COMPARATIVE STUDIES ON THE BIOLOGICAL EFFECT OF TOXOHORMONE AND BACTERIAL LIPOPOLYSACCHARIDE

II. Changes in Liver Catalase Subfractions Separated by Electrofocusing

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Introduction

The depression of liver catalase level has been known as the most outstanding and constantly demonstratable of the systemic changes in the tumor bearing animal. In 1948 Nakahara and Fukuoka (20) first demonstrated that the decrease of the liver catalase activity can be reproduced in normal mice by injection of toxohormone, which is a water soluble, thermostable and ethanol precipitable factor isolated from human and experimental tumors. Since then several other systemic changes occurring in tumor bearing animals were reproduced in normal animals by injection of toxohormone (7, 15, 19, 21).

In spite of the many favorable observations hitherto reported, there still remains some skepticism concerning the existence and the role played by this substance in the tumor bearing host. It has also been shown that the hepatic catalase activity of normal animals can be lowered by the induction of aseptic inflammation with turpentine oil injection (15),

by injection of RES stimulants such as talcum or zymosan (16), and even by injection of normal tissue homogenate (1). Kampschmidt (17) was also opposed to the toxohormone concept based on his observation in which a very minute amount of bacterial endotoxin can produce a marked depression of liver catalase activity following injection into normal animals. Although a number of workers succeeded in isolating potent catalase depressing fractions from aseptic malignant tumors (22, 25), the controversy cited above will continue until the fundamental mechanism involved in precipitating catalase depression in tumor bearing state is clarified.

In a previous report from our laboratory, a clear cut difference was described in the content of hepatic ferritin, which showed a marked lowering in rats treated with multiple subcutaneous injections of minute doses of toxohormone whereas normal amounts of hepatic ferritin were recovered from rats treated similarly with bacterial lipopolysaccharide (7). This work was extended further to demonstrate an alteration in ferrokinetics by ^{59}Fe-labeled colloidal iron, of which utilization mainly depends on the functional state of the reticuloendothelial system, and a marked retardation was found in the rate of red cell utilization of injected radioiron in tumor bearing as well as in the toxohormone treated animals (28, 29).

In the present report, the authors have examined the effect of toxohormone and those of bacterial endotoxins on the hepatic catalase subfractions of normal rats separated by electrofocusing technique.

Materials and Methods

I. Materials

1) Animals. Male albino mice, dd strain, weighing approximately 20 g and rats of Wistar strain weighing approximately 130 g were used. Animals were fed ad libitum for at least two weeks with commercial Oriental solid diet with water before the beginning of each experiment.

2) Toxohormone preparation. Crude toxohormone was prepared from non-necrotic portions of Yoshida sarcoma tumors produced in rats as an ethanol precipitate by the method of Nakagawa et al. (19). Subsequent fractionation on DEAE Sephadex A-50 column by the stepwise elution method was carried out as reported previously (7).

3) Bacterial lipopolysaccharide. Purified lipopolysaccharide extracted from Salmonella typhimurium Strain LT 2, by the method of O. Westphal was kindly donated by Dr. Kawakami of Department of Bacteriology, Faculty of Medicine, Gumma University, and lipopolysaccharide of E. Coli, 055:B5 was purchased from Difco Co. USA.

4) Crystalline liver catalase. Bovine liver catalase, 2 times crystallized and having a catalase activity of 42,000 Sigma units per mg, was purchased from Sigma Co. USA.

5) Ampholine. Carrier ampholytes Ampholine, 40% aquanous solution of pH range of 5 to 8 (Batch No. 11) was obtained from LKB produkter, Sweden, and used at the final concentration of 1% in a sucrose density gradient.

II. Methods

1) Preparation of enzyme solution for electrofocusing. Mice were killed by decapitation and exanguinated. Rats were bled from the abdominal aorta and perfused with 150 ml of physiological solution under application of mild pressure until the appearance of the liver returned to its original tan color. In either case, the liver was rapidly excised and weighed 1 g portion of the tissue was homogenized with 9 ml of ice cold 0.2% Triton X-100 solution. The homogenate was spun down at 105,000 x g for 60 minutes at 4°C. After removing the whitish lipid layer, the supernatant fluid was pipetted out and 1 ml aliquot was subjected to electrofocusing.

2) Electrofocusing. Electrofocusing was carried out by the method of Vesterberg and Svensson (27) using LKB Ampholine column 8101 of 110 ml capacity. Some minor modifications were made as described below. Formation of a linear sucrose gradient in the column was done by the use of peristaltic pump according to the method of Ayad et al. (3), employing dense and less dense solution as illustrated in Fig. 1. Sample application was done from an additional sample line into the mid portion of the column to avoid the direct contact of the enzyme solution to the electrode solutions. Electrolysis was carried out for 48 hours at 10°C ± 0.5°C, which was maintained by an electronic thermal regulator. Initial current was 300 volts, 6 mA. As the focusing of the carrier ampholytes proceeded, the current dropped steeply and the final condition of 1000 volts, 1.5 mA was maintained for at least 40 hours. At the end of each run, the current was disconnected and elution of the content was carried out from the

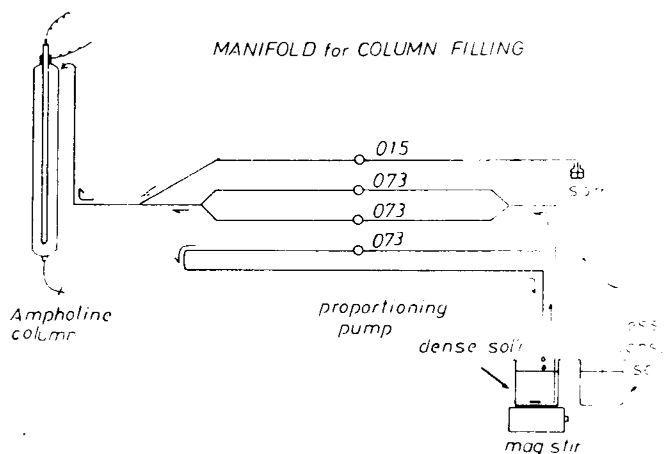

Fig. 1. The manifold used for an automated column filling.

bottom of the column through thin teflon tubing and fractionated using a peristaltic pump at a rate of 1 ml/min to yield 2 ml fractions.

3) Determination of catalase activities. Since the fraction number thus obtained from a single Ampholine column exceeded more than 60 fractions in each experiment, determination of catalase activity was made by the use of an automated catalase assay system as reported previously (8). Prior to catalase determination, pH of the individual fractions were measured using Heath EUW-301 pH recording electrometer connected to the combined pH electrode of Hitachi-Horiba 1028-10T at 20°C. The fractions were then transferred into sample cups and placed in the Sampler II. Since bound catalase was solubilized at the time of homogenization by surfactant Triton X-100, the solubilization coil was omitted from the manifold. The samples aspirated sequentially from

Sampler II at a speed of 40 samples per hour including wash out cups in between, was directly admixed with phosphate buffer solution and then mixed with substrate solution. The mixtures were then brought into the reaction coil immersed in an ice water bath and the reaction was terminated precisely after 60 seconds by adding 10 n H_2SO_4. The remaining peroxide in the reaction mixture was measured by an addition of titanyl sulfate reagent. After passing the time delay coil, the optical density of the reaction mixture was determined automatically by 15 mm flow cell and the results were recorded. Calculation of the catalase activity was done as reported previously, and expressed as k value per ml of the eluate (8).

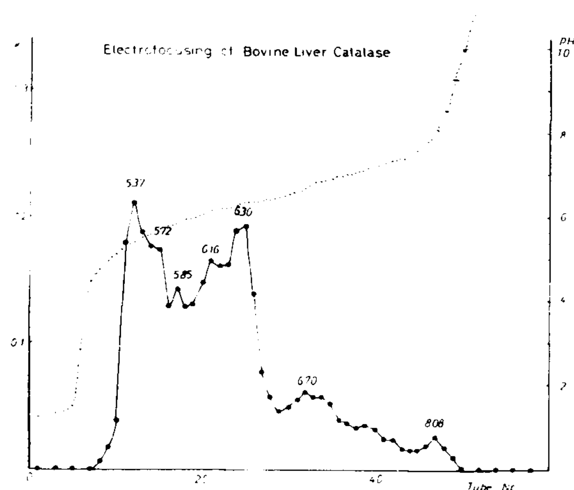

Fig. 2. Separation of bovine liver catalase by electrofocusing in 1% Ampholine of pH range of 5 to 8. The solid line and dotted line indicate enzyme activities and pH values, respectively.

Results

1. Fractionation of crystalline bovine liver catalase by electrofocusing

As shown in Fig. 2, 0.2 mg of two times crystallized bovine liver catalase separated into several activity peaks after electrofocusing. Catalase activity was found between 5.37 to pH 8.1 and the so-called acidic catalase fractions were predominant as compared with those of the neutral catalase fraction.

2. Fractionation of normal mouse liver catalase by electrofocusing

The catalase activity pattern obtained after electrofocusing of 105,000 x g supernatant fraction of the homogenate of the normal mouse liver is shown in Fig. 3. As apparent from the figure, the main peaks of enzyme

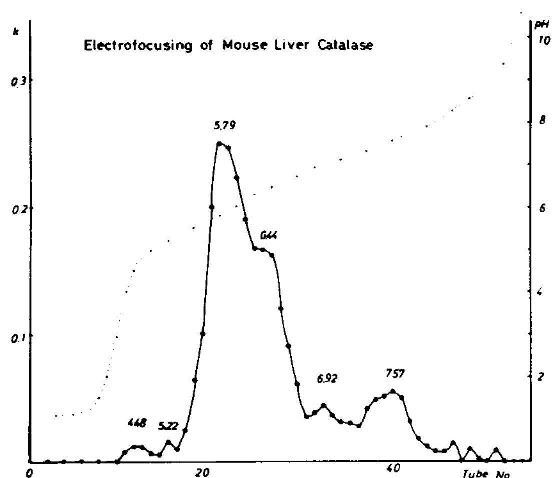

Fig. 3. Separation of mouse liver catalase by electrofocusing in 1% Ampholine of pH range of 5 to 8.

activity again emerged in the acidic catalase region of pH 5.8 to pH 6.4 and lesser activities were found in the range of pH 6.6 to 7.6. The validity of minor peaks appearing outside of these pH ranges is questioned due to their trace activities.

3. Fractionation of normal rat liver catalase by electrofocusing

As shown in Figs. 4 and 5, normal rat liver catalase also exhibited a marked heterogeneity, and was found to separate roughly into acidic and

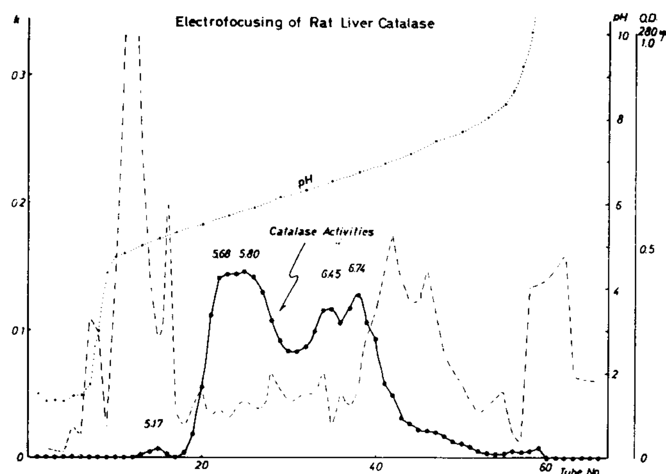

Fig. 4. Separation of liver catalase of normal rat by electrofocusing in 1% Ampholine of pH range of 5 to 8. The solid line, broken line and dotted line indicate catalase activities, optical densities at 280 mμ and pH values, respectively.

neutral fractions, and the pH values of the individual activity peaks differed from those of crystalline bovine liver catalase and from mouse

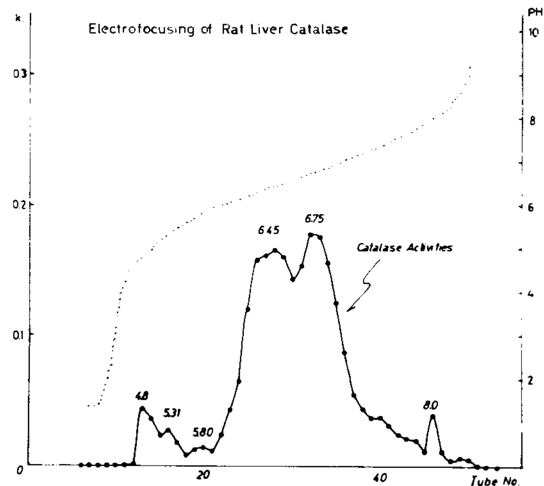

Fig. 5. Separation of liver catalase of normal rat by electrofocusing in 1% Ampholine of pH range of 5 to 8.

liver catalase. By repeating fractionation of liver catalase of normal Wistar rats, it was found that both pH values and relative activity of the acidic catalase fraction varied significantly from one experiment to the other while those of the neutral catalase fraction which emerged at pH 6.45 and 6.75 were rather constant when compared to the former fraction.

4. Liver catalase subfractions of rat treated with toxohormone or bacterial lipopolysaccharides

In order to assess the effect of toxohormone and bacterial endotoxins on rat liver catalase, the doses of the materials to be injected were first determined to produce a nearly 50% depression of total liver catalase by a single intraperitoneal injection, the dose was 4 mg for toxohormone

Fraction II and 0.1 mg for bacterial endotoins. After 24 hours of intraperitoneal injection, the animals were killed by bleeding from the abdominal aorta and enzyme preparation was obtained as described in the method.

The fractionated patterns of rat liver catalase injected with either preparation of bacterial lipopolysaccharide was illustrated in Fig. 6.

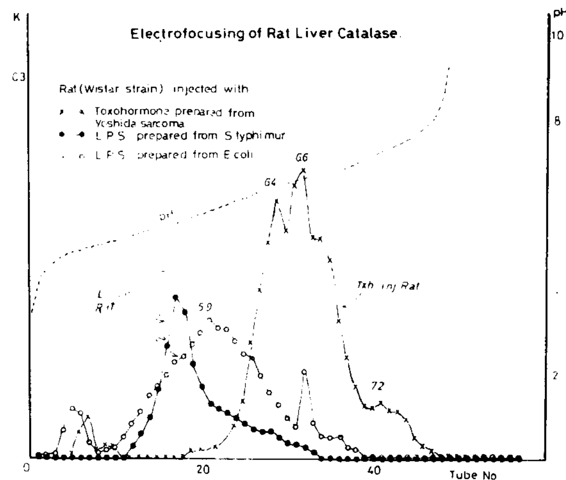

Fig. 6. Changes in isoelectric fractionation pattern of rat liver catalase by the injection of toxohormone and of bacterial lipopolysaccharide.

As appa ent from the figure, most of the catalase activity recovered after electrofocusing was found in a so-called acidic catalase region of pH 5.6 to 5.9. A weak activity was also found in a neutral catalase region. After 48 hours of endotoxin injection, total liver catalase

activity returned to their control values and neutral catalase activity reappeared in the pH region of pH 6.4 to 6.7.

on the other hand, patterns of catalase activity following toxohormone injection significantly differed from those of endotoxin injected animals. Most of the activity appeared in ranges from pH 6.4 to 7.2, and the two main peaks corresponded to pH 5.4 and 6.6. The acidic catalase was lacking in this enzyme preparation.

Discussion

The present experiment on isoelectric fractionation of liver catalase confirmed the existence of catalase subfractions showing different isoelectric points in rat, mouse and bovine enzyme preparations.

Although successful fractionations of liver catalase by electrofocusing were reported by a number of investigators (6, 9, 23), pI values of the individual catalase fractions differed from one report to the other. For example, Nakamura described the pI values of normal mouse liver catalase to be pH 6.33, 6.40, 6.53, 6.68 at 0 to 1°C, and Feinstein reported the pI value of particulate catalase of a normal mouse of Cs^a strain to be pH 6.7 and soluble catalase at even higher pH values of pH 8.0 to 8.5 using pH 3-10 carrier ampholytes. Our data on liver catalase subfractions of normal mouse of dd strain after electrofocusing also differed from these investigators and major catalase activities were found in the pH region of 5.37 to 6.30 with several activity peaks and minor activities in the higher pH region from pH 6.7 to 8.1.

As far as isoelectric fractionation of rat liver catalase are concerned, the present results roughly coincided with those reported by Nakamura et al. (23). The differences in pI values between these and ours may partially be attributable to the difference in experimental conditions such as preparation of the enzyme specimen, temperature of isoelectric fractionation and pH determination and so forth. There are, however, certain differences in the relative activities and pI values of acidic catalase fractions in normal rats. This may very well be due to the labile nature of acidic fractions as has been postulated by previous workers (23), other possibilities must also be considered.

Although similar heterogeneity of liver catalase was found by chromatographic (13, 26) as well as by electrophoretical means (12), the concept that the heterogeneity of catalase subfractions originates in catalase isoenzyme in vivo, was seriously criticized recently. Heidrich (10,11) working on three preparations of purified beef liver catalase, found 3 to 5 bands on polyacrylamide gel electrophoresis. Reduction or oxidation of these preparations resulted in the convergence of activity toward slowly or rapidly migrating fractions, respectively. Further it was demonstrated that carefully isolated peroxisomal catalase exhibited one homogenous band of the slowest mobility, whereas sonic oscillation of the same specimen readily resulted in the formation of five active fractions of different mobilities. Aebi and his coworkers (2, 4) studied erythrocyte catalase and concluded that the catalase isoenzymes separated by the column chromatographic method of Thorup et al. (26), are not true

isoenzymes in a strict sense but are merely oximers derived from the native catalase molecule by in vitro oxidation. These results seemed to indicate that due caution must be exercised in interpreting the experimental data on catalase subfractions separated by any of the prevalent separation methods.

Our experimental results on the depression of acidic catalase fractions of normal rat liver by injection of partially purified toxohormone preparation is consistent with the report of Nakamura et al.(23), who described a marked decrease of acidic catalase fraction in rats inoculated with Rhodamine sarcoma and in mice injected with in vivo catalase-depressing substances extracted from the tumor. On the contrary, neutral catalase fractions were markedly depressed in rats injected with small amounts of bacterial endotoxin. If catalase subfractions are merely the product of in vitro oxidation of native catalase molecules, the occurrence of different subfractions following administration of toxohormone or of endotoxin is difficult to interpret unless certain mechanisms other than oxidation in vitro were involved. At present, the site and mode of synthesis and destruction of intracellular catalase molecule is not known. However, Higashi (14) has reported recently that the depression of liver catalase in tumor bearing animals are caused by the depressed synthesis of catalase, of which the synthetic rate was assessed by the incorporation of C^{14}labeled leucine into nascent catalase contained in ribosomal fractions. On the other hand, Mazur (18) found that intracellular oxidation process is markedly enhanced in endotoxin treated rats.

To what extent the intracellular oxidation affects the catalase subfraction is not known as yet. Separation of peroxisomal catalase by electrofocusing under anaerobic conditions in the presence of SH protecting reagent is now in progress and will be reported shortly.

Conclusion

Isoelectric fractionation of liver catalase of different animals has disclosed a number of subfractions characteristic to each animal species. Rat liver catalase was found to separate roughly into so-called acidic and neutral catalase fractions of which the pH values were 4.8 to 6.0, and 6.4 to 6.8, respectively. The pI values and relative activity of the former fraction varied considerably among normal rat specimens.

Injection of toxohormone and bacterial endotoxin into normal rat yielded essentially different fractionation patterns and toxohormone depressed acidic catalase fractions whereas endotoxin administration caused a marked depression of neutral catalase fractions.

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