# INHIBITION OF CATALASE IN VITRO AND IN VIVO BY 4-HYDROXYPYRAZOLE, A METABOLITE OF PYRAZOLE

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## 1. Introduction

For many years it has been recognised that not all the properties of pyrazole are due to the molecule itself [1–4]. With the elucidation of the metabolism of pyrazole [5] it was appreciated that several metabolites could possess biological activity. In [6] an in vitro inhibition of catalase (EC 1.11.1.6) by 4-hydroxypyrazole (4-HP), one of the major metabolites of pyrazole was reported. This paper confirms the inhibition of catalase by 4-HP but also describes the importance of incubation time, a factor not studied earlier. We also describe the effects of 4-HP administration to mice on liver catalase activity.

## 2. Materials and methods

4-Hydroxypyrazole was kindly supplied by Eli Lilly Research Labs. (Indianapolis, IN). Catalase (suspension from beef liver, Boehringer, Mannheim) was diluted with 0.05 M phosphate buffer (pH 7.0). Catalase activity was followed spectrophotometrically, measuring the decrease in $A_{240}$ for 1 min after addition of substrate ($H_2O_2$, final conc. 10 mM) [7]. Preincubation times were calculated from addition of the enzyme to the cuvette containing 4-HP but not substrate.

In vivo inhibition was studied by injecting 4-HP (1 mmol/kg, i.p.) into male mice (25–35 g, NMRI/BOM strain). At set times after injection, the mice were decapitated, the liver quickly excised, rinsed in ice-cold phosphate buffer, weighed and homogenised using a Potter-Elvehjem homogeniser with a Teflon pestle. The crude homogenate was further diluted before measurement so that 1 g liver was diluted to 250 ml. Of this solution 17 μl was used for measurement, diluted with 650 μl phosphate buffer and 333 μl $H_2O_2$ (30 mM). Activity was assayed as described, each sample being determined in duplicate. Between the death of the animal and the measurement of the duplicate sample <5 min elapsed.

## 3. Results

We obtained a dose-dependent inhibition of catalase activity with 4-HP in vitro as in [6]. However, the extent of the inhibition was dependent, not only on the concentration of 4-HP, but also on the time of pre-incubation before assay. From plots of inhibition against time, it was possible to calculate the time required for various concentrations of 4-HP to inhibit catalase by 50%. There was a clear relationship between time and concentration (table 1). After storage for 96 h, 1 μM was sufficient to inhibit enzyme activity by 50% compared to a similarly stored con-

Table 1
The concentration of 4-hydroxypyrazole required to inhibit catalase activity by 50% at various times

| Time (h) | 1 | 2 | 3 | 4 | 24 | 48 | 72 | 96 |
|---|---|---|---|---|---|---|---|---|
| 4-HP (μM) | 150 | 83 | 60 | 34 | 24 | 17 | 8 | 1 |

For the first 4 h the tubes were stored at 22°C and thereafter at 4°C. The concentrations causing 50% inhibition were obtained by plotting log [4-HP] against % inhibition. During the course of the experiment, control activity declined from 156–124 units/min

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trol sample. This was 150-times less 4-HP than was needed to inhibit the enzyme to a similar degree after only 1 h incubation.

Exogenous $H_2O_2$ in the pre-incubation mixture did not accelerate the rate of inhibition. Once the inhibition of enzyme activity was complete increasing the substrate concentration was ineffectual in restoring activity. The irreversible nature of the inhibition was confirmed by the failure of both gel filtration (table 2) and overnight dialysis to prevent the inhibitory effects of 4-HP.

Injection of 4-HP (1 mmol/kg) into mice resulted in a rapid and almost complete block of liver catalase activity (fig.1). One hour after injection, the activity was negligible. There was a gradual recovery with time, but even 2 days after injection, liver catalase activity was still only 75% of control. In a separate

Table 2
The effect of gel filtration on the inhibition of catalase activity by 4-hydroxypyrazole

| Time (min) | Treatment | Activity ($\Delta A$/min) | | % of control |
|---|---|---|---|---|
| | | Control | 4-HP | |
| 0 | no filtration | 69 | 69 | 100 |
| | after filtration | 57 | 55 | 97 |
| 10 | no filtration | 69 | 23 | 33 |
| | after filtration | 58 | 15 | 26 |
| 30 | no filtration | 61 | 5 | 8 |
| | after filtration | 53 | 3 | 6 |
| 60 | no filtration | 67 | 0 | 0 |
| | after filtration | 50 | 0 | 0 |

Catalase was incubated with 4-HP (at 200 μM in the pre-incubation mixture) and at the times stated a sample from the incubation mixture was subjected to gel filtration by passage through a Sephadex G-25 column, a procedure which completely separated free 4-HP from that bound to the enzyme. Control samples did not contain 4-HP but were otherwise identical

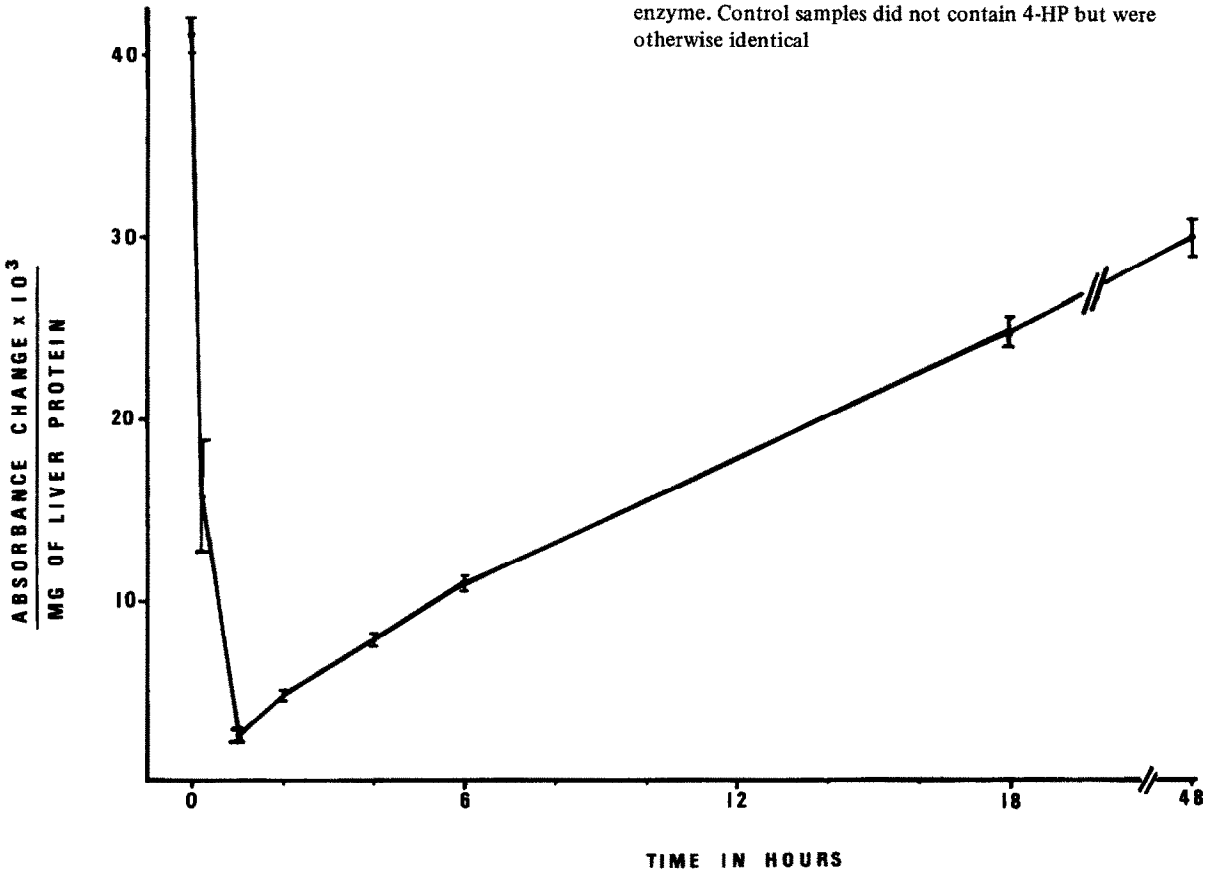


Fig.1. Effect of a single injection of 4-hydroxypyrazole (1 mmol/kg) on the activity of mouse liver catalase. Results are means ± SEM from groups of 5 mice with the exception of the 15 min group ($n$ = 3). Control mice ($n$ = 15) were killed at various times throughout the experiment but are pooled for the purpose of presentation. All values differ significantly from controls ($P$ <0.01).

experiment, a comparison was made between the in vivo and in vitro inhibitory properties of 4-HP. One hour after injection of 4-HP (1 mmol/kg), the activity in mouse liver was inhibited by 90%. It can be calculated that the [4-HP] in the assay system was between 68 nM (assuming equal distribution throughout the body) and 1.36 μM (assuming complete concentration of 4-HP in liver). In contrast, to obtain an equivalent inhibition of mouse liver catalase activity after 1 h of in vitro incubation, 4-HP at 133 μM was necessary.

## 4. Discussion

In the study on the inhibitory properties of 4-HP, [6] the effect of incubation time was not considered. They calculated that 4-HP at 3.38 mM was the concentration producing 50% inhibition. However, as we have shown, the time of incubation is as important a factor as concentration in determining the degree of inhibition and micromolar concentrations of 4-HP will cause total inhibition given sufficient time.

Although we have not performed detailed analysis of kinetics of the inhibition, it is clear that the inhibition is irreversible being reversed neither by gel filtration nor overnight dialysis. Evidence of its irreversible nature was also obtained in vivo, as recovery from a single injection of 4-HP seemed to depend on resynthesis of new enzyme [8]. The recovery was very similar to that seen after administration of pyrazole [2], the significant difference in the time course being the slow onset of the blockade with pyrazole, maximum inhibition occurring ~24 h after administration. Maximum inhibition with 4-HP occurred almost immediately after injection.

There are several explanations for the differences in [4-HP] inhibiting mouse liver catalase in vitro and in vivo. Nearly all liver catalase is located in microbodies termed peroxisomes [9] and there is evidence that drugs can penetrate their membranes [10]. If 4-HP is rapidly taken up by peroxisomes, which we cannot exclude, then the local [4-HP] inside the peroxisomes and in contact with the catalase may be considerably greater than that which we have estimated, even assuming [4-HP] in the liver as a whole. However, other explanations are possible. Metabolites of 4-HP have been identified [5] and it is possible that these may be even more potent inhibitors of catalase. 4-HP may have other effects either directly on the liver or indirectly, through the accumulation of toxic peroxide metabolites normally metabolised by catalase.

4-HP is a very potent inhibitor of catalase, 50% inhibition is obtained at μM levels, whereas most synthetic catalase inhibitors act at mM levels. It seems likely that 4-HP is not only responsible for the inhibition in vivo of catalase by pyrazole, but for many of its other toxic effects. This explains why 4-methylpyrazole, which cannot be metabolised to 4-HP, is much less toxic than pyrazole [11].

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