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Experiment 3: Reaction Kinetics.
Inhibition of the Enzyme \( o \)-Diphenol Oxidase

**KEYWORDS**
Catalysis, Competitive Reactions, Enzyme, Kinetics, Michaelis-Menten, \( o \)-Diphenol oxidase (oDPO), Tyrosinase.

**Aims**
The aim of this experiment is to investigate the kinetics of an enzyme-catalysed reaction, and the kinetics when there is a competing reaction due to the presence of an inhibitor.

**\( o \)-diphenol oxidase activity of potatoes**
The enzyme \( o \)-diphenol oxidase (oDPO), often called tyrosinase, is widely distributed in animals, fungi and plants; it is the cause of enzymic browning in damaged or cut plant tissue. Control of enzymic browning is of major importance in the food industry. DPOs may play a key role in plant defence mechanisms.

\( o \)DPO or tyrosinase is the only enzyme known to catalyse the direct aerobic oxidation of monophenol compounds. The enzyme was originally named because of its ability to catalyse the direct aerobic oxidation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). This is the first step in the formation of melanin (the pigment of hair, skin and eyes). \( o \)DPO in plants can also catalyse the aerobic oxidation of polyhydric phenols and substituted catechols.

\[
\begin{align*}
\text{catechol} & \xrightarrow{\text{tyrosinase}} \text{o-quinone} \\
\text{o-quinone} & \rightarrow \text{coloured compounds}
\end{align*}
\]

This is the reason for the dark colour occurring when some plant tissues are injured (eg cut apples, potatoes).

**Theory**
The behaviour of enzyme-catalysed reactions differs from that of non-catalysed reactions in that the reaction rate is **not simply** proportional to the power of the reactant (substrate) concentration \([S]\). The reaction kinetics changes from being first-order with respect to \([S]\) at low reactant concentration to being zeroth-order with respect to \([S]\) at high reactant concentration.
Michaelis-Menten model

This model assumes a simplified reaction scheme:  

\[
E + S \xrightleftharpoons[k_2]{k_1} ES \xrightarrow{k_3} E + P
\]

where E is an enzyme (a biological catalyst), S is the reactant (or substrate), ES is an association complex, and P are the products. Most biochemistry texts use the out-dated (circa 1950s) notation of “velocity” for the reaction rate, hence the use of \( V \) for the rate of reaction, and \( V_{\text{max}} \) for the maximum possible reaction rate.

Using the quasi-steady-state approximation (QSSA),\(^6\text{–}^8\) it is possible to show that the overall rate of reaction is given by:

\[
\text{Rate} = \frac{V_{\text{max}} [S]}{K_M + [S]}
\]

where

\[
K_M = \frac{k_2 + k_3}{k_1}
\]

is the Michaelis constant, which is related to the steady-state concentration of the complex ES. \( V_{\text{max}} \) is given by

\[
V_{\text{max}} = k_3 [ES] = k_3 [E]_0
\]

and \([E]_0\) is the total concentration of the enzyme in free (E) and complexed (ES) forms.

Inversion of Equation 2 gives the Lineweaver-Burk plot (Equation 3), from which \( V_{\text{max}} \) and \( V_{\text{max}}/K_M \) can be obtained from the intercept and slope of the double-reciprocal plot (see Figure 2).


**Inhibitors of enzyme-catalysed reactions**

A substance that decreases the rate of a biochemical reaction is called an *inhibitor*. There are four common mechanisms for inhibitor activity:\(^6\text{-}^{10}\)

- **Irreversible** inhibition;
- Reversible inhibition, which has the sub-categories:
  - **Competitive** inhibition
  - **Uncompetitive** inhibition;
  - **Mixed** inhibition.

**Irreversible inhibition**

*Irreversible inhibition* usually involves the destruction of one or more functional groups of the enzyme, thus destroying its catalytic activity. Irreversible inhibition cannot be modelled by any form of the Michaelis-Menten mechanism.

**Competitive inhibition**

In *competitive inhibition*, in addition to Equation 1, there is a second equilibrium

\[
E + I \rightleftharpoons EI
\]  

\[
K_{I,\text{comp}}
\]

![Lineeweaver-Burk Plot](image)

\[
\frac{1}{Rate} = \frac{1}{V_{\text{max}}} + \frac{K_{M}}{V_{\text{max}}} \frac{1}{[S]}
\]

Comparison of the value for \(K_M\) without and with the presence of inhibitor shows:\(^6\text{-}^{10}\)
Inhibition of the enzyme oxidation

\[ \left( \frac{V_{\text{max}}}{K_M} \right)_{\text{inhib}} = \frac{1}{\alpha_{\text{comp}}} \left( \frac{V_{\text{max}}}{K_M} \right)_{\text{uninhib}} \]

where

\[ \alpha = 1 + \frac{[I]}{K_I} \]

Here \([I]\) is the concentration of the inhibitor and \(K_I\) is the association equilibrium constant of the enzyme-inhibitor complex, \(\alpha\) and \(K_I\) can be either the values for competitive inhibition (Equation 5) or uncompetitive inhibition (Equation 8) (or both): see Table I.

**Uncompetitive inhibition**

In uncompetitive inhibition, in addition to Equation 1, there is a second equilibrium

\[ K_{I, \text{uncomp}} \]

\[ \text{ES} + \text{I} \xrightleftharpoons{} K_{I, \text{uncomp}} \text{ESI} \]

to form an association complex between the enzyme-substrate association complex and the inhibitor. Its effect is to effectively decrease \(V_{\text{max}}\) but the ratio \(V_{\text{max}}/K_M\) is unchanged.

\[ \left( \frac{V_{\text{max}}}{K_M} \right)_{\text{inhib}} = \frac{1}{\alpha_{\text{uncomp}}} \left( \frac{V_{\text{max}}}{K_M} \right)_{\text{uninhib}} \]

The effect of the inhibitor cannot be reduced by increasing the substrate concentration \([S]\). Thus the inhibitor lowers the observed \(V_{\text{max}}\).

**Mixed inhibition**

In mixed inhibition, the inhibitor interacts with both the enzyme and the enzyme-substrate complex (Equation 4 and Equation 7), to decrease both \(V_{\text{max}}\) and the ratio \(V_{\text{max}}/K_M\) (Equation 5 and Equation 8).

**Inhibited Michaelis-Menten kinetics**

In the competitive, uncompetitive, and mixed inhibition cases, \(V_{\text{max}}\), or the ratio \(V_{\text{max}}/K_M\), or both, are decreased from the uninhibited case by a factor of \(\alpha\) (Equation 5, Equation 6 and Equation 8).

---

\(^a\) The nomenclature in the literature is confused here because some textbooks refer to mixed inhibition as “non-competitive” inhibition. Note that in mixed inhibition, the ratio \(V_{\text{max}}/K_M\) is decreased by one \(\alpha\)-value (Equation 5), while \(V_{\text{max}}\) is decreased by another \(\alpha\)-value (Equation 8). Some textbooks (eg reference \(^b\)) only refer to the special case where the two different \(\alpha\)-values fortuitously are equal, adding to the confusion.
TABLE I.
INFLUENCE OF AN ENZYME INHIBITOR ON THE CONSTANTS $V_{\text{MAX}}$ AND $V_{\text{MAX}}/K_M$.

<table>
<thead>
<tr>
<th>Inhibition type</th>
<th>Effect of inhibitor on $V_{\text{MAX}}$</th>
<th>Effect of inhibitor on $V_{\text{MAX}}/K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Competitive inhibition</td>
<td>unchanged</td>
<td>decreased by $\alpha_{\text{comp}}$</td>
</tr>
<tr>
<td>Uncompetitive inhibition</td>
<td>decreased by $\alpha_{\text{uncomp}}$</td>
<td>unchanged</td>
</tr>
<tr>
<td>Mixed inhibition</td>
<td>decreased by $\alpha_{\text{uncomp}}$</td>
<td>decreased by $\alpha_{\text{comp}}$</td>
</tr>
</tbody>
</table>

$\alpha_{\text{comp}}$ and $\alpha_{\text{uncomp}}$ are both defined by Equation 6

Assay of oDPO activity
The coloured compounds produced by the oxidation of catechol absorb at 500 nm. Thus, the rate of production of these coloured compounds serves as an assay of oDPO activity.

Most assays of oDPO activity are conducted at pH 7 and 25 °C. At a laboratory temperature of about 20 °C, the reaction proceeds to equilibrium in about 30–60 minutes.

The oxygen concentration in the incubation medium must be maintained for the aerobic reaction, by frequent mixing of the reaction system.

The activity of oDPO can be determined by measurement of the initial rate of reaction under a given set of conditions. Estimates of the absorbance under different conditions provide sufficient information for the determination of (approximately) relative rates (in arbitrary units).

One or more of the inhibitors $p$-nitrophenol, sodium chloride and potassium cyanide will be studied.

Experimental Method

Organisation
Purified oDPO solutions containing in excess of 1 mg mL$^{-1}$ of enzyme show little loss of activity over several months if stored properly. However, highly diluted enzyme solutions can exhibit significant loss of activity in less than 20 minutes, even at 5 °C. oDPO solutions are degraded by light. Freshly prepared oDPO extracts, stored in the dark, must be used in this experiment.

The experimental work should be done in teams of two or three. One member from each team should help each other prepare the crude oDPO extract. The
oDPO extract will be shared between all teams. The other team member(s) should prepare the test tubes required for the experiment. At the conclusion of the laboratory work, all the results will be pooled.

Your demonstrator will advise you whether to use \( p \)-nitrophenol (25 mM), sodium chloride (2, 10 and 20 mM) or potassium cyanide (2.5 mM) inhibitor.

<table>
<thead>
<tr>
<th>Volumes (mL) of reagents for the first batch of rate measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test-tube</td>
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<tr>
<td>-----------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
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<td>4</td>
</tr>
<tr>
<td>5</td>
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<tr>
<td>6</td>
</tr>
</tbody>
</table>

5) At one-minute intervals, add the required amount of catechol substrate to test tubes 2-6 in turn. Mix the contents of each tube immediately after the addition of substrate and note the time of commencement. Also,
occasionally mix the contents of the testtubes which you have already made up. (This should bring you to the 5-minute mark.)

6) At the 6-minute mark, determine the absorbance at 500 nm of test-tube 1. Also, occasionally mix the contents of the testtubes which you have already made up.

7) Determine the absorbance of test tubes 2–6, in turn, at one-minute intervals. Also, occasionally mix the contents of the testtubes which you have already made up.

8) Repeat the above procedure (steps 2–6) for test tubes 7–12.

<table>
<thead>
<tr>
<th>Volumes (mL) of reagents for the second batch of rate measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test-tube</td>
</tr>
<tr>
<td>-----------</td>
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<tr>
<td>7</td>
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<td>8</td>
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<td>10</td>
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<td>11</td>
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<td>12</td>
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</tbody>
</table>

Analyses, Results and Discussion

Marks will be awarded for use of a spreadsheet program, programmable calculator program, or other computer program to plot and/or analyse numerical data where appropriate.

Your “Discussion” section should address the scientific significance and meaning of your results. The questions below are intended to guide your discussion, which should cover more than just the answers to the questions.

Marks will be awarded coherent, integrated discussion. Write your discussion so that the answers to any discussion questions are a natural part of the whole discussion, rather than a list of numbered answers, which have no connection with the rest of the discussion.

- Using test-tube 2 as a blank (reference) for the uninhibited rate measurements, correct the measured absorbances for test-tubes 3, and 6-9.
- Using test-tube 11 as a blank (reference) for the inhibited rate measurements, correct the measured absorbances for test-tubes 1, 4, 5, 10 and 12.
- Tabulate your results and plot both uninhibited and inhibited data on the same Lineweaver-Burk plot.
- Determine the $V_{max}$ and $K_M$ values for both the uninhibited and inhibited reactions.
- Determine if your inhibitor is a competitive, or non-competitive, or mixed inhibitor.
• By considering the chemical structure and/or chemical properties of the inhibitor, suggest why it behaves as a competitive, or non-competitive, or mixed inhibitor.
• Determine $K_{I,comp}$ and/or $K_{I,uncomp}$.
• Discuss why the instructions have arranged the test tubes in a non-systematic order.

The feedback form

The "Student Learning Experience" feedback form is used to evaluate the effectiveness of the laboratory teaching and learning. It is not used to assess your learning. The responses are used to improve the laboratory exercise and to improve the laboratory manual.

Improvements in teaching and learning practices are often discussed in public fora ("forums"). Anonymous comments from students are very useful in this regard. Your (anonymous) comments can contribute to this discourse.

• Please complete the "Student Learning Experience" feedback form.
• You may submit the feedback form in two ways:
  • You may attaching it to your report; or
  • You may submit the feedback form to the School office. Please ensure that Mrs. Ruth Henderson marks your name on the outside of the collection envelop (not your feedback form), so that you will get a class participation mark.

Your comments will not affect the marking of the laboratory report

The content of your answers in the feedback form will not affect your laboratory report marks; your willingness to complete and submit the feedback form will contribute to your class participation mark.

Extension exercises

Students that are more able should attempt one or more of these exercises for bonus marks

• Time permitting, you can repeat the experiment with another inhibitor — eg, benzoic acid or one of the inhibitors (p-nitrophenol, sodium chloride or potassium cyanide) which you have not used. You should prepare fresh oDPO extract and perform the kinetic runs both without and with inhibitor. (Why do you need fresh oDPO extract?)

• Using a spreadsheet (or otherwise) integrate the elementary rate equations associated with the Michaelis-Menten mechanism (Equation 1) to show that the quasi-steady-state assumption can be applied to the enzyme-substrate concentration [ES]. You will need to justify any assumptions you make in your calculations. See Assignment 1 for a brief description of the Euler method, which can be used for numerical integration of the appropriate rate equations.11-13

• Using the quasi-steady-state assumption, show that the Michaelis-Menten mechanism (Equation 1) will result in the Michaelis-Menten kinetic equation (Equation 2).
• Derive the Lineweaver-Burk plot (Equation 3) from the Michaelis-Menten kinetic equation (Equation 2).

• Show that the competitive inhibitor mechanism (Equation 1 and Equation 4) will result in the altered Michaelis-Menten kinetics (Equation 5 and Table 1).

• Show that the uncompetitive inhibitor mechanism (Equation 1 and Equation 7) will result in the altered Michaelis-Menten kinetics (Equation 8 and Table 1).

• Show that the mixed inhibitor mechanism (Equation 1, Equation 4 and Equation 7) will result in the altered Michaelis-Menten kinetics (Equation 5, Equation 8 and Table 1).

• Can you comment on the biological and medical significance of enzyme kinetics and inhibition? Can you find any discussion of inhibition of enzymatic browning of foodstuffs in the literature?

References
8 R. Chang, Physical Chemistry for the Chemical and Biological Sciences, University Science Books, Sausalito (CA), 2000.
Appendix: Learning objectives

By the end of the exercise, students should have learnt or reinforced the following concepts, knowledge, and/or skills:

- Students must understand and use the relationship between the transmitted light intensity of a “blank” and the transmitted light intensity of the sample, in order to determine the absorbance of the sample using a single-beam spectrophotometer.

- Students must understand and apply the Beer-Lambert Law to determine concentration (or, rather, concentration in arbitrary units) from absorbance.

- Students must understand the definition of reaction rate in order to determine rate (in arbitrary units) from the change in concentration (in arbitrary units) over time.

- Students should appreciate that reaction kinetics is applicable to real-life systems, not just systems involving methyl isocyanide and other “textbook” systems.

- Students should appreciate that complicated reaction mechanisms (like the Michaelis-Menten mechanism) will give rise to non-integer reaction orders.

- Students should be able to demonstrate judgement about
  - what is (or is not) relevant in the context of the exercise,
  - what is (or is not) significant in the context of the exercise, and
  - what is (or is not) important in the context of the exercise,
  by deciding what to include or omit from a formal written report.

- Students should be able to operate a simple spectrophotometer.

- Students should be able to handle light-sensitive reagents.

- Students should be able to use a spreadsheet package to collate, display, and analyse observed data.

- Students should be able to work in teams, and to plan and manage their time effectively.

- Students must be able to use and interconvert units correctly.

- Students should (further) develop communication and generic skills, including the ability to use appropriate computer programs.
Appendix: Michaelis-Menten Enzyme Kinetics

The kinetics of enzyme-catalysed reactions changes from being first-order with respect to reactant (substrate) concentration \([S]\) at low reactant concentration to being zeroth-order with respect to \([S]\) at high reactant concentration.\(^2\)\(^-\)\(^8\) This has been successfully explained by the Michaelis-Menten model for the reaction mechanism:

\[
\text{E} + \text{S} \overset{k_1}{\underset{k_2}{\rightleftharpoons}} \text{ES} \overset{k_3}{\to} \text{E} + \text{P}
\]

where \(E\) is an enzyme (a biological catalyst), \(S\) is the reactant (or substrate), \(ES\) is an association complex, and \(P\) are the products. Using the quasi-steady-state approximation (QSSA),\(^6\)\(^-\)\(^8\) it is possible to show that the overall rate of reaction is given by:

\[
\text{Rate} = \frac{V_{\text{max}} [S]}{K_M + [S]}
\]

where

\[
K_M = \frac{k_2 + k_3}{k_1}
\]

is the Michaelis constant, which is related to the steady-state concentration of the complex \(ES\). \(V_{\text{max}}\) is given by

\[
V_{\text{max}} = k_3 [ES] = k_3 [E]_0
\]

and \([E]_0\) is the total concentration of the enzyme in free (E) and complexed (ES) forms.

Inversion of Equation 2 gives the Lineweaver-Burk plot (Equation 3), from which \(V_{\text{max}}\) and \(V_{\text{max}}/K_M\) can be obtained from the intercept and slope of the double-reciprocal plot (see Figure 2).

\[
\frac{1}{\text{Rate}} = \frac{1}{V_{\text{max}}} + \frac{K_M}{V_{\text{max}}} \frac{1}{[S]}
\]

Inhibitors of enzyme-catalysed reactions

A substance that decreases the rate of a biochemical reaction is called an inhibitor. There are four common mechanisms for inhibitor activity:\(^6\)\(^-\)\(^10\)

- **Irreversible** inhibition;
- Reversible inhibition, which has the sub-categories:
  - **Competitive** inhibition
  - **Uncompetitive** inhibition;
- Mixed inhibition.

**Irreversible inhibition**

Irreversible inhibition usually involves the destruction of one or more functional groups of the enzyme, thus destroying its catalytic activity. Irreversible inhibition cannot be modelled by any (modified) Michaelis-Menten mechanism.

**Competitive inhibition**

In competitive inhibition, in addition to Eq.(1), there is a second equilibrium

\[
E + I \rightleftharpoons EI
\]

(EQUATION 4)

This forms an association complex between the enzyme and the inhibitor. Competitive inhibition effectively decreases \( V_{max}/K_M \) as inhibitor concentration [I] increases. The effect of the inhibitor can be reduced by increasing the substrate concentration [S], leaving \( V_{max} \) unchanged. Comparison of the value for \( K_M \) without and with the presence of inhibitor shows:

\[
\frac{V_{max}}{K_M}_{\text{inhib}} = \frac{\alpha}{\alpha_{\text{comp}}} \left( \frac{V_{max}}{K_M} \right)_{\text{uninhib}}
\]

(EQUATION 5)

where

\[
\alpha = 1 + \frac{[I]}{K_I}
\]

(EQUATION 6)

Here [I] is the concentration of the inhibitor and \( K_I \) is the association equilibrium constant of the enzyme-inhibitor complex, \( \alpha \) and \( K_I \) can be either the values for competitive inhibition (Equation 5) or uncompetitive inhibition (Equation 8) (or both): see Table I.

**FIGURE 3.** LINEWEAVER-BURK PLOT FOR COMPETITIVE INHIBITION

For any given (fixed) reactant concentration [S], the effect of a competitive inhibitor is to lower (inhibit) the overall rate. The substrate S directly competes
with the inhibitor to form an association complex with the enzyme. This usually occurs when the inhibitor has similar structure to the substrate: ie, the inhibitor and the substrate both compete for the same binding site on the enzyme. The effect of the inhibitor can be reduced by increasing the substrate concentration, leaving $V_{\text{max}}$ unchanged.

**Uncompetitive inhibition**

In **uncompetitive inhibition**, in addition to Equation 1, there is a second equilibrium

\[ K_{I, \text{uncomp}} \]

\[ E + I \rightleftharpoons ESI \]

Equation 7

to form an association complex between the enzyme-substrate association complex and the inhibitor. Its effect is to effectively decrease $V_{\text{max}}$ but the ratio $V_{\text{max}}/K_M$ is unchanged.

\[ \left( \frac{V_{\text{max}}}{V_{\text{max}}} \right)_{\text{inhib}} = \frac{1}{\alpha_{\text{uncomp}}} \left( \frac{V_{\text{max}}}{V_{\text{max}}} \right)_{\text{uninhib}} \]

Equation 8

The effect of the inhibitor cannot be reduced by increasing the substrate concentration [$S$]. Thus the inhibitor lowers the observed $V_{\text{max}}$.

**Mixed inhibition**

In **mixed inhibition**, the inhibitor interacts with both the enzyme and the enzyme-substrate complex (Equation 4 and Equation 7), to decrease both $V_{\text{max}}$ and the ratio $V_{\text{max}}/K_M$ (Equation 5 and Equation 8). The enzyme-inhibitor binding does not interfere with the enzyme-substrate binding but acts to decrease the free enzyme concentration and the enzyme-substrate association complex concentration.
Inhibition of the enzyme $O$-diphenol oxidase

$$E + S \overset{k_1}{\rightleftharpoons} ES \overset{k_3}{\rightarrow} E + P$$

$$+ \quad \quad +$$

$$I \quad \quad I$$

$$\frac{K_{I,\text{comp}}}{K_{I,\text{uncomp}}}$$

$$EI + S \overset{k_2}{\rightleftharpoons} ESI$$

**Figure 5. Lineweaver-Burk plot for non-competitive inhibition**

**Inhibited Michaelis-Menten kinetics**

In the competitive, uncompetitive, and mixed inhibition cases, $V_{\text{max}}$, or the ratio $V_{\text{max}}/K_M$, or both, are decreased from the uninhibited case by a factor of $\alpha$ (Equation 5, Equation 6 and Equation 8).6-10

**Table 1. Influence of an enzyme inhibitor on the constants $V_{\text{max}}$ and $V_{\text{max}}/K_M$.**

<table>
<thead>
<tr>
<th>Inhibition type</th>
<th>Effect of inhibitor on $V_{\text{max}}$</th>
<th>Effect of inhibitor on $V_{\text{max}}/K_M$</th>
</tr>
</thead>
<tbody>
<tr>
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<td>decreased by $\alpha_{\text{comp}}$</td>
</tr>
<tr>
<td>Uncompetitive inhibition</td>
<td>decreased by $\alpha_{\text{uncomp}}$</td>
<td>unchanged</td>
</tr>
<tr>
<td>Mixed inhibition</td>
<td>decreased by $\alpha_{\text{uncomp}}$</td>
<td>decreased by $\alpha_{\text{comp}}$</td>
</tr>
</tbody>
</table>

$\alpha_{\text{comp}}$ and $\alpha_{\text{uncomp}}$ are both defined by Equation 6.
Anonymous Student Learning Experience
Feedback

Introduction
Please help us to better tailor the laboratory experience to help your learning, by providing responses to the following questions.

Feedback
1. Did this experiment help you to understand the theory and concepts of the topic? If so, how, or if not, why not?

2. How is this experiment relevant to you in terms of your interests and goals?

3. Did you find this experiment interesting? If so, what aspects of this experiment did you find of interesting? If not, why not?

4. Can the experiment be completed comfortably in the allocated time? Is there time to reflect on the tasks while performing them?

5. Does this experiment require teamwork and if so, in what way? Was this aspect of the experiment beneficial?
6. Did you have the opportunity to take responsibility for your own learning, and to be active as learners?

7. Does this experiment provide for the possibility of a range of student abilities and interests? If so, how?

8. Did the laboratory notes, demonstrators’ guidance and any other resources help you in learning from this experiment? If so, how?

9. Are there any other features of this experiment that made it a particularly good or bad learning experience for you?

10. What improvements could be made to this experiment?

11. Any other comments?

Improvements in teaching and learning practices are often discussed in public fora (“forums”). Your (anonymous) comments can contribute to this discourse.

I do not give permission for my comments to be used outside Deakin University.
Experiment 3: Reaction Kinetics. Inhibition of the Enzyme o-Diphenol Oxidase

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