EFFECTS OF BACTERIAL ENDOTOXINS ON METABOLISM

VI. The Role of Tryptophan Pyrrolase in Response of Mice to Endotoxin*

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The ability of certain adrenocortical hormones to protect experimental animals against the lethal effects of lipopolysaccharides derived from Gram-negative bacteria is well established (1–4). The underlying basis for this protection has not been determined. Brooke *et al.* (5) recently reported for several adrenocorticoids a lack of correlation between their antiinflammatory properties and their ability to protect against endotoxins. It is perhaps a matter of faith that leads one to the view that all such hormonal capabilities (and of other substances with pharmacological actions as well) are ascribable to primary biochemical or metabolic effects. The problem becomes a matter of detecting the significant enzymic "needle" from among the mammalian "haystack" of enzymes. A number of enzymes may become altered by hormonal injections after a sufficient delay but most of these changes are probably secondary or adaptive rather than primary. A hopeful way out of this challenging situation is to find alternate ways of modifying an enzyme to see if by both methods a protective effect against endotoxins results.

Such an approach to the problem of corticoid protection against endotoxin is made possible by the work of Knox and Auerbach (6) and Feigelson *et al.* (7). These investigators have shown that an injection of cortisone into rats is followed within about 4 hours by a significant increase in liver tryptophan pyrrolase. The enzyme also becomes more active following an injection of tryptophan, probably as a result of induced enzyme formation.

Very briefly, tryptophan pyrrolase, with hematin as cofactor, oxidatively converts tryptophan into formyl kynurenine which, in turn, is further degradated into nicotinic acid. The ultimate metabolic fate of tryptophan *via* this pathway is the incorporation of nicotinamide into the di- and triphosphopyridine nucleotides (DPN and TPN), compounds of major significance in the energy release by organisms.

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If an elevation in activity of tryptophan pyrrolase results in metabolic reactions of survival value in endotoxin poisoning, then tryptophan alone should protect mice against endotoxin somewhat commensurate with the protection afforded by any one of several adrenocorticoids. Moreover, the products yielded by the enzyme should also act similarly to the corticoids and to tryptophan if the reactions are of importance in an animal's response to endotoxin. With these considerations serving to guide the research, the results to be presented in this report were obtained.

Methods

Endotoxins.—As in previous reports (8, 9), the standard material employed as endotoxin is a saline suspension of heat-killed (pasteurized) *Salmonella typhimurium*, strain SR-11. A sufficient quantity of material for several months of work is prepared, dispensed in sealed tubes in 10 ml portions, and stored at 5°C until ready for use. The dry weight of the suspension is 6.0 mg per ml and the number of bacteria, as estimated by a dilution count prior to heating, is approximately 8×10^9 per ml. A soluble purified lipopolysaccharide (LPS) derived from *Escherichia coli* 02:kl, lot 3712:92a, generously supplied by Dr. C. W. deWitt, Upjohn Company, Kalamazoo, was used for comparative purposes. In some experiments LPS derived from *Serratia marcescens* (generously supplied by Dr. A. Nowotny, Temple University, Philadelphia) was employed.

Protection Experiments.—Two types of protection experiments were carried out. In the first, the test material and the endotoxin were each injected intraperitoneally (cortisone was given subcutaneously) within a time interval no greater than 10 to 15 minutes. In the second series of experiments, the endotoxin was given 4 hours before the test material which was sometimes given again 8 hours after the endotoxin. In this situation, the aim was to improve the condition of an animal already showing symptoms of endointoxication, whereas with concurrent injections the objective was to prevent the onset of illness.

All injections were given in a volume of 0.5 ml and all solutions were made up in pyrogen-free saline (Baxter Laboratories, Morton Grove, Illinois). Cortisone acetate (Nutritional Biochemicals Corp., Cleveland) was given in 5 mg amounts. Nicotinamide, 10 mg, nicotinic acid, 10 mg, and L-tryptophan, 20 mg (all from Nutritional Biochemicals Corp.) were also injected. Diphosphopyridine nucleotide (DPN) (Sigma Chemical Company, St. Louis) was given in 5 and 10 mg amounts. In some experiments, glucose was combined with one of the above reagents in 45 mg quantities and in one series it was added to 5 mg of adenosine triphosphate (ATP) (Sigma Chemical Company). The survival data are based on an observation period of 48 hours.

Tryptophan Pyrrolase Assay.—The method used by Knox and Auerbach (6) for the assay of tryptophan pyrrolase in rat liver was modified for application to mice. Livers were removed and homogenized immediately with a teflon and Pyrex homogenizer in cold KCl (0.14 M) made alkaline with NaOH (0.0025 N). Homogenates were kept in an ice-water bath until time of addition of an aliquot to the incubation flasks.

Reaction vessels (25 ml Erlenmeyer flasks equipped with stoppers for gassing) containing reagents were permitted to equilibrate at 38°C in a water bath with reciprocating shaker (New Brunswick Instrument Company) during the time required for the homogenization of livers. The flasks contained phosphate buffer, 0.2 M and pH 7.0, L-tryptophan, 0.03 M and distilled water to a final volume of 8.0 ml. The liver homogenate to be assayed was added last and the flasks were gassed for 5 minutes with 100 per cent oxygen. At the end of 1 hour in the shaker, the reaction was stopped by the addition of metaphosphoric acid. A "blank" determination was carried through for livers in each experimental group by omitting the tryptophan sub-

strate from a flask. Spectrophotometric readings for these determinations were constant (within less than 2 per cent variation) except under experimental conditions where the tryptophan pyrrolase activity was extremely high. Under these conditions, the "blank" was increased to about 10 per cent of the control values. In all cases, flasks with substrate were read against those to which no substrate was added.

Known amounts of kynurenine sulfate (0.5 to 5.0 μM per flask) were equilibrated in the presence of liver homogenate from normal animals and carried through the entire procedure. All reaction filtrates were neutralized with NaOH to pH 6.5–7.5 and read in a Beckman DU spectrophotometer at 360 mμ. Dry weights were determined for each sample of liver homogenate and enzyme activity was expressed as micromoles kynurenine formed per gram dry weight of liver per hour.

Assay for Pyridine Nucleotides.—A sample of liver, 300 to 500 mg, was removed, weighed, and immediately homogenized in 5 ml of 5 per cent trichloroacetic acid in a glass tube with teflon pestle. The precipitated proteins were removed by centrifugation at 2200 *g* for 20 minutes and aliquots of the clear supernatant were used for fluorescence measurements.

The method of Kaplan *et al.* (10) was followed for the quantitative determination of the oxidized pyridine nucleotides. These substances form stable fluorescent compounds in the presence of strong (5 N) alkali. DPN (Sigma Chemical Company) was used for the standard curve in the range of 0 to 1 μg/ml. All solutions were heated in a boiling water bath for 5 minutes to develop maximum fluorescence. A Turner fluorometer with a primary filter, No. 7–60 (360 mμ exciting light) and a secondary filter, No. 75 (490 mμ transmitted light) was used to measure fluorescence.

This method does not detect the reduced pyridine nucleotides (DPNH and TPNH) since they are rapidly inactivated during the acid extraction of the tissues (11). However, nicotinamide mononucleotide and nicotinamide riboside, like DPN, form fluorescent products on treatment with strong alkali. Methyl nicotinamide also contributes some fluorescence but only about 1/60 that of DPN.

Exposure of Mice to Cold (5°C).—Mice were placed in individual plexiglas compartments free of bedding and with the floor of the cage made of ¼ inch hardware cloth so that no moisture accumulated. Each mouse was provided with food and water and the cages were placed on the shelves of a walk-in refrigerator maintained at 5° ± 1°C. The animals were protected from drafts. Twelve hours of light and 12 hours of dark were assured by an automatic switch. No control over relative humidity was attempted.

Mice.—CF-1 female mice (Carworth Farms, New City, New York) were received weekly when weighing 16 to 18 gm. They were housed 10 per cage with pine shavings as bedding and were fed Dietrick and Gambrill's (Frederick, Maryland) pathogen-free mouse food. After 1 to 2 weeks when they weighed 20 ± 1 gm they were subjected to the different experimental procedures. Confirmatory experiments were similarly carried out in limited areas with Swiss-Webster mice purchased from another dealer (Dierolf Farms, Boyertown, Pennsylvania). The animal room and the laboratory in which the mice were held during experimental observations were both maintained at 25° ± 2°C. Food and water were available at all times unless specifically stated.

RESULTS

Protection Experiments with Concurrent Injections of Endotoxin and Metabolites.—In Table I, a comparison is made of the ability of different substances to protect mice against graded amounts of endotoxin administered in the form of heat-killed *S. typhimurium*. The dosages of endotoxin administered to control mice range from, approximately, 20 to 100 per cent lethal. These

TABLE I

Protection of Mice against Heat-Killed S. typhimurium with Compounds Related to Tryptophan Pyrrolase Activity

All injections were given intraperitoneally except cortisone, which was given subcutaneously. The endotoxin and the protective agent were injected in rapid succession. The *P* values, compared with controls, were calculated by the rank order test of Wilcoxon (16).

Substance injected at time of endotoxin	No. of mice surviving /total injected with weight of heat-killed *S. typhimurium* shown			
	0.75 mg	1.5 mg	3.0 mg	4.5 mg
Control, 0.5 ml saline	8/10	15/30	2/30	0/20
Cortisone, 5 mg (19 μM)		20/20	15/20	9/20
		$P = 0.008$	$P < 0.008$	$P = 0.014$
Nicotinamide, 10 mg (82 μM)	10/10	20/20	13/20	5/20
	N.S.*	$P = 0.008$	$P < 0.008$	$P > 0.05$
Nicotinic acid, 10 mg (81 μM)	0/10	0/10		
	$P < 0.008$	$P < 0.008$		
DPN, 10 mg (15 μM)		20/20	14/20	5/20
		$P = 0.008$	$P < 0.008$	$P > 0.05$
DPN, 5 mg (7.5 μM)		18/20	8/20	
		$P = 0.028$	$P = 0.057$	

* N.S. = not statistically significant.

TABLE II

Protection of Mice against E. coli Lipopolysaccharide with Compounds Related to Tryptophan Pyrrolase Activity

All injections were given intraperitoneally except cortisone, which was given subcutaneously. The endotoxin and the protective agent were injected in rapid succession. The *P* values compared with controls, were calculated by the rank order test of Wilcoxon (16).

Substance injected at time of endotoxin	No. of mice surviving/No. injected with 175 μg *E. coli* lipopolysaccharide
Control, saline 0.5 ml	9/20
Nicotinamide, 10 mg (82 μM)	17/20
	$P = 0.028$
Nicotinic acid, 10 mg (81 μM)	2/20
	$P = 0.057$
Tryptophan, 20 mg (100 μM)	14/20
	N.S.*
DPN, 10 mg (15.1 μM)	17/20
	$P = 0.028$

* N.S. = not statistically significant.

animals were also injected with 0.5 ml saline since treated animals received this volume of fluid containing the protective agent. Results with cortisone, the one substance known for more than a decade to be highly protective against

TABLE III

Treatment of Mice Injected with Heat-Killed S. typhimurium with Tryptophan Administered 4 Hours before, at Time of, and 4 Hours after the Endotoxin

All injections were given intraperitoneally. The *P* values, compared with controls, were calculated by the rank order test of Wilcoxon (16).

Times of tryptophan and endotoxin injection	No. of mice surviving/No. injected with 1.5 mg of heat-killed *S. typhimurium*
Control, saline 0.5 ml at time of endotoxin	15/30
Tryptophan, 20 mg 4 hrs. before endotoxin	14/20 N.S.*
Tryptophan, 20 mg at time of endotoxin	11/30 N.S.
Tryptophan, 20 mg 4 hrs. after endotoxin	0/20 $P = 0.008$

* N.S. = not statistically significant.

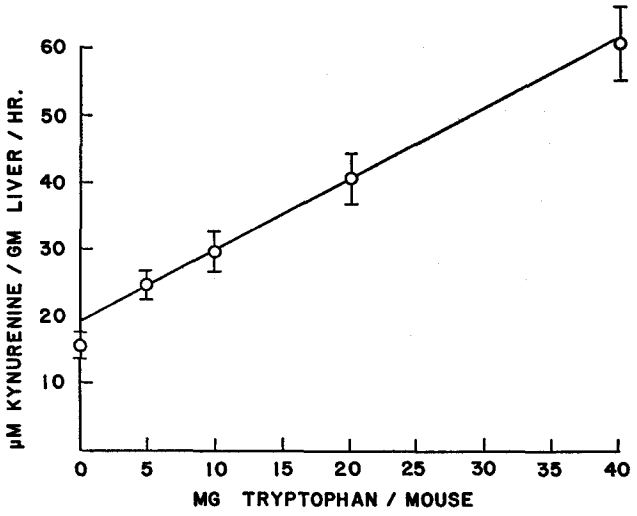

FIG. 1. Liver tryptophan pyrrolase activity in mice 4 hours after intraperitoneal injection of amounts of tryptophan shown.

the lethal and other manifestations of endotoxin poisoning, are shown in the second line of the table. Nicotinamide, at a molar quantity four times that of cortisone, affords almost the same degree of protection as the hormone. Nico-

tinamide is of interest because it is part of the molecule of the pyridine nucleotides and is synthesized along the metabolic pathway initiated by tryptophan pyrrolase. Interestingly enough, however, nicotinic acid, which is formed prior to the amide, not only fails to protect against endotoxin but potentiates it. Thus the dose that is only 20 per cent lethal in control mice is 100 per cent lethal in mice given nicotinic acid. Possibly the vasodilator effect of the acid results in a different distribution of the endotoxin to tissues, or it may be the result of a specific metabolic effect, as mentioned in a later section of the paper.

DPN at two dosage levels, about the same or slightly less in micromolar amounts than that of cortisone, affords good protection against endotoxin. This finding is consistent with the postulate that cortisone acts through its ability to increase the activity of tryptophan pyrrolase which, in turn, results in greater tissue levels of DPN and other nucleotides. This suggestion is further supported by the data presented in Table VIII.

When purified lipopolysaccharide derived from *E. coli* was substituted for heat-killed *S. typhimurium* as a source of endotoxin, the protective effect of the same compounds was observed. The results are summarized in Table II and show that both nicotinamide and DPN are equally active while nicotinic acid potentiates the lethal properties of the lipopolysaccharide. Tryptophan was without significant effect.

The inability of tryptophan to protect mice against endotoxin death when given either concurrently or 4 hours before heat-killed *S. typhimurium* is shown clearly in the data of Table III. These findings are contrary to expectations since tryptophan is known from the literature to inductively elevate pyrrolase activity in normal rats (6, 7). As Fig. 1 shows, this also occurs in unpoisoned mice. The increase in enzyme activity is linear with dosage of tryptophan over the range studied.

Of even greater interest is the obvious potentiation of endotoxin by tryptophan when the amino acid is given 4 hours after the heat-killed cells (last line of Table III). An LD_{50} in control mice becomes an LD_{100} following the delayed injection of tryptophan. A possible explanation for this action of tryptophan is given below.

In order to avoid the possibility that the results described above were unique for CF-1 mice, duplicate experiments were carried out with Dierolf mice. These animals were protected against *S. marcescens* LPS with nicotinamide, DPN, and cortisone to about the same degree as CF-1 animals. The lethal effect of endotoxin was potentiated by nicotinic acid and by tryptophan given 4 hours after the LPS. Thus, in respect to lethality, the Dierolf mice responded the same as CF-1 mice.

Irreversibility of Endointoxication.—It has been found that cortisone (and other glycocorticoids) have prophylactic value against endotoxin but are unable to protect against lethality after a delay of a few hours (9). Endointoxi-

cation appears to be irreversible, therefore, as far as cortisone is concerned. It was considered worthwhile, in light of the data of Table I, to examine the possible benefit that compounds formed beyond the metabolic conversion catalyzed by tryptophan pyrrolase might have after the animal begins to show symptoms of endotoxin poisoning. The results are presented in Table IV. No compound alone nor in combination with glucose was effective nor did any appear to potentiate endotoxin in a manner similar to tryptophan. Glucose

TABLE IV

Delayed Treatment of Mice Injected with Heat-Killed S. typhimurium.

All injections were given 4 and 8 hours after the endotoxin (cortisone 4 hours only) and all were injected intraperitoneally (cortisone, subcutaneously).

Substance(s) injected 4 and 8 hrs. after endotoxin	No. of mice surviving/No. injected with weight of heat-killed *S. typhimurium* shown	
	1.5 mg	3.0 mg
Control, saline 0.5 ml	19/30	4/30
Nicotinamide, 10 mg	13/20	5/20
	N.S.*	N.S.
Nicotinamide, 10 mg + glucose, 25 mg	10/20	5/20
	N.S.	N.S.
DPN, 5 mg	10/20	1/20
	N.S.	N.S.
DPN, 5 mg + Glucose, 25 mg	9/20	0/20
	N.S.	N.S.
ATP, 5 mg + Glucose, 25 mg	18/20	4/30
	N.S.	N.S.
Cortisone, 5 mg at 4 hrs. only	11/20	4/20
	N.S.	N.S.

* N.S. = not statistically significant.

was added to the metabolite since previous work from this laboratory (12) and elsewhere (13, 14) has established the fact that endotoxin rapidly depletes carbohydrate reserves in the mouse and in other laboratory animals. The possibility exists, therefore, that available metabolic energy in the poisoned animal would be increased by the addition of substrate. No improvement in survival of the mice is evident. Cortisone, also, fails to protect (last line, Table IV) when given 4 hours after endotoxin.

Tryptophan Pyrrolase Activity in Livers of Endotoxin-Poisoned Mice.—Fig. 2 presents in graphic form the tryptophan pyrrolase activity in livers of mice following a single injection of an LD_{50} dose of heat-killed *S. typhimurium.* An initial increase with 4 to 6 hours results in a significant rise in enzyme activity. At 17 hours, however, there is less than half the control level of pyrrolase

action and even after 48 hours the enzyme has not returned to normal. This sequence of changes is made more understandable by the results presented below.

Adrenalectomized mice, maintained on 1 per cent sodium chloride solution, were subjected to the water excretion test of Beatty *et al.* (15) as a measure of adrenal deprivement. Animals which were considered to be completely adrenalectomized on the basis of this test were then used for the determination of liver tryptophan pyrrolase activity 1 to 2 weeks postadrenalectomy. One group of mice was kept as controls and another group was injected intra-

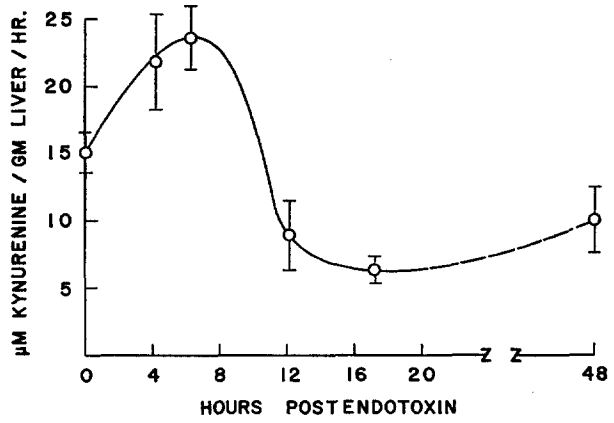

FIG. 2. Liver tryptophan pyrrolase activity in mice at times indicated following intraperitoneal injection of an LD_{50} of heat-killed *S. typhimurium*.

peritoneally with heat-killed *S. typhimurium* at an LD_{50} level for normal animals. This is approximately 250 times the LD_{50} for adrenalectomized mice. Four hours after the endotoxin was administered the livers were assayed for tryptophan pyrrolase. The data of Table V summarize the findings. The enzyme activity in the adrenalectomized mice is about one-half that found in intact animals and there is no change in the level of enzyme 4 hours after injection of endotoxin in contrast to the increase observed in the intact animal. Apparently, therefore, the sequence of changes seen in Fig. 2 is dependent in part on adrenal response. This is further borne out by the results now to be described.

Effect of Experimental Treatments on Tryptophan Pyrrolase Activity in Livers of Mice.—The first two lines of Table VI give the numerical values plotted graphically in Fig. 2 at time zero and 17 hours after an injection of an LD_{50} of endotoxin. The poisoned mouse at 17 hours shows typical symptoms of endointoxication: conjunctivitis, diarrhea, prostration, ruffled fur, weight loss, etc. At this time, tryptophan pyrrolase activity is less than one-half normal and

is not significantly different from that of the adrenalectomized mouse (Table V). Animals injected with the same amount of endotoxin and concurrently with 5 mg of cortisone acetate are completely protected against lethality (Table I) and also have normal tryptophan pyrrolase activity (line 3, Table VI). This is not observed, however, when 20 mg of tryptophan are substituted for cortisone. The mice are not protected (Table II) and, as line 4, Table VI shows, neither is tryptophan pyrrolase activity. The fact that both cortisone and tryptophan result in similar increases in the enzyme when given singly to control animals is evident from the values at the bottom of Table VI. These were measured 4 hours postinjection. Neither substance maintains enzyme activity 17 hours after it is injected. One may presume, therefore, that the

TABLE V

Effect of Heat-Killed S. typhimurium on Tryptophan Pyrrolase Activity of Liver from Mice Adrenalectomized 1 to 2 Weeks Previously

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Experimental treatment	Tryptophan pyrrolase activity of liver (μM kynurenine/gm liver/hr.)
Adrenalectomized mice	7.2 ± 0.8 (8)
Adrenalectomized mice 4 hrs. after 1.5 mg of heat-killed *S. typhimurium*	7.0 ± 1.0 (5)

low tryptophan pyrrolase level 17 hours after endotoxin alone or in combination with tryptophan may be associated with adrenal insufficiency. Cortisone, by contrast, permits normal enzyme levels and, by inference, normal adrenal function to be maintained in endotoxin poisoned mice.

Effect of Delayed Injection of Cortisone on Tryptophan Pyrrolase Activity in Endotoxin Poisoned Mice.—As shown previously (see Table IV), cortisone given 4 hours after endotoxin fails to protect mice against lethality. Results presented in Table VII show that tryptophan pyrrolase activity is neither maintained nor elevated by an injection of cortisone in mice already poisoned with endotoxin. On the basis of this evidence, the hormone is unable to induce protein synthesis under these conditions, an effect it is known to have in normal animals (7).

Tryptophan Pyrrolase Activity in Tolerant Mice.—Mice were made tolerant to endotoxin by a series of injections of heat-killed *S. typhimurium*. The first 2 days they were given 0.1 LD_{50} (0.15 mg dry weight). This was followed by 0.2 LD_{50} on days 3 and 4, and by 0.4 LD_{50} on days 5 and 6. When challenged on days 8 and 10 with *E. coli* lipopolysaccharide, the LD_{50} was increased

five-fold. Assays for tryptophan pyrrolase were carried out on day 8 on tolerant mice fasted for 17 hours and on another group 17 hours after an LD_{50} of *S. typhimurium* for non-tolerant mice. The results are presented in Table VIII and show no significant difference between the levels of enzyme activity as a result of endotoxin treatment. The control value for tolerant mice (12.4) is significantly less than that for normal mice (14.9, line 1, Table VI) at the 1 per cent probability level as determined by rank order test (17). The enzyme had apparently failed to return completely to normal.

Pyridine Nucleotide Levels in Liver.—A change in tryptophan pyrrolase activity may be expected to result in a change in total pyridine nucleotides. This has been found to be true as the data of Table IX show. Endotoxin-poisoned mice at 17 hours have about two-thirds the amount of nucleotides as control animals. Results similar to those shown in the table (CF-1 mice) were also obtained using Dierolf mice. This is a larger fraction than was found for the enzyme (see Table VI) but is perhaps dependent, at least in part, on the timing of the changes since a decrease in the enzyme should precede that of the coenzymes. Both cortisone and nicotinamide result in a significant increase in pyridine nucleotides but the latter is twice as effective as the former. This may be related to the fact that nicotinamide is part of the nucleotide molecule. Kaplan *et al.* (18) have reported similar findings and have also shown that nicotinic acid has little stimulatory effect on the biosynthesis of pyridine nucleotides. It may be that this effect of nicotinic acid plays an important role in potentiating endotoxin (see above).

TABLE VI

Effect of Heat-Killed S. typhimurium, Tryptophan, and Cortisone Singly and in Combination on Tryptophan Pyrrolase Activity of Liver from Mice

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Experimental treatment	Tryptophan pyrrolase activity of liver (μM kynurenine/gm liver/hr.)
Control, fasted 17 hrs.	14.9 ± 1.5 (16)
17 hrs. after an LD_{50} (1.5 mg) dose of endotoxin	6.0 ± 1.0 (17)
17 hrs. after endotoxin + 5 mg cortisone	13.3 ± 1.7 (6)
17 hrs. after endotoxin + 20 mg tryptophan	6.2 ± 1.0 (6)
4 hrs. after 5 mg cortisone	43.5 ± 2.8 (7)
4 hrs. after 20 mg tryptophan	39.7 ± 4.1 (4)

Concurrent injection of cortisone or nicotinamide with endotoxin protects mice against lethality (Table I), against a decrease in tryptophan pyrrolase activity (Table VI, cortisone only) and, as the last two lines of Table IX show, against a change in total oxidized pyridine nucleotides. The necessity of a normal level of coenzymes for survival of an endotoxin poisoned animal has not been established but is implied by these data.

Effect of Low Temperature (5°C) on Protection of Mice Against Endotoxin.—As the data of Table X show, mice exposed to 5°C and injected with about an LD_{25} of *S. marcescens* LPS fail to show 100 per cent survival when either nicotinamide, DPN, or cortisone is given at the same time. This is contrary

TABLE VII

Effect of Delayed Injection of Cortisone on Liver Tryptophan Pyrrolase Activity in Endotoxin-Poisoned Mice

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Experimental treatment	Tryptophan pyrrolase activity of liver (μM kynurenine/gm liver/hr.)
17 hrs. after LD_{50} endotoxin (heat-killed *S. typhimurium*)	6.3 ± 2.0 (8)
17 hrs. after LD_{50} endotoxin and 13 hrs. after 5 mg cortisone	7.5 ± 2.0 (8)
21 hrs. after LD_{50} endotoxin and 4 hrs. after 5 mg cortisone	6.0 ± 1.5 (8)

to what was reported in Tables I and II with data from mice maintained at 25°C. By increasing the quantity of LPS to slightly more than an LD_{80}, nicotinamide and cortisone were able to protect the mice but DPN was still ineffective. This finding with cortisone in which protection occurs with a large dose but not a small dose of endotoxin confirms an earlier observation reported by Previte and Berry (19, 20). These authors also observed that death from endotoxin in mice at 5°C occurs sooner and the animals fail to show the conjunctivitis, the ruffled fur, and the malaise seen in mice at 25°C. The vascular changes typically associated with endointoxication are believed not to occur in the cold-exposed mice.

Tryptophan Pyrrolase Activity in Mice at 5°C.—Four hours of exposure to 5°C, a time at which the body temperature of mice is one to two degrees lower than normal (20), the tryptophan pyrrolase activity of liver is significantly less than that of mice maintained at 25°C. This is seen in the first line of Table XI. By 17 hours, however, the enzyme is again normal, as is body temperature (second line, Table XI). The injection of an LD_{50} (at 25°C) of heat-killed *S. typhimurium* into mice at 5°C fails to alter after 4 hours the activity of

tryptophan pyrrolase while the same amount of crude endotoxin in mice at 25°C raises significantly the enzyme level (line 3, Table XI). This quantity of endotoxin is about 200 times the LD_{50} for mice at 5°C and hence is not directly comparable to the results found at room temperature. By reducing the amount of endotoxin to an LD_{50} or less, there are increases in tryptophan pyrrolase levels in livers of mice in the cold. This can be seen in the last three lines of Table XI. Increases follow an injection of either heat-killed *S. typhimurium* or *S. marcescens* LPS.

TABLE VIII

Effect of Heat-Killed S. typhimurium on Tryptophan Pyrrolase Activity of Liver from Tolerant Mice

Experimental treatment	Tryptophan pyrrolase activity of liver (μM kynurenine/gm liver/hr.)
Tolerant mice, 17 hrs. fasting	12.4 ± 2.4 (9)
Tolerant mice, 17 hrs. after 1.5 mg heat-killed *S. typhimurium*	11.8 ± 1.8 (6)

TABLE IX

Effect of Heat-Killed S. typhimurium, Cortisone and Nicotinamide Singly and in Combination on Total Oxidized Pyridine Nucleotides in Liver of Mice

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses. The *P* values were calculated by the rank order test of White (17).

17 hrs. after treatment with	Total oxidized pyridine nucleotides mg/gm liver	Significant difference (probability) *versus*	
		Control group	Endotoxin group
Control	0.75 ± 0.04 (15)	—	0.001
Endotoxin, 1.5 mg *S. typhimurium*	0.55 ± 0.06 (15)	0.001	—
Cortisone acetate	0.84 ± 0.08 (10)	0.001	0.001
Nicotinamide, 10 mg	1.95 ± 0.76 (9)	0.001	0.001
Cortisone + endotoxin	0.68 ± 0.09 (10)	N.S.*	0.01
Nicotinamide + endotoxin	0.78 ± 0.12 (10)	N.S.	0.001

* N.S. = not statistically significant.

TABLE X

Effect of Exposure of Mice to 5°C on Ability to Protect Against the Lethal Effect of S. marcescens Lipopolysaccharide

All injections were given intraperitoneally. The *P* values were caculated by the rank order test of Wilcoxon (16).

Substance injected at time of endotoxin	No. of mice surviving/No. injected with quantity of *S. marcescens* LPS	
	10 μg	30 μg
Control, saline 0.5 ml	22/30	5/30
Nicotinamide, 10 mg (82 μM)	24/30	15/30
	N.S.*	$P = 0.01$
DPN, 10 mg (17.1 μM)	26/30	7/30
	N.S.	N.S.
Cortisone, 10 mg (18 μM)	25/30	14/30
	N.S.	$P = 0.02$

* N.S. = not statistically significant.

TABLE XI

Tryptophan Pyrrolase Activity of Liver from Mice at 5°C Injected with Different Doses of Endotoxin

Each value is the mean ± the standard deviation for the number of separate determinations shown in parentheses.

Experimental treatment	Tryptophan pyrrolase activity of liver (μM kynurenine/gm liver/hr.) for mice exposed to	
	5°C	25°C
After 4 hrs. exposure	9.1 ± 1.7 (8)	14.9 ± 1.5 (16)
After 17 hrs. exposure	13.8 ± 3.2 (9)	14.9 ± 1.5 (16)
4 hrs. after 1.5 mg heat-killed *S. typhimurium*	11.3 ± 3.2 (14)	21.3 ± 3.7 (8)
4 hrs. after 60 μg heat-killed *S. typhimurium*	12.6 ± 2.6 (8)	—
4 hrs. after 6 μg heat-killed *S. typhimurium*	17.2 ± 2.6 (8)	—
4 hrs. after 50 μg *S. marcescens* LPS	14.7 ± 3.5 (8)	—

DISCUSSION

The changes found to occur in liver tryptophan pyrrolase activity in mice given toxic amounts of endotoxin suggest that this enzyme and the metabolic pathway in which it participates are involved in endointoxication. When

mice are protected against the lethal effects of endotoxin by cortisone, tryptophan pyrrolase activity remains normal. Tryptophan, on the other hand, augments the level of the enzyme in unpoisoned animals but fails to protect against lethality and to maintain the normal activity of the enzyme in the presence of endotoxin. Moreover, the ability of nicotinamide and DPN to protect against endotoxin is additional evidence for a significant role of tryptophan pyrrolase in the survival of these animals. The extent to which enzymic changes reflect adrenocortical function remains to be established but there seems to be little question that they are related. The rise in tryptophan pyrrolase activity about 4 hours after an injection of endotoxin (Fig. 2) fails to occur in the adrenalectomized mouse. In addition, the minimum level of enzyme activity seen about 17 hours after the endotoxin is the same as that observed in the adrenalectomized animal. It is not unreasonable to postulate that the high susceptibility of the adrenal-deprived animal to endotoxin is related to this metabolic deficiency.

The cold-exposed animal presents an interesting exception to the behavior of mice maintained at room temperature. They are not protected against endotoxin by DPN. In addition, the animals die from an LD_{50} dose sooner than mice at 25°C. It is known that the poisoned animals become severely hypothermic within a few hours (20) and their death may be a direct result of this change. A number of factors may contribute to this loss of body temperature. Of significance also is the suppression of gastric emptying (and hence cessation of food and water intake) which is demonstrable 5 minutes after as little as one-thousandth of an LD_{50} of endotoxin (21). Since mice in the cold eat nearly five times as much food as normal animals, it is apparent that inanition becomes an important consideration in survival.

The potentiating effect of a delayed injection of tryptophan (Table III) merits comment. Tryptophan is known to be degraded not only *via* its pyrrolase but it is also converted into 5-hydroxytryptamine (serotonin). Since this substance may sensitize an animal to endotoxin (22), transformation of a greater proportion of tryptophan to serotonin in poisoned mice than in controls might account for the observed effect. Experimental verification is possible and experiments are now underway.

Recent publications by Martini (23), Weissman and Thomas (24), and Janoff *et al.* (25) have strongly implicated lysosomes as a primary target of endotoxin action. Increased release of hydrolases from the so-called large granule fraction of liver was demonstrated only 5 minutes after an intraperitoneal injection of bacterial lipopolysaccharide. Endotoxin-tolerant animals, cortisone-pretreated animals, and rats made tolerant in traumatic shock all possess lysosomes from which less cathepsin and μ-glucuronidase are released following incubation or ultraviolet irradiation than in preparations derived from control animals. Apparently the development of stability of lysosomes is

related to adrenocortical integrity. Whether or not these observations are in any way related to our findings remains to be determined.

SUMMARY

Cortisone is known to protect mice against the lethal effects of endotoxin. It also elevates liver tryptophan pyrrolase (TP) activity, an enzyme that converts tryptophan into an intermediate which, in turn, is transformed in a series of reactions into nicotinamide, a component of the pyridine nucleotides. In the present report, results of experiments attempting to link the prophylactic action of cortisone in endointoxication to metabolism of tryptophan are described. It was shown first that both nicotinamide and diphosphopyridine nucleotide (DPN), compounds along the pathway initiated by TP, are each as effective as cortisone in protecting mice against lethality of different amounts of endotoxin. L-Tryptophan, which alone results in an increase in liver TP, fails to protect against endotoxin when it is given either 4 hours before or concurrently with the toxin while it potentiates the toxin when administered 4 hours later. Cortisone, nicotinamide, and DPN all fail to protect mice against lethality when given 4 hours after endotoxin but they do not potentiate it as does tryptophan.

Additional evidence linking tryptophan metabolism to endotoxin poisoning was derived from assays for TP. Activity of the enzyme in livers of mice 17 hours after injecting an LD_{50} of endotoxin is less than one-half the control value. It remains below normal for 48 hours. In adrenalectomized mice, TP activity is about the same as in mice 17 hours after endotoxin. Animals protected against lethality of endotoxin by cortisone have normal levels of TP but if the cortisone is given 4 hours after the toxin, TP activity is the same as in mice given endotoxin alone. Tryptophan is unable to maintain a normal level of TP when it is given concurrently with endotoxin. TP activity is not depressed when mice made tolerant to endotoxin are given an injection of endotoxin at the LD_{50} level for normal animals. Normal activity of the enzyme was always observed in livers of mice protected against endotoxin but not in those where protection failed.

The total amount of oxidized pyridine nucleotides (PN^+) in livers of mice 17 hours after an LD_{50} of endotoxin is about two-thirds the normal level. Animals injected with either cortisone or nicotinamide at the same time as endotoxin maintain the PN^+ level in liver.

Mice exposed to 5°C during the postinjection period can be protected with cortisone or nicotinamide against lethality of endotoxin but not with DPN. Changes in TP activity do not parallel those found in mice kept at 25°C. The toxic manifestations of endotoxin appear to be different, therefore, in animals stressed by cold.

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