TRYPTOPHAN OXYGENASE ACTIVITY AND SENSITIVITY TO TRYPTOPHAN IN ADRENALECTOMIZED MICE

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
MELBA JEAN BEINE
1970

ABSTRACT

TRYPTOPHAN OXYGENASE ACTIVITY AND
SENSITIVITY TO TRYPTOPHAN IN
ADRENALECTOMIZED MICE

By

Melba Jean Beine

A study was undertaken to examine the possible correlation between depressed tryptophan oxygenase activity and sensitivity of adrenalectomized mice to tryptophan. Adrenalectomized mice were injected with 20 mg of L-tryptophan, alone, in combination with 5 mg or 0.5 mg of allopurinol, or in combination with 1 LD_{50} endotoxin. Sensitivity to tryptophan was estimated by increased numbers of deaths within 8 hours following tryptophan and/or by increased deaths in 48 hours.

Tryptophan oxygenase activity, significantly lower in adrenalectomized mice than in intact mice, was further depressed with allopurinol; however, adrenalectomized mice were not sensitive to trypotophan when the amino acid was given alone or in combination with allopurinol. This suggests that lowered tryptophan oxygenase activity *per se* does not increase sensitivity of adrenalectomized mice to tryptophan. Significant numbers of adrenalectomized mice

died within 8 hours following trypotophan when given concurrently or delayed 4 or 10 hours after 1 LD_{50} endotoxin. At these times, tryptophan oxygenase activity was elevated above the levels due to endotoxin alone, but were significantly lower than following only tryptophan, especially when compared with tryptophan-treated intact mice. Following delayed injection of tryptophan, tryptophan oxygenase activity attained only approximately control levels. These results suggest that endotoxin not only results in impaired ability to clear tryptophan by tryptophan oxygenase, but also has other effects on the host so that the response of adrenalectomized mice to tryptophan is altered.

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By

Melba Jean Beine

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology
and Public Health

1970

DEDICATION

To my parents,

to my Sisters, and

to my major professor.

ACKNOWLEDGMENTS

The author wishes to thank Dr. Robert J. Moon for his guidance and friendship throughout this investigation.

I am also grateful to Sister Joyce Hibbert for constant encouragement and for her help in typing this thesis.

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INTRODUCTION

Dramatic changes in host metabolism are among the numerous parameters altered by endotoxin poisoning (5-8). An enzyme involved in amino acid metabolism which has been extensively studied in this context is trypotophan oxygenase. This enzyme is an adaptive liver enzyme which regulates the conversion of tryptophan to formylkynurenine in the metabolic pathway leading to the biosynthesis of nicotinamide adenine dinucleotide.

Tryptophan oxygenase (L-tryptophan:oxygen oxidoreductase, EC 1.13.1.12) activity is decreased both in adrenalectomized animals and in endotoxin-poisoned animals (6,7,33,34,41). Adrenalectomized rats and endotoxin-poisoned mice show sensitivity to tryptophan when the amino acid is given at a time when tryptophan oxygenase activity is lowered (6,33,34,41). Increased sensitivity to tryptophan is defined as death within 8 hours following injection of tryptophan and/or increased deaths in 48 hours when compared with appropriate control groups. Such sensitivity may reflect a reduced ability to metabolize tryptophan through the kynurenine pathway, resulting in funnelling of excess

tryptophan into alternate metabolic pathways producing potentially toxic products, including serotonin.

The aim of this project was to determine whether or not a correlation exists between depressed tryptophan oxygenase activity and the sensitivity of adrenalectomized mice to tryptophan and endotoxin. Treatments involved administration of L-tryptophan alone, or concurrent with, or 4 or 10 hours after 1 LD_{50} endotoxin. Similar observations were made in mice injected with tryptophan and either 5 mg or 0.5 mg allopurinol. The latter compound has been shown to decrease tryptophan oxygenase activity activity *in vivo*, as well as *in vitro* (3,12,13,31), and was used to simulate the endotoxin-induced depression of the enzyme activity.

LITERATURE REVIEW

Tryptophan oxygenase is an iron porphyrin enzyme which exists in both the ferrous and ferric states (60), as indicated by its absorption spectrum and inhibition reactions characteristic of iron porphyrin enzymes, such as light-reversible carbon monoxide inhibition, and inhibition by cyanide or ferricyanide. Inhibition of tryptophan oxygenase activity by cyanide and ferricyanide or by catalase could be lessened or prevented with ascorbic acid or peroxide (60). That the inhibition of tryptophan oxygenase by these compounds is reversible with reducing agents indicates the enzyme is active when in the reduced ferrous state and inactive as the oxidized ferric form (42,60).

Isolation and purification of tryptophan oxygenase indicate the existance of 3 forms: an apoenzyme (26), as well as an oxidized and reduced holoenzyme (60). Both the apoenzyme and oxidized holoenzyme require further activation to become catalytically active as the reduced holoenzyme. The overall <u>in vitro</u> activation process requires the presence of the substrate L-tryptophan, the prosthetic group hematin, and a reducing agent, such as ascorbic acid or H_2O_2(19,20,25,26,34,36,37,42,60). A sequential series of

events occurs in the substrate-mediated activation process (42). Inactive apotryptophan oxygenase formed from de novo synthesis is conjugated with its prosthetic group hematin to form the oxidized holoenzyme. This step requires the presence of L-tryptophan or certain analogues and can be inhibited by globin or thiol reagents. A second reaction involves the reduction of the oxidized holoenzymes to the catalytically active reduced holoenzyme. The reduction process involves ascorbic acid or H_2O_2 and has a specific requirement for L-tryptophan. Reversible loss of the active, reduced holotryptophan oxygenase to the inactive, oxidized holoenzyme can occur by oxidation in air following removal of L-tryptophan.

Tryptophan oxygenase from tryptophan-treated rats is more saturated with hematin than the enzyme from untreated or hydrocortisone-treated rats (18). This increased saturation with hematin significantly increases the ratio of holo- to apoenzyme following tryptophan treatment (25); this ratio is relatively unchanged by hydrocortisone treatment (18). Further elevation of tryptophan oxygenase activity beyond activation of latent enzyme occurs following administration of corticosteroids or tryptophan as a result of de novo apoenzyme synthesis (14,15,18,21,22,27,29,33,35,44-47).

Both corticosteroids and tryptophan increase the amount of apoenzyme present, as determined by radioactive label incorporation into proteins and RNA (18), by the

effects of inhibitors of protein synthesis (26,29,46), and by specific immunological reactions (21,22). Both cortisone and tryptophan increase ^{14}C-glycine incorporation into liver proteins which correspond with changing tryptophan oxygenase activities, but only cortisone stimulates ^{32}P-orthophosphate incorporation into RNA at a time corresponding to its effects on tryptophan oxygenase activity. Following tryptophan administration, there is no increase in RNA labeling while the enzyme activity is rising. These results imply that the mechanism of cortisone-mediated elevation of tryptophan oxygenase activity involves stimulation of RNA synthesis, whereas tryptophan-mediated elevation involves a different process, namely, decreased enzyme degradation. These conclusions were confirmed by the action of puromycin and actinomycin D on cortisone-mediated and tryptophan-mediated increases of tryptophan oxygenase activity. Both types of induction were greatly diminished by puromycin, an inhibitor of protein synthesis at the translation level. Only hormonal induction was inhibited by actinomycin D, a compound which exhibits DNA-dependent RNA polymerase, thus inhibiting RNA synthesis.

The decreased rate of enzyme degradation caused by tryptophan, as well as some of its analogues, is effective *in vitro* (15,18,47), as well as *in vivo* (44,46,47). *In vitro* stabilization can be measured by comparing the effect of L-tryptophan or its analogues on the loss of tryptophan

oxygenase activity normally occurring on incubation without tryptophan, or on loss of activity following inactivation with heat, ethanol, urea or trypsin (47). In vivo stabilization of tryptophan oxygeanse in rats was indicated by retention of pre-labeled tryptophan oxygenase levels following tryptophan administration, whereas in control rats without tryptophan, there was a rapid loss of total counts precipitated by the specific anti-tryptophan oxygenase antiserum.

Significant influence of corticosteroids on tryptophan oxygenase activity is also implied since the control levels of tryptophan oxygenase activity are significantly lower in adrenalectomized mice (6) and rats (33-35) than in intact animals. Additional factors may influence tryptophan oxygenase activity, such as stimulation of the hypothalmus (49), presence of pituitary growth hormone (16,38), tryptophan analogues (15,23,41,47) or tryptophan metabolites (10,23).

Previous reports have noted increases in tryptophan oxygenase activity by purines (11,13,31) and by 3', 5'-cyclic AMP (12). The probable action of purines is thought to occur through their conversion to hypoxanthine. This is indicated in that blocking the conversion of cyclic AMP to hypoxanthine with theophylline prevents activation of tryptophan oxygenase by cyclic AMP (12). It has recently been suggested that xanthine oxidase may be operative in

the regulation of tryptophan oxygenase activity (12,31). Xanthine oxidase, present in soluble (rat) liver preparations, activates tryptophan oxygenase when its substrate hypoxanthine is present. Removal of xanthine oxidase from the soluble fraction of rat liver preparations with specific antibody resulted in loss of tryptophan oxygenase by purines (31). Because activation of purines was not dependent on the presence of hematin (12) or hemoglobin (31), xanthine oxidase apparently activates tryptophan oxygenase by causing the reduction of the inactive holoenzyme, possibly due to the production of H_2O_2, one of the products of xanthine oxidase reaction (12).

Inhibition of xanthine oxidase with allopurinol inhibits tryptophan oxygenase activity *in vitro* (3,12,31). *In vivo*, allopurinol was found to be potent inhibitor of tryptophan oxygenase, in that, 4 hours after an injection of allopurinol (20mg/kg) to normal rats, tryptophan oxygenase activity was inhibited 90-95% (3,31). Mice were similarly inhibited by lower doses (3). Allopurinol-mediated inhibition of tryptophan oxygenase activity is not due to a direct effect on the enzyme, in that allopurinol does not inhibit tryptophan oxygenase activity in a purified preparation of the apoenzyme, thus implying allopurinol does not inhibit conjugation of the apoenzyme with hematin (31). Nor does allopurinol significantly inhibit hydrocortisone-mediated synthesis of tryptophan oxygenase, as measured by

precipitation of the enzyme with specific anti-tryptophan oxygenase antibodies (31). Addition of ascorbic acid prevented inhibition of tryptophan oxygenase by allopurinol (12), thus implying allopurinol inhibits tryptophan oxygenase activity at the step of the reduction of the oxidized holoenzyme, possibly by the inhibition of xanthine oxidase activation of tryptophan oxygenase.

Tryptophan oxygenase activity is depressed in endotoxin-poisoned mice (1,6,7.41). Because tryptophan oxygenase is the first enzyme in the pathway leading to the formation of pyridine nucleotides, its depression implies a block in the biosynthesis of such compounds, the consequence of which could be of biological significance in endotoxin poisoning. This concept is consistent with evidence that administration of NAD or nicotinamide concurrent with endotoxin protects mice against endotoxin lethality (6). A decrease in the levels of total oxidized pyridine nucleotide found in the liver of endotoxin-poisoned mice could be prevented by concurrent administration of nicotinamide which, when given alone, approximately doubled the control levels of total oxidized pyridine nucleotides in 17 hours.

Cortisone, known to elevate tryptophan oxygenase activity, (19,20,35) protects mice against endotoxin lethality (7,24,37,41) and also maintains the levels of total oxidized pyridine nucleotides (7). The protective role of cortisone, as well as its ability to elevate tryptophan oxygenase activity, is dependent on the time of

administration relative to the administration of endotoxin (6,7). Thus, 5 mg cortisone, given concurrent with 1 LD_{50} of endotoxin, increased survival of mice and elevated tryptophan oxygenase activity; however, if given more than one hour after endotoxin, cortisone failed to protect mice and failed to maintain tryptophan oxygenase activity at control levels.

Certain inhibitors of protein synthesis, namely actinomycin D, ethionine, 2-thiouracil, and 8-azaguanine, were found to potentiate endotoxin lethality and to prevent cortisone protection against endotoxin (7). These same compounds prevented cortisone elevation of tryptophan oxygenase activity in normal mice and prevented cortisone maintainance of tryptophan oxygenase activity at control levels in endotoxin-poisoned mice when endotoxin, cortisone and inhibitor were given concurrently. In the above instances, maintainance of tryptophan oxygenase activity at control levels during endotoxin-poisoning was associated with protection against the lethal effects of endotoxin.

Although tryptophan elevated tryptophan oxygenase activity in untreated mice, it failed to maintain control levels of activity when given concurrent with 1 LD_{50} endotoxin. Concurrent injections of tryptophan and endotoxin also failed to protect against lethality (6,41). Elevation of tryptophan oxygenase activity with α-methyltryptophan did not increase survival of endotoxin-poisoned

mice. Correspondingly, depression of tryptophan oxygenase activity with 5-hydroxytryptophan did not increase susceptibility of mice to endotoxin (41). These results imply that tryptophan oxygenase per se is not directly related to survival of endotoxin-poisoned mice.

A delayed injection of tryptophan, given 4 hours after endotoxin, at a time when tryptophan oxygenase activity was below control levels, potentiated the lethal effects of endotoxin, resulting in convulsive death, such that the LD_{50} became LD_{100} (6,41). This effect was prevented by prior treatment with cyproheptadine, an anti-serotonin drug (41). Convulsive deaths did not occur and cyproheptadine had no effect on mortality when endotoxin and tryptophan were given concurrently. Protection against convulsive death with cyproheptadine, at a time when tryptophan oxygenase activity was lowered by endotoxin, suggests that tryptophan is converted in excess to serotonin, possibly as a result of the lowered tryptophan oxygenase activity. Further implications of funnelling of tryptophan into serotonin synthesis come from protection by cyproheptadine against the hypothermia produced in mice kept at 15 C given serotonin alone or against increased hypothermia produced by tryptophan in endotoxin-poisoned mice (40). Although tryptophan oxygenase probably does not influence survival of mice given endotoxin, it may be a factor in convulsive death seen in endotoxin-poisoned mice given tryptophan.

MATERIALS AND METHODS

Mice: Eighteen to 20 gram, female CF-1 mice (Carworth Farms, Portage, Michigan) were used in all experiments. They were housed, 10 per cage, with pine wood chips as bedding. Food (Purina mouse chow, Ralston Purina Co., St. Louis, Missouri) and water were available ad libitum, unless otherwise noted. Adrenalectomized mice received 0.9% saline as drinking water.

Adrenalectomy procedures: Hair was removed from the backs of mice using electric hair clippers. Mice were anesthesized with 0.25 mg Nembutal (sodium pentobarbital) injected subcutaneously in 0.1 ml of 0.85% NaCl, followed 10-15 minutes later by light etherization. After etherization, the mice were restrained on a board with tape. The back of the mouse was disinfected with 0.2% iodine (Rexall). A 2-cm incision was made through the skin over the back bone, using small surgical scissors. The skin was pulled to the right side and a 1-cm incision was made through the muscle layer, beginning near the rib cage and extending down at a slight angle. The adrenal gland was removed using two pairs of straight point surgical forceps. The

skin was then pulled to the left side and the procedure repeated, removing the other adrenal gland. No stitches were used to close the muscle layer incisions. The medial dorsal incision was closed with two stitches of silk surgical thread, gauge 000, using a 4-0 suture needle.

Completeness of adrenalectomy was determined 24-48 hours prior to experimentation utilizing the water-retention test of Beatty (2). Mice were used for experimentation 6-8 days after surgery.

Tryptophan oxygenase assay: Tryptophan oxygenase was assayed by the procedure of Knox and Auerbach (35) as modified in our laboratory to insure activation of all latent enzyme. Mice were fasted overnight prior to experimentation, normal mice for 12-18 hours and adrenalectomized mice for 8-12 hours. Mice were sacrificed by cervical dislocation and liver removed. Approximately 1 gram of mouse liver (wet weight) was homogenized in 7 ml ice cold homogenizing medium, composed of 0.14 M KCl, 0.0025 M NaOH, and 0.01 M L-tryptophan, the latter served as substrate for the reaction. Two ml of each homogenate were dried in crucibles to determine dry weights. Additional 2-ml aliquots of each homogenate were added to two 25-ml Erlenmeyer flasks (a and b). Each reaction flask contained 3.0 ml distilled water, 2.0 ml $Na_2HPO_4 \cdot KH_2PO_4$ buffer, pH 7.0, and 1.0 ml methemoglobin (4 mg/ml). Methemoglobin (recrystalized bovine hemoglobin) was purchased from Nutritional

Biochemical Corp., Cleveland, Ohio. The flasks were incubated at 37 C for 30 minutes on a Forma Thermo-Shaker (Forma Scientific, Inc., Marietta, Ohio), after which the reaction in flask b of each pair was stopped by the addition of 4 ml of 15% metaphosphoric acid. The reaction in flask a was allowed to proceed for an additional 60 minutes, then stopped as stated above. The reaction mixture was filtered and 6.0 ml of each filtrate was neutralized by the addition of 2.0 ml of 1.5 N NaOH. The optical density of the filtrate was determined at 360 nm with a Beckman model B spectrophotometer (Beckman Instruments, Inc., South Pasadena). This wavelength is the maximum for kynurenine which was read as the product of tryptophan oxygenase since all formylkynurenine was converted immediately to kynurenine by the enzyme formylase present in excess in the homogenate. The amount of kynurenine formed per hour was calculated from the difference in optical density between flask a (stopped after 90 minutes reaction time) and flask b (stopped after 30 minutes). The quantity of product was estimated from a standard curve and the results expressed in umoles kynurenine per gram dry weight of liver per hour (uM K/gm/hr).

Endotoxin: Heat-killed cells of Salmonella typhimurium, strain SR-11, suspended in isotonic saline served as the source of endotoxin. Heat-killed cells were prepared in the following manner. Overnight cultures (18 hr) were grown in 500ml brain heart infusion broth to a

concentration of approximately 10^9 cells/ml. The cells were concentrated by centrifugation at 6000 x g for 5 minutes in a Sorvall SS-1 table centrifuge. Thirty ml aliquots of the culture were centrifuged, the supernant decanted, and additional culture added. This procedure was repeated until all the culture was centrifuged. The cells were washed twice with non-pyrogenic isotonic saline (Baxter Laboratory, Morton Grove, Illinois) and resuspended in saline to approximately 10 times the original concentration. The pooled cells were heat killed by autoclaving at 6 lb pressure and 222 F for 6 minutes. The preparation was judged sterile by lack of growth in 48 hours on subculture in brain heart infusion broth. The LD_{50} of this preparation for both normal and adrenalectomized mice were determined according to the method of Reed and Munch (43). The LD_{50} dose for normal mice was a 1:4 dilution of the final preparation and that for adrenalectomized mice was a 1:1000 dilution.

Chemicals: Nembutal (sodium pentobarbital), 50 mg/ml, was purchased from a local supply house. L-tryptophan was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Allopurinol, "Zyloprim" brand, Burroughs Wellcome and Co., Tuchahoe, New York, was obtained through the courtesy of Dr. Stanley T. Bloomfield, Medical Department. This latter preparation was injected as a suspension stabilized with 0.01% Methocel (Dow Chemical Co., Midland, Michigan).

RESULTS

Effect of single or repeated injections of tryptophan on tryptophan oxygenase activity and survival of adrenalectomized mice: The control level of tryptophan oxygenase activity was significantly lower ($P<0.01$) in adrenalectomized mice than in normal mice (Figure 1). Tryptophan oxygenase activity reached a maximum in adrenalectomized mice in 2 hours after injection of 20 mg of L-tryptophan and returned to control levels within 6 hours. Tryptophan induction of the enzyme in normal mice is greater in both magnitude and duration, as shown by a reference curve in Figure 1.

Further elevation of tryptophan oxygenase activity in adrenalectomized mice could be attained with a second injection of tryptophan given 2 hours after the first, at a time indicated by an arrow in Figure 1. Maximum activity occurred 2 hours after the second injection; the activity returned to control levels within 8 hours. The maximum activity attained after 2 injections was significantly higher ($P<0.01$) than after 1 injection.

No deaths occurred following a single injection of 20 mg of L-tryptophan in adrenalectomized mice. Although

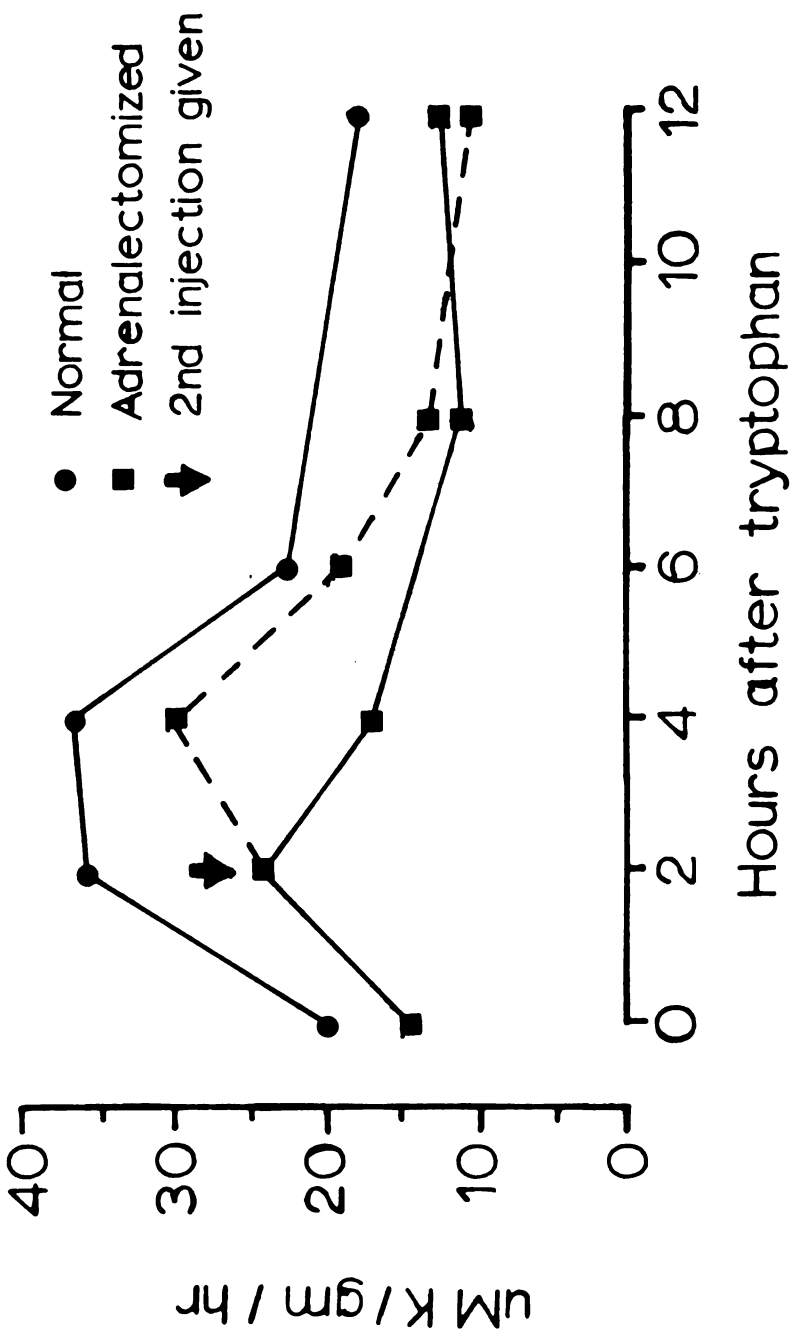

Figure 1. Tryptophan oxygenase activity in adrenalectomized and normal mice given single or repeated injection of 20 mg of L-tryptophan.

Table 1. Survival of adrenalectomized mice given a single injection of 20 mg L-tryptophan or 2 injections of 20 mg L-tryptophan 2 hours apart.

Experimental Treatment	# survivors / total injected		
	Hours		
	8	12	48
20 mg L-tryptophan alone	10/10	10/10	10/10[a]
20 mg L-tryptophan plus 20 mg L-tryptophan 2 hours later	7/7	7/7	5/7[b]

b vs a Not statistically significant

2 of 7 adrenalectomized mice died following 2 injections of L-tryptophan given 2 hours apart, the numbers were not statistically different from appropriate controls (Table 1). Single or repeated injections of L-tryptophan were not lethal for normal mice (30).

Effect of allopurinol, alone or in combination with tryptophan, on tryptophan oxygenase activity and survival of adrenalectomized mice: Five or 0.5 mg of allopurinol depressed tryptophan oxygenase activity in adrenalectomized mice to approximately the same levels within 2 hours (Figure 2). The depressed activity persisted in both cases for at least 12 hours. A similar depression was seen in normal mice given 5 mg of allopurinol. Approximately 50% of the adrenalectomized mice died in 48 hours following subcutaneous injection of 5 mg of allopurinol (Table 2). Only 1 of 10 adrenalectomized mice died following injection of 1 mg of allopurinol. There were no deaths among the group given 0.5 mg of allopurinol.

Concurrent injection of 5 mg of allopurinol and 20 mg of L-tryptophan elevated tryptophan oxygenase activity in adrenalectomized mice (Figure 3). The maximum enzyme activity attained 2 hours after this treatment was not significantly different from that attained after 20 mg of L-tryptophan alone. The enzyme activity returned to control levels by 4 hours and was below control levels by 6 hours. These results show no significant inhibition of tryptophan-

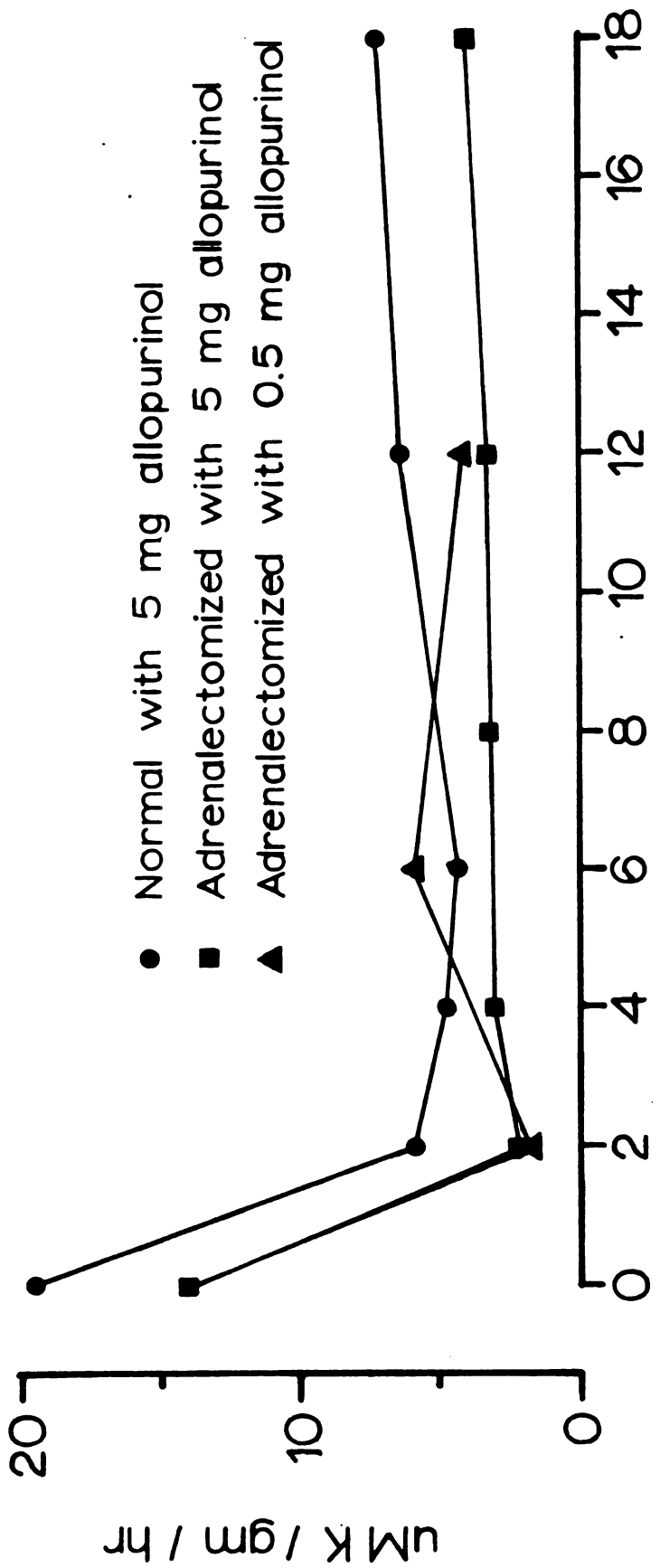

Figure 2. Tryptophan oxygenase activity in adrenalectomized mice given 5 mg or 0.5 mg of allopurinol and in normal mice given 5 mg allopurinol.

Table 2. Survival of adrenalectomized mice following various doses of allopurinol.

Experimental Treatment	# survivors / total injected		
	Hours		
	8	12	48
5 mg allopurinol	10/10	9/10	5/10
1 mg allopurinol	10/10	10/10	9/10
0.5 mg allopurinol	10/10	10/10	10/10

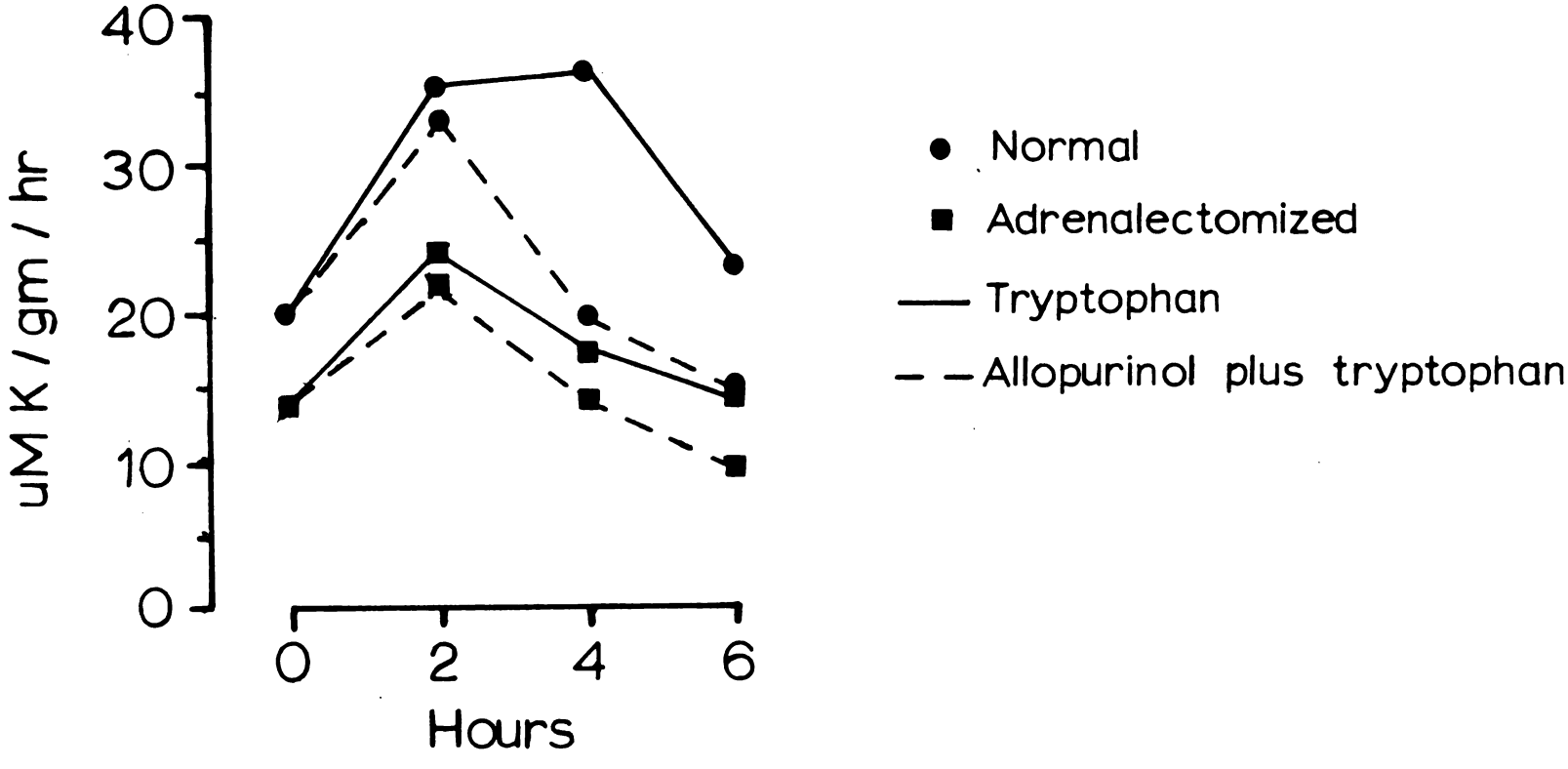

Figure 3. Tryptophan oxygenase activity in adrenalectomized and normal mice given concurrent injection of 5 mg of allopurinol and 20 mg of L-tryptophan. Reference curves following 20 mg of L-tryptophan are given.

mediated elevation of tryptophan oxygenase activity following concurrent injections of 5 mg of allopurinol and 20 mg of L-tryptophan in adrenalectomized mice, except for borderline significance at the 6-hour time period. A similar induction pattern of tryptophan oxygenase activity was seen in normal mice following concurrent injections of allopurinol and tryptophan. There was no significant difference in the activity at the 2-hour time period, but differences were highly significant at 4 and 6 hours.

Administration of allopurinol concurrent with or 4 hours prior to tryptophan did not increase sensitivity of adrenalectomized mice to tryptophan with either dose of allopurinol used (Table 3). Although approximately 10% of normal mice died within 48 hours following 5 mg of allopurinol, given alone or concurrent with or 4 hours prior to 20 mg of L-tryptophan, this treatment did not significantly increase the sensitivity of normal mice to tryptophan (data not shown).

Effect of endotoxin, alone or in combination with tryptophan, on tryptophan oxygenase activity and survival of adrenalectomized mice: Determination of LD_{50} doses of endotoxin for normal and adrenalectomized mice indicates a greater sensitivity of adrenalectomized mice to endotoxin (Table 4). Such an effect is consistant with previous observations (5). The LD_{50} dose for normal mice was 1 to 4 dilution of the endotoxin preparation, corresponding to

Table 3. Survival of adrenalectomized mice following administration of 5 mg or 0.5 mg allopurinol in combination with 20 mg L-tryptophan.

Experimental Treatment	# survivors / total injected		
	Hours		
	8	12	48
5 mg allopurinol alone	10/10	10/10	5/10
5 mg allopurinol plus 20 mg L-tryptophan concurrently	11/11	10/11	6/11
5 mg allopurinol plus 20 mg L-tryptophan 4 hours later	20/20	20/20	16/20
0.5 mg allopurinol alone	10/10	10/10	10/10
0.5 mg allopurinol plus 20 mg L-tryptophan concurrently	10/10	10/10	10/10
0.5 mg allopurinol plus 20 mg L-tryptophan 4 hours later	10/10	9/10	9/10

Table 4. Determination of LD_{50} doses of endotoxin for normal and adrenalectomized mice.

Experimental Treatment	# survivors (48 hours)	Percent survival	# cells / 0.5 ml
Normal mice			
undilute	0/10	0	2.9×10^{10}
1:2	1/10	10	1.4×10^{10}
1:4	4/10	40	7×10^{9}
Adrenalectomized mice			
1:1000	4/9	45	2.9×10^{7}
1:2000	6/9	67	1.4×10^{7}

approximately 7 x 10^9 bacterial cells per 0.5 ml dose. The LD_{50} for adrenalectomized mice was a 1 to 1000 dilution of the endotoxin preparation, corresponding to approximately 2.9 x 10^7 bacterial cells per 0.5 ml. This dose was equivalent to a 1/250th LD_{50} dose for normal mice.

Adrenalectomized mice showed increased sensitivity to tryptophan when the amino acid was given concurrent with or 4 or 10 hours after 1 LD_{50} endotoxin, as estimated by significant increases in deaths 8 hours after tryptophan was injected (Table 5). There was no significant alteration in the number of deaths after 48 hours in either of the three cases, as compared with endotoxin-poisoned mice.

Adrenalectomized mice given 1 LD_{50} endotoxin showed a transient increase in tryptophan oxygenase activity, followed by depressed activity which returned to near control levels within 18 hours (Figure 4). A similar effect was noted in normal mice; however, the activity remained depressed at least 18 hours.

Figure 5 shows the effect of an injection of 20 mg of L-tryptophan given to adrenalectomized mice at various times following administration of 1 LD_{50} endotoxin. When tryptophan was given concurrent with endotoxin or delayed 4 or 10 hours, tryptophan oxygenase activity was elevated above the level following endotoxin alone. Following concurrent injections, the enzyme activity remained significantly elevated for at least 8 hours above the activity

Table 5. Survival of adrenalectomized mice following 1 LD_{50} endotoxin, given alone or in combination with 20 mg L-tryptophan.

Experimental Treatment	# survivors / total injected			
	Hours			
	8	12	18	48
1 LD_{50} endotoxin alone	20/20[a]	20/20[c]	18/20[e]	10/20[g]
Endotoxin plus 20 mg L-tryptophan concurrently	12/20[b]	10/20	10/20	6/20[h]
Endotoxin plus 20 mg L-tryptophan after 4 hours	17/20	15/20[d]	14/20	13/20[i]
Endotoxin plus 20 mg L-tryptophan after 10 hours	20/20	19/20	13/20[f]	11/20[j]

b vs a P<0.005
d vs c P<0.01
f vs e P<0.05

h vs g N.S.S.
i vs g N.S.S.
j vs g N.S.S.

N.S.S. = Not statistically significant

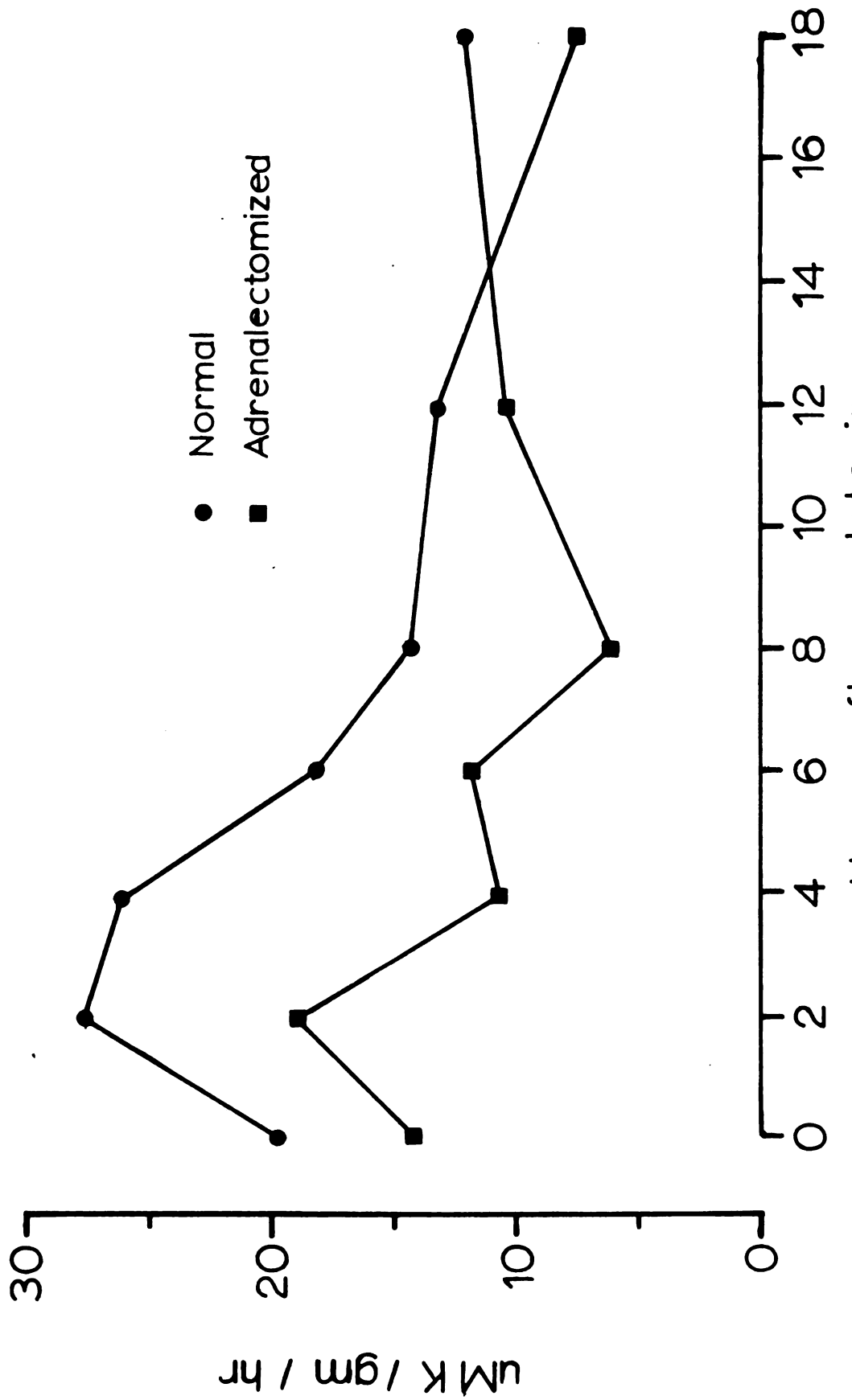

Figure 4. Tryptophan oxygenase activity in adrenalectomized and normal mice given 1 LD_{50} endotoxin.

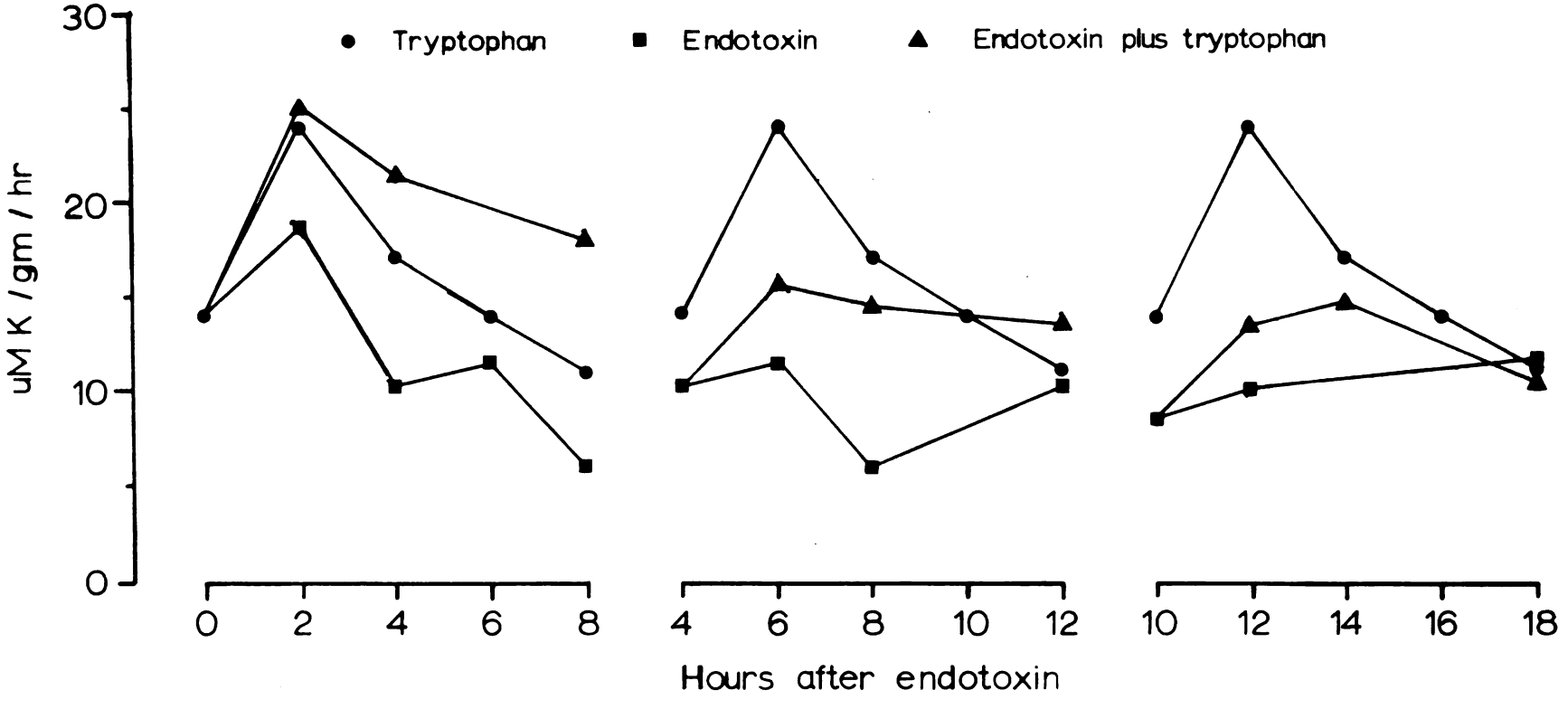

Figure 5. Tryptophan oxygenase activity in adrenalectomized mice given 20 mg of L-tryptophan concurrent with or 4 or 10 hours after 1 LD_{50} endotoxin.

due to either endotoxin or tryptophan alone. Delayed injection of tryptophan 4 hours after 1 LD_{50} endotoxin resulted in elevation of tryptophan oxygenase activity to approximately control levels in 2 hours after tryptophan; this level was maintained for at least 8 hours. Similar induction of tryptophan oxygenase activity to control levels followed injection of 20 mg tryptophan 10 hours after 1 LD_{50} endotoxin. These initial increases in tryptophan oxygenase activity decreased as the time span lengthened between injections of endotoxin and tryptophan, not only with respect to the level following endotoxin alone, but also with respect to levels following injection of tryptophan alone. These increases attained only control levels, except for concurrent injection of endotoxin and tryptophan, which elevated tryptophan oxygenase activity to a level similar to that following tryptophan alone.

DISCUSSION

Studies by Knox (33,34) indicate adrenalectomized rats are extremely sensitive to tryptophan, in that all died within 4-8 hours following an injection of 100 mg L-tryptophan / 100 gm body weight. A similar dose of tryptophan given as a single or repeated injection does not have this effect on adrenalectomized mice. Tryptophan oxygenase activity can be induced by substrate in both adrenalectomized rats (33,34) and adrenalectomized mice (5, Figure 1); however the enzyme remains significantly lower at all times when compared with normal animals, suggesting the participation of endogenous corticosteroids on the enzyme response on normal animals. That this enzyme is effected by corticosteroids has been well established (14,33,35,45).

Adrenalectomized mice are sensitive to 5 mg of allopurinol, a dose relatively nontoxic for normal mice. Decreasing doses of 1 mg and 0.5 mg of allopurinol were successively less toxic for adrenalectomized mice. A probable explanation of the toxicity of allopurinol is that allopurinol-mediated inhibition of xanthine oxidase causes an accumulation of xanthine beyond urine saturation levels which, in small

animals, results in crystallization of xanthine in renal tubules (30). It has been found that allopurinol exhibits a progressively greater toxicity, the smaller the animal being tested, from man to dog to rat to mouse.

Just as survival following allopurinol is dose-dependent, (30) the response of tryptophan oxygenase to allopurinol is also (3). Twenty-four hours after an injection of 10 or 20 mg/kg body weight of allopurinol, tryptophan oxygenase activity in rats was 60% of the control level, but that with smaller doses, the activity returned to normal in 8-10 hours. Tryptophan oxygenase activity in mice, inhibited with 2 or 8 mg/kg allopurinol, returned to control levels within 20-24 hours. Allopurinol-mediated inhibition of tryptophan oxygenase activity persists in both normal and adrenalectomized mice at least 18 hours following injection of 5 mg allopurinol and in adrenalectomized mice at least 12 hours after 0.5 mg allopurinol (Figure 2).

Concurrent injection of allopurinol and tryptophan elevated tryptophan oxygenase activity in both normal and adrenalectomized mice; however, these increases in 2 hours are not significantly different than following tryptophan alone. Julian and Chytil (31) have shown that in normal rats concurrent injection of allopurinol (20 mg/kg) and L-tryptophan (1.0 mg/kg) resulted in significant (50-60%) inhibition of tryptophan oxygenase activity in 4 hours. A possible explanation for the discrepency in the amount

of inhibition at a time of maximum tryptophan oxygenase activity lies in the dose of tryptophan used. The dose used for rats was 1.0 mg/kg body weight, whereas that for mice in these experiments was 20 mg/mouse, equivalent to 1000 mg/kg body weight. Thus, with a thousand-fold increase in the amount of tryptophan administered, one might expect less inhibition of tryptophan oxygenase activity by allopurinol.

Inhibition of tryptophan oxygenase activity with allopurinol did not increase the susceptibility of adrenalectomized mice to tryptophan (Table 3). This suggests that lowered tryptophan oxygenase activity *per se* does not increase sensitivity of adrenalectomized mice to tryptophan. A similar effect was noted in normal mice (Moon, submitted for publication).

Adrenalectomized animals are more susceptible to stress than normal animals, presumably due to their inability to respond with adrenal hormones (5,6,9,33,34). Thus, it is not surprising that the LD_{50} endotoxin for adrenalectomized mice was 1/250th the dose required for normal mice (Table 4).

Significant numbers of endotoxin-poisoned adrenalectomized mice given tryptophan died within 8 hours after administration of the amino acid (Table 5). Many of these mice were in convulsions prior to death. Although the cause of convulsive death is not known, several possible explanations have been offered. One is based on the

observation that endotoxin depletes liver glycogen and blood sugar levels (5,7,8,41,48). Normal endotoxin-poisoned mice given tryptophan are more hypoglycemic than mice given endotoxin alone (Moon, unpublished data). Thus one factor in convulsive death seen in endotoxin-poisoned mice given tryptophan may result from extreme hypoglycemia. Another possible explanation suggests that excess serotonin is produced from tryptophan due to inhibition of tryptophan oxygenase. This explanation is favored by evidence of protection against endotoxin-induced convulsive death in normal mice by cyproheptadine, an anti-serotonin drug (41).

Following 1 LD_{50} endotoxin, tryptophan oxygenase activity in adrenalectomized mice was initially elevated, then depressed below control levels. The activity returned to near normal levels in 18 hours. A similar response was observed in normal mice; however, the activity remained lowered for at least 18 hours. When tryptophan was given concurrently or 4 or 10 hours after endotoxin, its ability to elevate tryptophan oxygenase activity in adrenalectomized mice at each of these times was not completely destroyed by endotoxin treatment. The increases in activity decreased as the time between endotoxin treatment and administration of tryptophan lengthened, implying a progressive inhibition of tryptophan oxygenase by endotoxin.

The time relationship between tryptophan induction of tryptophan oxygenase activity and convulsive deaths in

adrenalectomized mice was similar to that observed in adrenalectomized rats (33,34): tryptophan oxygenase activity was elevated above control levels prior to and at the time of death. The evidence that the enzyme was elevated during this time suggests that either the activity of tryptophan oxygenase does not relate to survival of endotoxin-poisoned adrenalectomized mice given tryptophan or that the elevated activity is not accompanied by a corresponding increase in the efficiency of tryptophan oxygenase.

Kim and Miller (32) have shown that an increase in tryptophan oxygenase activity in intact or adrenalectomized rats was not associated with increased conversion of ^{14}C-tryptophan to $^{14}CO_2$ unless the substrate load was increased. Others (18) have shown that there is an increased amount of active holotryptophan oxygenase, as well as apoenzyme, following administration of tryptophan. In contrast, there was little increase in the amount of active holoenzyme following cortisone treatment, although this resulted in high levels of apoenzyme. Because the elevated tryptophan oxygenase activity in endotoxin-poisoned adrenalectomized mice was induced with tryptophan, rather than with cortisone, the latter possibility that increased tryptophan oxygenase activity was not accompanied by increased efficiency of clearing tryptophan, is lessened.

Because depression of tryptophan oxygenase activity with allopurinol did not increase susceptibility of adrenalectomized mice to tryptophan and because tryptophan

oxygenase activity was elevated by tryptophan in endotoxin-poisoned adrenalectomized mice at the time of death, tryptophan oxygenase activity apparently has little influence on survival following such treatment. This has been previously suggested (41). However, in spite of the elevation of tryptophan oxygenase by tryptophan in endotoxin-poisoned adrenalectomized mice, the overall increases in activity are greatly lessoned when compared to the capacity of normal mice given tryptophan (cf. Figure 1). This implies that the elevated activity may not be sufficient to handle the 20 mg dose of L-tryptophan, and thus, excess tryptophan may be funnelled into other pathways, including the one to serotonin.

Endotoxin-poisoned adrenalectomized mice were sensitive to both concurrent and delayed injections of tryptophan, whereas normal mice were sensitive to tryptophan only when tryptophan oxygenase activity was depressed with endotoxin (41). This suggests the possibility that endotoxin has effects on the host, in addition to those on tryptophan oxygenase, which are important in the response of such animals to tryptophan. These latter effects are not understood at this time.

SUMMARY AND CONCLUSIONS

Adrenalectomized mice do not exhibit increased sensitivity to tryptophan when the amino acid is given alone or in combination with allopurinol. These mice, die in greater numbers within 8 hours when tryptophan is given concurrently or delayed 4 or 10 hours after 1 LD_{50} endotoxin. At these times, tryptophan oxygenase is elevated above the levels following endotoxin alone, but is significantly lower than levels following tryptophan alone, especially in reference to the levels in tryptophan-treated intact mice. This suggests a reduced ability to metabolize tryptophan by tryptophan oxygenase, leading to possible funnelling into other pathways. Lowered tryptophan oxygenase activity *per se* is not responsible for increased sensitivity to tryptophan, in that allopurinol inhibition of tryptophan oxygenase activity did not sensitize adrenalectomized mice to tryptophan. Thus, endotoxin may exert other effects on the host which alter its response to tryptophan.

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