ON THE DIFFERENTIAL INHIBITION OF THE MULTIPLE FORMS OF CATALASE IN MOUSE TISSUE

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1. Introduction

It has been known for some time that 3-amino-1,2,4-triazole (AT) inhibits the catalase (EC 1.11.1.6) levels of mammalian liver and kidney when injected into these species; but that the activity in erythrocytes remains constant with this treatment [1]. Furthermore, subsequent studies have established that a parallel situation prevails under *in vitro* conditions: whereas the level of catalase in rat liver and kidney homogenates is similarly affected by AT, blood haemolysates and purified catalase preparations are inhibited by this agent [2].

In considering these reported properties of catalase, the possibility that the inhibitory characteristics might be utilized to shed further light on the isoenzyme status of this enzyme was examined; for although catalase has been extensively investigated in the past, a diversity of opinion remains evident in the literature as to the extent and nature of the multiplicity of this enzyme [3, 4]. The *in vivo* and *in vitro* effects of AT on the multiple forms [4] of catalase from various organs of the mouse have been studied, and evidence has been provided for a differential inhibition of these heteromorphs, with the more anodic electrophoretic forms being appreciably more susceptible to inhibition than slower migrating species. This data is considered to provide added confirmation of the reality of catalase multiplicity *in vivo*.

2. Methods

Adult female mice (Quackenbush strain) were used in these experiments. The AT was injected intraperitoneally as a sterile aqueous solution, at a dosage of 1 mg/gm of body weight. Control mice were injected with equal volumes of NaCl adjusted to the same freezing point depression as the AT solution. The animals were killed by exsanguination 6 hr after the injections, and the organs excised and washed in cold isotonic sucrose solution. Homogenization of the tissues was achieved in a Potter-Elvehjem homogenizer after the organs had been carefully dried by blotting and weighed. Liver homogenates were prepared in 10% sucrose/50 mM Tris-HCl buffer pH 7.2 for differential intracellular studies; whereas total liver homogenates and homogenates of all the other organs except brain were prepared in 50 mM Tris-HCl pH 7.2 aqueous buffer. Brain homogenates were prepared in the latter buffer, modified by the incorporation of 5% Triton X-100 to facilitate solubilization.

Liver homogenates were fractionated according to the differential centrifugation procedure of Hogeboom [5], with the nuclei/whole cell fraction being discarded and the resultant supernatant centrifuged to yield 2 fractions: large granules and the supernatant (extra particulate cytoplasm or EPC). The large granule fraction was extracted by shaking with 50 mM Tris-HCl buffer pH 7.2 in measured amounts, and centrifuged to yield a large granule extract, henceforth referred to as the peroxisomal aqueous extract (PAE). Blood was obtained by heart puncture and haemolysed by shaking with 50 mM Tris-HCl buffer pH 7.2. The homogenates and the haemolysate were all spun at

100,000 *g* on a Beckman L4 ultracentrifuge, and the supernatants extracted prior to electrophoresis.

Catalase activity was determined by a modification of Beer and Sizer's spectrophotometric method [6]. Hydrogen peroxide decomposition was followed by measuring the initial rate of decrease in absorbance at 240 nm in a 3 ml cuvette using a Unicam S.P. 800 spectrophotometer at 37°. The reaction mixture contained 90 ml of 50 mM Tris-HCl buffer pH 7.2 to which 0.1 ml of 30% H_2O_2 (w/v) had been added. Varying amounts of enzyme (5.40 μl) were added to the reaction mixture, and activities in I.U. were calculated, utilizing the reported value for the extinction coefficient of hydrogen peroxide at 240 nm of 43.6 $M^{-1}cm^{-1}$ [14] : I.U. = ΔA/min × 69 × dilution factor.

Supernatant fractions from the various organs were subjected to zone electrophoresis on vertical 12% starch gels at 4° with 30 mM Tris-glycine buffer pH 9.0 [4]. A potential difference of 20 V/cm was applied for 16 hr to ensure an adequate separation of the isoenzymic forms. Both the electrolytes and gel buffer contained 1 mM dithiothreitol and 1 mM EDTA; these additions allowing a stabilization of the banding pattern for catalase without interfering with the mobility. Following electrophoresis the gels were sliced, incubated in 100 mM phosphate buffer pH 6.0 for 30 min; stained for catalase activity after the method of Scandalios [7] and photographed.

The 10% sucrose supernatant (EPC extract) from both control and AT treated mice was subjected to electrophoresis under the same conditions. In order to facilitate the elucidation of an activity profile, however, about 1 ml of extract was applied in this case to a starch gel in a slot cut across the width of the gel. After electrophoresis (20 V/cm, 16 hr), the gel was cut transversely into equal pieces with a gel cutter. Each segment of gel was homogenized manually in 0.3 ml of 50 mM Tris-HCl buffer pH 7.2 and the homogenate spun at 6000 rpm in a Christ centrifuge. The supernatants were then assayed for catalase activity, and these values plotted against the fraction number of the gel slices.

3. Results

Table 1 demonstrates the differential effect of AT on organ catalase levels of the mouse. Whereas the injection of AT has only a small effect on the levels of blood catalase, the catalase levels from the PAE are markedly depressed. Catalase from the EPC responds in an intermediate position between these two extremes.

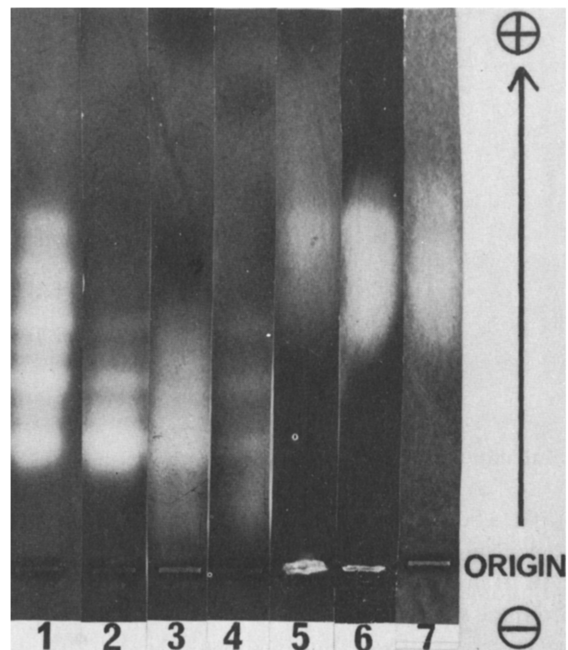

Fig. 1. Zymograms of mouse liver catalase activity obtained from different organs and different subcellular fractions for normal mice and for mice injected with AT. 1: Supernatant catalase from sucrose homogenate (EPC); 2: as in (1) but mouse previously injected with AT; 3: catalase from blood haemolysate (normal); 4: catalase from aqueous heart homogenate (normal); 5: catalase from aqueous spleen homogenate (normal); 6: catalase from aqueous kidney homogenate (normal); 7: catalase from aqueous extracted large granules of liver (PAE) (normal).

From fig. 1 it can be seen that a relationship exists between the occurrence of the more anodally migrating species of catalase and the percentage inhibition by AT. Spleen, kidney and PAE catalase have much more of the anodally migrating catalase species and are considerably depressed by AT treatment. The EPC extract contains more of the cathodally migrating catalase species and is less affected by AT, while blood and heart catalase have the least of the more anodally migrating forms and are, correspondingly, the least affected by AT treatment.

A more graphic visual representation of this effect

Table 1
The depression of catalase levels in mouse tissues by amino-triazole.

Organs	Normal	AT-treated	Depressed (%)
Liver (10% sucrose homogenate) (supernatant EPC)	8.4×10^3	4.28×10^3	49
Liver (Peroxisomal AE)	8.9×10^3	1.80×10^3	80
Liver (total aqueous homogenate)	15.8×10^3	7.7×10^3	51
Kidney (total aqueous homogenate)	1.9×10^3	414	78
Blood (aqueous haemolysate)	3.7×10^3	2.8×10^3	24
Heart (aqueous homogenate)	420	270	36
Spleen (aqueous homogenate)	720	205	72
Brain (0.5% TX-100 homogenate)	190	70	63

Results are expressed as mean values of 3 experiments, and in international units per gram wet tissue.

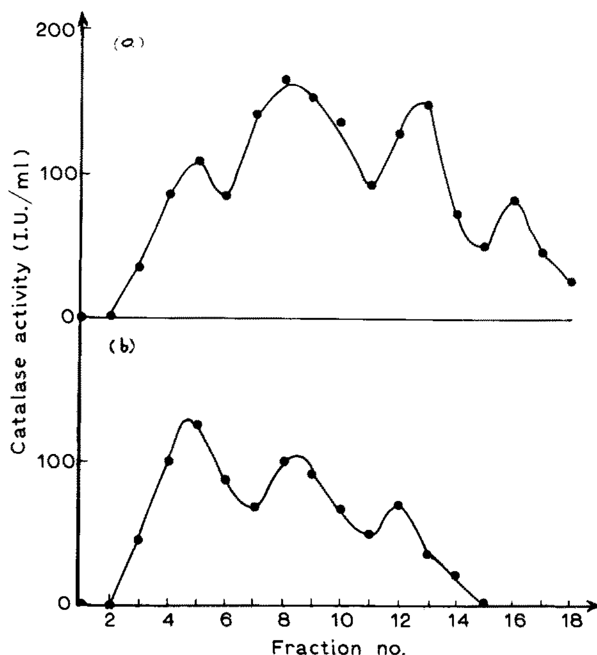

Fig. 2. Starch gel elution pattern of catalase activity in the extraparticulate cytoplasm of livers from (a) normal mice, and (b) previously injected with 3-amino-1,2,4-triazole.

can be seen by comparing slot (1) with slot (2), when it is evident that the injection of AT into the animal has resulted in a preferential inhibition of the more anodal bands of activity.

A similar type of effect is again evident from the gel elution studies which are compared in fig. 2. Four peaks of activity can be shown for the EPC catalase extracted from normal mice, whereas only 3 peaks, diminishing in intensity from the cathodal extreme, are shown for the EPC catalase extracted from mice treated with AT.

4. Discussion

This isoenzyme status of catalase from mammalian sources has been a keenly contested topic since the first fractionation of this enzyme in 1954 [8].

Holmes and Masters [3] have reported a complicated multiplicity of catalase in rat and mouse tissues and postulated that this might be ascribed to the presence of 2 types of subunit; a native sub-unit (A) occurring in the peroxisomes and the microsomes, and the other (A′) a more basic epigenetic modification [4]. The present results confirm the results of Holmes and Masters with respect to the isoenzyme patterns of mouse liver catalase but also bring to light a new complementary method of characterizing the different heteromorphs of this enzyme. The isoenzymic content

of catalase extracted from different organs of the mouse may be characterized in terms of the ratio of subunit types (i.e. A/A′) by the percentage depression of the catalase levels from that source following injection of AT (table 1, figs. 1 and 2).

Confirmatory evidence for the suggestion that an epigenetic modification takes place when catalase is released from the peroxisomes into the cytosol (EPC) may also be derived from the data presented. For example, the peroxisomal form is depressed to the extent of 80% by AT whereas the supernatant from (EPC) is depressed to a markedly lesser degree (49%). In normal mouse liver, the soluble catalase activity is shared to an approximately equal extent between the peroxisomes and the EPC [7]. In a total aqueous homogenate (peroxisomal + EPC) however, the depression by AT is only 51% or almost equal to the depression suffered by the EPC form (table 1). Taken in conjunction, these data indicate that the peroxisomal form is altered to a form which is less sensitive to inhibition, and more cathodal in migration on release into the cytoplasm.

In summary, then, the data in this communication provide confirmatory evidence for the existence of multiple forms of catalase in mouse tissues, furnishes a new chemical means of estimating the relative ratios of these isoenzymes, and draws attention to the parallel inhibitory responses of murine tissues to amino triazole under *in vitro* and *in vivo* conditions. Considered in toto, these facts would seem to place in question recent negations of the reality of catalase multiplicity in mammalian tissues [9].

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