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# STUDIES ON MECHANISMS OF TRYPTOPHAN PYRROLASE INHIBITION DURING ENDOTOXIN POISONING

George N. Eaves
L. Joe Berry

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ARCTIC AEROMEDICAL LABORATORY

AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
FORT WAINWRIGHT, ALASKA

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# STUDIES ON MECHANISMS OF TRYPTOPHAN PYRROLASE INHIBITION DURING ENDOTOXIN POISONING

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## FOREWORD

This report was prepared under contract AF 41(609)-1764 (Project 8241, Task 824101) with the Department of Biology, Bryn Mawr College, Bryn Mawr, Pennsylvania. The report covers research carried on from 1 December 1964 to 28 February 1965. Air Force program monitor is Mr. Robert Becker, ALRA, Arctic Aeromedical Laboratory.

This technical report has been reviewed and is approved.

Horace F. Drury

HORACE F. DRURY
Director of Research

## ABSTRACT

The inhibition of tryptophan pyrrolase *in vitro* by plasma and certain other native substances was investigated in an attempt to elucidate mechanisms responsible for the assumed decrease in activity of this enzyme *in vivo* during endotoxin poisoning. The inhibitor in plasma was present in normal mice, but increased significantly in endotoxin-poisoned, cortisone-protected, tolerant and challenged tolerant mice. On the basis of its physical properties and kinetics of inhibition, the plasma inhibitor was identified circumstantially as a globin. Results of kinetic studies eliminated the plasma inhibitor as a causal factor in the irreversible decrease in activity of tryptophan pyrrolase in whole homogenates of liver from endotoxin-poisoned mice. The enzyme was also found to be inhibited by citrate, the concentration of which increases substantially in the liver during endotoxicosis. Since inhibition by citrate could not be reversed by excess cofactor or substrate, it was concluded that at least part of the decreased activity of tryptophan pyrrolase in whole homogenates of poisoned mice was the result of inhibition by citrate.

# I

# INTRODUCTION

The decrease in activity of tryptophan pyrrolase in homogenates of liver from endotoxin-poisoned mice (1) is considered to have special significance since it implies an initial block in the pathway leading to the formation of pyridine nucleotides in vivo. Guided by the hypothesis that impaired biosynthesis of essential corequirements for the production of energy could represent a primary biochemical lesion in endotoxin poisoning, the investigations reported to date have been attempts to correlate tryptophan pyrrolase activity to survival of poisoned mice during various experimental treatments (2, 3, 4). A chance observation revealed that plasma from mice injected with heat-killed Salmonella typhimurium inhibited tryptophan pyrrolase activity in whole liver homogenates to a significantly greater extent than did plasma from normal mice (5). It was therefore considered relevant to investigate the inhibition of tryptophan pyrrolase by plasma and certain other native substances in an attempt to elucidate mechanisms responsible for the assumed decrease in activity of this enzyme in vivo during endotoxin poisoning.

# II

# MATERIALS AND METHODS

## Mice

Female Swiss-Webster mice (Dierolf Farms, Boyertown, Pa.) were housed 10 per cage with pine shavings as bedding in an animal room maintained at 25° ± 2° C. Tetracycline antibiotics (Polyotic, American Cyanamide Co., Princeton, N. J.) were added to the drinking water during the first two days after arrival of the mice from the dealer. The antibiotics were withdrawn at least one week before the mice were used experimentally. Water and D and G pathogen-free mouse food (Price-Wilhoite Co., Frederick, Md.) were given ad libitum until the beginning of an experiment, at which time the food was withdrawn. Mice weighing 23-25 gm were used in all experiments.

## Experimental Endotoxin Poisoning and Protection

Endotoxin was in the form of a saline suspension of heat-killed Salmonella typhimurium, strain SR-11, as described previously (1). The dry weight of the suspension was 5.6 mg per ml. In all experiments, one $LD_{50}$ (7.0 µg at 5° C, 0.7 mg at 25° C) endotoxin in a volume of 0.5 ml was injected intraperitoneally. Controls were injected intraperitoneally with 0.5 ml nonpyrogenic saline (Baxter Laboratories, Morton Grove, Ill.).

In protection experiments, 5 mg cortisone acetate (United Research Laboratories, Inc., Philadelphia, Pa.) in a volume of 0.5 ml, diluted with nonpyrogenic saline, was injected subcutaneously into the interscapular region immediately before the intraperitoneal injection of endotoxin.

## Induction of Tolerance

Mice were made tolerant to Serratia marcescens endotoxin, in the form of lyophilized S. marcescens cells (provided by Merck and Co., Rahway, N. J.) suspended in saline, by the following injection schedule. On successive days, 0.02, 0.02, 0.04, 0.04, 0.08 and 0.08 $LD_{50}$ endotoxin was injected intraperitoneally in a volume of 0.5 ml. Controls were injected intraperitoneally with 0.5 ml nonpyrogenic saline. Evidence of tolerance was established by challenging 20 mice from each experimental group, on the eighth day, with 3 $LD_{50}$ S. typhimurium endotoxin. The number of survivors was determined at the end of 48 hours.

## Environmental Studies

Mice exposed to cold were placed in a Modulab Room (Labline, Inc., Chicago, Ill.) at 5° C immediately after injection. The mice were protected from drafts by plastic sheeting which covered draft-exposed areas of the incubator shelves.

## Determination of Tryptophan Pyrrolase Activity

Tryptophan pyrrolase activity was determined by the method of Knox and Auerbach (6). Liver homogenates were prepared according to the method of Eaves and Berry (7). Total tryptophan pyrrolase activity was detected by adding 10 µg hematin according to the method of Eaves and Berry (8). The hematin solution was prepared immediately before use by dissolving twice-crystallized bovine hemin (Sigma Chemical Co., St. Louis, Mo.) in dilute sodium hydroxide. The flasks containing the reaction mixture were incubated at 38° C in a table model water bath shaker (Eberbach Corp., Ann Arbor, Mich.) equipped with a hood for controlled atmosphere. Oxygen was added during the first five minutes of incubation.

In some experiments, the assay for tryptophan pyrrolase activity was modified as follows. To 15 ml porcelain crucibles were added 0.2 ml 0.2 M sodium phosphate buffer, pH 7.0; 0.3 ml 0.015 M L-tryptophan; 1.0 ml distilled water (or 0.9 ml water and 6.5 µg hematin in 0.1 ml); and 0.5 ml supernatant fluid of liver homogenate from which cell debris and nuclei were removed by centrifugation at 600 x g for 30 minutes. The supernatant fluid was strained through cheesecloth to remove the lipid layer at the surface.

The enzyme reaction was stopped by the addition of 1 ml 15% $HPO_3$. After filtration, 1 ml of the filtrate was neutralized with 0.35 ml 1.5 N NaOH.

Enzyme activity is expressed as μM kynurenine formed per hour per gram liver (dry weight) under the conditions described. The fraction of tryptophan pyrrolase activity detected in whole liver homogenates without added hematin is considered to be controlled by the amount of coenzyme normally present and, hence, is presumed to represent the activity of tryptophan pyrrolase in vivo, i.e., the native holoenzyme. The addition of excess hematin to the assay which employs whole liver homogenates prepared according to the method of Eaves and Berry (7) activates the pool of inactive apoenzyme and hence reveals the total activity of the enzyme.

## Inhibition of Tryptophan Pyrrolase Activity of Whole Liver Homogenates

Solutions containing inhibitors were added to the assay mixture immediately before initiation of the enzymic reaction by addition of whole liver homogenate. Homogenates were prepared from livers of mice fasted for 12-15 hours. Bovine albumin (Sigma Chemical Co.; and Pentex Inc., Kankakee, Ill.), bovine alpha globulin, bovine beta globulin, bovine gamma globulin (all from Pentex, Inc.) and undenatured globin (Nutritional Biochemicals Corp., Cleveland, Ohio) were dissolved in 0.05 M sodium phosphate buffer, pH 7.0, containing 0.9% NaCl. Beef liver catalase and purified fungal glucose oxidase (Type II) were obtained from Sigma Chemical Co.

In plasma and serum inhibition studies, blood was obtained by retro-orbital puncture. Blood for plasma was withdrawn with Pasteur pipettes moistened with sodium heparin (United Research Labs., Inc.). Serum was removed, after centrifugation at 2° C, from blood which had been allowed to clot at room temperature for one hour and incubated for an additional hour at 5° C.

## Chloroform-methanol Extract of Serum

The method for isolation of total lipids from animal tissues was essentially that of Folch, Lees, and Stanley (9). To 1 ml serum pooled from five mice were added 19 ml 2:1 v/v chloroform-methanol mixture. After being stirred for 5 minutes in warm water (50° C - 60° C), the flocculent precipitate was concentrated by centrifugation at 400 x g for 10 minutes and subsequently removed by filtration. To the supernatant fluid was added 0.2 ml 0.05 N NaCl. The upper of the resulting two phases was removed by siphoning and discarded. The interphase was rinsed with methanol-water-chloroform (48:47:3). The resulting mixture was made monophasic by the addition of methanol and dried

in a flash evaporator (Rinco Instrument Co., Inc., Greenville, Ill.). The extract was resuspended in 1 ml 90% ethanol.

## Determination of Serum Proteins

The protein concentration of serum was measured by the method of Lowry et al (10), with bovine serum albumin as the standard.

## Statistical Analyses

Statistical significance was determined by the "t" test.

# III

# RESULTS

Titration of hematin with liver homogenates revealed that the amount of hematin required for maximum detection of total tryptophan pyrrolase activity was the same in homogenates of livers from both normal and endotoxin-poisoned mice. In addition, the accumulation of the product of both total and native holo-tryptophan pyrrolase activity was linearly proportional to the concentration of homogenate, over a wide range, from livers of normal and endotoxin-poisoned mice. The possibility that the measurable enzyme activity in homogenates of liver from poisoned mice is a reflection of the lag period in initiation of the enzymic reaction was eliminated by the experiments presented in Table I. The proportional reduction in total and native holoenzyme activity of homogenates from poisoned mice was not changed when the incubation time of the enzymic reaction was increased or when the lag period was reduced by ascorbic acid. The accumulation of kynurenine remained proportional to enzyme concentration or activity throughout the two hours of incubation. The linearity of the reaction has been shown previously to persist during 3.5 hour incubation of normal mouse liver homogenates (11). The optimum pH for tryptophan pyrrolase activity of mouse liver homogenates is around pH 6.5; however, the level of tryptophan pyrrolase activity in homogenates from poisoned mice was not elevated proportionally more than that of homogenates from normal mice when the assay mixture was pH 6.2. Urea (final concentration 2M, pH 7.0) inhibited the enzyme reaction in homogenates of both normal and poisoned livers about 50%.

The foregoing data suggested that the reduced activity of tryptophan pyrrolase associated with endotoxin poisoning was neither a kinetic phenomenon nor a deficiency in the amount of available cofactor or the reportedly (12) essential peroxide generating system (Table II). That the reduction in tryptophan pyrrolase activity during endotoxicosis may be associated with an

inhibitor was suggested by the data presented in Table III, which shows a decrease in specific activity of the enzyme during fractionation of the liver homogenate.

TABLE I

Effect of Increased Incubation Time, Reduced Lag and Acid pH on the Assay for Tryptophan Pyrrolase Activity of Whole Liver Homogenates from Endotoxin-Poisoned Mice

| Alteration in assay | Native holoenzyme activity (μM kynurenine/gm liver/hr) | | Total enzyme activity (μM kynurenine/gm liver/hr) | |
|---|---|---|---|---|
| | Poisoned[a] | Control[b] | Poisoned | Control |
| 1.0 hr incubation (control) | 8.1 | 12.0 | 12.5 | 20.6 |
| 1.5 hr incubation | 11.2 | 17.0 | 16.7 | 28.2 |
| 2.0 hr incubation | 16.5 | 21.7 | 22.0 | 35.6 |
| 0.8 mM ascorbic acid | 9.7 | 17.4 | 15.5 | 32.0 |
| pH 6.2 | 10.1 | 17.1 | 21.3 | 28.6 |

[a] 17 hr after endotoxin ($LD_{50}$).
[b] 17 hr after saline.

Normal plasma or serum inhibited the activity of tryptophan pyrrolase in homogenates of whole liver; however, plasma or serum from endotoxin-poisoned mice inhibited the enzymic reaction to a significantly greater extent ($0.005 < P > 0.001$). Demonstration of increased concentration of plasma inhibitor in poisoned mice requires blood obtained by retro-orbital puncture. Plasma of blood obtained by decapitation of normal mice depresses tryptophan pyrrolase activity to the same extent as does that from poisoned animals, which suggests that an inhibitory substance normally present in tissue fluids is released into the blood during endotoxicosis. The increased inhibitory activity of plasma from poisoned mice was detectable within 4 hours after injection of an $LD_{50}$ of heat-killed S. typhimurium cells (Figure 1) and the concentration increased gradually during the 24-hour period following injection of endotoxin. The plasma inhibitor of tryptophan pyrrolase was increased significantly ($P > 0.001$) in mice protected from the lethal effects of endotoxin by cortisone (Table IV). The inhibitor was also increased significantly ($0.01 < P > 0.0005$) in tolerant mice injected with an $LD_{50}$ of endotoxin (Table V). It can also be seen in Table V that the small amounts of S. marcescens endotoxin used to induce tolerance were sufficient to elevate the level of plasma inhibitor. There was no difference in inhibitory activity of plasma from

PERCENT INHIBITION OF TP ACTIVITY

TABLE II

Tryptophan Pyrrolase Activity of Whole Liver Homogenates Supplemented with Catalase-Glucose Oxidase[a]

| Units[b] glucose oxidase | Relative tryptophan pyrrolase activity | |
|---|---|---|
| | Liver from poisoned mice[c] | Liver from control mice[d] |
| 0 | 100 | 100 |
| .07 | 91 | 97 |
| .14 | 61 | 74 |
| .42 | 37 | 44 |
| .70 | 19 | 27 |
| 1.40 | 10 | 15 |

[a] Assay: 0.1 ml 1M glucose, 6.5 μg hematin, 25 Sigma units catalase and the indicated units glucose oxidase added to the microassay.

[b] One unit of glucose oxidase will oxidize 1 μM glucose to gluconic acid and $H_2O_2$ per min at pH 5.1, 35° C.

[c] 17 hr after injection of endotoxin ($LD_{50}$).

[d] 17 hr after injection of saline.

TABLE III

Specific Activity of Tryptophan Pyrrolase During Fractionation of Liver Homogenates from Endotoxin-Poisoned Mice

| Homogenate fraction | Total tryptophan pyrrolase activity (μM kynurenine/gm liver/hr)[a] | |
|---|---|---|
| | Endotoxin poisoned[b] | Control[c] |
| Whole homogenate | 11.5 | 31.7 |
| 600 x g (30 min) supernatant fluid | 17.0 | 51.5 |
| 12,000 x g (20 min) supernatant fluid | 14.5 | 63.8 |
| 105,000 x g (50 min) supernatant fluid | 8.4 | 63.4 |

[a] Modified assay, i.e., 2.0 ml volume.

[b] 17 hr after $LD_{50}$.

[c] Uninjected, fasted 17 hr.

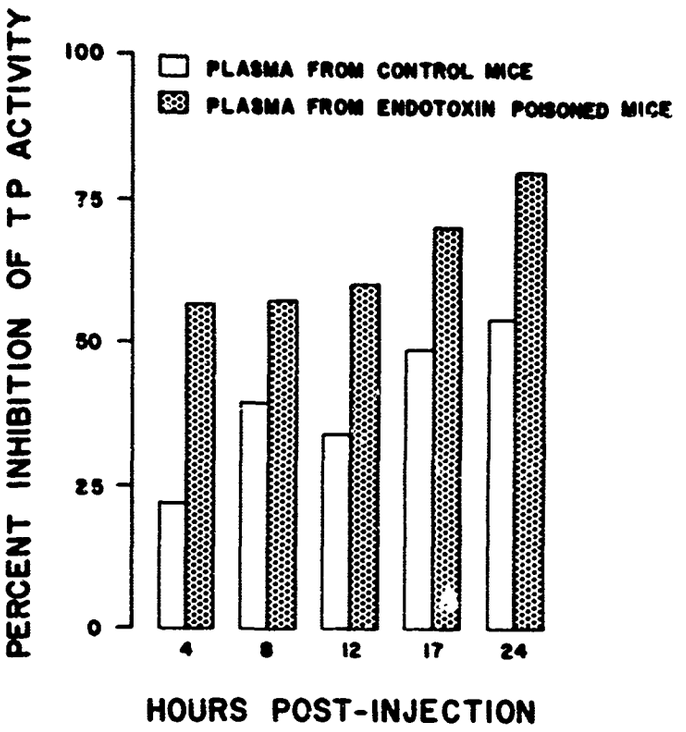


FIGURE I

The increase in tryptophan pyrrolase inhibiting activity of plasma during endotoxicosis and fasting.

TABLE IV

Inhibition of Tryptophan Pyrrolase Activity by Plasma from Cortisone-Protected Mice

| Plasma from mice injected 17 hr before with | Per cent inhibition of tryptophan pyrrolase activity[a] | |
|---|---|---|
| Saline | 34 ± 6.4 | (12)[b] |
| Endotoxin ($LD_{50}$) | 58 ± 3.7 | (12) |
| Cortisone (5 mg) | 38 ± 5.2 | (8) |
| Endotoxin + cortisone | 63 ± 2.2 | (8) |

[a] 0.1 ml plasma added to assay mixture.

[b] Values represent the mean ± S. E. for the number of individual animals indicated in parentheses.

TABLE V

Inhibition of Tryptophan Pyrrolase Activity
by Plasma from Tolerant Mice

| Experimental treatment | Per cent inhibition of tryptophan pyrrolase activity[a] | |
|---|---|---|
| Plasma from tolerant mice 17 hr after | | |
| Endotoxin ($LD_{50}$) | 70 ± 1.2 | (9)[b] |
| Saline | 58 ± 5.1 | (8) |
| Plasma from control mice 17 hr after | | |
| Endotoxin ($LD_{50}$) | 57 ± 3.9 | (7) |
| Saline | 44 ± 2.9 | (8) |

[a] 0.1 ml plasma added to assay mixture.

[b] Values represent the mean ± S. E. for the number of individual animals indicated in parentheses.

mice that were exposed to a 5° C environment immediately after injection of endotoxin ($LD_{50}$ 5° C) or saline. Blood was taken from the cold-exposed animals four hours after injection and exposure, at which time the lethal effects of sudden cold exposure were apparent (survivors/number injected: controls, 11/13; endotoxin poisoned, 13/16). There was no difference in tryptophan pyrrolase activity of mice which had been injected intravenously, two hours before assay, with 0.3 plasma from poisoned or normal mice or with 0.3 ml saline.

In Figure 2 it can be seen that the inhibition of tryptophan pyrrolase by plasma from normal and endotoxin-poisoned mice was proportional, at low concentrations, to the amount of plasma added to the assay mixture. Suspensions of thrice saline-washed red blood cells from blood of normal or poisoned mice had no effect on tryptophan pyrrolase activity.

The inhibition of tryptophan pyrrolase by plasma did not occur when excess hematin was added to the assay mixture. The reversibility by hematin of this inhibition is shown in Figure 3, which also illustrates the proportionality of inhibitor concentration to amount of hematin required to reverse the reaction. The plasma from poisoned mice contained twice the concentration of inhibitor

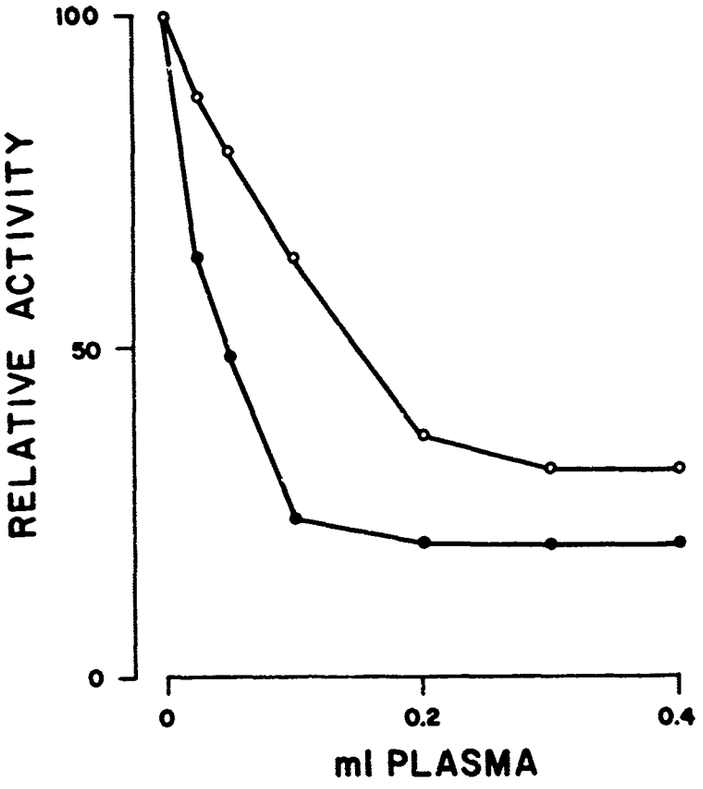


FIGURE 2

The inhibition of tryptophan pyrrolase activity of whole liver homogenates by plasma from normal (o) and endotoxin-poisoned (●) mice.

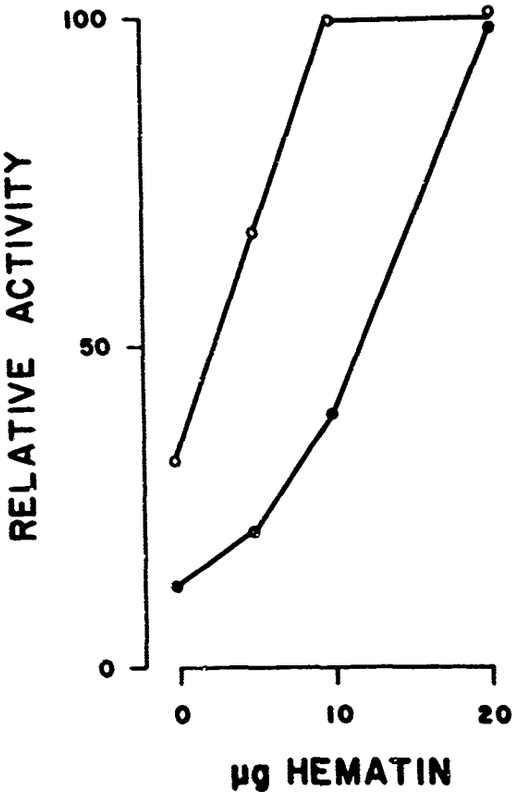


FIGURE 3

The effect of hematin on the inhibition of tryptophan pyrrolase by plasma from normal (o) and endotoxin-poisoned (●) mice.

as was in normal plasma and required twice the amount of hematin to reverse the reaction. The inhibition by plasma was not reversed when substrate in concentrations of $7.5 \times 10^{-3}$ to $4.5 \times 10^{-2}$ M was used in the absence of added hematin.

Figures 4 and 5 show the effect of heat on the plasma inhibitor of tryptophan pyrrolase activity. The inhibitor was active after 60 minutes at 55° C (Figure 4), but was not demonstrable after 60 minutes at 60° C (Figure 5). The increased activity observed when heated plasma was added to the assay mixture was probably the result of the releasing or unmasking of hematin and/or 3', 5'-cycle adenosine monophosphate (3', 5'-AMP) during heating.

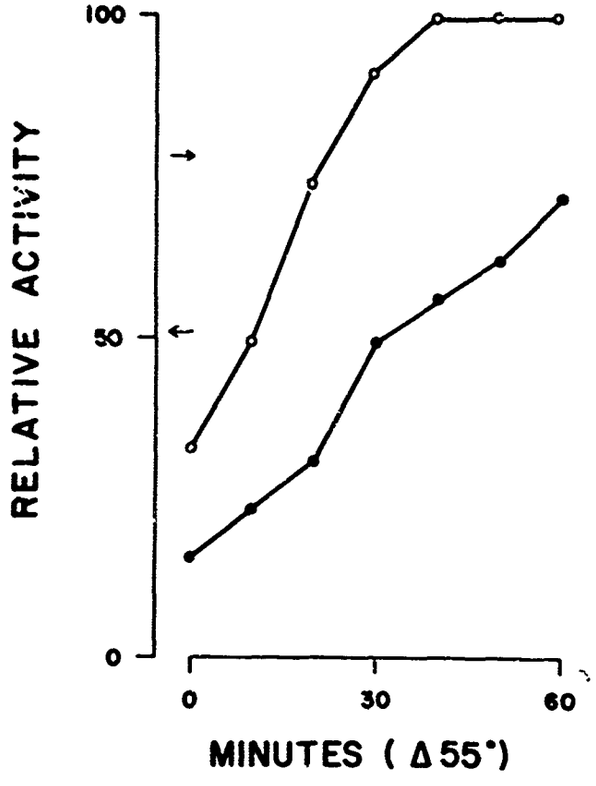


FIGURE 4

The stability of the plasma inhibitor of tryptophan pyrrolase during heating at 55° C and the concomitant release of an activator. Symbols: o = plasma from controls; ● = plasma from endotoxin-poisoned mice; ← = native holoenzyme activity; → = total enzyme activity.

The plasma inhibitor was stable at refrigerator temperature for several days and could be frozen and thawed repeatedly without loss of activity. The inhibitor was also non-dialyzable, which together with the forementioned heat-labile properties, suggested that it was protein in nature.

Table VI shows the concentration of serum proteins in normal and endotoxin-poisoned mice 17 hours after injection. Albumin, which decreases in individuals following injections of typhoid vaccine (13), was decreased in the sera of mice injected with endotoxin. The concentration of gamma globulins was unchanged; however, there was an increase in the alpha and beta globulins in the sera of endotoxin-poisoned mice. The possibility of

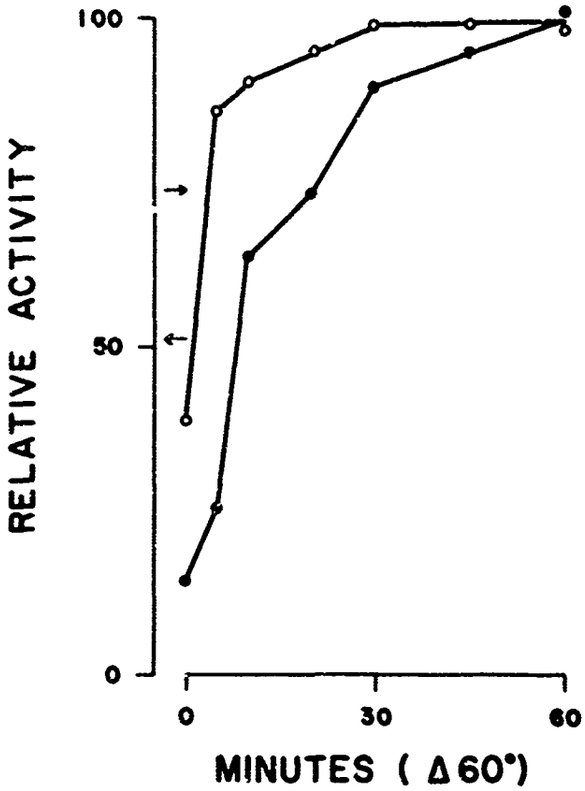


FIGURE 5

The inactivation of the plasma inhibitor of tryptophan pyrrolase at 60° C and the concomitant release of an activator. Symbols: o = plasma from controls; ● = plasma from endotoxin-poisoned mice; ← = native holoenzyme activity; → = total enzyme activity.

TABLE VI

Concentration of Proteins in Sera of Endotoxin-Poisoned Mice 17 Hours after Injection of $LD_{50}$ Heat-Killed Salmonella typhimurium Cells

| | Protein concentration (gm/100 ml serum) | |
|---|---|---|
| | Endotoxin poisoned | Control[a] |
| Total protein[b] | 3.36 ± 0.17 (6)[c] | 3.94 ± 0.19 (6) |
| Electrophoretic separation: | (3)[d] | (2) |
| albumin | 1.14 (1.07-1.21)[e] | 1.66 (1.51, 1.80) |
| $\alpha_1$ globulin | 0.35 (0.29-0.46) | 0.22 (0.20, 0.25) |
| $\alpha_2$ globulin | 0.40 (0.35-0.47) | 0.18 (0.16, 0.21) |
| $\beta_1$ globulin | 0.40 (0.37-0.43) | 0.20 (0.19, 0.22) |
| $\beta_2$ globulin | 0.39 (0.30-0.44) | 0.20 (0.20, 0.21) |
| gamma globulin | 0.28 (0.22-0.32) | 0.28 (0.24, 0.31) |
| Total | 2.96 | 2.74 |

[a] Injected with saline.
[b] Method of Lowry, et al;(10)
[c] Values represent the mean ± S.E. for number of individual determinations indicated in parentheses.
[d] Number of individual determinations.
[e] Values represent the mean (range) for number of individual determinations indicated at head of column.

non-specific increase of proteins by anhydremic concentration was eliminated by determinations of total protein, shown also in Table VI. There was a significant decrease (0.025 <P> 0 05) in total serum proteins of poisoned mice.

The effect of selected serum proteins on tryptophan pyrrolase activity of whole liver homogenates is shown in Table VII. Of the proteins used, only undenatured globin, which binds metalloporphyrins (14), exhibited inhibitory properties. Undenatured globin mimiced the inhibition of tryptophan pyrrolase by plasma in that its activity was proportional, at low concentrations, to the amount added to the assay mixture (Figure 6). In addition, the inhibition by undenatured globin was reversed by excess hematin.

TABLE VII

Inhibition by Serum Proteins of Tryptophan Pyrrolase Activity of Whole Liver Homogenates

| Serum protein | Per cent inhibition of tryptophan pyrrolase activity |
|---|---|
| albumin, bovine[a] | |
| 0.5 and 1 mg/ml | 0 |
| albumin, bovine[b] | |
| 0.5 and 1 mg/ml | 0 |
| α globulin, bovine[b] | |
| 0.4 mg/ml | 0 |
| β globulin, bovine[b] | |
| 0.4 mg/ml | 0 |
| gamma globulin, bovine[b] | |
| 0.4 mg/ml | 0 |
| globin, undenatured[c] | |
| 0.4 mg/ml | 64 |
| globin, denatured (60° C, 1 hr) | |
| 0.4 mg/ml | 0 |

[a] Sigma Chemical Co., St. Louis, Mo.

[b] Pentex, Inc., Kankakee, Ill.

[c] Nutritional Biochemicals Corp., Cleveland, Ohio

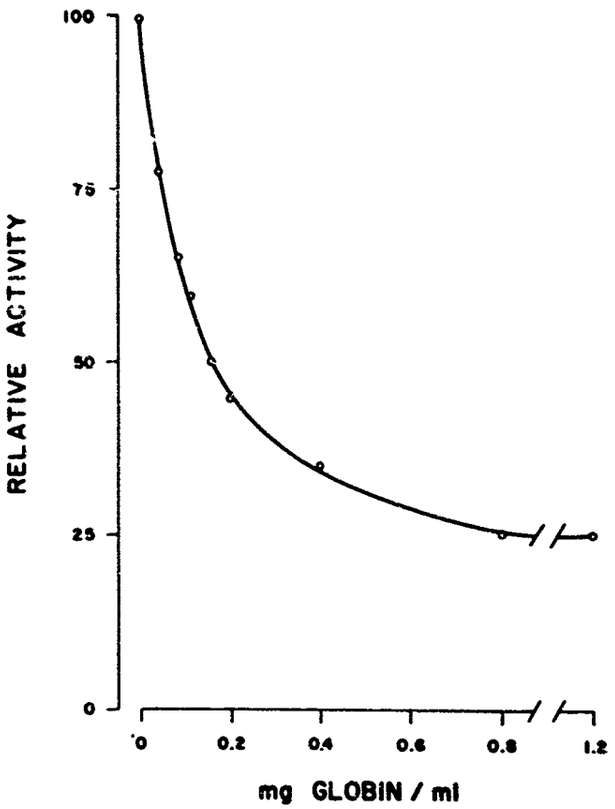


FIGURE 6

The inhibition of tryptophan pyrrolase activity of whole liver homogenates by undenatured globin.

Tryptophan pyrrolase activity of whole liver homogenates was also inhibited by citrate (Figure 7) and disodium ethylenediamine tetra-acetic acid, both at pH 7.0. The inhibition of enzymic activity by these compounds, which inhibit to the same extent on a molar basis, was not affected by the addition of excess hematin.

In order to test the hypothesis that a deficiency in an essential activator may be responsible for the decrease in tryptophan pyrrolase activity of liver homogenates from endotoxin-poisoned mice, 3', 5'-AMP and plasma which had been heated for 60 minutes at 60° C were added separately to assay mixtures containing homogenates of whole liver from normal and endotoxin-poisoned mice. The addition of 0.1 mM 3', 5'-AMP (15) had no effect on the activity of tryptophan pyrrolase in whole homogenates, either with or without added hematin, of liver from normal or poisoned mice. The effect of heated plasma on the tryptophan pyrrolase activity of these homogenates is shown in Table VIII. Although enzyme activity in homogenates of both normal and poisoned livers was increased by the addition of heated plasma, the activity of tryptophan pyrrolase in homogenates of liver from poisoned mice was not elevated to normal levels.

The effect of the chloroform-methanol extract of serum on tryptophan pyrrolase activity of whole liver homogenates is shown in Table IX. This

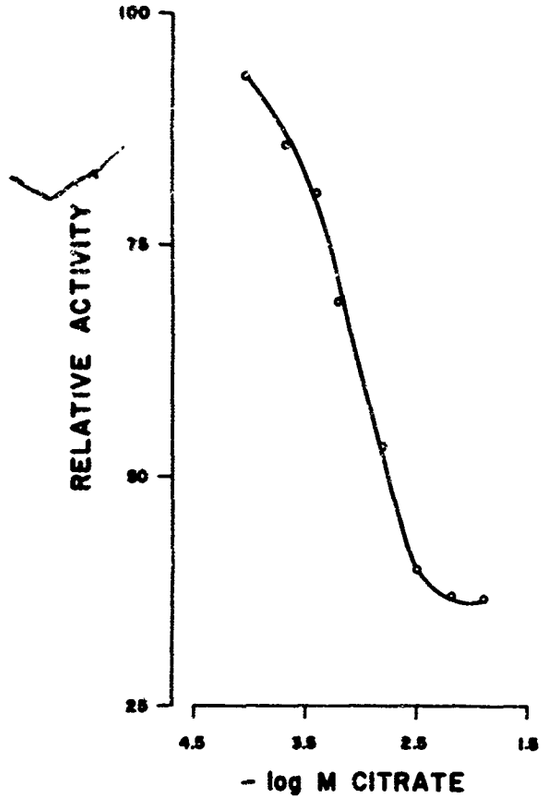


FIGURE 7

The inhibition of tryptophan pyrrolase activity of whole liver homogenates by sodium citrate at pH 7.0.

TABLE VIII

Effect of Heated Plasma (60° C, 1 hr) on Tryptophan Pyrrolase Activity of Whole Liver Homogenates from Endotoxin-Poisoned Mice

| Addition to assay mixture[a] | Tryptophan pyrrolase activity (μM kynurenine/gm liver/hr) |
|---|---|
| Liver homogenates from endotoxin-poisoned mice ($LD_{50}$): | |
| No addition | 6.0 ± 1.4[b] |
| Heated plasma[c] | 7.8 ± 2.7 |
| 10 μg hematin | 8.2 ± 2.5 |
| Heated plasma + 10 μg hematin | 6.0 ± 2.2 |
| Liver homogenates from control (saline injected) mice: | |
| No addition | 10.0 ± 1.2 |
| Heated plasma | 18.3 ± 5.1 |
| 10 μg hematin | 15.6 ± 2.9 |
| Heated plasma + 10 μg hematin | 15.9 ± 4.5 |

[a] 0.1 ml plasma.
[b] Values represent the mean ± S.E. of two duplicate experiments.
[c] Pool of plasma from 5 mice fasted 17 hr.

TABLE IX

Effect of Chloroform-Methanol Extract of Serum on Tryptophan Pyrrolase Activity of Whole Liver Homogenates

| Addition to assay mixture[a] | Tryptophan pyrrolase activity (μM kynurenine/gm liver/hr) |
|---|---|
| Serum[b] from endotoxin-poisoned mice ($LD_{50}$): | |
| Serum | 4.8 |
| Serum + 10 μg hematin | 12.5 |
| Chloroform-methanol extract | 11.3 |
| Chloroform-methanol extract + 10 μg hematin | 12.2 |
| Serum[b] from control (saline injected) mice: | |
| Serum | 6.1 |
| Serum + 10 μG hematin | 13.2 |
| Chloroform-methanol extract | 12.2 |
| Chloroform-methanol extract + μg hematin | 10.1 |
| No addition | 9.3 |
| 10 μg hematin | 13.2 |

[a] 0.1 ml sera or extract.

[b] Pool of serum from 5 mice.

extract contained, in addition to total nonsaponifiable lipids, a reddish-brown precipitate, probably protohemin IX (16), which was insoluble in ethanol, but soluble in water. The chloroform-methanol extract increased the activity of tryptophan pyrrolase in homogenates of liver from normal and endotoxin-poisoned mice to the same extent as did hematin.

## IV

## DISCUSSION

The most obvious explanations for the decrease in activity of tryptophan pyrrolase during endotoxicosis are as follows: (1) the synthesis of the enzyme may be depressed, (2) an inhibitor may be present, or (3) an essential activator may occur in sub-optimal concentration. There is no

evidence to date which suggests that protein synthesis is inhibited during endotoxin poisoning. To the contrary, it is known that the concentration of certain proteins, e.g., globulin and fibrinogen (13), increases following injection of endotoxin. In addition, injecting rabbits with endotoxin derived from S. marcescens, S. typhosa, and meningococcus enhances antibody production to certain protein and polysaccharide antigens (17).

The inability to elevate the activity of tryptophan pyrrolase associated with endotoxicosis to normal levels by adding 3', 5'-AMP, heated plasma or chloroform-methanol extract or by supplementing the assay with the peroxide generating system implies that the essential co-requirements for enzyme activity are present in both normal and poisoned livers. Similarly, the results of kinetic studies seem to eliminate the possibility that decreased enzyme activity in poisoned livers is a reflection of either variations in lag period (18) or phenomena resulting from assay conditions which are not optimum.

That the decreased tryptophan pyrrolase activity of whole liver homogenates from poisoned mice is the result of increased concentration of a compound with inhibitory activity was suggested initially by the results of purification studies and subsequently by the demonstration of increased inhibitory activity of plasma from poisoned mice. It is highly unlikely that the plasma inhibitor is of any significant consequence in lowering tryptophan pyrrolase activity in poisoned mouse liver, since the activity of the enzyme in liver homogenates from endotoxin-poisoned mice could not be elevated to normal levels with hematin. The in vitro inhibition of tryptophan pyrrolase activity by the plasma inhibitor was reversible with hematin. Citrate inhibition, however, could not be reversed by adding excess hematin to the assay mixture. Previous investigations have shown that the concentrations of citric acid in certain tissues increases during fasting (19) and following injection of endotoxin (20). The citric acid concentration of liver 15 hours after intraperitoneal injection of heat-killed S. typhimurium was four times normal. In contrast, there was very little change in concentration of blood citric acid. It is therefore conduded that at least a part of the irreversible decrease in tryptophan pyrrolase activity which occurs in homogenates of liver from poisoned mice can be attributed to inhibition by citrate.

Regardless of what circumstantial evidence may suggest as the explanation for decreased tryptophan pyrrolase activity in homogenates of whole liver from endotoxin-poisoned mice, it must be recognized that the in vitro assay for activity of this enzyme requires the use of a highly artificial, but necessarily essential, form of liver, i.e., a completely homogenized organ. There is always the possibility that disruption of cells brings together certain normally non-reacting cellular constituents. If such were the case here, then the inhibition of tryptophan pyrrolase in homogenates, by any inhibiting substance which is not in contact with the enzyme in an intact liver,

would have little or no significance in studies on the physiology of endotoxicosis. Therefore, the assumption that tryptophan pyrrolase has a role in endotoxin poisoning must await proof that activity of the enzyme is depressed in vivo following injection of endotoxin.

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13 ABSTRACT The inhibition of tryptophan pyrrolase in vitro by plasma and certain other native substances was investigated in an attempt to elucidate mechanisms responsible for the assumed decrease in activity of this enzyme in vivo during endotoxin poisoning. The inhibitor in plasma was present in normal mice, but increased significantly in endotoxin-poisoned, cortisone-protected, tolerant and challenged tolerant mice. On the basis of its physical properties and kinetics of inhibition, the plasma inhibitor was identified circumstantially as a globin. Results of kinetic studies eliminated the plasma inhibitor as a causal factor in the irreversible decrease in activity of tryptophan pyrrolase in whole homogenates of liver from endotoxin-poisoned mice. The enzyme was also found to be inhibited by citrate, the concentration of which increases substantially in the liver during endotoxicosis. Since inhibition by citrate could not be reversed by excess co-factor or substrate, it was concluded that at least part of the decreased activity of tryptophan pyrrolase in whole homogenates of poisoned mice was the result of inhibition by citrate.

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