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Inactivation of catalase with 3-amino-1,2,4-triazole: an indirect irreversible mechanism

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The metabolism of oxygen in biological environments generates a number of reactive chemical species which, if not controlled, can cause severe localized injury to the tissue [1]. Of these reactive oxygen metabolites, it is probably hydrogen peroxide which is the most toxic since it is able to oxidize proteins directly or can act as a precursor to produce the extremely reactive hydroxyl radical [2]. It is therefore not surprising that the hydroperoxidases, particularly catalases, are considered to play a pivotal role in protecting many tissues from the toxic effects of hydrogen peroxide [3].

Elucidation of the biological role of catalase has been limited by the absence of specific pharmacological inhibitors. While 3-amino-1,2,4-triazole is a compound which has been used to inhibit catalase in a number of studies [4–6], there is, in fact, some evidence to suggest that it does not have a direct effect upon catalase [7]. Nevertheless, systemic administration of 3-amino-1,2,4-triazole has been shown to result in inhibited hepatic, renal and ocular catalase activity [4–6]. *In vivo* experiments with 3-amino-1,2,4-triazole have provided the basis for the suggestion that catalase in the ocular tissues may fulfill a role in the maintenance of lens transparency [5, 6]. In contrast, the use of 3-amino-1,2,4-triazole in short term, *in vitro*, studies with ocular tissues has proved to be ineffective [8].

In this study, we have further characterized the mechanism of action of 3-amino-1,2,4-triazole upon purified catalase and the catalase activity of rabbit liver and rabbit iris-ciliary body. Our findings suggest that 3-amino-1,2,4-triazole has little direct influence upon catalase but is able to irreversibly inactivate a significant amount of catalase activity via an indirect mechanism.

Methods and materials

Chemicals. 3-Amino-1,2,4-triazole was purchased from the Sigma Chemical Co. (St. Louis, MO). Purified bovine liver catalase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Hydrogen peroxide was purchased as a 30% analar grade solution from the Baker Chemical Co. (Phillipsburg, NJ). All other chemicals were reagent grade unless otherwise stated. Tissue incubations were undertaken in modified Tyrode's solution which contained 145 mM NaCl, 5 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and 5.5 mM dextrose at a pH of 7.4.

Measurement of catalase activity. Catalase causes the breakdown of hydrogen peroxide to water and oxygen according to the overall reaction:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

The liberation of oxygen can be monitored polarographically using an oxygen electrode, enabling a straightforward method of measurement to determine catalytic activity in biological samples [9, 10]. This method was employed in the present study using a Clark type oxygen electrode (Yellow Springs, CA): 2 ml of potassium phosphate buffer (50 mM, pH 6.0) containing 10^{-3} M hydrogen peroxide was placed in a continuously stirred constant temperature (25°) chamber containing the oxygen electrode. The release of oxygen following introduction of the purified catalase or tissue homogenates (50 μl volumes) was recorded graphically on a chart recorder and used to compute the rate of breakdown of hydrogen peroxide.

Tissue preparation. The tissues (liver and iris-ciliary body) were obtained from albino rabbits (2–3 kg) which were killed painlessly by an overdose of sodium pentobarbital. The liver was excised and a portion washed in ice-cold Tyrode's solution to remove excess blood. The iris-ciliary body was carefully dissected from the enucleated eyes as described in detail previously [11]. The tissues were gently blotted dry on filter paper, weighed, and then thoroughly homogenized in ice-cold Tyrode's solution. Since the liver has considerable catalase activity, less concentrated homogenates of liver tissue were prepared (25 mg wet weight/ml Tyrode's) relative to the iris-ciliary body homogenates (50 mg wet weight/ml Tryode's).

Incubations with 3-amino-1,2,4-triazole. (1) Purified catalase. Commercially obtained catalase was diluted with Tyrode's solution to a final concentration of 325 units/ml. The diluted solution of catalase was then incubated for 4 hr at 37° in either the absence or the presence of 3-amino-1,2,4-triazole (10–100 μg/ml). At given periods of time (5 min and 4 hr) during the incubation, the catalase activity was determined polarographically as described above.

It has been suggested that 3-amino-1,2,4-triazole might inhibit catalase in the presence of hydrogen peroxide [7]. To examine this issue, additional experiments were undertaken in which 3-amino-1,2,4-triazole was incubated with catalase in the presence of hydrogen peroxide. In these experiments, catalase (162.5 units/ml) was incubated with 3-amino-1-2-4-triazole (100 μg/ml) for 4 hr at 37° in phosphate buffer containing 10^{-3} M hydrogen peroxide. The final concentration of catalase was less than used in the experiments described above to ensure that the hydrogen

peroxide would remain in solution long enough to potentially react with 3-amino-1,2,4-triazole and/or catalase.

(2) Liver and iris-ciliary body homogenates. The liver and iris-ciliary body homogenates were incubated at 37° or on ice either in the absence or presence of 3-amino-1,2,4-triazole (100 μg/ml). During a 4-hr incubation period, 100-μl aliquots of the homogenates were removed hourly and the catalase activity was determined as described above. After incubation for 4 hr, 5 ml of each of the incubated samples was placed in dialysis tubing and dialyzed overnight in a large volume of Tyrode's solution at 4°. This procedure removed 3-amino-1,2,4-triazole which passes through the dialysis membrane. The catalase activity of the non-dialyzable material was determined and compared to the catalase activity prior to dialysis.

Results and discussion

The activity of purified catalase was not influenced by 3-amino-1,2,4-triazole at concentrations up to 100 μg/ml (Fig. 1a). Incubation of catalase with 3-amino-1,2,4-triazole at 37° for time periods of up to 4 hr resulted in no measurable change in the activity of the purified enzyme (Fig. 1b). These findings demonstrate clearly that 3-amino-1,2,4-triazole has no significant direct inhibitory effect upon catalase activity.

In 1958, Margoliash and Novogrodsky [7] presented experimental evidence which suggested that 3-amino-1,2,4-triazole inhibited catalase in the presence of hydrogen peroxide. This contrasts with the present study; we found that 3-amino-1,2,4-triazole had no effect on catalase activity in the presence of hydrogen peroxide (Table 1). However, while Margoliash and Novogrodsky found that 3-amino-1,2,4-triazole inhibited catalase in the presence of hydrogen peroxide, it should be noted that their studies, although appropriate at the time, were nevertheless undertaken using a non-specific technique to measure catalase activity. Thus, there is a strong possibility that the discrepancy between the present study and that of Margoliash and Novogrodsky can be explained purely on the basis of our ability to measure catalase activity in a much more specific manner using the oxygen electrode.

Table 1. Influence of 3-amino-1,2,4-triazole (3-AT) on catalase activity following incubation in the presence of 10^{-3} M hydrogen peroxide*

Incubation (4 hr at 37°)	H_2O_2 metabolized (μmoles/min)
Catalase	3.3 ± 0.5
Catalase + H_2O_2	3.7 ± 0.13
Catalase + H_2O_2 + 3-AT	3.5 ± 0.5

* Each value is the mean ± S.E.M., N = 3.

When 3-amino-1,2,4-triazole was incubated at 37° with a homogenate of liver or iris-ciliary body, the catalase activity measured in the homogenate decreased substantially with time relative to controls containing no 3-amino-1,2,4-triazole (Fig. 2). Such an observation suggests that 3-amino-1,2,4-triazole might be converted to an effective inhibitor of catalase activity during incubation with the homogenized tissue. This suggestion is supported by the finding that the catalase activity was inhibited to a greater extent by incubation of 3-amino-1,2,4-triazole in homogenates of liver, a tissue known to be rich with enzymes capable of metabolizing foreign compounds. Incubation of the 3-amino-1,2,4-triazole at 0° in the presence of tissue homogenates for up to 4 hr did not diminish the initial catalase activity. Interestingly, 3-amino-1,2,4-triazole has been reported to exhibit a similar inhibitory effect on catalase when incubated with rat liver homogenates at 37°, but, again, this compound had no effect on the partially purified

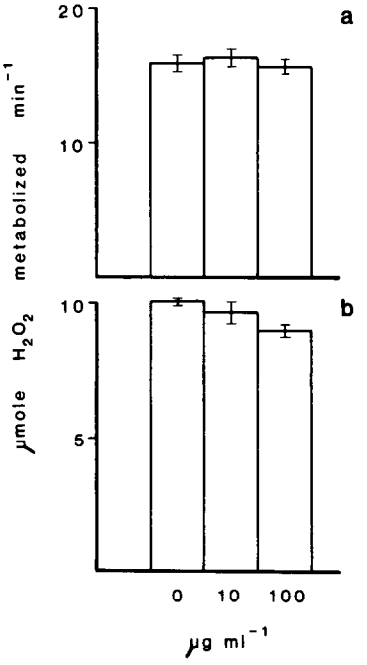

Fig. 1. Effect of 3-amino-1,2,4-triazole on purified catalase activity. Catalase activity was measured after incubation with 3-amino-1,2,4-triazole for 5 min (a) and 4 hr (b). Each value is the mean ± S.E.M. for three separate experiments.

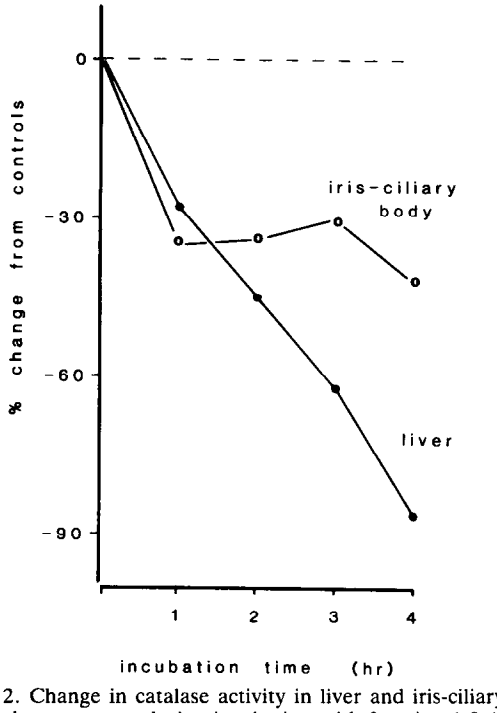

Fig. 2. Change in catalase activity in liver and iris-ciliary body homogenates during incubation with 3-amino-1,2,4-triazole. Tissue homogenates were incubated in the absence (controls) or presence of 3-amino-1,2,4-triazole (100 μg/ml) at 37°. The catalase activity in the homogenates containing 3-amino-1,2,4-triazole is expressed as a percentage of the activity in the control homogenates.

enzyme [7]. Therefore, it appears the 3-amino-1,2,4-triazole does not directly inhibit catalase and probably only affects the enzyme following its metabolic transformation.

We examined whether incubation of 3-amino-1,2,4-triazole in the presence of homogenized tissue resulted in a reversible inhibition of catalase activity by removing the 3-amino-1,2,4-triazole or its metabolites by dialysis. The dialysis tubing used in these experiments had a molecular weight cut off of 12,000, and therefore 3-amino-1,2,4-triazole (molecular weight 94) or metabolites derived from this compound would be expected to pass freely through this membrane. It was observed that dialysis of either liver or iris-ciliary body homogenates in which catalase activity had been inhibited by incubating with 3-amino-1,2,4-triazole did not restore the catalase activity (Fig. 3). The fact that catalase activity of the iris-ciliary body and liver homogenates could not be restored by dialysis suggests that the inhibition of catalase, induced indirectly by 3-amino-1,2,4-triazole, might be irreversible. This contention is supported by the studies of Jones and Masters [12], who showed that, after systemic administration of 3-amino-1,2,4-triazole, the restoration of catalase activity in the tissues followed a time course which corresponded to the synthesis of new enzyme.

In summary, the evidence presented in this study supports the concept that 3-amino-1,2,4-triazole does not directly inhibit catalase but, rather, undergoes metabolic transformation to form an irreversible inactivator of the enzyme. Consequently, experiments involving the systemic administration of 3-amino-1,2,4-triazole in order to examine catalase activity *in vivo* should be cautiously interpreted, and pains should be taken to differentiate between the direct and indirect effects of 3-amino-1,2,4-triazole upon catalase. Furthermore, studies using 3-amino-1,2,4-triazole to inhibit catalase *in vitro* may be misleading unless the experimental design incorporates a demonstration of the ability of the tissue to transform the compound into a catalase inactivator. Finally, it is clear that identification of the activity principle derived from 3-amino-1,2,4-triazole would provide a powerful pharmacological tool to further examine the biological role of catalase.

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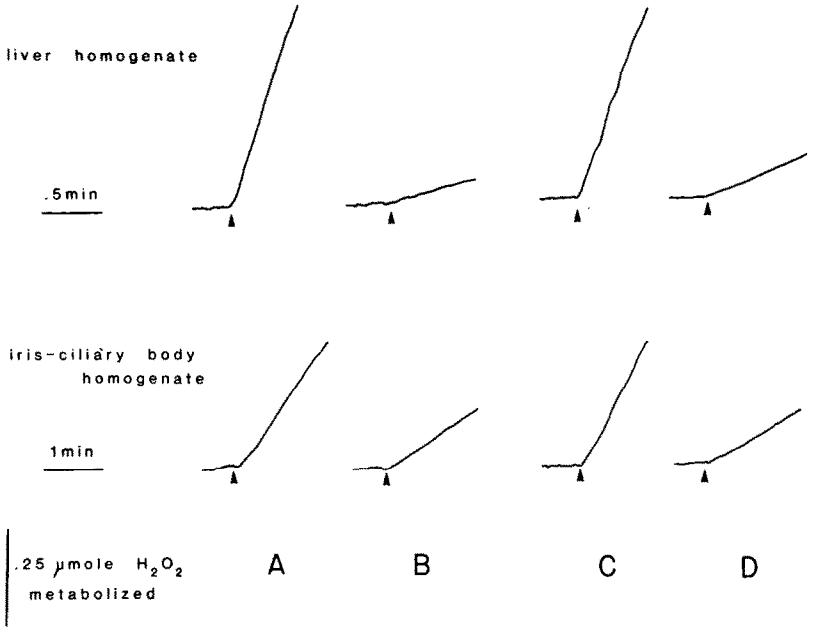

Fig. 3. Effect of dialysis on liver and iris-ciliary body catalase activity following inactivation by 3-amino-1,2,4-triazole. Each tissue sample (50 μl) was added (▲) to a solution of H_2O_2 (10^{-3} M), and the rate of peroxide metabolized was measured using an oxygen electrode connected to a chart recorder. The catalase activity in each tissue is shown following incubation for 4 hr at 37° in the absence (A) and presence (B) of 3-amino-1,2,4-triazole. These samples (A and B) were then dialyzed for 24 hr at 4°, and the catalase activity was again measured as shown in C and D respectively.

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Cytochrome P-450 activity in hepatocytes following cryopreservation and monolayer culture

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Hepatocytes in suspension or in primary culture are useful for the *in vitro* study of drug and carcinogen metabolism [1]. One of the biggest practical requirements is the ability to prolong the useful lifetime of hepatocyte preparations. Effective techniques for storing hepatocytes would be particularly useful when applied to human liver, for which availability is limited. Frozen hepatocytes may also be valuable for transplantation in the treatment of liver injury [2]. Certain studies have indicated that the viability and efficiency of drug metabolism of frozen and thawed rat hepatocytes was reasonably comparable with that of freshly prepared cells [3, 4]. Other studies, however, indicate marked abnormal morphology and metabolism following a similar cryopreservation protocol [5]. We have further investigated cell viability and cytochrome P-450-catalysed drug metabolism in post-mitochondrial supernatants derived from hepatocytes, in suspension and in primary monolayer culture, following cryopreservation. The effect of urea as a possible neutralizer of cryoprotectant toxicity [6] was also investigated.

The animals used were adult male Wistar rats (approximately 250 g) which were provided with food (422-modified diet, Heygates and Sons Ltd., Northampton, U.K.) and water *ad lib*. The procedure for isolation of hepatocytes was based on that of Seglen [7] with the following modifications. The rat was anaesthetized with an intraperitoneal injection of sodium pentobarbitone (60 mg/kg). The liver was preperfused at 37° with 100 ml of Krebs–Henseleit (KH) buffer pH 7.4 which was Ca^{2+}-free and contained *N*-tris-[hydroxymethyl]-methyl-2-amino ethane sulfonic acid (TES; 32 mM). The liver was then perfused with re-circulating KH buffer containing Ca^{2+} (2 mM), TES (32 mM) and 0.075% (w/v) collagenase (type IV Sigma or *Clostridium histolyticum*; BCL). The perfusion flow rate was 20 ml/min throughout. The cell suspension so prepared was kept on ice in KH and TES buffer prior to use and cells were counted using a Neubauer haemocytometer. This method provided freshly isolated hepatocytes which were approximately 85% viable (by trypan blue exclusion tests [8]) and approximately 90% viable (by lactate dehydrogenase (LDH) leakage tests [8]). Attachment of these cells to culture dishes was 86 ± 8%.

Isolated hepatocytes were suspended in culture medium to allow distribution of 5 ml of cell suspension (containing 2.5×10^6 hepatocytes) to each sterile 60 mm × 15 mm culture dish (Nunc). The culture medium consisted of modified Eagles medium (Gibco) with the following alterations: methionine (1 mM), δ-aminolaevulinic acid (77 μM), dexamethasone (260 nM), foetal calf serum (2.5%), horse serum (10%), insulin (13.25 IU/l), total glutamine (2.0 mM) and gentamycin (50 μg/ml). Cysteine was omitted. The culture dishes were then incubated for 20 hr in a 5% CO_2, humidified incubator at 37°. At 4 hr, the culture medium and non-attached cells were replaced with fresh medium (5 ml) and the number of cells removed were counted in a haemocytometer.

For freezing of cells in suspension, the washed hepatocyte preparation was centrifuged at 50 g for 1 min and the cells were resuspended in modified Eagles medium (MEM, Gibco) containing 1% bovine serum albumin and kept on ice in 2 ml polypropylene cryotubes (Nunc). Addition of cryoprotectant was based on the method of Van der Meulen *et al.* [9]. Thus 0.9 ml of cell suspension (approximately 1.0×10^7 cells/ml) was diluted over a period of 10 min with an equal volume of ice-cold MEM containing 20% dimethylsulphoxide (DMSO) with or without urea (1.1 M). After a further 10 min, the samples were suspended, via an insulating platform, in the vapour phase of a flask containing liquid nitrogen. The rate of cooling was between 1° and 2° per min. After 3 hr, the tubes were transferred into the liquid phase of liquid nitrogen for storage. The samples were removed from liquid nitrogen after 7–10 days and rapidly thawed in a water bath at 37°. The cells were then washed three times in KH buffer containing TES (32 mM).

To prepare post-mitochondrial supernatants (S9), hepatocytes were homogenized in 0.15 M KCl, 3 mM Tris/HCl buffer (pH 7.2 at 4°) until >90% of the cells were disrupted. Cultured cells were rinsed twice with buffer and the cells were scraped off the plates using a plastic wedge prior to homogenization in a Potter–Elvehjem apparatus with a close-fitting PTFE plunger. The homogenates were then centrifuged at 9000 *g* for 20 min and the supernatant (S9) was used for determination of cytochrome P-450 activity. 7-Ethoxycoumarin *O*-deethylase activity was measured by the method of Greenlee and Poland [10] and aminopyrine *N*-demethylase activity was assessed by the method of Lake *et al.* [11] but using a total incubation volume of 1.5 ml. S9 protein was estimated by the Lowry method [12].

Pilot studies, in accord with other findings [3], indicated that recovery of viable cells from cryopreservation was greater using DMSO as a cryoprotectant than was found using glycerol. The recovery of cells frozen in a medium containing 10% DMSO was 56.0% (Table 1). The viability of recovered cells as measured by trypan blue exclusion and LDH release was 75.4 and 74.9% respectively of the cell viability prior to freezing. Ability of hepatocytes to attach to culture dishes (also a measure of viability) was markedly reduced following cryopreservation (66% of the values derived from freshly isolated cells). Thus, in summary, the recovery of viable cells was estimated to be 42.2, 41.9 and 37% as measured by trypan blue exclusion, LDH release and attachment to culture dishes respectively.

The monooxygenase activity of S9 fractions from the recovered cells was measured towards 7-ethoxycoumarin (a relatively non-specific marker of cytochrome P-450 activity which is induced by phenobarbitone and 3-methylcholanthrene [13]) and aminopyrine (a marker more specific for cytochrome P-450 inducible by phenobarbitone [14])