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Immunological Quantitation of Hepatic Tryptophan Oxygenase in Endotoxin-Poisoned Mice

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An apparent inhibition of induction of mouse hepatic tryptophan oxygenase by endotoxin has been reported previously, as evidenced by low catalytic activity. This could be due either to decreased tryptophan oxygenase levels or to inactivation of existing enzyme molecules. To resolve this question, the enzyme was quantitated immunologically in control and endotoxin-poisoned mice. Tryptophan oxygenase was purified and used as an antigen to prepare anti-enzyme antibodies. The antiserum was shown to be monospecific by immuno-electrophoresis. Addition of the antiserum to high-speed supernatant fluids of liver homogenates of control or endotoxin-poisoned mice resulted in precipitation of the enzyme. Radial immunodiffusion assays revealed that there was less enzyme in livers of mice that received 1 mean lethal dose of endotoxin. It was concluded that endotoxin interfered with the synthetic process that results in enhanced levels of tryptophan oxygenase.

Bacterial endotoxins are known to inhibit the induction of some hepatic enzymes in mice, namely, tryptophan oxygenase (TO) (2, 3) and phosphoenolpyruvate carboxykinase (PEPCK), (5, 9). It was postulated by Berry (1) that the lost of inducibility of these and possibly other enzymes may result in metabolic impairment and subsequent loss of available energy, which then leads to irreversible shock. In light of this possibility, it becomes important to establish how endotoxin interferes with catalysis. Lower enzyme activities, as measured by formation of product, could be the result of either lower enzyme levels or inactivation of existing molecules. In the former case, the actual number of enzyme molecules would be diminished in poisoned animals, whereas in the latter, the number of molecules would remain the same in control and poisoned mice, but the activity would be less by virtue of the enzyme inactivator. A previous report from this laboratory indicated that endotoxin prevents the synthetic process that brings about higher levels of PEPCK (10). Evidence presented in this communication points to a similar effect on tryptophan oxygenase.

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MATERIALS AND METHODS

Purification of TO. Twenty-five outbred male Swiss mice (Texas Inbred Mouse Co., Houston, Tex.) were injected with 1 mg of cortisone acetate (United Research Laboratories, Philadelphia, Pa.), and on the following day they were given 25 mg of tryptophan (Sigma Chemical Co., St. Louis, Mo.) 3 h before sacrifice. The livers were excised and homogenized in 2 volumes of 0.15 M KCl containing 10^{-3} M tryptophan. This and all subsequent steps, unless otherwise stated, were performed at 4 C. The homogenate was centrifuged at 100,000 × *g* for 1.5 h and the precipitate was discarded. The supernatant fluid was heated at 56 C for 5 min and cooled rapidly in an ice bath. The resulting precipitate was centrifuged at 10,000 × *g* and discarded. Samples (15 ml each) of the supernatant fluid were filtered through a Sephadex G-100 column (4 by 50 cm) equilibrated in 0.05 M phosphate buffer, pH 7.0, containing 10^{-3} M tryptophan. Fractions with TO activity (which eluted in the void volume) were pooled and applied to a diethylaminoethyl (DEAE)-cellulose (type 52, Whatman) column at a concentration of 5 mg of protein per cm^3 DEAE bed. Removal of attached protein was accomplished with a 0.1 to 0.4 M linear NaCl gradient. TO eluted at approximately 0.2 M NaCl. Hydroxylapatite (HT) (Bio Rad, Richmond, Calif.) was added to the enzyme solution in sufficient amounts to remove TO activity from the supernatant fluid. The gel was centrifuged at 2,000 × *g* for 10 min and the supernatant fluid was discarded. The pellet was washed with 30 ml of 0.25 M phosphate buffer, pH 7.0, and recentrifuged. TO was

then extracted from the pellet with 30 ml of 0.5 M phosphate buffer, pH 7.0. The solution was concentrated to 4 ml under nitrogen pressure in an Amicon Diaflow ultrafiltration chamber fitted with a PM-20 membrane. TO was then subjected to isoelectric focusing in an LKB 8101 column. The sucrose gradient containing the pH 3–10 ampholine solution was poured manually. The initial voltage was 500 V and the current was 8 mA. Electrofocusing was allowed to continue until the current decreased to 0.8 mA. Fractions were collected and assayed for pH, protein content, and TO activity. The enzyme was solubilized in 0.02 N NaOH prior to immunization of the rabbits.

TO determination. Kinetic assays were performed according to Cho Chung and Pitot (6). Activity was expressed as micromoles of formylkynurenine formed per hour per milliliter of enzyme solution. Immunization of rabbits, TO-anti TO titrations, radial immunodiffusion, and immunoelectrophoresis were carried out as described previously for PEPCK (10). The anti-whole mouse liver homogenate and anti-TO antisera were treated with ammonium sulfate (9) to isolate the immunoglobulin fraction.

RESULTS

Enzyme recoveries and activities at different stages of purification are presented in Table 1. Usually, a 100-fold increase in specific activity was obtained after absorption to hydroxylapatite (line 5) followed by a decrease after isoelectric focusing (line 6). The isoelectric point (pI) of the enzyme is approximately 5 (Fig. 1). A heavy precipitate was always observed at this pH, which could account, in part, for the loss in activity.

TABLE 1. *Purification of mouse-hepatic tryptophan oxygenase*

Determination	Total protein (mg)	Total activity	Specific activity
High-speed centrifugation	3,500	350	0.10
Heating at 56 C	1,700	320	0.20
Sephadex G-100	180	180	1.00
DEAE-cellulose	40	140	3.00
Hydroxylapatite	10	120	12.00
Isoelectric focusing	3	24	8.00

Only one constituent was detected in the purified preparation when tested with antibodies against whole mouse liver homogenate (Fig. 2a). Furthermore, the antiserum prepared against purified TO recognized a single component in both supernatant fluids of whole liver homogenates and purified TO (Fig. 2b). Addition of anti-TO antiserum to supernatant fluids of whole liver homogenates from normal and endotoxin-poisoned mice selectively removed TO activity (Fig. 3). As the antibody concentration increased, enzymatic activity decreased. Normal rabbit IgG had no effect when added to supernatant fluids (not shown here). The antiserum precipitated TO equally well in control

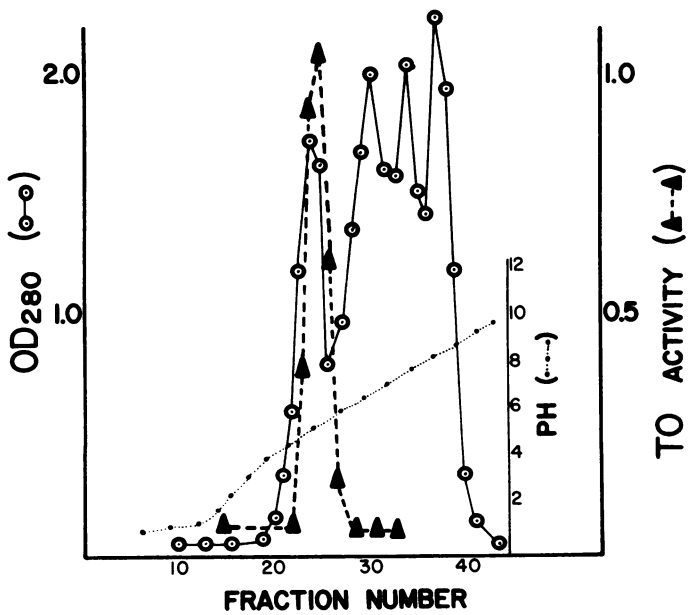

FIG. 1. *Isoelectric focusing pattern for TO. Samples, 12 mg each, of protein (TO after hydroxylapatite absorption) were electrofocused for 48 h in an LKB 8101 column at 500 V using a pH 3–10 ampholine solution. Proteins were collected in 1.5-ml samples. Symbols: ○, absorbance at 280 nm; ▲, TO activity; ●, pH.*

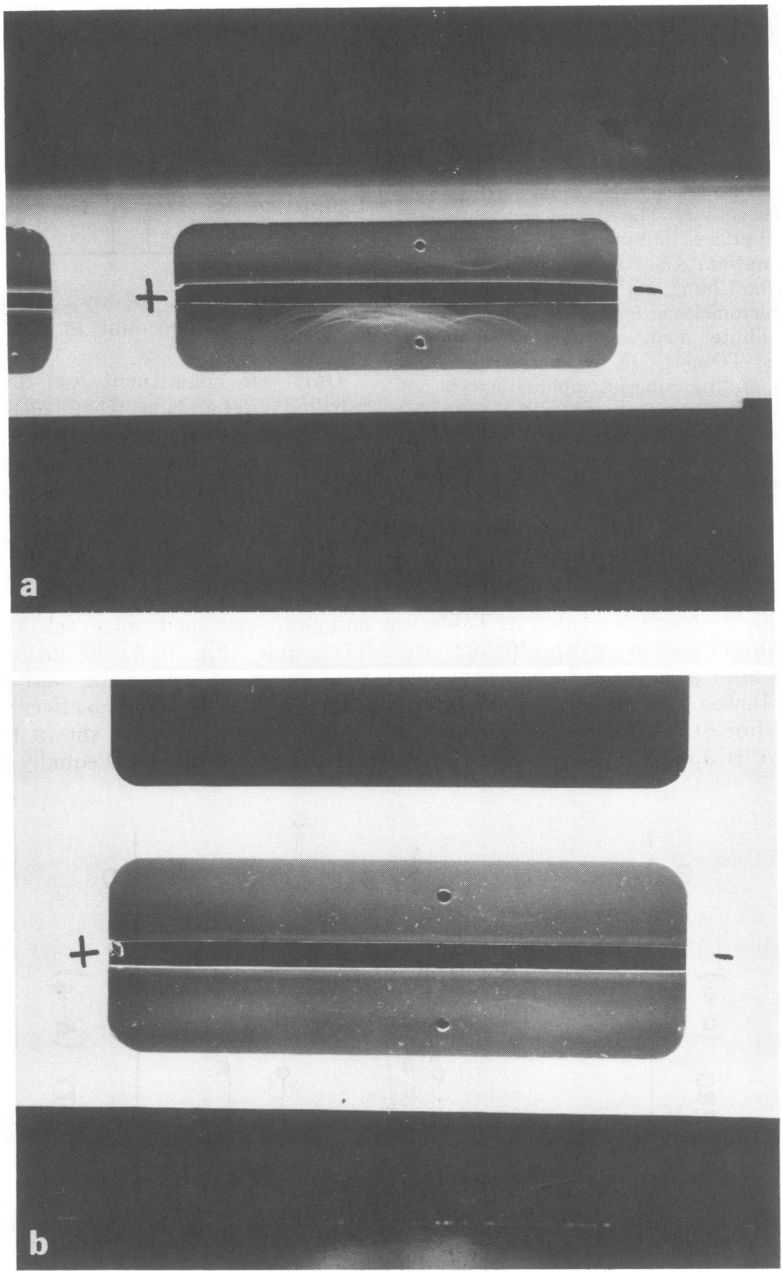

FIG. 2. (a), *Immunoelectrophoretic pattern of purified TO (top) and high-speed supernatant fluid of whole liver homogenate (bottom). Center trough contains rabbit anti-whole liver homogenate.* (b), *Same as above; center trough contains rabbit anti-TO immunoglobulin.*

and poisoned mice, as witnessed by the similarity of slopes in both curves.

Quantitation of TO in control and poisoned mice was performed by radial immunodiffusion (Fig. 4). The top row contains serial dilutions of a 33% homogenate from a pool of livers of five normal mice. The bottom row contains similar dilutions of liver homogenates from mice that received one mean lethal dose of endotoxin 20 h before sacrifice. It is apparent that livers from the control group gave rise to larger rings than livers from the poisoned group. This means that endotoxin causes a reduced output of tryptophan oxygenase in poisoned mice.

A linear graph is obtained when the $\log_{10}$ of TO activity (assayed catalytically) is plotted against the diameter of precipitin rings (Fig. 5). When TO activity is measured enzymatically in samples from endotoxin-poisoned mice, the values obtained are within 10% of those calculated from the standard curve (Table 2). A sensitive assay for enzymatic activity based of the size of precipitin rings was, therefore, developed.

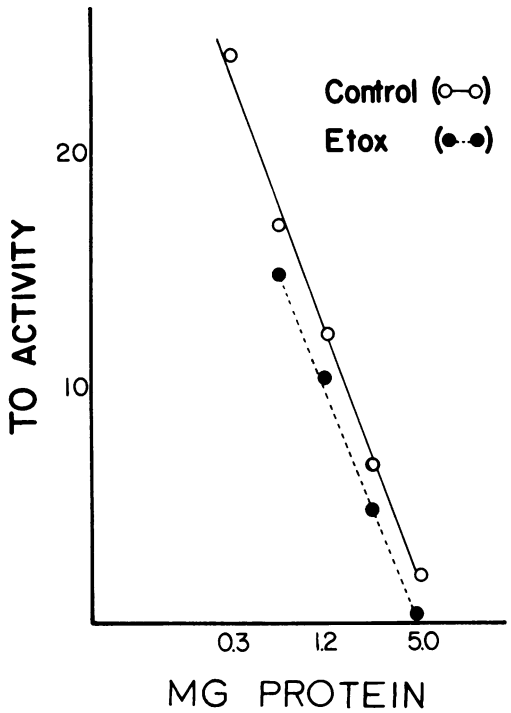

FIG. 3. *TO precipitation from liver supernatant fluids by anti-TO. Increasing amounts of anti-TO immunoglobulin were added to samples of high-speed supernatant fluids of liver homogenates of control* (O) *or poisoned* (●) *mice. After incubation and centrifugation of the mixture, the supernatant fluid was assayed for enzymatic activity. TO activity is expressed as micromoles of formylkynurenine per hour per 2 ml of supernatant fluid.*

DISCUSSION

The criteria for the validity of radial immunodiffusion assays for TO as described here are (i) antigenic purity of the enzymatic preparation, and (ii) specificity of the antiserum for TO. Both of these conditions are satisfied by immunoelectrophoretic patterns showing a single precipitin line and by the precipitability the enzyme from supernatant fluids after addition of anti-TO antibodies. Keeping in mind the first condition, it must be noted that although isoelectric focusing partially inactivated TO it also fractionated the enzyme preparation into at least four discrete protein components, only one of which had enzyme activity. Isofocusing, therefore, may not be recommended if one wishes to perform kinetic assays with TO; on the other hand, this step is very helpful if the enzyme is to be used as an antigen, regardless of its catalytic power at the time of injection.

Measurement of enzyme activities by radial immunodiffusion has several applications. It should be stressed that this method permits the correlation of catalytic activities from the standard with enzyme levels from properly prepared samples. Since small volumes are needed for the assay, it can be used to measure enzymatic activities in small samples such as fetal tissues. Furthermore, it is also possible to adapt this method for clinical assays of serum enzymes when dealing with a large number of test samples.

Immunological data presented in this paper indicate that there is less TO in livers of endotoxin-poisoned mice. This corroborates and explains Moon and Berry's finding that the in vivo catalytic potential of TO is decreased by endotoxin (7). It can only be speculated as to how the synthesis of the enzyme is inhibited, since it was previously shown that endotoxin does not enhance the rate of tryptophan oxygenase degradation (4). Hepatic messenger ribonucleic acid and total protein synthesis increase (11) at the time when TO is inhibited, hence it seems that endotoxin poisoning does not elicit a total impairment of homeostasis.

Endotoxin does not inhibit TO or PEPCK directly (4, 9), but rather it seems to operate via the release of mediators. As yet, the "target cells" have not been identified, but they presumably release or produce a substance(s) that controls levels of selected liver enzymes. Reticuloendothelial cells are prime suspects. Experiments are now in progress to selectively obliterate B cells, T cells, and Kupffer cells in vivo, to

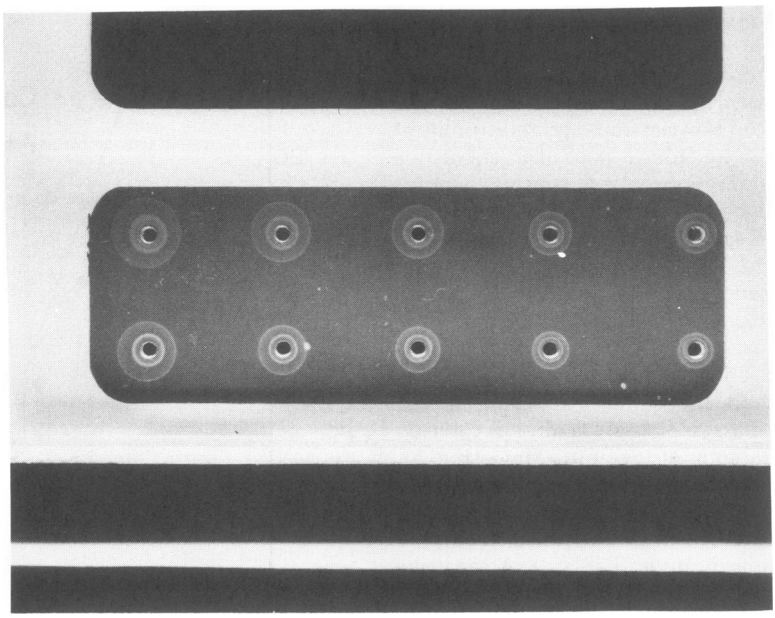

FIG. 4. *TO radial immunodiffusion assay. Top row: serial doubling dilutions of high-speed supernatant fluids of 33% liver homogenates from normal mice fasted for 20 h. Bottom row: serial doubling dilutions of high-speed supernatant fluids of 33% liver homogenates of mice injected with 1 mean lethal dose of endotoxin 20 h before sacrifice.*

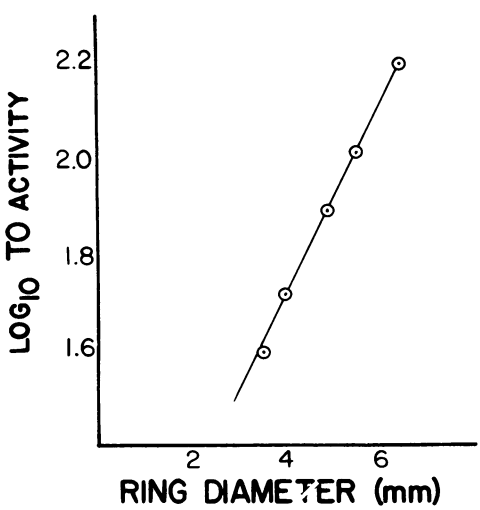

FIG. 5. *Quantitation of radial immunoassay. The diameter of precipitin rings from controls (Fig. 4) is plotted against the $\log_{10}$ of TO activity assayed enzymatically in samples of the same preparation. TO activity is expressed as micromoles of formylkynurenine formed per hour per 2 ml of supernatant fluid.*

see whether the inhibitory effect of endotoxin is diminished. It is hoped that in this way a clearer picture of the metabolic changes during endotoxin poisoning will emerge.

TABLE 2. *Theoretical and actual to activity in endotoxin-treated mice*

Sample dilution	Enzymatic assay	Calculated from plot
Undiluted	96	103
1:2	43	48
1:3	22	25
1:4	11	12

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