 hours later. Figure 9 shows that administration of 50 ug
LPS followed 6 hours later with 150 ug of LPS results in a
mortality rate of 53% (16/30) at 24 hours and 77% (23/30) at
48 hours after challenge with the second dose. Also, 50 ug of
RD-LPS sensitizes mice to this second injection of LPS as 58%

Figure 8: Protective effect of hydrocortisone on survival of tolerant mice subjected to GAF-rich serum during stress to heat. Percent mortality was calculated and plotted along the ordinate against various times (hours) after challenge plotted along the abscissa. HC = hydrocortisone given sc, 50 mg/kg (7 mice per group). CpES = C. parvum endotoxin serum given iv, 0.4 ml (10 mice per group). HC + CpES = hydrocortisone given sc, 50 mg/kg, 2 hours prior to CpES given iv, 0.4 ml (27 mice per group). Fasted mice were housed, 5 per cage in a walk-in incubator at 37°C.
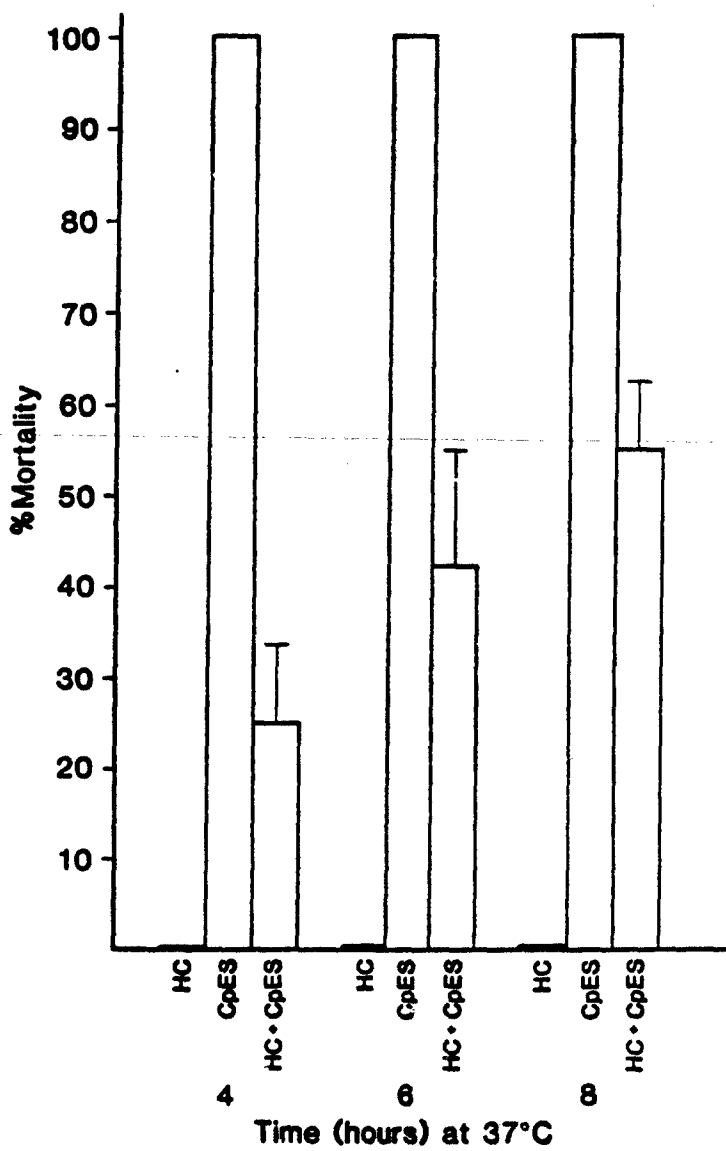
(10/17) and 70% (12/17) of these mice were dead at 24 and 48 hours, respectively. Initial stress of the mouse must be caused by the first 50 ug dose-level because the combined total of both dose levels (ie, 200ug) results in a mortality rate of only 7% (1/15) at both 24 and 48 hours after challenge (Figure 9).

Exogenous hydrocortisone, 50 mg/kg, repressed this lethality and its effect was more intense when given concurrent with the 50 ug priming dose of LPS than when given concurrent with the second 150 ug dose of LPS 6 hours later. Mice given hydrocortisone concurrent with the priming dose of LPS exhibited a mortality rate of 15% (4/26) at 24 hours and 23% (6/26) at 48 hours (Figure 9). This is significantly less than the mortality rates of 58% (10/17) and 70% (12/17) at 24 and 48 hours, respectively, seen in mice which did not receive hydrocortisone. However, when the same dose of the hormone was given 6 hours after the priming dose of LPS and concurrent with the second dose of LPS, it was less protective. The mortality rates for these mice were 38% (5/13) at 24 hours and 54% (7/13) at 48 hours (Figure 9).

These data suggest that the priming dose of LPS stimulates the production of a glucocorticoid antagonizing factor which impairs the effects of exogenous hydrocortisone and sensitizes mice to an otherwise sublethal dose of LPS given 6 hours later. This initial glucocorticoid antagonizing activity can be opposed when hydrocortisone is given with

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Figure 9: Sensitization of mice to the lethal effects of LPS following a small dose of LPS or RD-LPS 6 hours earlier. Percent mortality was calculated and plotted along the ordinate against various treatments as shown along the abscissa. The clear bar in a given treatment group represents an elapse of 24 hours and the shaded bar an elapse of 48 hours. All LPS injections were given iv in a total volume of 0.2 ml. Hydrocortisone was given in a dose of 50 mg/kg, sc, in a total volume of 0.2 ml.

[a]
50 ug LPS given 6 hours prior to 150 ug LPS (30 mice/group).

[b]
50 ug RD-LPS given 6 hours prior to 150 ug LPS (17 mice/group).

[c]
200 ug LPS given as a bolus control dose (15 mice/group).

[d]
50 ug LPS given concurrent with 50 mg/kg of hydrocortisone 6 hours prior to 150 ug LPS (13 mice/group).

[e]
50 ug LPS given 6 hours prior to 150 ug LPS given concurrent with 50 mg/kg of hydrocortisone (13 mice/group).
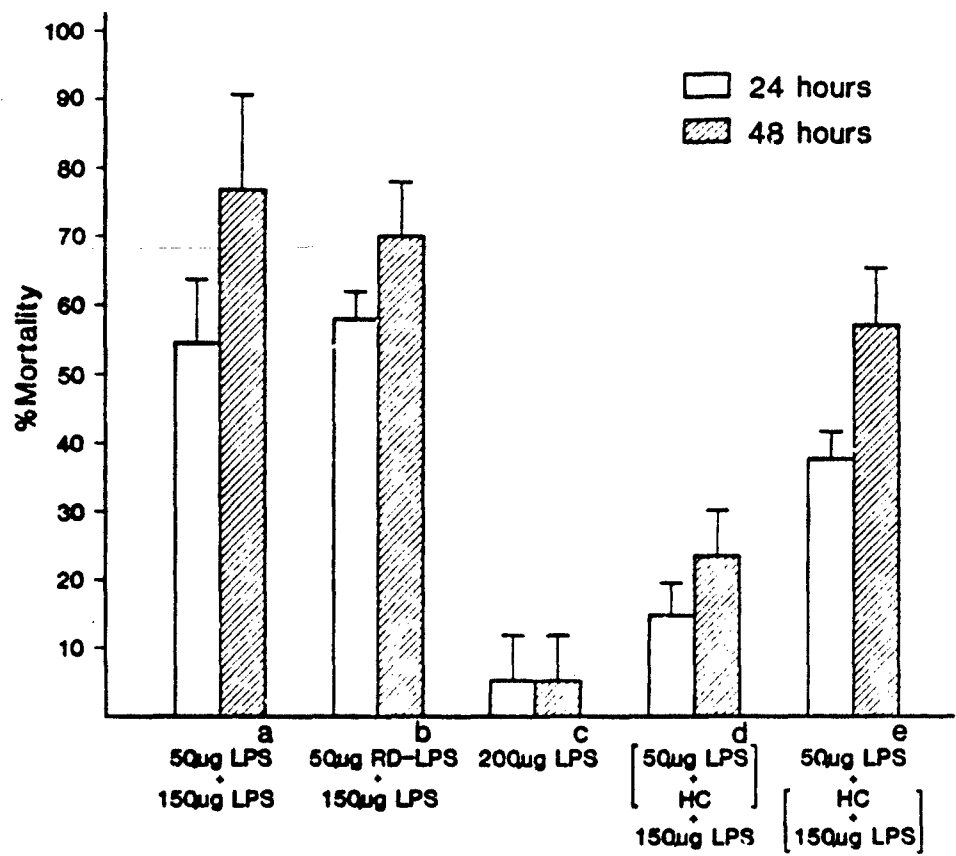
the priming dose of LPS. Since GAF has been shown to pre-
dispose mice to the lethal effects of LPS (43), it seems
reasonable to suggest that the priming dose of LPS elicits
the production of GAF which renders the animal susceptible
to a second exposure to LPS. This is further support for the
requirement of an adrenal response during stress and the
ability of GAF to block this response.

EFFECT OF TOURNIQUET SHOCK ON SENSITIZATION TO LPS
AND GAF: In addition to heat stress, cold stress, and stress
induced by priming mice with a sublethal dose of LPS 6 hours
prior to challenge, the effect of stress due to tourniquet
shock on sensitization of mice to LPS or GAF was tested.
Tourniquet shock was described in 1943 when Rosenthal (136)
showed maximum lethality in mice after the tourniquet had
been applied for 2 hours. If tourniquet stress could be in-
duced without the associated lethality, it would provide
a system by which mice could be sensitized to the lethal
effects of LPS or serum rich in GAF (CpES). The application
of the tourniquet is discussed in detail in the materials
and methods section of this thesis.

Figure 10 shows that at 24 hours all of the mice
(6/6) stressed by tourniquet application for 2 hours were
dead. However, when the tourniquet was applied for only 1
hour, none of the mice (0/13) had died. Therefore , a 1 hour
application time was selected as a means of stress. When LPS

was given at the time of tourniquet application, the mice survived poorly (Figure 10). At 24 hours, all of the mice died (10/10) when given 25 ug of LPS, 91% (10/11) were dead after receiving 5 ug of LPS, and 1 ug of LPS was lethal for 48% (10/21) of the mice (Figure 10, clear bars). Exogenous hydrocortisone (50 mg/kg) provided partial protection against lethality due to endotoxin when it was given concurrently with LPS at the time of the tourniquet application. At 24 hours the mortality rates for these mice were 21% (3/14) and 43% (6/14) with an LPS dose of 1 ug or 5 ug, respectively.

Mice placed under the stress of tourniquet shock were also highly sensitive to the lethal effects of serum containing GAF (CpES, Figure 10). By 24 hours, all of the mice (10/10) treated with CpES had died. Serum which does not contain appreciable levels of GAF (ES for endotoxin serum and CpS for C. parvum serum) were essentially nontoxic, exhibiting mortality rates of 7% (1/14) and no mortality (0/7), respectively. In sharp contrast to protection against LPS, hydrocortisone did not protect mice during tourniquet stress against lethality due to CpES. All mice (8/8) treated with 50 mg/kg of hydrocortisone concurrent with 0.4 ml of CpES at the time of application of the tourniquet were dead at 24 hours. These data indicate that hydrocortisone can afford some protection when given prior to the production and/or release of GAF, but pre-existing GAF completely blocks protection afforded by this hormone.

Figure 10. Effect of tourniquet shock on sensitization of

mice to the lethal effects of LPS or GAF. Percent mortality

was calculated and plotted along the ordinate against various

treatments shown along the abscissa. All LPS injections were

given iv in a total volume of 0.2 ml. All serum injections

were given iv in a total volume of 0.4 ml. Hydrocortisone

was given as a 50 mg/kg dose. sc in a volume of 0.2 ml.

In the LPS treatment group, the clear bars represent LPS

given alone and the shaded bars represent LPS given con-

currently with hydrocortisone.

a
 Tourniquet application for 2 hours (6 mice/group).

b
 Tourniquet application for 1 hour (13 mice/group).

c-e
    LPS given at the time of tourniquet application (for c,
    7 mice/ group; for d, 11 mice/group; for e, 21 mice/group).

f,g
    LPS given concurrently with hydrocortisone at the time of
    tourniquet application (14 mice/group).

h
 Serum collected 2 hours after challenge with 25 ug LPS.
 Given at the time of tourniquet application (14 mice/group).

i
 Serum collected 6 days after challenge with C. parvum.
 Given at the time of tourniquet application (7 mice/group).

j
 Serum collected 2 hours after challenge with 25 ug LPS given
 to mice treated 6 days earlier with C. parvum (8 mice/group).

k
 As in j. Given concurrent with hydrocortisone (8 mice/group).
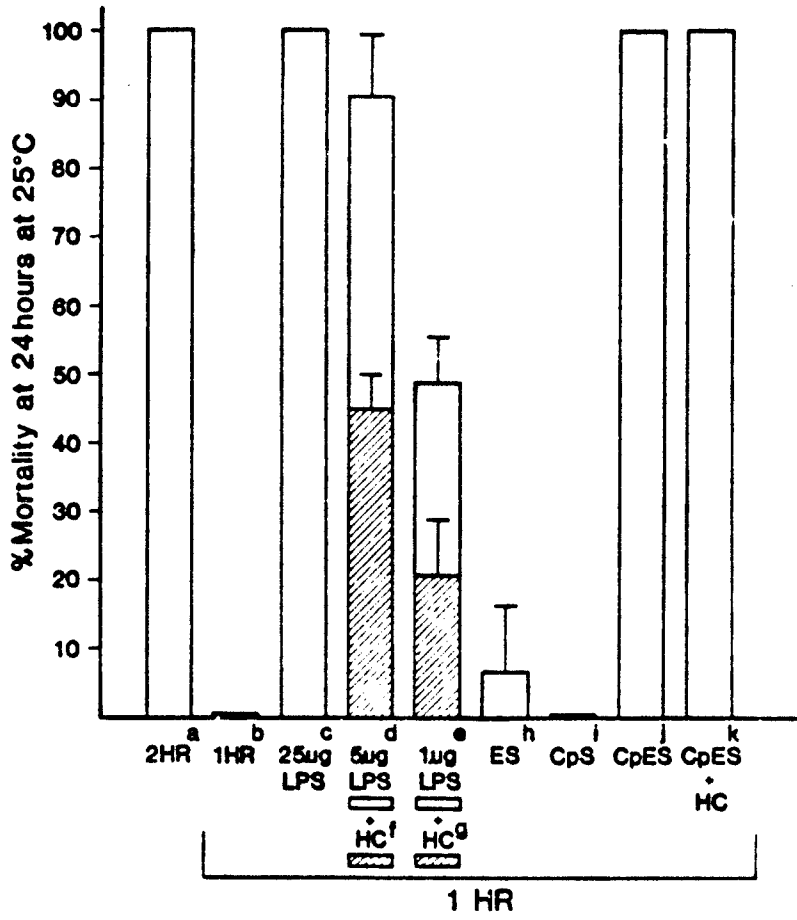
PURIFICATION OF PEPCK AND ACQUISITION OF ANTI-PEPCK ANTIBODY:

The purification of this enzyme is detailed in the Material and Methods section of this thesis. Enzyme activity was measured at various steps of the purification process as shown in Table 2. Precipitation between 45% and 65% ammonium sulfate saturation resulted in a 3-fold purification of the enzyme as seen in the specific activity column in Table 2. Proteins precipitated by ammonium sulfate were then desalted by passage through a gel filtration column in which proteins larger than 6 kilodaltons eluted with the void volume while the passage of smaller proteins through the column was delayed. This resulted in an additinal 1.5-fold purification of PEPCK. Further purification was achieved by ion exchange chromatography. Proteins which adsorbed to a DEAE cellulose resin were eluted with a linear gradient of 0-300 mM NaCl resulting in a further 3.5-fold purification. The final purification step involved affinity chromatography. This column consisted of GTP coupled to an acyl hydrazide derivative of agarose. Guanosine triphosphate is a substrate of PEPCK and the enzyme binds to this column-bound ligand in the presence of manganese (86). Purified PEPCK was then eluted from the column with a 1 mM GTP solution. Affinity purification resulted in an additional 3-fold purification of the enzyme (Table 2). Based on total activity, 10% of the enzyme was recovered in a highly purified form. The final specific activity was 16 U/mg which is comparable to that

Table 2: Purification of PEPCK.

	Total Protein mg/ml	Total Activity U/ml	Specific Activity U/mg [a]	% Yield
Mouse liver cytosol	641	252	0.39	100
Ammonium sulfate precipitate	180	205	1.1	81
BioGel P6 gel filtration column	116	188	1.6	75
DEAE ion exchange column	16.8	98	5.8	39
GTP-hexane-agarose affinity column	1.5	24	16.0	10

[a] Defined as nanomoles $^{14}CO_2$ fixed per minute per mg protein.

used by Ballard and Hanson (5).

In order to determine enzyme purity, the affinity column eluate was analysed by SDS-PAGE. There is a distinct and single band (Figure 11). Its molecular weight was calculated by the method of Weber and Osborn (165) to be 71,500 daltons. This is in good agreement with the value of 70,600 daltons reported by Iynedjian et al (86). The purified enzyme was used to immunize a New Zealand white rabbit in order to obtain anti-PEPCK antibody. As shown in Figure 12, an antibody response was elicited against the purified enzyme preparation as the immune serum precipitated a single protein from a high-speed (100,000 x g), 15% (w/v), mouse liver supernatant fraction.

In order to further show that a unique protein reacted with the immune serum, the methods of immunoelectrophoresis and Western blotting were employed. The presence of two distinct bands that differ in electrophoretic mobility are shown in Figure 13. The presence of two distinct proteins was confirmed by the observation of a doublet band upon autoradiography of a Western blot (Figure 14). The immune serum was derived following immunization with purified PEPCK; therefore, the reason for the presence of two distinct antigens is unclear. It is possible that during the preparation of the liver supernatants some mitochondria may have been ruptured and leakage of mitochondrial PEPCK (mPEPCK) occurred. This isoenzyme is immunologically distinct from cytosolic

Figure 11: SDS-polyacrylamide gel electrophoresis of purified phosphoenolpyruvate carboxykinase (PEPCK). Details of the purification scheme are detailed in the Materials and Methods section. Lane a = molecular weight standards: thyroglobin (half unit), 330K; ferritin (half unit), 220K; lactate dehydrogenase, 140K; phosphorylase b, 94K; albumin, 67K; catalase, 60K; ovalbumin, 43K; lactate dehydrogenase (subunit), 36K; carbonic anhydrase, 30K; trypsin inhibitor, 20.1K; and lactalbumin, 14.4K. Lane b = high-speed (100,000 x g) mouse liver supernatant, 15% (w/v). Lane c = purified PEPCK.

330K
220K
140K

94K

67K
60K

43K

36K
30K

20K

14K

— PEPCK

a   b   c
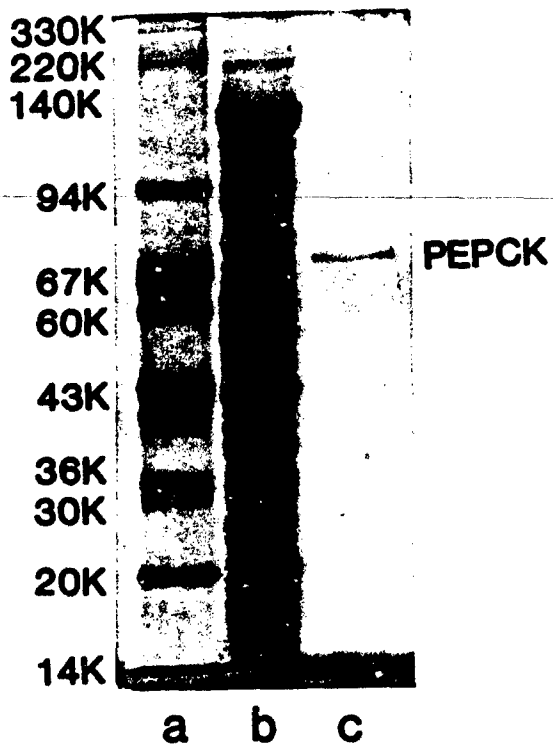
72

Figure 12. Immunodiffusion pattern showing an immunoprecipi-
tate. Immune serum, obtained from a New Zealand white rabbit
after immunization with purified PEPCK, is diluted from 1:1
to 1:128, counterclockwise, in the outer wells. A high-speed
(100,000 x g) mouse liver supernatant, 15% (w/v), is in the
center well.
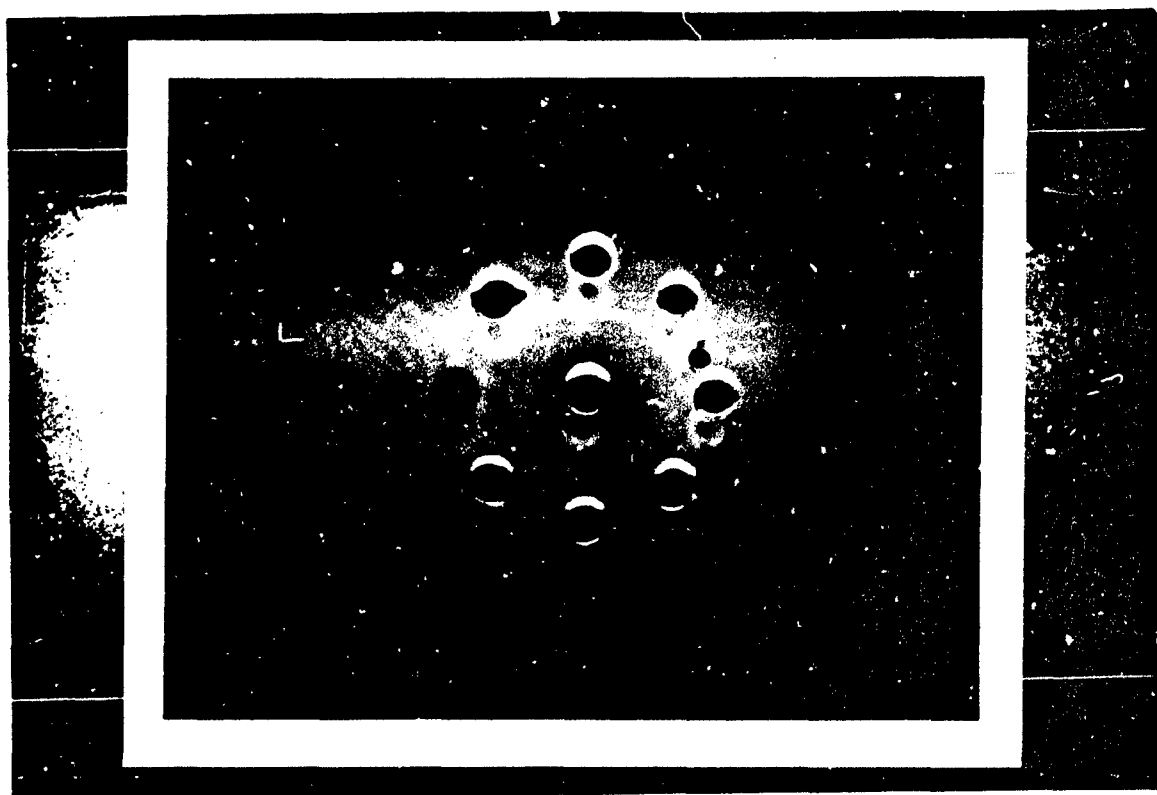
74

Figure 13: Immunoelectrophoresis of a high-speed (100,000 x g) mouse liver supernatant, 15% (w/v), for 90 minutes at 3 mA. An immunoprecipitate was observed 24 hours after incubation of the electrophoresed antigen with rabbit anti-mouse PEPCK placed in the center trough.
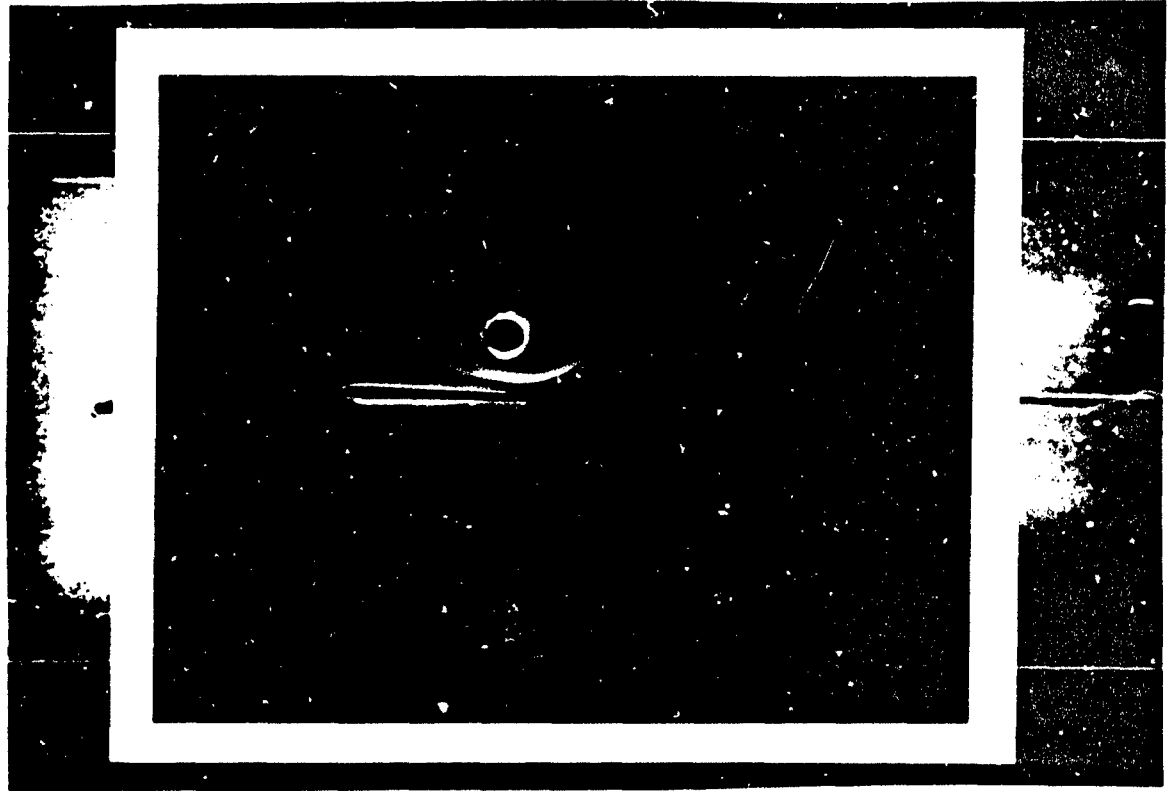
Figure 14: Western blot following SDS-PAGE. Details are provided in the Materials and Methods section. Lanes a-c represent one-half of the SDS-PAGE gel which was stained by conventional methods. Lane d represents the other half of the SDS-PAGE gel which was transferred to nitrocellulose and incubated with antiPEPCK antibody and ^{125}I-labelled protein A. Lane a = molecular weight standards (refer to figure 13 for details). Lane b = 40 ul of purified PEPCK. Lane c = 20 ul of purified PEPCK. Lane d = high-speed (100, 000 x g) mouse liver supernatant, 15% (w/v), blotted onto nitrocellulose.

330K
220K
140K

94K

67K
60K

43K

36K
30K

20K

14K

PEPCK

a b c d
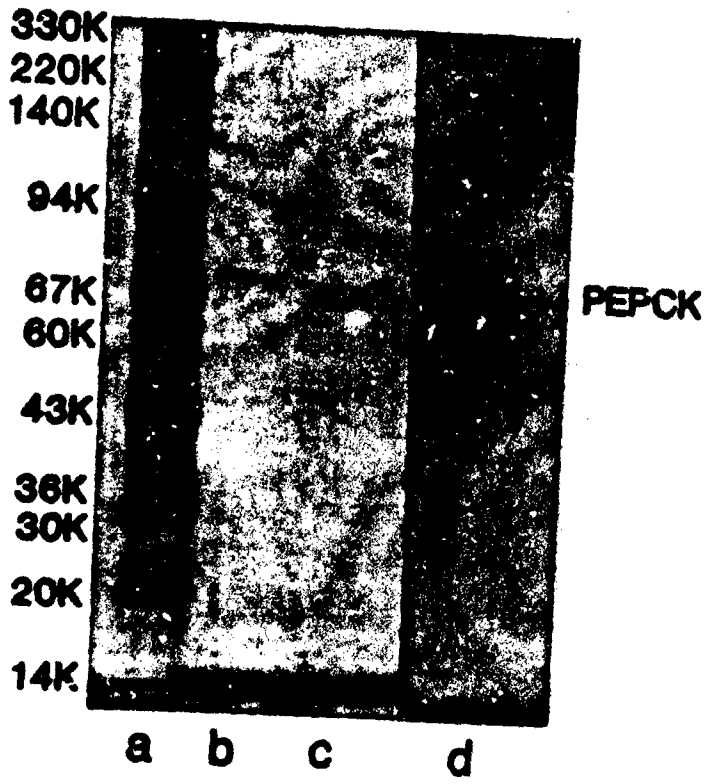
PEPCK (cPEPCK) (86). A small amount of mPEPCK may have contaminated the purified enzyme used in the present study and although undetectable by the conventional stain employed in SDS-PAGE, it may have elicited an immune response. Therefore, the two proteins may represent the presence of both anti-mPEPCK and anti-cPEPCK antibodies in the immune serum. It is also possible that, although immunologically distinct, residual amounts of mPEPCK in the liver due to leaky mitochondria may cross-reacte with anti-cPEPCK in the immune serum. However, based upon the intensity of both bands in the Western blot, this appears unlikely, unless mPEPCK is more antigenic than cPEPCK. An alternative explanation is that anti-PEPCK cross-reacts with a liver protein, other than PEPCK, which exhibits similar immunoelectrcphoretic mobility and comparable molecular weight to that of PEPCK (Figures 13 and 14).

To further clarify the specificity of the immune serum against PEPCK, a PEPCK-antiPEPCK immune complex was precipitated with protein A of Staphylococcus aureus. Following immunoprecipitation the supernatant was assayed for the presence of PEPCK. The absence of activity with increasing concentrations of antibody verify the specificity of the immune serum (Figure 15). Addition of preimmune serum, in the presence of protein A, had no effect on decreasing the activity level of PEPCK; therefore, nonspecific immunoprecipitation was not a factor in the assay.

Figure 15: Titration of rabbit anti-mouse PEPCK IgG. A fixed amount of PEPCK (24 mU/mg) in a high-speed (100,000 x g) mouse liver supernatant was incubated with anti-PEPCK. the immune complex was then precipitated with protein A, S. aureus, Cowan I strain. Following centrifugation, the supernatant from immune serum (•---•) and preimmune serum (o---o) incubations were assayed for PEPCK activity.
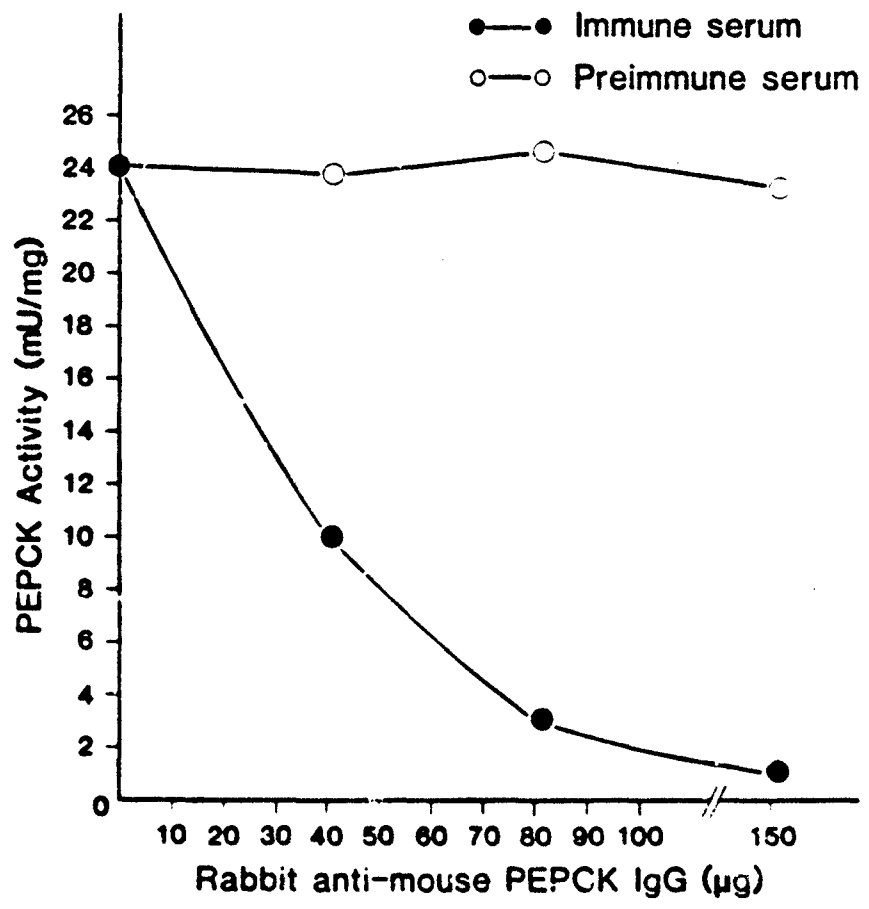
MEASUREMENT OF AMOUNT OF MOUSE HEPATIC PEPCK BY
ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA) AND COMPARISON
WITH ACTIVITY LEVELS MEASURED BY CARBOXYLATION WITH RADIO-
LABELED BICARBONATE: Hepatic PEPCK is readily induced by
hydrocortisone (126,158), cAMP (169,170), and fasting (59,
148). LPS and GAF are known to inhibit the induction of PEPCK
by hydrocortisone (18,130) and fasting (131), but not in-
duction by cAMP (66). Inhibition of this key gluconeogenic
enzyme may be directly related to the animals increased sensi-
tivity to stress. In the above studies, quentitation of PEPCK
is measured by activity, defined as nanomoles of $^{14}CO_2$ fixed
per minute. Only one study has attempted to directly quanti-
tate the amount of enzyme and this was performed using the
method of radial immunodiffusion (91). The purpose of this
experiment was to design an enzyme linked immunosorbant assay
(ELISA) for direct quantitation of hepatic PEPCK and compare
the OD readings obtained by ELISA with the activity levels
of the enzyme. Rabbit anti-mouse PEPCK IgG was obtained by
immunizing a New Zealand white rabbit with purified PEPCK
and isolating the IgG fraction (complete details are given
in the Materials and Methods section). the specificity of
this anti-PEPCK antibody was illustrated previously (Figure
15).

Hepatic PEPCK from the liver supernatant of normal
mice had an OD 490 of 0.093 by ELISA (Table 3). An OD of
0.084 was seen when mice were treated with 25 ug of LPS 4

Table 3: Catalytic activity vs ELISA antigenicity of hepatic
PEPCK. Numbers in parenthesis represent the number of mice
in that group.

a
Defined as nanomoles of $^{14}CO_2$ fixed per minute per mg protein.

b
Defined as OD 490 of oxidized OPD after 30 minute reaction
time with HRP and H O .

c
Ratio of specific activity to OD 490 (ELISA).

d
Normal, uninduced mice.

e
25 ug LPS given (iv) 5 hours prior to hepatectomy.

f
50 mg/kg Hydrocortisone given (sc) 4 hours prior to hepa-
tectomy.

g
24 Hour fast prior to hepatectomy.

h
25 ug LPS given (iv) 1 hour prior to 50 mg/kg hydrocorti-
sone (sc) and 5 hours prior to hepatectomy.

i
Serum collected 6 days after RES activation with 700 ug
C. parvum. Given (0.4 ml,iv) concurrent with hydroco tisone.

j
Serum collected 2 hours after challenge with 25 ug LPS.
Given (0.4ml,iv) concurrent with hydrocortisone.

k
Serum collected 2 hours after challenge with 25 ug LPS in
mice primed 6 days earlier with C. parvum. Given (0.4 ml,
iv) concurrent with hydrocortisone (sc).

Table 3:

Treatment	Specific Activity [a] mU/mg	ELISA [b] OD 490	Specific Activity [c] ───────────── ELISA
Uninduced [d]	19.9 1.0 (17)	.093 .002 (17)	214
LPS [e]	20.0 1.1 (14)	.084 .004 (14)	238
Hydrocortisone [f]	42.4 1.8 (17)	.154 .004 (17)	275
Fasted [g]	45.0 3.6 (12)	.168 .005 (12)	268

Hydrocortisone:

Treatment	Specific Activity [a] mU/mg	ELISA [b] OD 490	Specific Activity [c] ───────────── ELISA
LPS [h]	18.3 1.1 (5)	.090 .002 (5)	203
Cps [i]	36.2 2.2 (11)	.139 .005 (11)	260
ES [j]	36.0 3.0 (11)	.137 .005 (11)	262
CpES [k]	22.4 2.8 (8)	.094 .007 (8)	238

hours prior to hepatectomy. Thus, normal and LPS-treated
mice have essentially the same levels of hepatic PEPCK
(P > 60). In addition, the specific activity of PEPCK did
not differ significantly between normal mice and mice pre-
treated with LPS, 19.9 mU/mg and 20.0 mU/mg, respectively
(Table 3). An increase in both the activity and the amount
of PEPCk was shown upon treatment with a dose of 50 mg/kg
of hydrocortisone given 4 hours prior to liver extraction.
An OD of 0.154 was measured by ELISA in this group. This is
a significant increase in the amount of enzyme when compared
to normal mice (P < 0.05). Likewise, the specific activity
of PEPCK was substantially elevated by hydrocortisone from
a baseline level of 19.9 mU/mg to 42.4 mU/mg. Table 3 also
shows the effects of a 24 hour fast on hepatic PEPCK. These
mice exhibited the greatest rise in the amount of enzyme
and corresponding activity, 0.168 OD by ELISA and 45.0 mU/mg
by carboxylation, respectively. Although this increase is
significant with respect to normal levels of PEPCK (P < 0.05)
it is only slightly higher than that obtained by hydrocorti-
sone (P = 58).

To show that the induction of PEPCK by hydrocortisone
was inhibited by a serum factor, mice made tolerant to LPS
were treated with 50 mg/kg of hydrocortisone that was given
concurrently with serum rich in GAF (CpES). CpES completely
blocked the induction of PEPCK by hydrocortisone (Table 3).
As measured by ELISA the OD was 0.094 for the group

treated with hydrocortisone plus CpES and 0.093 OD for the normal group (P - 96). The activity levels of PEPCK were also similar for these two treatment groups, 19.9 mU/mg for normal mice and 22.4 mU/mg for mice treated with hydrocortisone and CpES (P - 52). Thus, there is no significant difference between measurable PEPCK in normal mice and in mice given hydrocortisone concurrent with CpES. Control serum was also tested to show that inhibition of PEPCK by hydrocortisone was due neither to residual LPS nor to some nonspecific inhibitor in the serum. Endotoxin serum (ES) collected 2 hours after challenge with 25 ug of LPS and C. parvum serum (CpS) collected from mice receiving C. parvum 6 days earlier did not significantly alter the induction of PEPCK by hydrocortisone. Measurements by ELISA show that mice given CpS or ES plus hydrocortisone had PEPCK levels corresponding to 0.139 and 0.137 OD readings, respectively (Table 3). These results were slightly lower than those for hydrocortisone induction. This difference, though statistically insignificant (P < 0.05), appears to be consistent. The activity levels were also lower than results obtained by hydrocortisone alone (42.4 mU/mg). The activity of PEPCK was 36.2 mU/mg in mice treated with hydrocortisone plus CpS and 36.0 mU/mg in mice treated with hydrocortisone plus ES. The reason for the slight inhibition of induction of PEPCK by ES and CpS may be due to the presence of slightly elevated amounts of GAF (65).

A linear relationship between carboxylation activity

levels and amount of enzyme (ELISA) can be established by comparing the ratio of activity to the amount of enzyme (Table 3). This is statistically significant as calculated by the Spearman rank correlation coefficient ($r = 0.93$). Therefore, induction of PEPCK by hydrocortisone or by fasting resulted in enhanced levels of activity of the enzyme and this correlates directly with increased amounts of the enzyme as measured by ELISA.

DISCUSSION

The antagonism between glucocorticoids and endotoxin has been known for more than 30 years (49). Although much research has been conducted during this time, only recently has the basis for this conflict between these hormones and LPS been attributed to a serum factor produced by cells of the RES (21,111,114,115). Since an adrenal cortical response is necessary for survival of animals that are or have been subjected to stress (140), the nature of how LPS antagonizes the protection afforded by the adrenals was elucidated by subjecting mice to stressful environments. It should be noted that both glucocorticoids and mineralcorticoids are released during the stress-induced cortical response, but the role played by mineralcorticoids is believed to be of little importance to survival (6). The discussion that follows will focus, therefore, on the role of glucocorticoids.

Surgically adrenalectomized animals are extremely sensitive to the lethal effects of LPS (33,37,128), the stress of cold or heat (74), trauma (142), and other types of stress. Accordingly, glucocorticoids are necessary for an animal's survival under these conditions. When the gluco-corticoid response is blocked by LPS, the deliterious effects of stress are magnified in the endotoxinemic animal. Previte and Berry (125) showed that mice housed at 5°C are killed, while mice housed at 25°C survive infection with

<u>Salmonella</u> <u>typhimurium</u> strain Sr-11-A. This observation was
extended to purified endotoxin when Berry (15) showed that
the LD_{50} for LPS was dramatically reduced in mice exposed
to environmental temperatures of 5°C or 37°C. Couch et al
(43) also showed that mice exposed to 5°C are sensitized to
lethality due to LPS. This present study confirm these early
reports. In addition, it was found that mice given a small
dose of LPS, detoxified by irradiation with 150 kiloGrays
from a ^{60}Co source (RD-LPS) at the beginning of cold exposure
also led to poor survival. Mice were sensitized 700-fold to
LPS and 40-fold to RD-LPS after 10 hours of cold exposure
(Figure 1). At 37°C, lethality was even more pronounced. At
10 hours, a 10,000-fold and a 2,000-fold sensitivity was seen
for LPS and RD-LPS, respectively (Figure 2).

The reason for enhanced sensitivity to LPS and RD-LPS
at 37°C is not clear. Heroux and Hart (74) reported that the
amount of adrenal cortical extract required by adrenalecto-
mized rats was the same at 5°C and at 37°C. Therefore, the
greater sensitivity of mice to LPS or RD-LPS at 37°C, rela-
tive to 5°C, cannot be explained on the basis of a difference
in demand for glucocorticoids. A hyperthermic response of
only 2-3°C has been measured at about the time the animals
die when housed at 37°C (Figure 4). By comparison, a de-
crease in body temperature, by as much as 18°C, occurs at the
time of death when mice are housed at 5°C. Thus the degree of
cooling is much greater than the degree of a rise in body

temperature. This suggests that regulation of hyperthermia
may be more critical .an regulation of hypothermia, at
least in terms of the number of degrees regulated. This
difference in response during exposure to heat or cold may
account for the greater sensitivity to heat.

Another difference in how animals respond to heat and
cold may involve the release of B-endorphins. These endo-
genous opiates appear to play a role in adaptation to heat
but not to cold. Holaday et al (82) showed that rats exposed
to heat exhibit a greater hyperthermic response, by about
$0.5^{\circ}C$, when treated with the opiate antagonist naloxone.
This small difference in body temperature was associated with
an increase in weight loss and an increase in the number of
escape attempts (82). Goldstein (64) did not observe a signi-
ficant difference in the hypothermic response of rats during
exposure to cold in the presence or absence of naloxone. The
results presented in this thesis also show that naloxone does
not alter the hypothermic response of mice during exposure
to cold (Figure 3), in agreement with the work of Goldstein
(64). However, this work does not confirm the conclusion made
by Holaday (82) since treatment with naloxone did not result
in increased hyperthermia in mice kept at $37^{\circ}C$ (Figure 4).
One obvious difference between these studies and those of
Holaday is the fact that in this work animals were poisoned
with LPS. Since LPS induces the release of B-endorphins (81),
the dose level of naloxone which was administered may not

have been sufficient to antagonize the increased level of
B-endorphin. The dose of naloxone used was 5 mg/kg which is
50 times greater than that used by Faden et al (52,53) to
reverse LPS-induced hypotensive shock, but is only one-half
the dose used by Holaday (82). We also measured colonic tem-
perature over a period of hours whereas Holaday measured
colonic temperature over a period of minutes. These obvious
differences in experimental design could account for the
different results. In addition, naloxone failed to alter the
mortality rate of mice that were poisoned with LPS and then
housed at either 5°C or 37°C (Figures 3 and 4). This obser-
vation is important since it negates several conclusions
which suggest that B-endorphins may serve as lethal factors.
First, the levels of mRNA for the B-endorphin and adreno-
corticotropin hormone (ACTH) precursor, proopiomelanocortin
(POMC), are increased following adrenalectomy (24). Also,
adrenalectomized rats have elevated levels of B-endorphins
in their blood (1) and these animals are sensitized to stress
(33,37,74,142). Exogenous dexamethasone, acting as a feedback
inhibitor of POMC (48) prevents hyperendorphinemia in
adrenalectomized rats (24) and also affords protection
against stress (Figures 3,4 and 9). Finally, B-endorphins
are produced in response to LPS and they have been implicated
in hypotensive shock (52,81,98). Increased levels of these
opiates can thus be correlated directly with a decrease in
the survival of animals unable to elicit an adrenal cortical

response during stress. It can be postulated that glucocorti-
coids, endogenous or exogenous, may act by decreasing the
level of B-endorphin and hence, increase survival. However,
this study does not show increased survival of endotoxinemic
mice during cold or heat stress when pretreated with the
endorphin antagonist naloxone (Figure 3 and 4). The role of
these opiates remains enigmatic in unstressed mice as well.
Treatment with naloxone results in increased survival of LPS-
poisoned dogs (52) and monkeys (83), but not in rats (81),
piglets (145) or Yucatan minipigs (57).

The lethal effects of LPS and RD-LPS were also
studied during stress conditions other than temperature
stress. When mice are primed with a subcutaneous dose of LPS
or RD-LPS they become sensitive to a second, normally sub-
lethal dose, of LPS 6 hours later (Figure 8). Since serum
rich in GAF sensitizes mice to the lethal effects of LPS
(43), it is probable that the priming dose of LPS or RD-LPS
may cause the release of GAF from cells of the RES and sub-
sequent sensitization to a later dose of LPS. Figure 9 also
shows that when hydrocortisone is given concurrently with the
priming dose of LPS, mortality rates, due to the second dose
of LPS, are reduced. This indicates that hydrocortisone
inhibits the release of GAF by the priming dose, although
antagonism of GAF at some other metabolic level cannot be
excluded. When hydrocortisone is given 6 hours after the
priming dose and concurrently with the second dose of LPS

the protection afforded is considerably less (Figure 9).

This implies that the anti-glucocorticoid effect of GAF is

more intense when it is released prior to exposure to hydro-

cortisone. Thus the antagonism between endotoxin and gluco-

corticoids appears to be bidirectional and the early effects

of one may block the later effects of the other. This may be

a critical factor during stress where functional adrenal-

ectomy is fatal. Support for the pharmacological action of

GAF was unknowingly introduced by Geller (61) in an earlier

study. He observed that both adrenalectomized mice and mice

whose RES had been stimulated by zymosan were sensitized to

the same extent to LPS. In agreement with the work of Geller

Table 1 shows that mice whose RES is activated with heat-

killed Corynebacterium parvum are sensitized to the lethal

effects of LPS and Rd-LPS. Since elevated levels of GAF are

released following treatment with zymosan and LPS (65), it

is likely that a decrease in survival is due to functional

adrenalectomy by GAF. This is comparable to the sensitization

of surgically adrenalectomized mice to the lethal effects of

endotoxin.

Another type of stress observed during this study was

that of trauma, introduced by tourniquet shock. Mice whose

hind limbs were subjected to tourniquet application for 1

hour were sensitized to the lethal effects of LPS by more

than 300-fold (Figure 10). At 24 hours after trauma, the LD_{50}

for LPS was 1.3 ug. Deaths were mediated by the effects of

endotoxin as the tourniquet application alone was nonlethal
in unpoisoned mice (Figure 10). Since adrenalectomized
animals are also sensitized to tourniquet shock (142), it
seems reasonable to suggest that LPS has, through the release
of GAF, sensitized the mice to this trauma by antagonizing
the adrenals. This suggestion is reinforced by the obser-
vation that lethality could be attenuated by exogenous hydro-
cortisone (Figure 10).

A direct analogy between surgical adrenalectomy and
adrenal antagonism by LPS c: RD-LPS can be ascertained by
the findings that treatment with either results in poor
survival of animals subjected to stress. However the degree
of adrenal suppression is not the same since LPS is more
lethal than RD-LPS during exposure to cold or heat (Figures
1 and 2). Radiodetoxification of LPS results in decreased
levels of ketodeoxyoctonate (KDO) groups and altered glyco-
samine and fatty acid groups (23) generating a more hydro-
phobic molecule this may alter the time course of the inter-
action of RD-LPS with cells of the RES. Although macrophages
are believed to be the cells responsible for the production
of LPS-induced mediators (19,20), the interaction between
these cells and LPS is ill-defined and further research in
this area may provide some insight into the decreased, or
residual toxicity seen with RD-LPS. Nonetheless. this data
shows that RD-LPS is a potent toxin in mice during stress
(Figures 1,2 and 9) and this lethality, as well as lethality

due to toxic LPS under similar conditions, is mediated by a
serum factor which may act to functionally adrenalectomize
the animal.

It is known that serum collected 2 hours after chal-
lenge with LPS in mice whose RES has been activated contains
elevated levels of glucocorticoid antagonizing activity
(111,112,114). The responsible factor was referred to as
glucocorticoid antagonizing factor (GAF) based on its ability
to inhibit the induction of PEPCK by hydrocortisone (111,
114). GAF has been partially characterized as a protein with
a molecular weight of approximately 150,000 daltons and it
is sensitive to both heat and trypsin (114). This study shows
that GAF-rich serum can be obtained when either LPS of RD-LPS
is used as the inducing agent in mice whose RES has been
primed with heat-killed Corynebacterium parvum. This serum
was designated CpES for C. parvum-LPS treatment and CpdES
for C. parvum-RD-LPS treatment. As shown in Figures 5 and 6,
both CpES and CpdES are highly toxic for mice during exposure
to cold or heat. In addition, this serum also sensitizes
mice to an otherwise nonlethal traumatic shock (Figure 10).
All mice treated with this serum were made tolerant to the
effects of LPS, it can therefore be concluded that lethality
was indeed due to a humoral factor, presumably GAF, and not
due to LPS. It becomes apparent that serum collected after
proliferation of cells of the RES followed by exposure of
these cells to the toxin, results in a yield of GAF which is

approximately the same whether RD-LPS or LPS is injected.
The relative significance of these findings remains to be
clarified. When RD-LPS was used as a stimulant for inducing
nonspecific resistance (NSR) without hypotensive effects
associated with the toxic preparation, Bértók (23) showed
that the administration of RD-LPS, given at least 48 hours
prior to challenge resulted in the protection of dogs against
hemorrhagic shock and protected rats against tourniquet or
septic shock. In contrast, these studies showed RD-LPS to be
lethal when given at the time stress is initiated (Figures
1,2 and 9). This lethaltiy was also shown to be mediated by
a serum factor (GAF) produced in response to RD-LPS (Figures
3 and 4). It is likely that the deliterious effects of GAF
occur early. In support of this, Moore et al (115) showed
that the maximal level of GAF is obtained 2 hours after
challenge with LPS and this level rapidly subsides to normal
levels after about 3 hours. Consequently, by the time Bértók
applied stressor stimuli 48 hours after challenge with RD-LPS
the biological effects of GAF may have disappeared. Therefore,
the time between challenge with RD-LPS and the induction of
stress is crucial for the sensitization of the animal to,
or the protection of the animal against, stress. Since mice
are sensitized to the antagonism of their adrenal response
by GAF, it seemed logical to study the effect of exogenous
glucocorticoids in protecting these mice.

Glucocorticoids are known to attenuate lethality

when LPS is given to mice housed at 25°C (12,14,61,90,91,
109), at 5°C (125) and at 37°C (15). This work has shown
that exogenous hydrocortisone results in increasd survival
of mice poisoned with LPS at 5°C (Figure 3) and 37°C (Figure
4). Hydrocortisone was also shown to protect against a sub-
lethal dose of LPS given to mice which had been primed
earlier with a small dose of LPS or RD-LPS (Figure 9) and
against traumatic shock (Figure 10). Thus glucocorticoids
are efficacious in protecting mice during a variety of con-
ditions where the animal is poisoned with LPS and also sub-
jected to various conditions of stress. This exemplifies the
importance of glucocortcoids and their potential antagonism
of LPS. In order for these exogenous glucocorticoids to
afford protection, they must be given no later than 1 hour
after challenge with LPS (14). This is most likely due to
immunosuppression of macrophages (47) with diminished release
of serum mediators, such as GAF. Once these humoral factors
have been released and are allowed to exert their biological
effects, treatment with hydrocortisone is ineffective. This
was shown where hydrocortisone given 2 hours prior to CpES
afforded protection (Figures 7 and 8) while hydrocortisone
given concurrently with CpES did not protect (Figure 10).
In addition to immunosuppression, glucocorticoids may afford
protection by other means.

Another mechanism for the action of glucocorticoids
may be due to maintenance of carbohydrate homeostasis. Wolfe

et al (171) showed that the hypotensive effects of LPS in
dogs was not severe enough to account for their deaths.
However, the hypoglycemic state manifested by these animals
was incompatible with life. In addition, all dogs which
survived poisoning with LPS were able to maintain their
blood glucose levels. Menten and Manning (108) were the first
to observe total depletion of carbohydrates in rabbits
poisoned with LPS. This was later shown to be due to a combi-
nation of factors, including depletion of hepatic glycogen
stores (10,13,58,94) and hyperinsulinemia (173). In order
to maintain a sufficient caloric balance the animal must
rely on increased consumption of exogenous carbohydrates or
an increase in the rate of gluconeogenesis. Both of these
parameters are blocked by LPS. Exogenous carbohydrate
intake is blocked because LPS severely limits the con-
sumption of food and water by inhibiting gastric emptying
(159). Also, LPS blocks the synthesis of glucose from non-
carbohydrate sources (11,102,147) even though protein
catabolism is unaltered (11). Thus caloric insufficiency may
be a critical factor in the demise of endotoxinemic animals.
It is interesting to note that the LPS-induced glycogen de-
pletion in rats is abolished by adrenalectomy (139), yet
adrenalectomized animals are still sensitized to cold (74).
The most likely explanation for this is that glycogenolysis
is regulated by the cholinergic response from the adrenals
(139) and adrenalectomized animals in the cold may have

sufficient glycogen stores, but these are unobtainable and thus of little value in caloric conservation.

Endotoxinemic mice treated with hydrocortisone and exposed to cold or heat survive longer and are able to regulate their body temperature more efficiently than mice treated with LPS alone (Figures 3 and 4). In a previous study Previte and Berry (125) also showed the ability of cortisone to protect mice poisoned with LPS during exposure to cold. This protection may be due to the maintenance of carbohydrate reserves and preservation of energy as hydrocortisone induces protein catabolism (100) and gluconeogenesis (50). The importance of glucocorticoids during exposure to cold is illustrated by the findings that mice housed at 5°C demonstrate increased levels of endogenous glucocorticoids (92) and PEPCK (43), the rate-limiting enzyme in gluconeogenesis. This enables sequestration of carbohydrate reserves in order to cope with the additional energy requirements during stress to cold, and possibly other types of stress. Kun (94) showed that rats poisoned with LPS are unable to convert glucose or pyruvate into hepatic glycogen. In addition, Berry (13) observed that hepatic glycogen dropped significantly below normal levels in the presence or absence of cortisone. The situation is compounded further by the finding that LPS inhibits gluconeogenesis (10,102,147). This is due to the inhibition of PEPCK by GAF, as Couch et al (43) showed that endotoxin tolerant mice housed at 5 C retain fully inducible

levels of PEPCK when given LPS but that addition of serum
rich in GAF completely blocked this induction and hypogly-
cemia ensues. This confirms the work of others who have shown
the ability of GAF to block induction of PEPCK by hydrocor-
tisone (65,111,112,114). Carbohydrate homeostasis may thus
be a crucial factor for the survival of the endotoxinemic
animal, especially during stress where enhanced sensitization
to GAF is observed.

The question remains as to whether LPS sensitizes
the animal to stress or whether the stressed animal is sensi-
tized to LPS. This is made difficult by the fact that
adrenalectomized animals are sensitized to both stress (74,
142) and LPS (33,37). This study shows that the lethal effect
of LPS during stress is mediated by a serum factor whose
effects can be reduced by prior treatment of hydrocortisone
(Figures 7 and 8) but not by hydrocortisone given concur-
rently with serum that contains the serum factor GAF (Figure
10). The anti-glucocorticoid activity of GAF is demonstrated
by its ability to inhibit the induction of hepatic PEPCK by
hydrocortisone (Table 3). The ability of GAF to suppress the
animals adrenal response suggests that LPS or RD-LPS may
sensitize the animal to stress, where an adrenal cortical
response is obligatory for survival (140). Most of the pre-
ceeding work has dealt with the biological aspects of the
antagonism of glucocorticoids by endotoxin, specifically
the LPS-induced serum factor GAF. Experiments of this type

are often difficult to assess due not only to differences in response of animals of different genera, but to individual differences in response of animals in the same genus as well. Environmental conditions, general handling, and overall care may also be important to the animal's biological response. Thus the mechanism by which GAF exerts adrenal steroid antagonism must be resolved not only in terms of death or survival of animals, but also at the cellular level.

In order to understand how GAF antagonizes glucocorticoids, it is essential to understand how these hormones produce their effects. Glucocorticoids enter target cells and bind noncavalently and reversibly to cytosolic receptor proteins (138). Following dephosphorylation, these receptor proteins associate with nuclear chromatin to initiate transcription (138). Higgins and Gehring (75) showed this to be site specific. Thus it would follow that antagonism by GAF may also be site specific. In support of this, it has been shown that GAF blocks the glucocorticoid induction of PEPCK but not tyrosine aminotransferase (TAT) in mouse peritoneal exudate cell cultures (65). By using [^{3}H]dexamethasone as a stimulant, Berry and Shackleford (22) showed that LPS neither altered the entry of the hormone into hepatic cells nor inhibited the binding of the hormone to cytosolic receptor proteins. Adrenalectomized mice were used to limit the effect of endogenous glucocorticoids. This suggests that the hormone exerts its effects at a transcriptional or post-

transciptional level. It has been shown that induction of PEPCK by fasting (131) or hydrocortisone (Table 3) is the result of an increase in amount of enzyme and not altered enzyme activity. Rippe and Berry (131) also showed by radial immunodiffusion that the amount of PEPCK, induced by fasting, could be blocked by LPS. Employing the method of ELISA, this study shows that hydrocortisone induces an increase in the amount of PEPCK and this could be inhibited with either LPS or the serum rich in GAF (CpES) (Table 3). This is due to inhibition of synthesis and not a result of modification of the enzyme resulting in dysfunctional enzyme activity as the amount of PEPCK measured by ELISA correlated directly with the activity of the enzyme (r = 0.93 by the Spearman rank correlation coefficient shown by the activity to ELISA ratio in Table 3). Induction of PEPCK requires active protein synthesis as actinomycin D inhibits the in-crease in the amount of enzyme (131). In addition de novo synthesis of PEPCK is probable since the amount of mRNA for PEPCK is increased following induction by glucocorticoids (2,8,38,39,85,107,164). The fact that levels of mRNA for PEPCK are not induced by hydrocortisone during endotoxinemia (22) strongly suggest that antagonism by GAF occurs at the level of transcription. Further studies involving gene expression of readily available DNA have provided additional information in this area of research.

The transcription of mammary tumor virus (MTV) can

be stimulated 100-fold by glucocorticoids (129). A 1450 base
pair fragment of viral DNA containing a long terminal repeat
(LTR) is necessary for this induction to occur (54). This is
highly suggestive of a direct interaction of glucocorticoids
with DNA at the level of transcription. Also, binding of the
promoter region appears to be the rate-limiting step for in-
duction of transcription of MTV DNA by glucocorticoids as
the rate of initiation of transcription was unaltered (160).
This same study showed that post-transcriptional events,
splicing and polyadenylation, were also unaffected. It does
not seem unreasonable to propose the same mode of action by
glucocorticoids acts in the regulation of PEPCK, as well as
other target enzymes. If glucocorticoids act at site specific
promoter regions of target DNA, the antagonism by GAF may
also occur at this level. Several possibilities need to be
examined. GAF may alter the dephosphorylation of the cyto-
solic receptor protein, inhibit the transport of the acti-
vated receptor protein across the nuclear membrane, or block
the binding of the phosphoprotein to site specific DNA
sequences. Isolation and purification of GAF should lead
to more meaningful experiments on the mechanism by which it
antagonizes glucocorticoids.

These studies clearly show that a serum borne
mediator (GAF), produced by cells of the RES in response
to LPS or RD-LPS, antagonizes the effects of hydrocortisone
in inducing levels of hepatic PEPCK and subsequently gluco-

neogenesis. This is associated with a severe and terminal
hypoglycemia which may be incompatible with life. Animals
placed under conditions of stress where an adrenal cortical
response is required for survival are highly sensitized to
the lethal effects of LPS or RD-LPS and their respective
GAF-rich sera (CpES and CpdES, respectivley). Purification
of GAF would enable further research into the specific
physiological and molecular derangements incurred by this
glucocorticoid antagonist.

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