3-Hydroxyanthranilic acid oxidase is known to catalyze the oxidation of 3-hydroxyanthranilic acid to a very unstable aliphatic compound, which may either be spontaneously transformed into quinolinic acid or undergo enzymic conversion to picolinic or nicotinic acid, or both (1-3).

According to Mason (4), this enzyme has been classified as an “oxygen transferase,” since the oxygen consumed, 1 molecule per molecule of substrate, appears in the oxidation product.

Almost all the work so far published on the properties of 3-hydroxyanthranilic acid oxidase has been carried out with an extract of acetone-dried powder of ox liver. Very recently Decker et al. (6) obtained an 1800-fold increase in the specific activity and studied factors enhancing the enzymic activity.

In preceding papers from this laboratory (7, 8) successful attempts to purify this enzyme (approximately 3000-fold increase in specific activity) and some general properties of the purified enzyme have been reported.

The present work describes a new simplified method of further purification of 3-hydroxyanthranilic acid oxidase from calf liver, and some other properties of the purified enzyme.

EXPERIMENTAL PROCEDURE

Materials—Fresh calf liver was obtained from the local slaughterhouse and immediately used or stored after freezing: sucrose, acetone, and ammonium sulfate were purchased from C. Erba S. A., Milan, Italy; 3-hydroxyanthranilic acid was supplied by the California Corporation for Biochemical Research; anthranilic acid and anthranilic acid analogues were obtained from the British Drug Houses, Ltd., England; carboxymethyl cellulose Selectacel, with a capacity of 0.83 meq per g, was supplied by the California Corporation for Biochemical Research; and other salts were purchased from E. Merck, G. A., Darmstadt, Germany.

Assay of Enzymic Activity—Enzymic activity was measured at room temperature in a Beckman model DK-2 ratio recording spectrophotometer, by following the increase in optical density at 360 mp, due to the formation of the primary oxidation product. The reaction mixture contained 0.3 mM 3-hydroxyanthranilic acid (9), in a 1-cm light path cell. The reaction mixture contained 0.3 mM 3-hydroxyanthranilic acid (sodium salt) and 1 mM FeSO₄, dissolved in 0.033 M collidine buffer, pH 6.5, prepared according to Gomori (10), in a final volume of 3 ml. The reaction was started by the addition of the enzyme. Measurements at pH 8.0 were performed with the same buffer, in the absence of FeSO₄, since at this pH ferrous ion is readily oxidized to ferric ion; the precipitation of the latter interferes with the spectrophotometric readings.

The activity unit used in the present work is defined as millimicromoles of the primary oxidation product formed per minute in the incubation mixture (7). The molar absorbancy of this product is assumed to be 4.7 x 10⁴ at 360 mp (2). This activity unit is 15.6 higher than that used by Decker et al. (6). The specific activity is given as units per milligram of protein.

Spectrofluorometric measurements were carried out in a Farrand double-grating spectrofluorometer; pH was determined with a Beckman Zeromatic pH meter.

Protein was determined by the biuret method (11) and by the method of Lowry et al. (12).

Chromatography—CM-cellulose was thoroughly washed first with 1 N NaOH and then with water until the excess alkali had been removed; it was carefully deaerated under vacuum and poured into a column. The column (1 x 25 cm) was packed and then saturated with Fe²⁺, with a solution of 3 mM FeSO₄ and 2 mM cysteine in 6.6 M collidine buffer, pH 6.5. The operation was performed at 3°, and N₂ was bubbled into the buffer during the whole chromatographic procedure.

RESULTS AND DISCUSSION

Enzyme Purification—The enzyme was extracted by homogenizing 250 g of fresh calf liver in 2 volumes of 0.25 M sucrose, in aServall Omni Mixer for 1 minute at 0°. The homogenate was centrifuged in a Servall centrifuge at 18,000 x g for 45 minutes, and the sediment was discarded. 3-Hydroxyanthranilic acid, cysteine, and FeSO₄ were added to the supernatant fluid up to a final concentration of 0.3 mM, 1.5 mM, and 1 mM, respectively. Then acetone (previously stored at -15°) was added dropwise under gentle mechanical stirring to a final concentration of 45%, volume for volume. During the acetone fractionation the temperature was kept at -10°. The suspension was centrifuged at -10° in an International model PR-2 centrifuge for 10 minutes at 3500 x g and the precipitate was discarded. Additional acetone was added to the supernatant fluid to a final concentration of 55%, volume for volume. The precipitate was collected by centrifugation at 3500 x g and dissolved in 0.033 M collidine buffer, pH 6.5, containing 3-hydroxyanthranilic acid, FeSO₄, and cysteine at the same final concentration as described above. The solution was dialyzed at 3° against the same buffer, previously saturated with N₂, to remove excess acetone.

The enzyme solution was then heated in a water bath, under
mechanical stirring, until the temperature of the solution reached 35°, and was kept at this temperature for 5 minutes. The heating procedure was performed in the absence of oxygen. The exclusion of oxygen from the medium proved to be essential in order to prevent inactivation of the enzyme, possibly due to the oxidation of ferrous ions to ferric ions (see below). The solution was then rapidly cooled in an ice bath and the precipitate removed by centrifugation. This step resulted in a considerable activation of the enzyme.

The ammonium sulfate fractionation was carried out in the enzyme solution as described (8); the precipitate obtained between 35 and 52% saturation was collected, dissolved in 0.6 M collidine buffer, pH 6.5, containing 1.5 mM cysteine and 1 mM FeSO₄, and dialyzed against the same buffer until all ammonium sulfate was removed from the solution.

The solution was poured on top of a CM-cellulose column, prepared as described in “Experimental Procedure.” Then 0.6 M collidine buffer, pH 6.5, containing 1 mM FeSO₄ and 1.5 mM cysteine was run through the column. Fractions of 2.5 ml were collected at a flow rate of 5 ml per hour, without modifying the pH and ionic strength of the effluent; after approximately 10 hours the fractions contained enzymic activity. Solid ammonium sulfate was added up to 75% saturation to the fractions exhibiting the highest specific activity; the precipitate was collected by centrifugation at 15,000 × g for 20 minutes, dissolved in 1 to 2 ml of collidine buffer, and dialyzed against the same buffer until all ammonium sulfate was removed from the solution.

The eluting solution was 0.6 M collidine buffer, pH 6.5, containing 1 mM FeSO₄ and 1.5 mM cysteine. Temperature 3°. The solid line denotes protein concentration, determined by the method of Lowry et al. (19); the dashed line indicates enzymic activity, as units per ml.

A summary of the complete purification procedure is reported in Table I. Fig. 1 shows a typical chromatography on CM-cellulose.

It is to be noticed that the yield of enzyme total units is approximately 80% after the ammonium sulfate fractionation, owing to the considerable activation which follows the heating step, but it falls to 90% after the CM-cellulose chromatography. A similar increase in enzyme total units during the purification procedure of 3-hydroxyanthranilic acid oxidase was recently observed by Decker et al. (9). However, according to the data of these authors, the activation of the enzyme took place through several purification steps, whereas in our procedure the whole activation was due to the heating treatment. From our observations, it appeared that the extent of activation during heating was dependent on the presence of ferrous ions in the solution. Decker et al. (6) have reported an 1800-fold purification of 3-hydroxyanthranilic acid oxidase, starting from crude liver homogenate. The purified enzyme had a specific activity of 32,000 and a Kₘ value of 21 μM. By comparing the above data with those reported here, the following conclusions may be drawn: (a) The activity unit used in the present work is 15.6 times that described by Decker et al. (6). Thus the specific activity of 53,200, reported in Table I, corresponds to a value of 380,000 when calculated on the basis of the units of Decker et al. (6). (b) The 3500-fold purification of the enzyme reported in Table I, calculated starting from the activity of the liver homogenate supernatant, must be considered as an apparent value since, as shown previously, during the purification procedure, a strong activation occurs during heating. (c) The Kₘ value of our enzyme preparation (see below) is approximately 3 times lower than that calculated by Decker et al. (6).

Effect of Fe⁺⁺ Ions—It is known that Fe⁺⁺ is a specific activator of 3-hydroxyanthranilic acid oxidase (4, 6, 8). Experiments to be reported below indicate that Fe⁺⁺ also has a stabilizing effect on the enzyme. As shown in Fig. 2, the specific activity was constant when measured at different enzyme concentrations, provided that the concentration of Fe⁺⁺ was kept constant during the dilution of the enzyme. On the other hand, if the enzyme was diluted without maintaining the concentration of Fe⁺⁺ constant, a considerable decrease in specific activity was observed at lower enzyme concentrations. In the experiment reported in Fig. 2A, the enzyme activity was measured at equal Fe⁺⁺ concentrations (1 mM) in the incubation mixture. Under these conditions, however, it was impossible to understand whether a stronger inactivation of the enzyme had taken place.

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units per ml</th>
<th>Volume</th>
<th>Total units</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Times purified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Homogenate supernatant</td>
<td>371</td>
<td>500</td>
<td>185,000</td>
<td>24</td>
<td>15.4</td>
<td>100</td>
<td>34.4</td>
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<tr>
<td>2. Acetone fraction</td>
<td>8,500</td>
<td>21</td>
<td>179,000</td>
<td>16</td>
<td>550</td>
<td>97</td>
<td>34.4</td>
</tr>
<tr>
<td>3. Heat-treated supernatant</td>
<td>82,500</td>
<td>18.5</td>
<td>1,590,000</td>
<td>14</td>
<td>5,900</td>
<td>830</td>
<td>377</td>
</tr>
<tr>
<td>4. (NH₄)₂SO₄ fraction</td>
<td>227,000</td>
<td>6</td>
<td>1,370,000</td>
<td>15</td>
<td>15,200</td>
<td>740</td>
<td>980</td>
</tr>
<tr>
<td>5. Column peak</td>
<td>80,000</td>
<td>2.1</td>
<td>168,000</td>
<td>1.5</td>
<td>53,200</td>
<td>91</td>
<td>3450</td>
</tr>
</tbody>
</table>

**FIG. 1.** Chromatography on CM-cellulose. The eluting solution was 0.6 M collidine buffer, pH 6.5, containing 1 mM FeSO₄ and 1.5 mM cysteine.

**Table**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units per ml</th>
<th>Volume</th>
<th>Total units</th>
<th>Protein</th>
<th>Specific activity</th>
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<td>5,900</td>
<td>830</td>
<td>377</td>
</tr>
<tr>
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<td>6</td>
<td>1,370,000</td>
<td>15</td>
<td>15,200</td>
<td>740</td>
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</tr>
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<td>80,000</td>
<td>2.1</td>
<td>168,000</td>
<td>1.5</td>
<td>53,200</td>
<td>91</td>
<td>3450</td>
</tr>
</tbody>
</table>
FIG. 2. Relationship between enzyme concentration and specific activity of 3-hydroxyanthranilic acid oxidase. A, The enzyme solution was diluted with 0.033 M collidine buffer, pH 6.5, either with or without 1 mM FeSO₄. The activity was measured after 1 hour of incubation at 0°C; the composition of the reaction mixture was as described in “Experimental Procedure.” B, The dilution was performed as in a. The assay system contained 0.033 M collidine buffer, pH 8.0, 0.3 mM 3-hydroxyanthranilic acid, and no FeSO₄. Final volume, 3 ml.

during dilution, and whether this inactivation had been partially reversed by Fe⁺⁺ in the assay system. In order to test this possibility, the activity assay of the enzyme after dilution was carried out at pH 8.0, without Fe⁺⁺ in the test system. In fact the enzyme activities at pH 6.5 in the presence of Fe⁺⁺, and at pH 8.0 in the absence of Fe⁺⁺, were approximately equal, as previously described (8). As shown in Fig. 2B, dilution of the enzyme in the presence of Fe⁺⁺ resulted in practically no variation in enzymatic activity, whereas dilution in the absence of Fe⁺⁺ resulted in a strong inactivation of the enzyme. The extent of this inactivation was higher than that observed when the pH of the assay system was 6.5. In control experiments, it was ascertained that the small amount of Fe⁺⁺ introduced together with the enzyme solution in the incubation mixture at pH 8.0, had no effect on the rate of the enzymic activity. By comparing the inactivation curves reported in Fig. 2, A and B, it appears that addition of Fe⁺⁺ to the assay system diminished the extent of inactivation or, in other words, that the inactivation due to dilution in the absence of Fe⁺⁺ was partially reversed during the assay.

Further evidence for this stabilizing effect of Fe⁺⁺ is given by the data reported in Fig. 3. The decay of enzymatic activity was followed during time at room temperature and compared with the decay of another sample, from which Fe⁺⁺ had been removed by dialysis. It appears from Fig. 3 that the decay was much slower when the enzyme was stabilized by Fe⁺⁺; after 150 minutes the activity of the sample incubated in the absence of Fe⁺⁺ resulted in only 15% of the value of the sample incubated in the presence of Fe⁺⁺.

Stability of Enzyme—Previous work on 3-hydroxyanthranilic acid oxidase has pointed out the instability of this enzyme (3, 5). The instability appears to parallel the purification. The pH of the medium and the temperature are among the factors influencing the stability of the enzyme. At room temperature and at pH 8.0, the activity was lost almost completely in a matter of minutes. The minimal rate of inactivation was found to be at pH 6.5. Storage of the purified enzyme for 72 hours resulted in a loss of activity of 50% at 0°C and of 70% at −15°C. Decker et al. (6) reported a higher stability of their preparation during storage at −13°C. It is possible that this difference is to be attributed to the much higher purity (26 times) of our enzyme preparation.

Effect of Metal Ions—The effect of several metal ions on the activity of 3-hydroxyanthranilic acid oxidase, in the presence of its specific activator (Fe⁺⁺), is reported in Table II. Nearly all metals tested showed some extent of inhibition. Since Fe⁺⁺ proved to be one of the strongest inhibitors, it seemed of interest to investigate the type of inhibition due to this ion. The Line-Weaver and Burk (13) plots obtained with two different concentrations of Fe⁺⁺ are reported in Fig. 4, and indicate a non-
competitive inhibition. The $K_i$ value, calculated from the data of Fig. 4, is 8.3 μM. The value of $K_i$, calculated according to Dixon and Webb (14), is 79 μM.

The inhibition by Fe+++ may prove to be of considerable help in the future in understanding the mechanism of operation of the enzyme. In this regard it should be noticed that homogenizable acid oxidase, which, like 3-hydroxyanthranilic acid oxidase, is considered an "oxygen transferase" (4) and requires Fe++ and -SH groups for enzymic activity (15, 16), is not inhibited by Fe+++ (16). It has been suggested that the -SH groups of homogenizable acid oxidase are involved in binding the ferrous ions to the enzyme (17, 18). It is possible that the inhibition by Fe+++ in the case of 3-hydroxyanthranilic acid oxidase, is due to the oxidation of the -SH groups of the enzyme molecule. It would then follow that the -SH groups of 3-hydroxyanthranilic acid oxidase are more sensitive to Fe+++ either because they are not involved in the binding of Fe+++ or because this binding is easily cleaved in the presence of Fe+++.

Inhibition of 3-Hydroxyanthranilic Acid Oxidase by Anthranilic Acid Analogues—Table III shows the effect on the enzymic activity of some compounds with a chemical structure similar to that of 3-hydroxyanthranilic acid. With the exception of m-aminophenol, all these substances proved to be inhibitors, both at pH 6.5 and 8.0. The $K_i$ values for each compound are also reported.

None of these substances acts as substrate of 3-hydroxyanthranilic acid oxidase, since no modification in their typical fluorescence was observed upon addition of the enzyme.

The type of inhibition exerted by these substances on the enzyme has also been investigated. A competitive inhibition was observed only in the case of anthranilic acid; all the other compounds acted as noncompetitive inhibitors. Fig. 5 shows the Lineweaver-Burk plots (13) obtained with anthranilic acid at two different concentrations; the noncompetitive inhibition of the compounds tested, it appears that only 3-hydroxyanthranilic acid oxidase is reported in Fig. 6.

The data reported in Table III and in Figs. 5 and 6 provide some information about the active site of 3-hydroxyanthranilic acid oxidase. From the examination of the structural formulas of the compounds tested, it appears that only 3-hydroxyanthranilic acid and anthranilic acid have a -COOH and a -NH$_2$ group in the ortho position. It would seem, therefore, that this configuration is a requirement for the formation of the linkage between enzyme and substrate.

**Table II**

*Effect of some cations on 3-hydroxyanthranilic acid oxidase*

The enzyme was added to the reaction mixture after 5 minutes of incubation at 0° with the cation.

<table>
<thead>
<tr>
<th>Cation</th>
<th>Final concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{++}$</td>
<td>1 mM</td>
<td>21</td>
</tr>
<tr>
<td>Ca$^{++}$</td>
<td>1</td>
<td>15.9</td>
</tr>
<tr>
<td>Mn$^{++}$</td>
<td>1</td>
<td>10.5</td>
</tr>
<tr>
<td>Fe$^{+++}$</td>
<td>0.5</td>
<td>73.7</td>
</tr>
<tr>
<td>Zn$^{++}$</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cu$^{++}$</td>
<td>1</td>
<td>41.3</td>
</tr>
<tr>
<td>Ni$^{++}$</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>Co$^{++}$</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cu$^{++}$</td>
<td>0.5</td>
<td>76.3</td>
</tr>
</tbody>
</table>

**Fig. 4.** Noncompetitive inhibition of 3-hydroxyanthranilic acid oxidase by Fe$^{+++}$. Reciprocal plots by the method of Lineweaver-Burk (13). The composition of the incubation mixture was as described in "Experimental Procedure;" 0.5 μg of enzyme was added to the system.

Effect of Oxygen—It was previously reported that the rate of enzymic activity was dependent on the partial pressure of oxygen (7). It was also concluded that excess of oxygen inhibited 3-hydroxyanthranilic acid oxidase. However, since the experiments supporting the above conclusion were performed at an Fe++ concentration of 0.1 mM, the possibility existed that the concentration of Fe++ was rate-limiting at high oxygen pressure, i.e., under conditions facilitating the oxidation of Fe++ to Fe+++.

In fact, when the effect of varying oxygen pressures was tested on the rate of enzymic activity at 1 mM Fe++, a close proportionality was found between oxygen pressure and enzymic activity (Fig. 7). No inhibition was observed at the highest partial pressures of oxygen.

Inhibition by Excess Substrate—With a 4-fold purified enzyme preparation, Stevens and Henderson (5) observed a substrate inhibition phenomenon at a substrate concentration of 0.15 mM; on the other hand, Decker et al. (6) did not confirm this effect at a substrate concentration as high as 1 mM.

Fig. 8 provides evidence of the effect of high substrate concentration on the enzymic activity; it is shown that inhibition by excess substrate takes place both at pH 6.5 and 8.0. Such inhibition appears at a 3-hydroxyanthranilic acid concentration of approximately 0.4 mM.

Spectrum of Purified 3-Hydroxyanthranilic Acid Oxidase—In a preceding paper from this laboratory (7) an absorption spectrum of a partially purified enzyme preparation has been reported showing two absorption bands at 287 μm and at 400 μm, respectively. In a subsequent paper (8) the spectrum of the more purified enzyme preparation showed no maximum at 400 μm and only a single peak at 267 μm.

The spectrum of the present preparation of 3-hydroxyanthranilic acid oxidase, however, reveals a typical protein spectrum with a single peak at 280 μm and with an A$_{280}$ to A$_{260}$ ratio of 1.5.

It seems obvious, therefore, that the purification procedure described in the present paper eliminates some impurities ab
TABLE III
Effect of anthranilic acid analogues on activity of 3-hydroxyanthranilic acid oxidase

The reaction mixture, in a final volume of 3 ml, contained 0.3 mM 3-hydroxyanthranilic acid and the inhibitor at the final concentration indicated in the Table. When measuring the activity at pH 6.5, 1 mM FeSO₄ was added. Values for $K_i$ were calculated from Lineweaver-Burk plots (13) similar to those showw in Figs. 5 and 6.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Name</th>
<th>% of Inhibition</th>
<th>% of Inhibition</th>
<th>Type of inhibition</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>$[C_i] = \text{mm}$</td>
<td>$[C_i] = 0.1\text{mm}$</td>
<td>$[C_i] = \text{mm}$</td>
<td></td>
</tr>
<tr>
<td>OH</td>
<td>o-Aminophenol</td>
<td>68</td>
<td>42</td>
<td>34</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\text{pH 6.5}$</td>
<td>$\text{pH 6.0}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OH</td>
<td>m-Aminophenol</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>OH</td>
<td>p-Aminophenol</td>
<td>68</td>
<td>94</td>
<td>Noncompetitive</td>
<td>69</td>
</tr>
<tr>
<td>OH</td>
<td>Anthranilic acid</td>
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<td>45</td>
<td>47</td>
<td>Competitive</td>
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<td>m-Aminobenzoic acid</td>
<td>95</td>
<td>41</td>
<td>20</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td>COOH</td>
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<td>94</td>
<td>36</td>
<td>43</td>
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</tr>
<tr>
<td>OH</td>
<td>Anthranilic acid</td>
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<td>50</td>
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<tr>
<td>OH</td>
<td>Anthranilic acid</td>
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</tr>
<tr>
<td>OH</td>
<td>p-Hydroxybenzoic acid</td>
<td>100</td>
<td>29</td>
<td>19</td>
<td>Noncompetitive</td>
</tr>
</tbody>
</table>
sorbing in the region of visible and ultraviolet light which were present in the preceding enzyme preparations (7, 8).

SUMMARY

1. A simplified purification method of 3-hydroxyanthranilic acid oxidase is described. The steps are acetone fractionation, heating at 55° for 5 minutes, ammonium sulfate fractionation, and chromatography by means of a carboxymethyl cellulose column. A 3500-fold purification has been achieved starting from the supernatant fluid of calf liver homogenate. Spectrophotometric measurements on the purified enzyme reveal a single absorption band in the ultraviolet, with a maximum at 278 μM and an A250 to A260 ratio of 1.5.

2. The purified enzyme is rather unstable and is rapidly inactivated at pH 8.0 and at room temperature. The stabilizing effect of ferrous ions at different enzyme concentration has been analyzed. Other metal ions are reported to inhibit the enzyme. The inhibition exerted by ferric ions is particularly effective. The inhibition by ferric ions has been shown to be of a noncompetitive type.

3. Compounds structurally related to 3-hydroxyanthranilic acid act as inhibitors. Anthranilic acid has been shown to com-
pete with the substrate; a noncompetitive inhibition has been observed with all other compounds. High substrate concentration also lowers the reaction rate; the inhibition becomes evident at a concentration of 0.4 mM.

4. The influence of increasing oxygen partial pressure on enzymic activity has been investigated in the presence of 1 mM ferrous sulfate. The rate of enzymic activity is proportional to the oxygen pressure in the 0 to 1 atmosphere range.

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Alessandro Vescia and Guido di Prisco


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