INHIBITION OF CATALASE BY HYDROXYLAMINE AND
p-HYDROXYLAMINOBENZENESULFONAMIDE AND THE
REVERSAL OF INHIBITION BY SERUM, CRYSTALLINE
SERUM ALBUMIN, AND HEMIN*

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In a previous communication (1) it was shown that p-hydroxylamine-
benzenesulfonamide (HONHR) did not inhibit catalase in the presence of
serum. In contrast in a later experiment it was observed that HONHR
inhibited catalase in the absence of serum. The present study deals with
the reversible inhibition of catalase by hydroxylamine and its benzene-
sulfonamide derivative in the presence of serum, crystalline serum albumin,
and hemin.

p-Hydroxylaminobenzenesulfonamide was obtained in two forms (1),
one melting at 141.5° and the other at 161.5°. These two forms manifest
different chemical and physical properties. A paper dealing with these
differences will be published elsewhere. The experiments in this study
were carried out with the substance which melts at 141.5°. It oxidizes
with relative ease; a study of its biological and chemical action therefore
involves certain complications which must be strictly controlled to measure
its effects accurately. 1 mole of this substance consumes 1 mole of oxygen,
yielding 1 mole of p-nitrosobenzenesulfonamide and 1 mole of hydrogen
peroxide. The former reaction product combines with 1 mole of p-hy-
droxylaminobenzenesulfonamide forming azoxydibenzenesulfonamide.
The rate of oxidation and, therefore, the amount of hydrogen peroxide
formed are dependent on the pH of the reaction system. At pH 4.5 it is
practically non-oxidizable; on the other hand in alkaline solution the
volume of oxygen consumed corresponds to 90 to 95 per cent of the theo-
retical value. At neutrality the degree of oxidation occupies an inter-
mediary point.

In the inhibition experiments if catalase is present in excess, hydrogen
peroxide which is produced by the oxidation of p-hydroxylaminobenzenesul-
fonamide (inhibitor) is not to be found in the reaction system. Even
though the catalase may be completely inhibited, the oxidation of the
inhibitor is not inhibited. This inhibitor thus provides the catalase it in-
hibits with the specific substrate and thereby offers an excellent method of studying its effect on catalase under various conditions (Table I). Addition of hydrogen peroxide to the system does not affect the inhibition results. These methods have been employed in this study in the presence and absence of serum, crystalline serum albumin, and hemin, on blood and crystalline liver catalases (Tables II and III).

**EXPERIMENTAL**

*Crystalline Liver Catalase*—The sample of crystalline liver catalase kindly supplied by Professor J. B. Sumner of Cornell University contained 23.4 mg. of non-dialyzable total solids per cc. of stock solution. In inhibition experiments the solutions of catalase used were prepared by diluting the undialyzed stock sample. The expressed molarity was calculated from the molecular weight of 248,000 as determined by Sumner and Gralén (2). Since 1 molecule of catalase contains four heme groups, a molar solution of catalase would be equal to $4M$ with respect to heme.

*Crystalline Serum Albumin*—The dry weight of a dialyzed solution of crystalline serum albumin was determined by analysis for micro-Kjeldahl nitrogen. The molecular weight of 70,000 was used to calculate the molarity of albumin per vessel. The absence of hemin in serum albumin was shown with benzidine as follows: 1.63 mg. of serum albumin in 0.5 cc. of phthalate buffer of pH 4.4 were treated with 3 drops of 3 per cent $H_2O_2$ solution and 2 drops of saturated alcoholic benzidine solution. The test was negative. As control, 0.5 cc. of a solution containing $3.6 \times 10^{-3}$ mg. of hemin ($1.1 \times 10^{-7} M$) gave a positive peroxidative test.

*Hemin*—The hemin (mol. wt. 651.3) used in this study was a crystalline preparation kindly supplied by Dr. D. L. Drabkin of the Department of Physiological Chemistry.

*Blood Catalase*—The solution of blood (rabbit) catalase was water-clear. In the preparation of the stock solution care was taken to remove the last traces of serum and hemoglobin as previously described (3). 1 cc. of catalase solution was obtained from 0.1 cc. of the rabbit whole blood.

*Normal Serum*—The normal horse serum was heated at 56° (water bath) for 1 hour to inactivate traces of catalase that might have been present. Though it was free from apparent traces of hemolyzed blood, it gave a positive peroxidative test with benzidine, showing the presence of hematin derivatives. Dialysis of the serum against running 0.02 $N$ HCl solution and distilled water for several days did not eliminate the substance responsible for the positive peroxidative test.

*Measurement of Inhibition of Catalase*—The inhibition of catalase was measured manometrically with the Barcroft-Warburg set-up, and at the end of the experiment the contents of the vessels were also analyzed for $H_2O_2$ (iodometric method).
The manometric measurements were carried out in vessels with two side arms. The reaction system consisted of 2.5 cc. of \( \text{M/15 phosphate buffer of pH 7.4} \), 0.2 cc. of catalase solution, and 1.0 cc. of either serum, serum albumin, or hemin solution. One side arm contained the inhibitor, the other side arm the \( \text{H}_2\text{O}_2 \) (0.0063 \( \text{M} \) final concentration). Distilled water was added to the vessel to bring the final volume to 5.8 cc. After the equilibration of the temperature the contents of the side arms were mixed in the order desired.

At the end of the manometric measurements the contents of the vessels and the side arms were thoroughly washed into Erlenmeyer flasks to which were added 2.0 cc. of 5 per cent \( \text{H}_2\text{SO}_4 \) solution, 2.0 cc. of 2 per cent potassium iodide solution, and 1.0 cc. of 1 per cent ammonium molybdate solution (containing 5 cc. of 25 per cent \( \text{H}_2\text{SO}_4 \) solution). After a 5 minute interval the liberated iodine was determined by titrating with 0.01 \( \text{N} \) sodium thiosulfate solution delivered by a micro burette. Near the endpoint 2 drops of 1 per cent soluble starch solution were used.

RESULTS AND DISCUSSION

The results given in Table I show that \( p \)-hydroxylaminobenzenesulfonamide (HONHR) inhibited rabbit blood and liver catalase.\(^1\) The inhibition caused by \( 9.17 \times 10^{-3} \text{M} \) HONHR was completely reversed by 1.0 (or 0.01) cc. of clear catalase-free serum.\(^2\) In other experiments similar to those presented in Table I, \( 1 \times 10^{-4} \text{M} \) HONH\(_2\) inhibited 1.63 \( \times 10^{-9} \text{M} \) crystalline liver catalase 60.0 per cent, and \( 2.72 \times 10^{-10} \text{M} \) liver catalase was inhibited 59 per cent by \( 1 \times 10^{-5} \text{M} \) HONH\(_2\). These inhibitions were similarly reversed by serum.

It is evident from these data that the serum contains certain substances which compete with catalase for the above inhibitors. In peroxidative tests serum showed the presence of a hematin compound which could not be eliminated by dialysis. It was thought that either the serum proteins or the hemin, or both, present in serum might be responsible for the complete reversal of the inhibition of catalase by the above inhibitors. As will be seen from the results given in Tables II and III, this assumption was confirmed. Table II shows that \( 2.72 \times 10^{-10} \text{M} \) crystalline liver catalase was inhibited 89 per cent by \( 1 \times 10^{-5} \text{M} \) HONH\(_2\) and 89 per cent by \( 4.58 \times 10^{-5} \text{M} \) HONHR. These inhibitions were reversed to the extent of 77 and 96.0 per cent, respectively, by \( 4.0 \times 10^{-4} \text{M} \) crystalline serum.

\(^1\) Collier (4) had previously shown that \( p \)-hydroxylaminobenzenesulfonamide inhibited liver catalase.

\(^2\) The present study shows that in the presence of serum 10 mg. (9.17 \( \times 10^{-3} \text{M} \)) of \( p \)-hydroxylaminobenzenesulfonamide are incapable of inhibiting 0.02 cc. of catalase (corresponding to 0.002 cc. of whole blood). In other words, 5 gm. of \( p \)-hydroxylaminobenzenesulfonamide are incapable of inhibiting 1.0 cc. of whole blood.
albumin. The inhibition by $1 \times 10^{-5}$ M HONH$_2$ was reversed 46 per cent also with as little as $6.2 \times 10^{-8}$ M crystalline serum albumin. This indicates that HONH$_2$ has about the same affinity for serum albumin that it has for catalase. The question as to what group in the protein reacts with the inhibitors to account for the reversal of the inhibition of catalase was investigated.

Carbohydrates such as mannose, galactose, levulose, arabinose, polysaccharides of pneumococcus Type I, and rabbit liver glycogen were added

<table>
<thead>
<tr>
<th>Reaction systems</th>
<th>O$_2$ (c.mm.)</th>
<th>H$_2$O$_2$ (micromoles)</th>
<th>Inhibition (%)</th>
<th>Reversal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No catalase</td>
<td>350</td>
<td>8.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. &quot; &quot; + 1 cc. serum</td>
<td>459</td>
<td>11.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 0.02 cc. blood catalase†</td>
<td>354</td>
<td>8.6</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>4. 0.02 &quot; &quot; + 1 cc. serum</td>
<td>260</td>
<td>0.0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5. 0.2 &quot; &quot; + 1 cc. serum</td>
<td>253</td>
<td>1.25</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>6. 0.2 &quot; &quot; + 1 cc. serum</td>
<td>269</td>
<td>0.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7. 1.63 $\times 10^{-9}$ m liver catalase</td>
<td>331</td>
<td>7.65</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>8. 1.63 $\times 10^{-9}$ &quot; &quot; + 1 cc. serum</td>
<td>267</td>
<td>0.0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>9. 3.62 $\times 10^{-9}$ m liver catalase</td>
<td>310</td>
<td>3.15</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>10. 3.62 $\times 10^{-9}$ &quot; &quot; + 1 cc. serum</td>
<td>264</td>
<td>0.0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

In comparison to Systems 1, 2, 3, 7, and 9, Systems 4, 5, 6, 8, and 10 show smaller volumes of oxygen consumed. This is due to the fact that in the latter systems catalase is not inhibited and therefore it decomposes H$_2$O$_2$ formed according to $2H_2O_2 \rightarrow 2H_2O + O_2$. The liberated oxygen returning to the system causes a positive pressure and therefore the measured volume of consumed O$_2$ is smaller. 47 to 60 per cent of the reactions were completed after 30 minutes in all the above systems. After 60 minutes the completed reactions varied from 60 to 90 per cent.

* Cc. of 0.01 Na$_2$S$_2$O$_3 \times 5$

† 0.02 cc. of blood catalase corresponds to 0.002 cc. of whole rabbit blood.

to the reaction systems. There was no trace of reversal of the inhibition of catalase, indicating either the absence of a carbohydrate prosthetic group in crystalline serum albumin (5) or the inability of the inhibitors to react with the protein-bound carbohydrates under these experimental conditions. To test the possible reactivity of the keto group in dipeptide linkages for the inhibitors hippuric acid was used, but no effect was observed. On the basis of these results it would seem that the protein molecule itself manifests affinity for HONH$_2$ and HONHR.
Table III showed that $2.72 \times 10^{-10}$ M crystalline liver catalase was inhibited 84.0 per cent by $1 \times 10^{-5}$ M HONH$_2$ and about 70 per cent by $4.58 \times 10^{-6}$ M HONHR. The inhibition by HONH$_2$ was reversed 52 to 64.0 per cent by $4.73 \times 10^{-6}$ M hemin. These results show that HONH$_2$

and HONHR manifest affinities towards hemin as well. The evidence that HONH$_2$ combines with hemin was derived also from experiments in which the catalase activity of higher concentrations of hemin per se was inhibited 50 per cent by HONH$_2$.

$2.72 \times 10^{-10}$ M liver catalase contains $1.1 \times 10^{-9}$ M hemin. The inhibi-
tion of catalase was reversed 46.0 to 50.0 per cent by $4.73 \times 10^{-6}$ M hemin. A direct comparison between the affinities of catalase hemin and the crystalline hemin towards HONH$_2$ and HONHR is difficult to make because

<table>
<thead>
<tr>
<th>Reaction systems</th>
<th>2.72 $\times 10^{-10}$ M liver catalase (hemin content = $1.1 \times 10^{-9}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>reacting with</td>
</tr>
<tr>
<td></td>
<td>oxygen</td>
</tr>
<tr>
<td>1. No inhibitor</td>
<td>545</td>
</tr>
<tr>
<td>2. $' ' + 9.5 \times 10^{-7}$ M hemin</td>
<td>92</td>
</tr>
<tr>
<td>3. $' ' + 4.73 \times 10^{-6}$ M hemin</td>
<td>164</td>
</tr>
<tr>
<td>4. $1 \times 10^{-5}$ M HONH$_2$ hemin</td>
<td>299</td>
</tr>
<tr>
<td>5. $1 \times 10^{-5} ' ' + 9.5 \times 10^{-7}$ M hemin</td>
<td>152</td>
</tr>
<tr>
<td>6. $1 \times 10^{-5}$ M HONH$_2$ + 4.73 X $10^{-6}$ M hemin</td>
<td>166</td>
</tr>
<tr>
<td>7. $4.58 \times 10^{-5}$ M HONHR</td>
<td>384</td>
</tr>
<tr>
<td>8. $4.58 \times 10^{-5} ' ' + 9.5 \times 10^{-7}$ M hemin</td>
<td>48.3</td>
</tr>
<tr>
<td>9. $4.58 \times 10^{-5}$ M HONHR + 4.73 $\times 10^{-6}$ M hemin</td>
<td>47.3</td>
</tr>
</tbody>
</table>

Catalase absent. Controls

|                  | 6 | 48.3 |
| 10. $1 \times 10^{-5}$ M HONH$_2$ hemin | 6 | 47.3 |
| 11. $1 \times 10^{-5} ' ' + 9.5 \times 10^{-7}$ M hemin | 29 | 45.8 |
| 12. $1 \times 10^{-5}$ M HONH$_2$ + 4.73 X $10^{-6}$ M hemin | 8 | 49.0 |
| 13. $4.58 \times 10^{-5}$ M HONHR hemin | 7 | 48.3 |
| 14. $4.58 \times 10^{-5} ' ' + 9.5 \times 10^{-7}$ M hemin | 33 | 44.8 |
| 15. $4.58 \times 10^{-5}$ M HONHR + 4.73 $\times 10^{-6}$ M hemin | 52 | 64 |

* In Systems 4 to 9, the inhibitions after 8 minutes were respectively 83, 79, 64, 66, 67, and 29 per cent.

of the fact that they combine with both the protein and the hemin components of catalase. From the fact, however, that $6.2 \times 10^{-5}$ M crystalline serum albumin and $4.73 \times 10^{-6}$ M hemin reverse the inhibition of catalase
to the same degree, it would appear that HONH₃ manifests about 100-fold
greater affinity towards protein than it does towards hemin.

Stern (6) found that hydroxylamine did not bring about a change in the
spectrum of catalase. He stated that the stability of the enzyme spectrum
towards hydroxylamine is of interest in view of the fact that this substance
inhibits the activity of the enzyme. Keilin and Hartree (7) found likewise
that HONH₂ does not appreciably change its color or the general pattern
of its absorption spectrum.

Smythe (8) stated that hydroxylamine inhibited yeast fermentation.
Sevag and Shelburne (1) found that hydroxylamine (likewise HONHR)
inhibited aerobic bacterial respiration and the anaerobic glycolysis of glu-
cose by bacteria. Frank and Gaffron (9) stated that hydroxylamine in-
hibited the photosynthetic reactions by plants and bacteria. There were
lacking, however, definite data as to the point of attack by HONH₂ on the
above enzyme systems. The results of the present study show that
hydroxylamine and its derivatives are capable of combining both with the
heme type of enzyme systems and those enzyme systems which do not
involve heme groups.

SUMMARY

The inhibition of blood and crystalline liver catalase by hydroxylamine
and p-hydroxylaminobenzenesulfonamide is completely reversible in the
presence of serum, crystalline serum albumin, and hemin.

Hydroxylamine and p-hydroxylaminobenzenesulfonamide combine with
both hemin and crystalline serum albumin free from hemin. These in-
hibitors are, therefore, capable of inhibiting both the heme and non-heme
type of enzyme systems.

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