Inhibition of the reaction kinetics of the enzyme o-diphenol oxidase: An APCELL experiment.*

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Introduction

Chemical kinetics is a fundamental component of chemistry. Traditional chemistry-laboratory exercises have concentrated on the study of non-biological organic or inorganic reaction kinetics. Historically, these experiments are chosen for their perceived didactic value — they usually exhibit simple kinetics, without complicating factors — rather than for any "relevance" to the world outside the chemistry laboratory. The same lack-of-relevance is applicable to many "traditional" experiments in other areas of chemistry. Therefore, the fact that students are avoiding the study of chemistry (*1*), should not be surprising.

The aim of this experiment is to investigate the kinetics of an enzyme-catalysed reaction, and the kinetics in the presence of an inhibitor (*2-15*). This relates physical chemistry to a "real world" application — the action of a biological catalyst, o-diphenol oxidase (oDPO). Better students need to be challenged (*16*) by extentions to the experiment, which is easily achieved since the complexity of biological systems offers many avenues for exploration.

Enzyme kinetics is usually described by the Michaelis-Menten model, which can be used to illustrate several concepts in the curriculum. The behaviour of enzyme-catalysed reactions is **not simply** proportional to the power of the reactant (substrate) concentration [S]. The kinetics changes from being first-order with respect to [S] at low [S], to being zeroth-order at high [S], with non-integer order at intermediate [S] (*3-10*). The derivation of the Michaelis-Menten kinetic equations involves use of the quasi-steady-state approximation (*3-6*). Furthermore, the laboratory is an opportunity to learn how to handle photo-sensitive reagents.

The original version of this laboratory exercise used *p*-nitrophenol and cyanide anion inhibitors. Other inhibitors (*15*), with smaller health and safety risks, can be used: eg NaCl (10 mM stock) under the conditions described in the notes, has been successfully used at Deakin University.

This exercise has the potential to be further developed as an affordable high-school experiment using the browning of mashed raw fruit or potatoes as the enzyme-catalysed reaction and a home-made colorimeter for under $15 (*17*). In this configuration, the most expensive items of equipment or reagents would be a sturdy kitchen knife and food processor!

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Educational Template

Section 1 - Summary of the Experiment

1.1 Experiment Title

Reaction Kinetics. Inhibition of the Enzyme *o*-Diphenol Oxidase

1.2 Description of the Experiment

Chemical kinetics is a fundamental component of physical chemistry. 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 (*2-15*). This relates physical chemistry to a "real world" application — the oxidation of an organic compound through the action of a biological catalyst.

1.3 Course Context and Students' Required Knowledge and Skills

The School of Biological and Chemical Sciences at Deakin University has students enrolled in the biology, biotechnology, forensic and wine science streams as well as the chemistry stream: the physical chemistry unit has to cater for a wide range of student interests and mathematical skills.

One of the foci of the School is the promotion of the

*The complete documentation for this experiment is freely available on the APCELL web site [www.apcell.org]. It includes the educational template, a set of student notes, demonstrator notes and technical notes to allow ready implementation into a new laboratory.

interdisciplinary nature of modern chemistry (via "biologically relevant" chemistry) and of modern biology (via molecular biology). Instead of doing a "traditional" kinetics experiment, an enzyme kinetics reaction is studied, also with inhibition via a competing equilibrium (here "competing equilibrium" is used in the usual chemical sense, meaning alternative pathway).

This kinetics experiment is the third experimental exercise (total of five such experimental exercises) done during the semester-long 2nd year physical chemistry laboratory.

- Students are expected to have knowledge of 1st year kinetics and 1st year equilibrium topics.
- Prior to the start of this laboratory exercise, students have been introduced to the use of a spreadsheet package. Nevertheless, students are told that use of a spreadsheet is part of the computer laboratory component of the exercise and should consult their demonstrator if they encounter difficulties.
- Students are not expected to have knowledge of kinetics or equilibrium topics at 2nd year level. An appendix introducing enzyme kinetics is included in the student notes for those students who are unfamiliar with the topic.

Some students may have encountered enzyme kinetics in Biochemistry during the first semester of second year. Those students would have done a biochemistry laboratory exercise on enzyme kinetics (Michaelis-Menten kinetics), but not an exercise on *inhibited* enzyme kinetics. Over half the students attempting *this* laboratory exercise would *not* have attempted the biochemistry laboratory exercise.

1.4 Time Required to Complete

Prior to Lab	1 hour reading
In Laboratory	2 hours "wet" laboratory and 2 hours "dry" laboratory for analysis of results
After Laboratory	2-3 hours report writing

1.5 Providence

The original source of this experiment is unknown.

Kieran Fergus Lim (Deakin University) and Robert Learmouth (University of Southern Queensland) both have versions of this experiment derived from an experiment run by Robert Learmouth when he was at University of New England (*c*. 1990). Robert Learmouth has given permission for the experiment to be adapted and used.

Verbal tradition at University of New England suggests that the experiment came from the University of Sydney (*c*. 1960s? 1970s?), but current Sydney staff (*c*. 2000) have no knowledge of the experimental exercise.

Many similar experiments (mostly on ***un***-inhibited Michaelis-Menten kinetics) have been published in the literature: for example, (*14,15,18-23*).

1.6 Other Comments

J.R.L. Walker (retired, University of Canterbury, New Zealand) has run a similar experiment using the same enzyme extracted from mushrooms and bananas. Extraction from other fruit is also possible. The University of Canterbury experiment usually used the kinetics to determine comparative concentrations of *o*-diphenol oxidase enzyme (oDPO or tyrosinase) in different parts of the mushroom, and hence was not a kinetics experiment as such. Walker has published a paper on the inhibition of o-diphenol oxidase by phenolic acids (*14*) and gives a list of inhibitors of o-diphenol oxidase activity in (*15*).

In the student notes, *p*-nitrophenol and cyanide anion inhibitors were described for historical reasons. One of the referees has pointed out that use of these inhibitors poses health and safety risks: we have since used NaCl (10mM stock) as an inhibitor: other inhibitors of o-diphenol oxidase activity (*15*) are also possible.

ionic lattice, and use the formula $[Fe(H_2O)_6](NH_4)_2(SO_4)_2$.

But we have to be careful. How should we represent the formula of nickel chloride-6-water whose composition is represented by $NiCl_2.6H_2O$? In view of what has been said above, we might think that the formula $[Ni(H_2O)_6]Cl_2$ provides structural information. Well it would - but incorrect information! Crystallographers tell us that each nickel ion forms a complex with four water molecules and two chloride ions. The other two water molecules in the lattice are not directly bound to the nickel ions. So perhaps $[Ni(H_2O)_4Cl_2].2H_2O$ is the most appropriate structural formula.

Where do we stop? Presumably our best guide is provided by the purpose for which a formula is being used.

The really important message in this discussion is that how we interpret the formula of a substance usually depends on prior knowledge about the substance. And it is often the case that students don't have the knowledge that allows them to interpret formulas in the same way that experts do. And perhaps it's not surprising that sometimes they are confused!

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Section 2 - Educational Analysis

Learning Outcomes *What will students learn?*	**Process** *How will students learn it?*	**Assessment** *How will staff know students have learnt it?* *How will students know they have learnt it?*

Theoretical and Conceptual Knowledge

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.	These three fundamental concepts underpin the entire exercise	Students are able to convert their light intensity (transmittance or "absorbance") measurements into reaction rates
Students must understand and apply the Beer-Lambert Law in order to use absorbance to measure concentration (or at least concentration in arbitrary units).		
Students must understand the definition of reaction rate in order to measure rate (in arbitrary units) by 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 isocynide and other "textbook" systems.	Students will measure kinetics associated with a food stuff	
Students should appreciate that complicated reaction mechanisms (like the Michaelis-Menten mechanism) will give rise to non-integer reaction orders.	Use of the Michaelis-Menten reaction kinetics.	Students are able to see to see that a rate versus reactant (substrate) concentration curves from linear proportionality (first order) to being constant (zero order) at high reactant concentration. Staff will know from the students' discussion of the Michaelis-Menten reaction kinetics.
Students should be able to exercise judgement about what is (or is ***not***) relevant in the context of the exercise, judgement about what is (or is ***not***) significant in the context of the exercise, and judgement about what is (or is ***not***) important in the context of the exercise.	Students must decide what to include or omit from a formal written report. They are given the demonstrator's assessment and feedback *pro forma*. They are encouraged to seek help from the demonstrator.	There must be sufficient data, details and discussion in the main body of the report, so that a student (classmate) who has done everything as the student writer, except this exercise (or this unit), can understand the report.

Scientific and Practical Skills

Students should be able to operate a simple spectrophotometer.	Students prepare sample solutions and use the spectrophotometer to make measurements.	Spectrophotometer will be within the expected ranges (ie **not** off-scale). Students will record consistent measurements. Linear Lineweaver-Burk plots will result from proper use of the instrument when the proper "blank corrections" have been made.
Students should be able to handle light-sensitive reagents.	Students will protect reagents and sample solutions from light.	Students will record measurements at the end of the exercise (eg test-tubes 10, 11, 12) that are consistent with measurements at the start of the exercise (eg test-tubes 1, 2, 3).
Students should be able to use a spreadsheet package to collate, display, and analyse observed data.	Students will use a spreadsheet package to collate, display, and analyse observed data.	Students will obtain linear Lineweaver-Burk plots, similar to those in the student notes.

Generic Skills

Students should be able to work in teams, and to plan and manage their time effectively.	Students must divide tasks between themselves at different stages of the laboratory exercise.	Students will complete the allocated tasks with minimal conflict.
Students must be able to use and interconvert units correctly.	Students should be aware of and convert between molar (mol L^{-1}) and millimolar (mmol L^{-1}) quantities.	Students should be aware of and convert between molar (mol L^{-1}) and millimolar (mmol L^{-1}) quantities.
Students should (further) develop communication and generic skills (*24,25*), including the ability to use appropriate computer programs (*26*). *Note: The semester-long physical chemistry laboratory program at Deakin University is one of a series of laboratory programs specifically intended to foster report-writing skills. Students are given the opportunity to submit draft reports for comment. This aspect of the curriculum is not an integral component of the current exercise.*	Students are given the opportunity to submit draft reports for comment. Students are encouraged to consult their demonstrator on the report writing style and use of appropriate computer programs	Students will present a formal written report, which satisfies the criteria set out on a assessment and feedback *pro forma*.
All of the above knowledge and skills	By preparing a clear, well-structured formal report, students will organise their knowledge and understanding and to consolidate learning (*27*)	Students demonstrate that their knowledge, skills and understanding … satisfy the stated and implied criteria and they have met [or exceeded] all the other requirements … *Note: This criterion is an extract from the Faculty guidelines on grading and assessment. It is clearly communicated to students during the semester and is the basis for assessment of all laboratory exercises and assignments.*

Section 3 - Student Learning Experience

Explainatory notes to Student Learning Experience
In response to student feedback in 1999 and 2000, the exercise was revised (*28,29*) and presented to the APCELL workshop (*30*) in early 2001. The exercise and associated documentation was again revised, incorporating suggestions from workshop participants. The student responses in this section document improvements to the exercise over the last 2 years (S1-4, 2000; S5-8, 2001). The version presented here includes further changes in response to the 2001 student feedback, and to comments from the APCELL referees. In the following responses, omission of a particular student (eg S7 and S8 for question 3.2) indicates that the student did not respond to that particular question.

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

S1: Yes, gives practical example in which the observed occurrences aid understanding.
S2: No, not easy to understand the kinetics. Therefore didn't understand final results.
S3: Yes, a little. I got values and I know what these values mean but concepts where a little hard to understand at first.
S4: Yes, it demonstrated how Michaelis-Menton kinetics can be applied to enzyme activity.
S5: I personally struggled a little with the concepts and theory involved. I think this was partly due to my very limited background in biology/ biochemistry.
S6: Yes, the write-up helped me understand the theory.
S7: Yes, it overlapped Biochem A from last term, but in more depth
S8: Yes.

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

S1: Enzyme kinetics isn't my major area of interest. But it is good to be able to understand.
S2: Not very relevant.
S3: Not that relevant, but I do think it is interesting.
S4: It helps to increase my background knowledge of reaction kinetics.
S5: My planned major is environmental engineering. I feel some biochem may be useful for me.
S6: Kinetics is used extensively for chemistry in pyrotechnics and explosives development. *Note: This student had commented previously (in feedback for another exercise) that he wanted a career in pyrotechnics and explosives development.*

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

S1: Yes. understanding the principles behind the prac.
S2: Yes, it was interesting to observe the biological effects of catalysts and inhibitors and the effects they have on reactions.
S3: Yes, getting to use "real" food not just chemicals in jars was something different.
S4: Yes, it was good to use a potato instead of just chemicals.
S5: I find chemistry work much more interesting when it has a 'real world' relevance. Here we used potatoes and talked about bruising.
S6: Nope, sorry. Didn't find much at all interesting. Didn't appeal to me.
S7: In between! It had interesting things and boring things.
S8: Yes.

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

S1: Yes, there is time to reflect as the prac can be covered quite quickly
S2: Yes, there was enough time to complete the prac but when adding the inhibitor in one-minute intervals, it was difficult to reflect on what was happening.
S3: Yes, we had plenty of time.
S4: Yes.
S5: Yes & no.
S6: Yes plenty of time.
S7: Yes.
S8: Yes.

3.5 Does this experiment require teamwork and if so, in what way? Was this aspect of the experiment beneficial?

S1: Teamwork is required to ensure the experiment runs smoothly during analysis section. Working as a team is very beneficial as it can lead to quick and effective work.
S2: Not much teamwork was required, only when recording absorbances in one-minute intervals was it better to work with someone. This was beneficial as it allowed timing to be more accurate.
S3: Working in groups is usually more beneficial as you can discuss what is happening with team members and it speeds up the prac as you don't have to do all the boring pipetting yourself.
S4: Yes, to help understanding.
S5: Exp. definitely runs faster when working in a group.
S6: Yes. teamwork is needed to get the potato and enzyme ready in the right time intervals.
S7: Yes, teamwork for this experiment was beneficial.
S8: Yes — In order to make it a quick and easy experiment.

3.6 Did you have the opportunity to take responsibility for your own learning, and to be active as learners?

S1: Yes, you have to take responsibility otherwise understanding of the topic will be harder.
S2: No, more focussed on getting good results.
S3: Yes, I had to do some research to understand the concepts.

S4: Yes.
S5: Yes, to an extent.
S7: Yes.
S8: Yes.

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

S1: Yes, there was a reasonable diversity of elements that needed to be understood.
S2: Yes, it incorporates a bit of biology with a bit of kinetics.
S3: Yes, because it is more Biochemistry / Biology related.
S4: It's kind of a bit of biochemistry mixed with physical chemistry which is good.
S5: Yes, it has biochem links to phys. chem concepts.
S6: I guess, student who had done biochem had a definite head start.
S8: Yes — chemical kinetics and biological reactions.

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

S1: Gives an adequate guide as to where to start.
S2: No. Not enough information was given on K. The notes showed what to expect, but not how to get there or how to interpret results.
S3: Yes.
S4: Yes, the laboratory notes were very helpful.
S5: Yes, although demonstrators explanation was important for my understanding.
S6: Yes. they were my only reference. Very good notes.
S7: Yes.
S8: Yes.

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

S1: No.
S2: Relatively simple experiment but the theory behind it not well understood.
S3: The manual should more clearly explain how Abs is related to Rate. It was unclear how to interpret results to start off.
S4: The results did not work which was a bit of a drawback.
S5: I found the procedure for calculations a little confusing. I wasn't sure what to do with results. ie: Abs a c
S6: The calculations, write-up, and plotting of graphs takes way too long.
S7: None.
S8: To much theory on enzyme kinetics to learn that I didn't know of.

3.10 What improvements could be made to this experiment?

S3: A little more about K_I could be included in the theory section.
S4: The actual experiment could be enhanced to facilitate good results.
S5: More detail related to calculations in method or introduction.

3.11 Other Comments

No comments were received from students for this question.

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