estrus. Among the variables that could play a role in this phenomenon are 1) components of the inflammatory response including endogenous breakdown products of the disintegrating vaginal epithelium, bacteria and their endotoxins, and neutrophilic leukocytes and 2) circulating hormone levels.

In view of the ability of injected bacterial endotoxins to evoke splenic erythropoiesis (6), it is possible that the bacteria found in the vagina may play some role in this regard. It would be useful to know to what extent bacteria and bacterial products enter the tissues during the various phases of the estrous cycle. Another approach to this problem might be found in the examination of germ-free mice. Such mice have been shown to display a typical leukocytic inflammatory response in the vagina following progesterone (8) and this suggests that germ-free mice may also display vaginal changes comparable to those seen in conventional mice.

Another puzzling fact is that splenic erythropoiesis was highest during estrus, a time when estrogen levels in the animal are increased, because estrogens are generally regarded as depressors of both erythropoiesis and circulating red blood cell numbers(9). A meaningful evaluation of the aforementioned variables requires more information about the time required for a given stimulus to evoke its effect. Thus it is possible that a stimulus applied during one part of a cycle may not find its full expression until some time later. Studies currently in progress to determine the effects of ovariectomy, injected hormones and pregnancy upon splenic erythropoiesis may help to elucidate some of the questions that have been raised.

Summary. Ferrokinetic and cytologic studies in female mice reveal that splenic erythropoiesis is heightened during proestrus, estrus, and metestrus-1 as compared to metestrus-2 and diestrus.

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A Possible Explanation for Decreased Tryptophan Pyrrolase Activity In Homogenates of Liver from Endotoxemic Mice.* (31171)

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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, related investigations reported to date have been attempts to correlate tryptophan pyrrolase activity to survival of poisoned mice during various experimental treatments, *e.g.*, Berry and Smythe(2) and Berry(3). Eaves and Berry(4) subsequently reported that plasma from mice injected with heat-killed *Salmonella typhimurium* inhibited tryptophan pyrrolase activity in whole homogenates of

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normal liver to a significantly greater extent than did plasma from normal mice. This report summarizes the results of investigations on native inhibitors of tryptophan pyrrolase and suggests a possible explanation for the decreased activity of this enzyme in homogenates of liver from endotoxin poisoned mice.

Methods and materials. 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 Cyanamid Co., Princeton, N. J.) were added to the drinking water during the first 2 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 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 g were used in all experiments.

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} (0.7 mg) 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 all experiments, mice were injected with endotoxin or saline 17 hours before sacrifice by cervical dislocation.

Tryptophan pyrrolase activity was determined by the method of Knox and Auerbach (5) with liver homogenates prepared according to the report of Eaves and Berry(6). Total tryptophan pyrrolase activity was detected by adding 10 μg hematin according to the method of Eaves and Berry(7). 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 5 minutes of incubation.

Solutions containing inhibitors were added to the assay mixture immediately before initiation of the enzymic reaction by addition of whole liver homogenate. Homogenates prepared from livers of normal mice fasted for 12-15 hours were used as the source of enzyme in the inhibition studies. The effect of supplementing the assay with a peroxide generating system was determined by adding 0.1 ml 1 M glucose, 6.5 μg hematin, 25 Sigma units beef liver catalase and 0, 0.07, 0.14, 0.42 and 0.70 units glucose oxidase (fungal, Type II; Sigma Chemical Co.) to the assay mixture which was proportionally reduced to a final volume of 2 ml. One unit of glucose oxidase oxidized 1 μM glucose to gluconic acid and H_2O_2 per minute at pH 5.1, 35°C.

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 holo-enzyme. The addition of excess hematin to the assay which employs whole liver homogenates prepared as reported previously(6) activates the pool of inactive apoenzyme and hence reveals the total activity of the enzyme.

Results and discussion. Titration of hematin with liver homogenates revealed that the amount of cofactor 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 was related to variations in the lag period in initiation of the enzymic reaction was eliminated by increasing the time of incubation and/or adding

TABLE I. Specific Activity of Tryptophan Pyrrolase During Fractionation of Liver Homogenates from Normal and Endotoxin Poisoned Mice.

Homogenate fraction	Total tryptophan pyrrolase activity (μM kynurenine/g homogenate fraction/hr)	
	Endotoxin poisoned	Control
Whole homogenate	11.5	31.7
600 × *g* (30 min) supernatant fluid	17.0	51.5
12,000 × *g* (20 min) supernatant fluid	14.5	63.8
105,000 × *g* (50 min) supernatant fluid	8.4	63.4

0.8 mM ascorbic acid. The proportional reduction in total and native holo-enzyme 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 2 hours of incubation. The linearity of the reaction had been shown previously to persist during 3.5 hours incubation of normal mouse liver homogenates(8). The optimum pH for tryptophan pyrrolase activity of mouse liver homogenates was found to be around pH 6.5; however, the level of enzyme activity in homogenates from poisoned mice was not elevated proportionally more than that of homogenates from normal mice when the assay mixture was at the optimal pH. Whole liver homogenates from both normal and poisoned mice were supplemented with the reportedly essential(9) catalase-glucose oxidase system. A decrease in tryptophan pyrrolase activity of both types of homogenates was proportional to the increase in units of glucose oxidase added. Enzyme activity was optimal in the absence of an extraneous peroxide generating system. Similarly, the addition of 0.1 mM 3′,5′-cyclic adenosine monophosphate(10) had no effect on the activity of whole homogenates of livers from normal or poisoned mice.

The results of these kinetic studies seem to eliminate the possibility that decreased enzyme activity in poisoned livers reflected either variations in the lag period(11) or phenomena resulting from assay conditions which were not mutually optimal to both poisoned and normal livers. Similarly, the inability to elevate the activity of tryptophan pyrrolase associated with endotoxicosis to normal levels by adding 3′,5′-cyclic adenosine monophosphate, varying amounts of the hematin cofactor, or supplementing the assay with the peroxide generating system implies that the essential corequirements for enzyme activity are operative optimally in both normal and poisoned livers.

That the decreased tryptophan pyrrolase activity of whole liver homogenates from poisoned mice may result from an increased concentration of a substance with inhibitory activity was suggested by the results of the fractionation experiments summarized in Table I. The specific activity of the enzyme in normal liver increased with partial purification by differential centrifugation, whereas the specific activity of the enzyme in livers from endotoxin poisoned mice decreased. These findings infer the presence of a soluble inhibitor in liver homogenates from endotoxin poisoned mice. The plasma inhibitor reported previously(4) has subsequently been identified inferentially by comparative kinetics as a globin, which inhibits activity of the enzyme by competing for the hematin cofactor. The *in vitro* inhibition of tryptophan pyrrolase activity in normal liver homogenates by the plasma inhibitor could be reversed by addition of a stoichiometric excess of hematin. It is, therefore, highly unlikely that the plasma inhibitor is of any significant consequence in lowering tryptophan pyrrolase activity in the poisoned mouse liver, since the activity of the enzyme in liver homogenates from poisoned mice could not be elevated to normal levels, or reversed to any extent, by hematin.

As shown in Fig. 1, tryptophan pyrrolase activity was inhibited by relatively small concentrations of citrate. The enzymic reaction was also inhibited to approximately

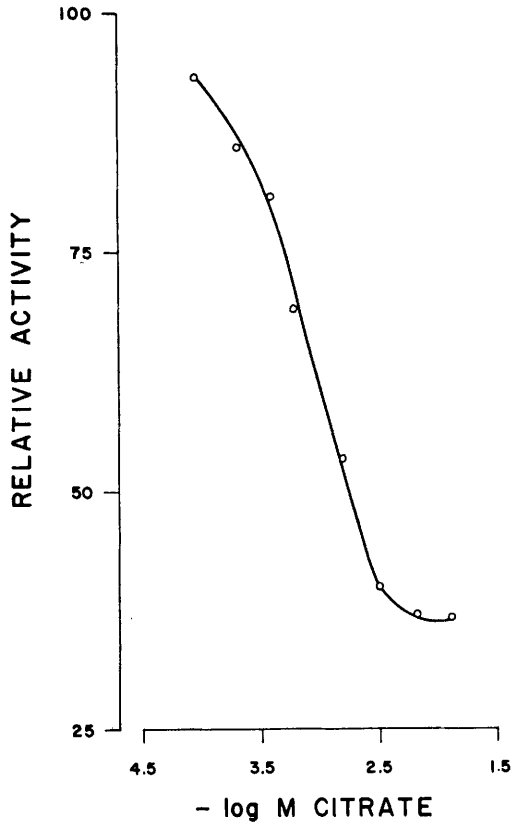

FIG. 1. Inhibition of tryptophan pyrrolase activity of whole liver homogenates by sodium citrate at pH 7.0.

the same extent on a molar basis by ethylenediamine tetraacetic acid at pH 7.0. Unlike inhibition by plasma, neither citrate nor EDTA inhibition could be reversed by adding excess hematin to the reaction mixture. The possibility that citrate inhibition may be a mechanism by which tryptophan pyrrolase activity is irreversibly decreased in liver homogenates from endotoxemic mice is suggested by previous and concurrent investigations which demonstrated that the concentration of citric acid in certain tissues increased following injection of endotoxin. Berry *et al*(12) have shown that the citric acid concentration of liver 15 hours after intraperitoneal injection of heat-killed *Salmonella typhimurium* was 4 times normal. In contrast, there was very little change in concentration of blood citric acid. It is therefore concluded that at least a part of the irreversible decrease in tryptophan pyrrolase activity which occurs in homogenates of liver from poisoned mice may be attributed to inhibition by citrate.

Regardless of what circumstantial evidence may suggest as an 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 form of liver, *i.e.*, a completely homogenized organ. There is a distinct possibility that disruption of cells brings together certain normally non-interacting cellular constituents. If such were the case here, then the inhibition of tryptophan pyrrolase in homogenates by any inhibiting substance not in contact with the enzyme in a physiologically 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.

Summary. 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. 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 endotoxemic mice. The enzyme was found to be inhibited by citrate, the concentration of which increased substantially in the liver during endotoxicosis. Unlike inhibition by plasma, citrate inhibition could not be reversed by excess hematin cofactor. It was therefore concluded that at least part of the decreased activity of tryptophan pyrrolase in whole homogenates of poisoned mice may be the result of inhibition by citrate.

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Studies on the Mechanism of Action of Thymic Leukemogenic Virus.* (31172)

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At the present time little is known of the mechanisms by which leukemia develops in man. By the time clinical signs first appear, the disease is relatively far advanced. Fortunately, the laboratory mouse offers a convenient model in which to study leukemogenesis(1). Potent viral agents are available which induce leukemia in high incidence after a short latency(2). When these viruses are inoculated intraperitoneally into susceptible mice, a thymic lymphoid leukemia develops. When the same virus is given to thymectomized mice, a myeloid leukemia occurs(3,4).

The dynamics of the evolution of the thymic lymphoma are currently under study and several steps are already known. Within a day after virus inoculation, the titer of infectious (input) virus in tissues is significantly reduced. This period of "eclipse" is followed by a generalized viremia, at which time virus is present in the blood and may be found in all tissues examined. This viremia occurs many weeks before the earliest histologic appearance of tumor. Under these conditions it would appear that each of the 2 thymuses is equally exposed to the virus. It was therefore surprising to observe that the leukemogenic response was almost always unilateral, occurring in only one of the 2 thymuses(5,6).

In the development of thymic lymphatic leukemia, two types of mechanisms may be considered. The leukemogenic effect of the virus may be *direct,* acting on the cells of the individual thymus where the tumor first appears, or the virus may act *via* an *indirect* systemic leukemogenic action, perhaps mediated through immunologic mechanisms. The unilateral development of tumor in the face of a generalized viremia suggested the possibility of an indirect leukemogenic action(6). Recent studies by Lieberman, Haran-Ghera, and Kaplan suggested that a direct viral effect was operative(7). If the virus acts *directly* on the thymic cells, then inoculation of virus into one of the thymuses should produce the tumor in that thymus and not in its mate. In the present quantitative study, we have confirmed and extended the report of Lieberman *et al,* and present additional data which support the hypothesis that the leukemogenic virus acts directly *within* the inoculated thymus.

Materials and methods. A murine leukemia virus (Rich) was employed as previously described(8). A 100 μl syringe was fitted with a 30 gauge needle which had been reground

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