

Mystrica

colorimeter

The following pages contain details of many of the applications for the colorimeter.

More details, suggestions for investigations and sample results can be viewed on the Mystrica website, www.mystrica.com

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SAFETY

The investigations described here have been developed by teachers with experience in schools and colleges worldwide.

The techniques, equipment and chemicals used are, as far as possible, simple and present few hazards.

Where hazards exist they are minimal, are clearly identified and the precautions for safe working are indicated.

Students and teachers should

- follow good laboratory practices.
- complete their own risk assessments.
- students should work under adequate supervision.

BASIC APPARATUS AND CHEMICALS

With a few exceptions simple laboratory apparatus is sufficient to carry out the investigations. Most schools will have access to basic equipment of various degrees of sophistication, but there is little here that could not be done in most kitchens.

Other than this an accurate balance is important if solutions, buffers, etc are to be made up. A small number of the investigations benefit from the use of a bench centrifuge. Volumes can be measured with measuring cylinders or pipettes.

Low cost 'supermarket' solutions such as using bottled water instead of distilled water and disposable plastic cups instead of laboratory glassware are perfectly acceptable.

More details, suggestions for methods, investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com

Beer's Law

Introduction

For most applications it is preferable to measure absorbance rather than transmittance since there is a linear relationship between absorbance and the concentration of the coloured substance being measured, though there are qualifications to be considered, (see below).

Background

Beer's Law (or Beer-Lambert, Lambert-Beer or Beer-Lambert-Bouger) states that there is a logarithmic relationship between the transmission of light through a sample and the product of the absorption coefficient of the absorbing substance times the distance of the light path through the sample.

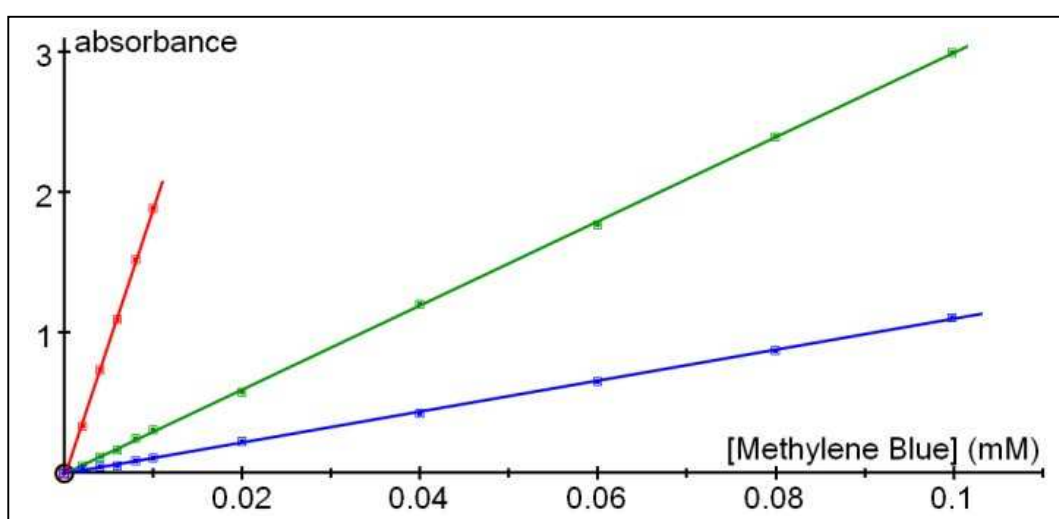
In practice this means that there is a directly proportional straight line relationship between concentration and absorbance.

There are certain conditions that must be met for the law to apply. The most important of these in the context of colorimetry is that the incident light should be as near monochromatic as possible, or at least should have be equally absorbed throughout its range of wavelengths. If this condition is not met the relationship will be non-linear. In general colorimeters, whether they use LEDs or coloured filters to create their light beam, do not have the narrow wavebands that can be achieved with spectrophotometers. So precise linear relationships are not always achievable and depend on a good match between the incident waveband and the absorbed waveband.

Most substances will give good straight line responses in the range 0 – 0.5 absorbance units.

Example

Absorbance versus concentration of methylene blue using red, green or blue LEDs



More details, suggestions for investigations and sample results can be viewed on the Mystrica website, www.mystrica.com/Experiment.aspx?PagelId=66

Amylase

Introduction

The digestion of starch by amylase is likely to be one of the first enzyme reactions encountered by students, probably using iodine solution to show the disappearance of starch when it is mixed with amylase.

Background

Amylase is a general term for several different enzymes that hydrolyse starch. There are many natural sources of amylase, the most commonly used being those from mammalian saliva and pancreas; plant amylase from sweet potatoes or from grains such as barley; fungal amylase and bacterial amylase.

Starch is a mixture of **amylose**, a straight chain of glucose molecules and **amylopectin**, a branching chain. Most starches are about 20% amylose and 80% amylopectin.

α -amylases digest starch by randomly breaking the glycosidic bonds between glucose molecules. The product is therefore a mixture of small molecules containing two, three or more glucose molecules, (i.e. a mixture of maltose and dextrans).

β -amylases digest starch by cleaving every second bond starting from one end, producing maltose.

Salivary amylase, (ptyalin), is an α -amylase, as are fungal and bacterial amylases. β -amylases are obtained from plant sources, such as barley or sweet potato.

Suggestions for investigations

- compare amylase from different sources
- measure optimum temperature and pH of amylases
- investigate the products of the reaction

Investigations with α -amylase

α -amylase is present in saliva. Salivary amylase, (*ptyalin*), is readily available and is considered safe to use when simple precautions are followed. There are a number of different bacterial and fungal α -amylases available in liquid or powdered form.

SAFETY

Each student should collect and use only THEIR OWN saliva.

After use test-tubes and other laboratory equipment that has been in contact with saliva should be soaked in a sterilising solution such as dilute bleach before being washed up.



If powdered enzymes from bacteria or fungi are used then precautions must be taken when preparing solutions as the enzyme powders may provoke allergic reactions. Solutions should be prepared in a fume cupboard, or with care while wearing a dust mask. Skin contact with powdered enzymes and prepared solutions should be avoided.

The reaction between soluble starch and α -amylase can be followed by monitoring the disappearance of the blue/black starch-iodine complex or the appearance of reducing sugars using DNSA reagent or Benedict's reagent.

Salivary amylase is good for showing the effects of temperature and pH on enzyme activity as it is relatively sensitive to both conditions, though the optimum temperature is considerably higher than is usually given in school textbooks.

To collect salivary amylase

- Rinse the mouth out first with water.
- Take a little water into the mouth, swirl it around and spit out into a container labelled to identify the person whose saliva it contains.

Reaction mixture

A suitable reaction protocol for α -amylase is as follows;

- 0.5cm³ of a 5% solution of soluble starch
- 4.4cm³ of pH7 buffer, (the reaction will work with water instead of buffer).
- 0.1cm³ of amylase, (salivary amylase or a 0.1% solution of bacterial or fungal amylase).

Before adding it to the enzyme the substrate/buffer mixture should be equilibrated to the reaction temperature; 35°C works well, but lower temperatures, though slower, may be easier to maintain.

At 30 second intervals remove 0.1cm³ of the reaction mixture and add it to 3cm³ of iodine solution, (2% iodine stock solution in 0.1M HCl)

Read absorbance using red light.

More details, suggestions for investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PageId=13

Investigations with β -amylase

Sweet potato is an excellent source of β -amylase.

Enzyme extraction

Sweet potato (*Ipomoea batatas*) is crushed in a pestle and mortar, or blended, with water using 1cm³ of water for each gram of sweet potato. 30g of sweet potato gives a useful amount of extract and we have found that large sweet potatoes give a better yield of enzyme than small, young potatoes, though small ones still give good yields.

The extract is filtered through several layers of butter muslin.

If a centrifuge is available it can be used to remove cell debris but the extract will give good results without this stage.

The extract will keep for several weeks refrigerated without much loss of activity.

Enzyme activity can be shown using soluble starch as the substrate and DNSA reagent or Benedict's reagent, to measure the maltose produced. Soluble starch

will not be completely broken down by β -amylase as the enzyme can only digest straight chains of glucose and is stopped by branches.

An alternative method is to use **amylose** prepared from potato starch as the substrate as this is completely broken down to maltose, (since there are no branches in the chains to stop the progress of the enzyme), and the blue colour obtained with iodine solution will disappear completely. The method for preparing amylose is given on the *Mystrica* website,

www.mystrica.com/Experiment.aspx?PagelId=14

Reaction mixture

Suitable conditions for this reaction are a temperature of 35 °C in pH6 buffer. The buffer/substrate mixture should be allowed to equilibrate to the reaction temperature before being added to the enzyme.

Using soluble starch as the substrate and measuring the production of maltose.

Mix 1.5cm³ of a 5% solution of soluble starch with 1.5cm³ of buffer.

Add this to 0.1cm³ of the enzyme extract to start the reaction. At intervals of 1 minute remove 0.3cm³ and add to 0.3cm³ DNSA reagent in a test tube.

Stand the tube in boiling water for 5 minutes then add 3cm³ of cold water and read the absorbance using green light.

Using amylose as the substrate and measuring the colour of the blue complex formed between amylose and iodine.

Mix 2cm³ of amylose from potato starch with 2cm³ of buffer.

Add this to 0.1cm³ of the enzyme extract to start the reaction.

At intervals of 30 seconds remove 0.1cm³ into 3cm³ of iodine solution, (2% iodine stock solution in 0.1M HCl)

Read absorbance using red light

More details, suggestions for investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PagelId=14

Catechol oxidase

Introduction

Catechol oxidase is the enzyme responsible for the browning of fruit. It is easy to prepare from a number of different sources – bananas are particularly good – and the reaction is readily followed using a colorimeter.

Background

Catechol oxidase has a number of alternative names, (Polyphenol oxidase, Diphenol oxidase, Tyrosinase, etc.) The reaction catalysed is the oxidation of catechol to the yellow product 1,2-benzoquinone. On exposure to air there is a further reaction in which the yellow benzoquinone is converted to dark brown melanin.

SAFETY

The volumes and concentrations of catechol and its inhibitors, (if used), present minimal hazards. Good laboratory practices should be observed and the wearing of gloves is advised when preparing working solutions.

Suggestions for investigations

- Effect of pH on enzyme activity
- Effect of temperature on enzyme activity
- Effect of competitive and non-competitive inhibitors
- Enzyme kinetics

Enzyme extraction

Catechol oxidase can be extracted from fruits that brown on exposure of their cut surfaces to air, potato and apple are commonly used. We have found banana to be easiest to use, and the extract retains its activity for weeks in the fridge.

To prepare the enzyme blend banana with two volumes of water or just squash the banana with a fork and crush it in a pestle and mortar with two volumes of water. 20g of banana with 40cm³ of water will give plenty of extract for most purposes.

Filter the extract through several layers of butter muslin and store refrigerated.

Reaction mixture

For continuous reading put 0.1cm³ of the enzyme extract in a cuvette and add 2.9cm³ of a mixture containing 0.5cm³ of catechol solution and 2.4cm³ of buffer. The reaction can be performed at room temperature

The optimum pH is around 6.8 and catechol concentrations between 0.01M and 0.05M (final concentration in the cuvette) should give a good linear response when the concentration is plotted against the rate of reaction.

More details, suggestions for investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PagelId=17

Dopa oxidase

Introduction

The conversion of L-dopa to dopachrome is a reaction that is rarely encountered in schools. It is simple, reliable, interesting, safe, colourful and inexpensive and can be used to introduce the concept and properties of enzymes to secondary pupils or to carry out sophisticated studies of enzyme kinetics.

L-dopa (3,4-dihydroxy-L-phenylalanine) is a colourless metabolite synthesised from the amino acid tyrosine. It is enzymatically converted to dopaquinone by the oxidation of two hydroxyl groups followed by spontaneous conversion of dopaquinone to red dopachrome which slowly and spontaneously converts to melanin.

Background

Phenol oxidase enzymes that catalyse this reaction are ubiquitous in plants, animals and fungi. It is not entirely clear how many enzymes and polymorphs are involved. The enzyme system is essentially the same as catechol oxidase.

The advantages of this system are:

- students can easily extract the enzyme for themselves
- the reaction can be carried out quickly using simple methods and is wonderfully reliable and predictable
- there are no safety issues
- the cost is negligible
- for more advanced studies the reaction can be followed by continuous colorimetry so that rates of reaction can be determined and sophisticated investigations of enzyme kinetics carried out
- it has relevance to a number of important and interesting reactions and is a very good way of introducing the concept of interlinked metabolic pathways.

Suggestions for investigations

The system is ideal for qualitative demonstrations of enzyme action, effect of boiling, addition of acid or alkali to show the effect of pH, etc. Using the colourimeter detailed studies of optimum temperature, pH, enzyme kinetics, effects of inhibitors, are all relatively straightforward.

Enzyme extraction

The enzyme can be prepared from a variety of vegetable sources. Banana and potato work well, the yield from potato peelings being greater than from the flesh. The tissue simply needs to be crushed with cold water, (2cm³ of water per gram of tissue). For preference it should be filtered through muslin. For advanced work careful preparation with measured quantities, centrifugation, etc may be desirable.

Reaction mixture

One of the reasons why this is such a good system is the ease with which the reaction can be carried out. L-dopa can be dissolved in water; the maximum solubility at 25 °C is 0.5g in 100cm³, (25mM). [*L-dopa* M.Wt =197.2] 0.1cm³ of enzyme extract in 3cm³ of 5mM dopa will give a good reaction at room temperature.

In solution dopa will slowly decompose and darken, particularly at pH above 7. Prepare solutions fresh before use and store them cold.

More details, suggestions for investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PageId=59

Invertase

Introduction

Invertase is easy to obtain as a commercial preparation or from a culture of yeast and can be used for a variety of safe and simple yet interesting and rewarding investigations

Background

Invertase converts the disaccharide sucrose into the monosaccharides glucose and fructose by hydrolysing the bond between them.

Sucrose is a non-reducing sugar and gives negative results with Benedict's and DNSA reagents. These can therefore be used to monitor the enzyme by measuring the appearance of the reducing sugars glucose and fructose.

Invertase is found in the lining of the small intestine but the usual commercial source is yeast. Commercial preparations are available but the enzyme activity can easily be shown by culturing yeast.

Suggestions for investigations

- Effect of temperature on rate of reaction.
- Effect of pH on rate of reaction.
- Isolation and partial purification of enzyme.
- Experiments with immobilised enzymes

Preparation of invertase

Invertase can be obtained from dried yeast sold for home baking. We have obtained the best yield using the following recipe and growing for six hours at 35°C.

- Dried yeast 10g
- Sucrose 5g
- Yeast extract* 1g
- Peptone* 1g
- Water to 50cm³.

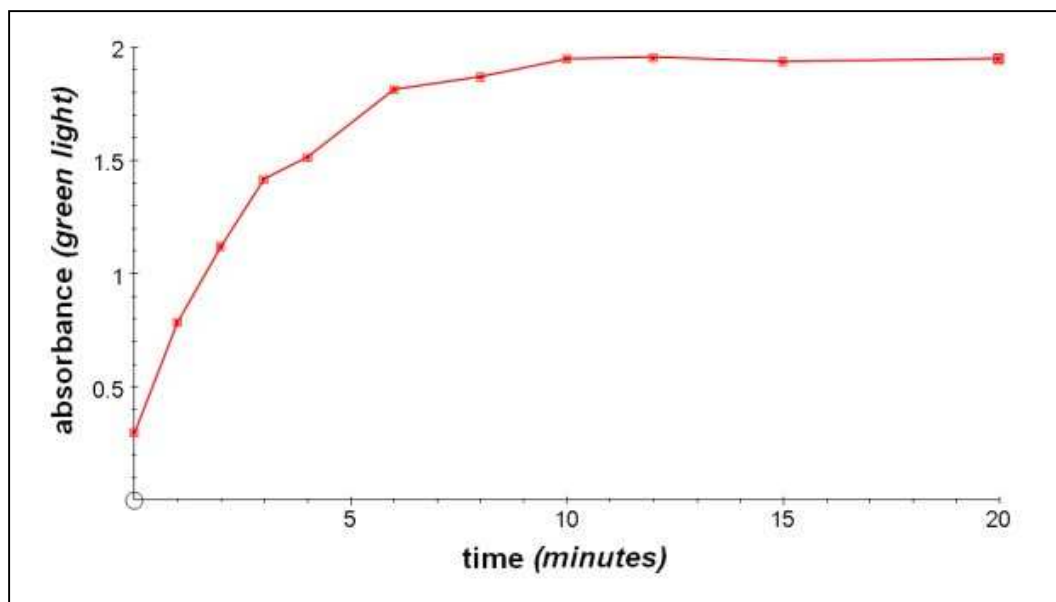
*Yeast extract and peptone can be replaced with 2g Marmite or Vegemite

Once growth has finished and the culture has separated out the clear liquid on top should contain a high concentration of invertase.

If a centrifuge is available a short spin will remove any remaining yeast cells.

Reaction mixture

Incubate 1cm^3 of invertase solution prepared from yeast with 9cm^3 of 0.4M sucrose solution in citrate buffer pH6 at 35°C . After about 10 minutes the sucrose has all been converted to the reducing sugars glucose and fructose. This method gave the results shown in the graph below using DNSA to detect reducing sugar.



More details, suggestions for investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PageId=18

Lactase

Introduction

Lactase activity can easily be demonstrated using the artificial substrate ONPG which is converted to a yellow product by lactase.

Background

Lactase, is responsible for the hydrolysis of lactose, forming glucose and galactose. It is secreted by the intestinal villi in humans, though the ability to produce the enzyme is lost by many people after childhood and leads to lactose intolerance, an inability to digest lactose.

Lactase, (usually fungal), is readily available in solution; a few drops added to milk help to make it digestible by people with lactose intolerance. This is not quite the same as the mammalian enzyme, being a β -galactosidase.

The method described here uses an artificial substrate, o-nitrophenol-galactopyranoside, (ONPG). Lactase splits colourless ONPG into galactose and o-nitrophenol, (ONP), which is bright yellow.

Suggestions for investigations

The reaction of β -galactosidase, (lactase), with ONPG is particularly good for investigating the effects of varying substrate and enzyme concentration on rate of reaction. Continuous colorimetry can be used to obtain graphs of reaction rates.

Galactose can be used to show end-product inhibition.

The colour of the product ONP is affected by pH so buffering is important and investigations of the effect of pH are not recommended.

Reaction mixture

We have used ONPG dissolved in 0.1M phosphate buffer, pH 7.0 and a 1% solution of a commercial liquid lactase from *Aspergillus*. The buffering is important as the colour of the product (ONP) depends on the pH.

2.9cm³ of the ONPG in buffer solution was added to 0.1cm³ of lactase in a colourimeter cuvette and the absorbance of blue light recorded continuously.

Room temperature (approximately 20 °C) was used throughout.

Stored refrigerated the undiluted liquid retained over 60% of its activity after 2 years. Diluted preparations do not keep well but it is easy and inexpensive to prepare fresh dilutions as required.

More details, suggestions for investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PageId=20

Pepsin

Introduction

The slow breakdown of albumen proteins gives good results with continuous colorimetry and is well suited for the study of the effect of pH on enzyme activity.

Background

Pepsin is a proteolytic (protein splitting) enzyme produced in the gastric glands lining the stomach of vertebrates. It is produced as pepsinogen, a larger molecule that is activated by hydrochloric acid to the smaller pepsin molecule. Pepsin can only work in a low pH environment, (optimum pH~1.8) Pepsin does not break the bonds between every amino acid so the products of the reaction are polypeptides of varying lengths.

Pepsin can conveniently be assayed using a cloudy suspension of egg white (albumen), which will slowly clear as the albumen protein is broken down into small, soluble polypeptides.

Suggestions for investigations

It is particularly suitable for demonstrating the effect of pH. Pepsin's low optimum pH is noteworthy and interesting comparisons can be made with other enzymes which usually have much higher pH optima.

The effect of temperature on enzyme activity can also be investigated, though this cannot be done with continuous colorimetry because of difficulties maintaining temperatures very different from ambient in the colorimeter. The results may be affected by changes in pH with changing temperature. Each new temperature should have its pH individually adjusted using a pH meter.

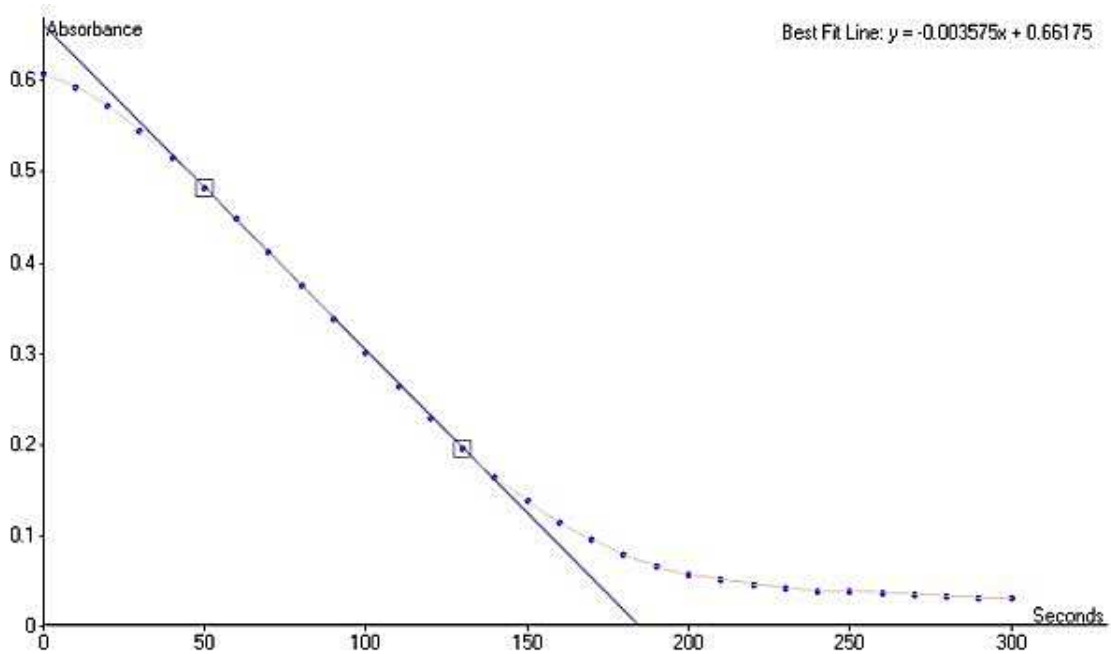
Preparation of an egg white suspension

- separate the white from the yolk – depending on the size of the egg this will probably be about 30cm³
- add 9 volumes of water – 270cm³ to a 30cm³ egg (this seems to work well with a fresh egg, but older eggs may need less water to achieve a good, dense suspension.)
- stir gently to disperse the egg white
- stand the mixture in a bath of hot water, with gentle stirring, for 5-10 minutes, (do not boil this – the suspension will form at around 70°C)
- pour the mixture through 2-4 layers of butter muslin cloth to remove large particles.

You should be left with an opaque suspension. This can be diluted 1:1 with water or buffer for use.

Reaction mixture

The results illustrated below were obtained with 2.8cm³ egg white suspension adjusted to pH2.1 at 25°C + 0.2cm³ of 5% pepsin solution. The absorbance was measured using blue light.



More details, suggestions for investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PageId=21

Phosphatase

Introduction

A phosphatase is an enzyme that releases a phosphate group from its substrate by hydrolysis. The assay method described here is for an acid phosphatase that can be obtained from germinated seeds.

Background

An acid phosphatase (i.e. a phosphatase with an optimum pH below 7) is readily obtained from germinated mung beans, (beansprouts), though other sources could be investigated.

To measure the activity of the enzyme a good substrate is phenolphthalein phosphate which is colourless until it is hydrolysed to phenolphthalein. Phenolphthalein is pink in alkaline solution and the absorbance can be measured using the colorimeter and green light. Adding the reaction mixture to an alkali also serves to stop the reaction.

This reaction is particularly suitable for studying the effects of end product inhibition since the addition of small amounts of phosphate has a marked effect on the rate of the reaction.

Suggestions for investigations

End product inhibition: The action of phosphatase releases phosphate which has a powerful inhibitory effect on the reaction. This is easily demonstrated by adding small amounts of phosphate buffer at the same pH to the citric acid-sodium citrate buffer.

For example:

0.1M Citric acid-Sodium citrate buffer pH6 (cm ³)	0.1M Phosphate buffer pH6 (cm ³)	1% phenolphthalein diphosphate (cm ³)	Enzyme solution (cm ³)	[Phosphate] mM
2.5	0	0.5	0.5	0
2.4	0.1	0.5	0.5	2.8
2.3	0.2	0.5	0.5	5.7

Enzyme extraction

To prepare an extract of acid phosphatase crush 20g of germinated mung beans, (beansprouts), in a pestle and mortar and add 10cm³ of water. (Alternatively add 10cm³ of citric acid-sodium citrate buffer pH6. Do not use a buffer containing phosphate.) Filter the mixture through several layers of muslin and store refrigerated.

Reaction mixture

The following method works well but there are many potential modifications of the volumes, timing and temperature. The end point should turn pink on addition to sodium carbonate.

- 2.5cm³ citric acid-sodium citrate buffer pH6 0.5cm³ 1%
- 0.5cm³ 1% phenolphthalein diphosphate tetrasodium salt in water
- 0.5cm³ enzyme preparation

Incubate for 10 minutes at 25°C then add 0.5cm³ of the reaction mixture to 2.5cm³ 10% sodium carbonate solution.

Read absorbance using green light.

More details, suggestions for investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PageId=22

Phosphorylase

Introduction

Potato phosphorylase can be used to demonstrate the synthesis of starch. It can also be used to investigate the equilibrium in a reversible reaction.

Background

Phosphorylase, is usually associated with the breakdown of starch or glycogen by the release of terminal glucose molecules as glucose-1-phosphate. However, if provided with glucose-1-phosphate and in the absence of inorganic phosphate, the reaction proceeds in the opposite direction and starch is synthesised by the addition of successive glucose molecules to pre-existing polysaccharide chains.

Suggestions for investigations

Starch phosphorylase extracted from potato can be used to demonstrate the synthesis reaction in the presence of glucose-1-phosphate. The reaction requires priming with small fragments of partially hydrolysed starch which can grow with the addition of further glucose molecules. It also requires the absence of inorganic phosphate since this will cause the reaction to proceed in the opposite direction.

This system can also be used as a good demonstration of reversibility and equilibrium in an enzyme reaction. The direction of the reaction depends on the ratio of glucose-1-phosphate : inorganic phosphate. By adding phosphate during the course of the reaction the direction can be reversed and synthesis of starch becomes degradation of starch.

In both of these investigations the reaction can be followed using a colorimeter to measure the starch-iodine complex.

Extraction of potato phosphorylase

Try to keep everything as cold as possible during this extraction.

Use a blender, or a pestle and mortar to liquidise small pieces of potato with 0.1M citric acid-sodium citrate buffer pH6 in the ratio 2g potato : 1cm³ buffer.

Filter the liquid through four layers of muslin and allow the filtrate to stand to let residual starch settle. Alternatively the liquid can be filtered through filter paper. The resulting liquid can be used as it is or centrifuged to remove suspended particles.

Primer

The synthesis of starch by potato phosphorylase can only proceed if the reaction mix is primed by the addition of small fragments of starch at least four glucose molecules in length. A satisfactory primer can easily be prepared by acid hydrolysis of soluble starch. We have found the following method gives good results.

- Place 2cm³ of a 10% solution of soluble starch in a test tube.
- In a second test tube put 1cm³ of 2M hydrochloric acid.
- Stand both test tubes in boiling water for a few minutes to heat up then mix the contents and let the mixture stand in boiling water for 4 minutes.
- Remove the test tube and add 1cm³ of 2M sodium hydroxide to stop the reactions and neutralise the pH.
- Add 0.1cm³ of this primer to 5cm³ of the reaction mixture

Reaction mixture

We have used the reaction mixture given here successfully.

- 4.4cm³ of 0.01M glucose-1-phosphate in citric acid-citrate buffer, (0.1M, pH6).
- 0.1cm³ primer
- 0.5cm³ enzyme
- 0.5cm³ samples were taken and added to 3cm³ of iodine solution containing 0.1M HCl,

The reaction can be carried out at room temperature, the results reported here were all obtained at 25 °C.

The direction of the reaction, (i.e. synthesis or degradation of starch), depends on the ratio of glucose-1-phosphate to inorganic phosphate. The equilibrium point occurs at a phosphate:G-1-P ratio of about 7:1.

Using a phosphate-free buffer the reaction can be run in the direction of starch synthesis, then by adding a phosphate buffer it can be reversed and the starch begins to be degraded.

Comparing this with the effect of the addition of a phosphate-free buffer acts as a control, showing that in this case the reaction will continue to run in the direction of the synthesis of starch.

The buffers used were 0.1M citric acid-sodium citrate buffer pH6 and 0.1M phosphate buffer pH6.

More details, suggestions for investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PagelId=23

Urease

Introduction

There are a number of different ways of measuring urease activity. The method described here uses bromothymol blue indicator which changes from yellow to blue as ammonia is released during the reaction.

Background

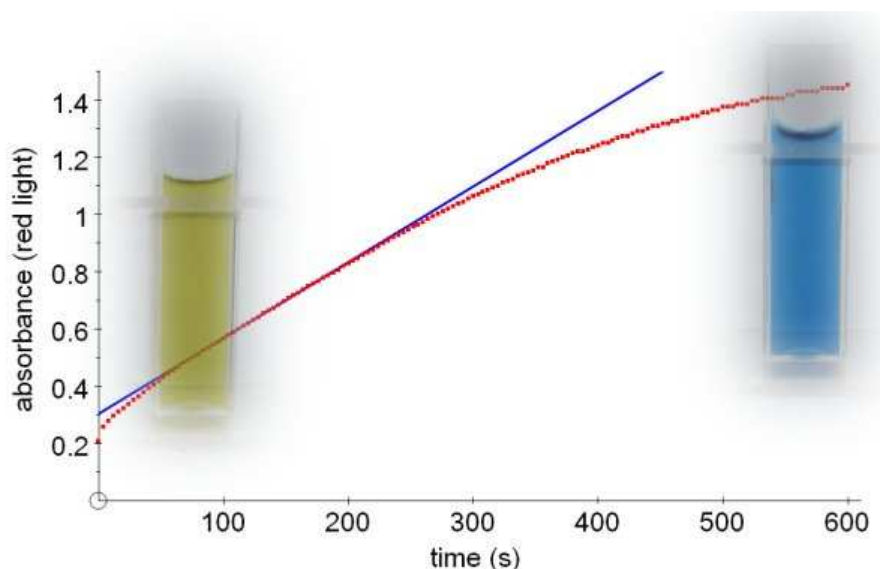
Urease from the jack bean (*Canavalia ensiformis*) was the first enzyme to be crystallised, for which discovery James Sumner was awarded a Nobel Prize for Chemistry in 1946. It breaks down urea into carbon dioxide and ammonia. A number of tests for urease utilise the fact that the ammonia released causes a rise in pH which can be detected with suitable indicators. The method described here gives good results with continuous colorimetry using bromothymol blue indicator which undergoes a change from yellow to blue as the pH rises from 6 to 7.6. The reaction can be followed by measuring the change in absorbance of red light with the colorimeter

Reaction mixture

We have obtained good results using a 0.01% w/v solution of bromothymol blue adjusted so that it is just yellow, pH about 6.0.

Jack bean urease is commercially available in powder or tablet form - the enzyme should be freshly prepared as it does not seem to keep well.

The results shown (*absorbance versus time*) were obtained at 25 °C using 3cm³ of 10mM urea containing 0.01% bromothymol blue and 0.2cm³ of an enzyme solution made by adding one urease tablet to 80cm³ of water.



More details, suggestions for investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PageId=64

Chlorine

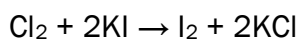
Introduction

The method described here can be used to determine the free chlorine content of unknown solutions such as swimming pool water and is sensitive enough to measure the chlorine content of most domestic tap water

Background

When water is treated by chlorination, either in swimming pools or in the treatment of drinking water, it is present mainly as hypochlorous acid (HOCl). Depending on the pH there are also varying amounts of chlorine (Cl₂) and hypochlorite ions (ClO⁻). These are the *free active chlorine* compounds in the water as opposed to the bound chlorine that has reacted with other compounds such as ammonia.

The free chlorine compounds react with potassium iodide to release iodine.



The intensity of the colour produced can be measured using the colorimeter (blue light) and compared with standards of known concentration to measure free chlorine.

The sensitivity of this method is increased by adding soluble starch solution which reacts with the iodine released giving an intense blue colour that can be measured using the green or red light on the colorimeter.

Preparing standard solutions of known chlorine concentration

Standard solutions can be prepared from household chlorine bleaches which typically contain 3-6% sodium hypochlorite (NaClO). We used 'Milton' sterilising fluid which is 2% sodium hypochlorite. To prepare a solution containing 8ppm (8mg/l) chlorine we diluted the Milton 1/100 then added 4.2cm³ of this to 80 cm³ distilled water. This was acidified by the addition of 0.5cm³ of ethanoic acid and made up to a final volume of 100cm³.

For other sources of chlorine divide 0.084 by the percentage concentration of sodium hypochlorite. This volume (cm³) in 100cm³ of distilled water acidified with 0.5cm³ ethanoic acid will give a chlorine concentration of 8ppm (8mg/l). (This is best done in two steps, initially a 1% dilution so that a reasonable volume can be measured in preparing the final dilution.)

Test method

Add 2cm³ of the sample to be tested to 0.2cm³ of a 2% solution of soluble starch in 0.1M potassium iodide solution.

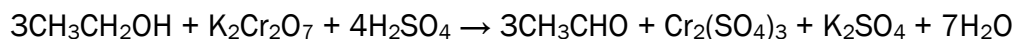
Read the absorbance with green light. (See Sample results *on the toolbar*)

More details, suggestions for investigations and sample results can be viewed on the Mystrica website, www.mystrica.com/Experiment.aspx?PageId=69

Ethanol

Background

Under acidic conditions ethanol is oxidised to acetaldehyde by potassium dichromate.



The acetaldehyde is itself oxidised to acetic acid.



As the dichromate oxidises the reactants it is reduced to trivalent chromium Cr^{3+} which is green.

The intensity of the green colour can be measured with the colorimeter and compared to known concentrations to measure the concentration of ethanol in the original sample.

It should be noted that other organic molecules can also be oxidised by dichromate with similar results, though the only example we have found where this is significant is the oxidation of glucose.

Method

The test sample is acidified with H_2SO_4 and heated. Different concentration of the acid and different incubation temperatures can be tried and will give different results.

The method we have used and described in the example illustrated used 0.5cm^3 of sample to which was added 1cm^3 of $1\text{M H}_2\text{SO}_4$.

Incubation was at 50°C for 30 minutes.

The colour change can be measured at this point or after the addition of 2cm^3 of 2M NaOH (corrosive) to remove the remaining dichromate and reveal the green chromium Cr^{3+}

SAFETY

$1\text{M H}_2\text{SO}_4$ should be labelled 'Irritant'

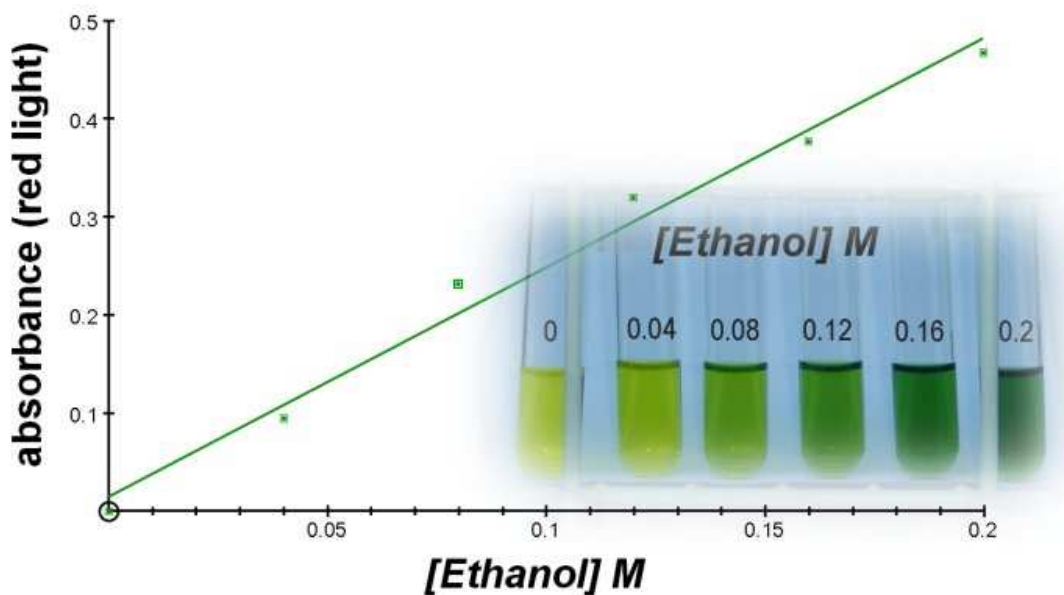


2M NaOH should not be used with pupils before Yr9 (S3, age 14) and should be labelled 'Corrosive'.
The method works without the addition of NaOH though the green colour is less apparent.



Standard curve

Using the method described above the results shown below were obtained. (0.1M = 0.46%). For higher concentrations of ethanol, e.g to measure the ethanol in of alcoholic drinks, less powerful reagents can be used to reduce the safety issues.



More details, suggestions for investigations and sample results can be viewed on the Mystrica website, www.mystrica.com/Experiment.aspx?PagelId=68

BIURET TEST

Qualitative or quantitative test for protein

Background

Biuret reagent reacts with peptide bonds, turning from blue to purple. Unlike the Bradford test it will give equally good results with any protein, but it is unable to detect the low concentrations that can be achieved with the Bradford test

A qualitative test can be performed simply by adding equal quantities of 1% sodium (or potassium) hydroxide and a 1% solution of copper sulphate to the sample. If the solution turns purple it means that protein is present.

The method described here uses quantitative Biuret solution which will give a good estimate of protein concentrations in the range 0.1-10 mg/cm³.

SAFETY



Biuret reagent contains 0.75M sodium hydroxide.
THE USE OF EYE PROTECTION MUST BE STRICTLY ENFORCED
In the event of eye contact flood the eye gently with running water for ten minutes and seek medical attention



Solution 1

Copper sulphate.5H ₂ O	0.75g
Sodium potassium tartrate	0.3g
Dissolve in 250cm ³ H ₂ O	

Solution 2

Sodium hydroxide	15g
Dissolve in 150cm ³ H ₂ O	



Add 2 to 1 mixing thoroughly and make up to 500cm³.

If a precipitate forms 0.5g potassium iodide can be added

Method

- To perform the quantitative test add 2 cm³ of Biuret reagent to 0.5 cm³ of sample.
- Allow the mixture to stand for 10 minutes then read absorbance in green light.

More details and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PagelD=27

BRADFORD TEST

Quantitative test for protein

Background

The Bradford assay is a very good, and simple, method of detecting microgram quantities of protein.

However the test is specific for certain amino acids, principally arginine, so not all proteins give the same reaction. For example albumin, casein and gelatin all give different responses. Gelatin has a very weak response to Bradford reagent since the protein, which is partially hydrolysed collagen, contains very few of the amino acids to which the reagent is sensitive. The standard usually employed is bovine serum albumin, (BSA). This is relatively expensive. We have used powdered egg white from the home-baking section of our local supermarket as the standard with results comparable to BSA.

The reagent contains Coomassie Blue dye which is light brown in the reagent but blue when bound to protein.

SAFETY



The stock solution contains phosphoric acid (50%).



Diluted for use the reagent is irritant.
The dye will stain skin and clothes.



Stock solution

- Dissolve 50mg Coomassie Blue in 20cm³ methanol
- Add this to 60cm³ phosphoric acid
- Make up to 100cm³ with water
- Label the stock solution 'CORROSIVE'



Method

Add 2.5cm³ of the reagent, (stock solution diluted 1+4 with water), to 0.25cm³ of the sample solution. Allow the mixture to stand for ten minutes then read the absorbance using red light. Ten minutes allows full development of the colour, longer intervals will not affect the result.

Microassay: The sensitivity can be increased by adding 0.5cm³ of undiluted stock solution to 2cm³ of the solution to be tested. The method will detect concentrations down to about 5µg per cm³.

More details and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PagelId=26

BENEDICT'S TEST

Qualitative or quantitative test for reducing sugars

Background

Benedict's solution reacts with reducing sugars on heating and reduces the Cu(II) ion to Cu(I) producing a precipitate of red copper oxide. The resulting colour change depends on the type and concentration of sugar, so this test can be used semi-quantitatively to indicate approximate concentrations.

An alternative version of Benedict's reagent for quantitative testing (QBS) contains potassium thiocyanate and does not form red copper oxide. Instead the presence of reducing sugar is measured by the loss of the blue colour of copper sulphate and a white precipitate is formed which will settle out or can be removed by filtration before measuring the colour of the filtrate.

Using a colorimeter you can obtain accurate, fully quantitative determinations of concentration down to 0.001M, (180 μ g of glucose/cm³). This is about 5 times lower than the concentrations detectable with test strips.

Lower concentrations can be detected rather more easily and in smaller volumes using DNSA reagent.

SAFETY

WEAR EYE PROTECTION
TAKE CARE WITH BOILING WATER

<u>Benedict's reagent</u>	<u>Quantitative Benedict's reagent</u>
<p>Solution 1 Sodium citrate 86.5g Sodium carbonate (anhydrous) 50g Dissolve in 400mls H₂O</p> <p>Solution 2 Copper sulphate.5H₂O 8.7g Dissolve in 50mls H₂O.</p> <p>Add 2 to 1 with rapid stirring then dilute to 500mls Positive result on boiling with reducing sugars</p> <p><i>The stock solution does not require a hazard warning label.</i></p>	<p>Solution 1 Sodium citrate 100g Sodium carbonate (anhydrous) 32.5g Potassium thiocyanate 62.5g Dissolve in 400mls H₂O</p> <p>Solution 2 Copper sulphate.5H₂O 9g Dissolve in 50mls H₂O. Add 2 to 1 with rapid stirring</p> <p>Add 0.13g potassium hexacyanoferrate (II) then dilute to 500mls</p> <p>For colourimetric use dilute 35mls of this solution to 100mls with water.</p> <p><i>The stock solution does not require a hazard warning label.</i></p>

Methods

Qualitative

- Add about 5cm³ of the reagent to a small amount of sample in a test tube.
- Stand the test tube in boiling water for a few minutes.
- A colour change through green to yellow, brown and finally to red indicates the presence of reducing sugar.

Quantitative

- Add 2cm³ of QBS to 4cm³ of sample in a test tube.
- Stand the test tube in boiling water for 5 minutes
- Allow the tubes to stand until the precipitate settles, or filter to remove the precipitate.
- Measure the absorbance using red light

More details and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PageId=19

DNSA REAGENT

Quantitative test for reducing sugars

Background

On boiling with reducing sugars 3,5 dinitrosalicylic acid (DNSA) reagent changes from yellow to red. Small volumes of the reagent and test sample are boiled for 5-10 minutes, then diluted with water and the colour read using a colorimeter. Concentrations of reducing sugar down to below 0.5mM, (90 μ g of glucose/cm³), can be detected using this test. There is no need to filter after boiling and small volumes of test solutions can be used, (typically 0.3cm³ for a standard cuvette).

SAFETY

WEAR EYE PROTECTION
TAKE CARE WITH BOILING WATER



DNSA reagent
contains 0.4M NaOH

To prepare 100cm³ of reagent

- dissolve 1g of 3,5-dinitrosalicylic acid in 50cm³ of water.
- slowly add 30g sodium potassium tartrate tetrahydrate, (KNaC₄H₄O₆·4H₂O)

- add 20cm³ of 2N NaOH.



- dilute to a final volume of 100cm³ with water.

- label the stock solution 'IRRITANT'



Method

- Add 0.3cm³ of the sample to be tested to 0.3cm³ of DNSA reagent in a test tube.
- Stand the test tube in boiling water for 5 -10 minutes.
- Add 3cm³ of water and read absorbance with green light (525nm).

More details and sample results can be viewed on the *Mystrica* website,
www.mystrica.com/Experiment.aspx?PagelD=16

Vitamin C

Introduction

The determination of the vitamin C content of different drinks, fruits and vegetables is an easy practical and gives good quantitative results. Using known concentrations of ascorbic acid to produce standard curves allows accurate determination of concentration and opportunities for students to practice techniques such as the preparation of dilution series, accuracy and precision. The practical generates results that students find interesting and relevant.

Background

Vitamin C, (ascorbic acid), was the first vitamin to be artificially synthesised in 1935, though its history goes back to ancient folk knowledge of the need for fresh plant material and raw animal flesh to prevent disease.

The most important role of vitamin C is in the formation of collagen, the ubiquitous structural protein component found in connective tissues, skin, bones, cartilage, ligaments and elsewhere. Lack of the vitamin eventually leads to the symptoms associated with scurvy resulting from collagen failure. Early symptoms include spongy bleeding gums, liver spots on the skin and lethargy followed in the later stages by loss of teeth, failure of wounds to heal, immobilisation and eventual death.

The RDA (Recommended Dietary Allowance) for vitamin C is 40mg/day (UK), 45mg/day (WHO), and 60-95mg/day in USA depending on age and sex. The Tolerable Upper Intake level in USA is 2000mg/day for a 25 year old male, though independent sources have recommended daily intakes higher than this.

Method

The methods for detecting vitamin C generally make use of its anti-oxidant property. A method often used in schools is the reduction of DCPIP from blue to colourless. The method recommended here is the decolourisation of the blue/black starch-iodine complex which we have found as accurate, cheaper and easier than the alternative.

- To prepare a stock solution of starch-iodine dissolve 0.2g of soluble starch in about 50cm³ of boiling water.
- Add 2cm³ of stock iodine solution* and make the final volume up to 100cm³ with water.
- For colorimetry this should be diluted 1:4 with water to give an absorbance between 1 and 1.5 using red light.
- Add 0.5cm³ of test solution to 3cm³ of the starch-iodine solution. For a standard curve a range of concentrations between 0 and 0.1g/l are suitable.

More details, suggestions for investigations and sample results can be viewed on the Mystrica website, www.mystrica.com/Experiment.aspx?PagelId=28

* Iodine stock solution - To prepare 100cm³ of stock solution put 1g of potassium iodide into a mortar and dissolve this in the smallest amount of water possible. Add 1g of iodine and grind to a smooth mixture.

Make up to 100cm³ with water and store in a glass bottle.

Iodine solution will not keep well after the stock solution has been diluted or in plastic containers.

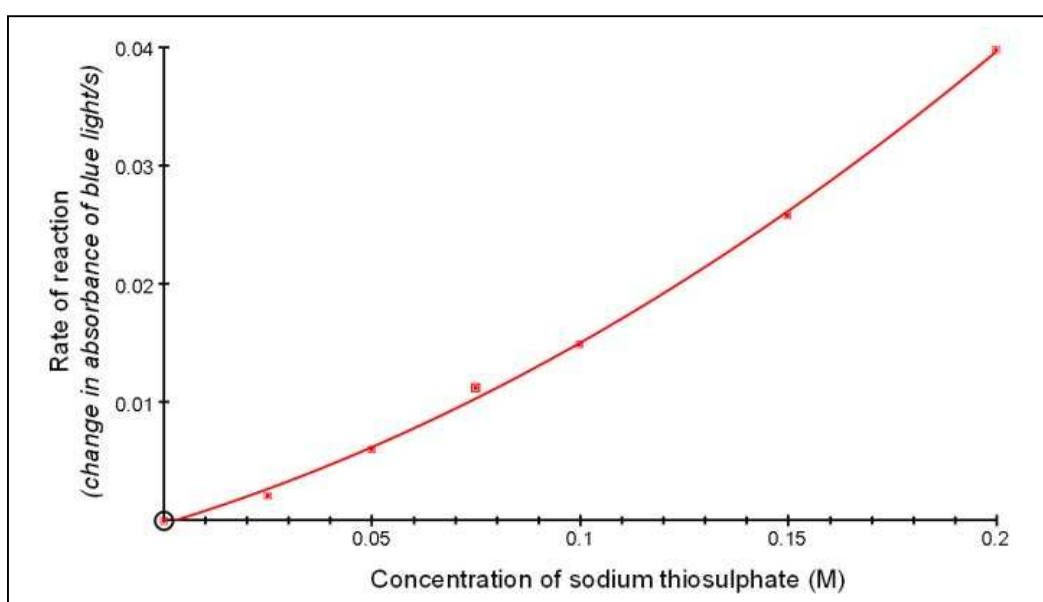
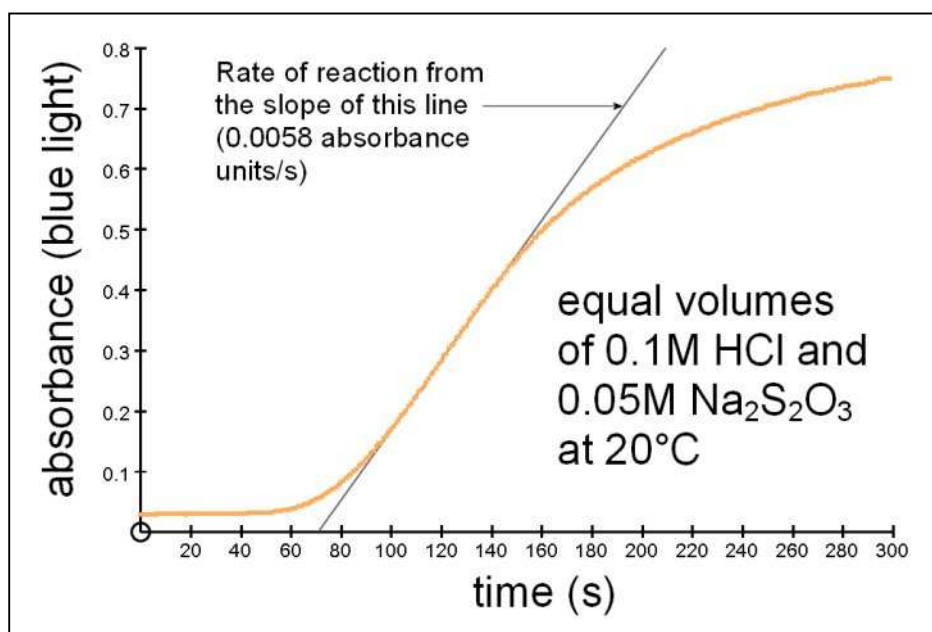
REACTION OF SODIUM THIOSULPHATE WITH ACID

Introduction

This is a well-known reaction found in most chemistry books and often carried out at GCSE level. Sodium thiosulphate reacts with acid to produce sulphur which is deposited in the solution causing it to become opaque.



The colorimeter can be used to measure the rate of this reaction since the increasing opacity scatters the light passing through.



More details, suggestions for investigations and sample results can be viewed on the *Mystrica* website, www.mystrica.com/Experiment.aspx?PageId=67

Biological membranes

Introduction

Use beetroot to show how membrane integrity is affected by treatments that damage either the protein or the phospholipid component of the membrane.

Background

The cell membrane, (plasma membrane), is composed primarily of a bilayer of phospholipid molecules with a mosaic of protein molecules embedded in and attached to it. Both these components are necessary for the membrane to fulfil its role of maintaining the integrity of the cell contents while allowing selected substances to cross the membrane. The effect of treatments that damage either the phospholipids or the protein components can be investigated using beetroot. The red pigment of beetroot is retained in the cells and only escapes into the surrounding medium if the membrane is damaged.

Method

Use small pieces of beetroot cut using a cork borer. Cylinders about 10mm long with a diameter of about 5mm are ideal. (If you do not have a cork borer just cut pieces about this size with a knife.) The pieces must be thoroughly washed in running water to remove all surface pigment released from damaged cells.

The amount of pigment leaking through the membrane into the surrounding liquid can be observed qualitatively by eye or measured accurately using the colorimeter.

More details, suggestions for investigations and sample results can be viewed on the Mystrica website, www.mystrica.com/Experiment.aspx?Pageld=44

Buffers

0.1M Citric acid-Sodium citrate buffer buffer – pH range 3.0 – 6.2

Prepare a 0.1M solution of citric acid monohydrate, $C_6H_8O_7 \cdot H_2O$ (21.01g/l) and a 0.1M solution of trisodium citrate dihydrate, $C_6H_5O_7Na_3 \cdot 2H_2O$ (29.41g/l). Mix the volumes shown in the table.

Or dissolve the masses shown and make up to 100cm³ with water

pH	0.1M citric acid (cm ³)		0.1M trisodium citrate (cm ³)	
	Volume of 0.1M solution (cm ³)	Mass in 100cm ³ (g)	Volume of 0.1M solution (cm ³)	Mass in 100cm ³ (g)
3.0	82	1.72	18	0.53
3.2	77.5	1.63	22.5	0.66
3.4	73	1.53	27	0.79
3.6	68.5	1.44	31.5	0.93
3.8	63.5	1.33	36.5	1.07
4.0	59	1.24	41	1.21
4.2	54	1.13	46	1.35
4.4	49.5	1.04	50.5	1.49
4.6	44.5	0.93	55.5	1.63
4.8	40	0.84	60	1.76
5.0	35	0.74	65	1.91
5.2	30.5	0.64	69.5	2.04
5.4	25.5	0.54	74.5	2.19
5.6	21	0.44	79	2.32
5.8	16	0.34	84	2.47
6.0	11.5	0.24	88.5	2.60
6.2	8	0.17	92	2.71

Citric acid-Sodium phosphate buffer – pH range 2.6 – 7.6

Prepare a 0.1M solution of citric acid monohydrate, $C_6H_8O_7 \cdot H_2O$ (21.01g/l) and a 0.2M solution of $Na_2HPO_4 \cdot 12H_2O$ (71.64g/l) Mix the volumes shown in the table. Or dissolve the masses shown and make up to $100cm^3$ with water

pH at 25 °C	Citric acid		$Na_2HPO_4 \cdot 12H_2O$	
	Volume of 0.1M solution (cm^3)	Mass in $100cm^3$ (g)	Volume of 0.2M solution (cm^3)	Mass in $100cm^3$ (g)
2.6	89.1	1.87	10.9	0.78
2.8	84.15	1.77	15.85	1.14
3.0	79.45	1.67	20.55	1.47
3.2	75.3	1.58	24.7	1.77
3.4	71.5	1.50	28.5	2.04
3.6	67.8	1.42	32.2	2.31
3.8	64.5	1.36	35.5	2.54
4.0	61.45	1.29	38.55	2.76
4.2	58.6	1.23	41.4	2.97
4.4	55.9	1.17	44.1	3.16
4.6	53.25	1.12	46.75	3.35
4.8	50.7	1.07	49.3	3.53
5.0	48.5	1.02	51.5	3.69
5.2	46.4	0.97	53.6	3.84
5.4	44.25	0.93	55.75	3.99
5.6	42.0	0.88	58.0	4.16
5.8	39.55	0.83	60.45	4.33
6.0	36.85	0.77	63.15	4.52
6.2	33.9	0.71	66.1	4.74
6.4	30.75	0.65	69.25	4.96
6.6	27.25	0.57	72.75	5.21
6.8	22.75	0.48	77.25	5.53
7.0	17.65	0.37	82.35	5.90
7.2	13.05	0.27	86.95	6.23
7.4	9.15	0.19	90.85	6.51
7.6	6.35	0.13	93.65	6.71

High pH buffer – pH range 2.6 –7.6

Prepare 0.1M solutions of sodium carbonate ($\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$) (28.62g/l) and sodium hydrogen carbonate (NaHCO_3) (8.4g/l)

Mix these in the volumes shown in the table.

Or dissolve the masses shown and make up to 100cm^3 with water

pH		Na_2CO_3		NaHCO_3	
at 20°C	at 37°C	Volume of 0.1M solution (cm^3)	Mass in 100cm^3 (g)	Volume of 0.2M solution (cm^3)	Mass in 100cm^3 (g)
9.2	8.8	10	0.29	90	0.76
9.4	9.1	20	0.57	80	0.67
9.5	9.4	30	0.86	70	0.59
9.8	9.5	40	1.14	60	0.50
9.9	9.7	50	1.43	50	0.42
10.1	9.9	60	1.72	40	0.34
10.3	10.1	70	2.00	30	0.25
10.5	10.3	80	2.29	20	0.17
10.8	10.6	90	2.58	10	0.08

Delroy & King, Biochem. J. 39, 245 (1945)

Low pH buffer – pH range 1.0 –2.2

Prepare a 0.2M solution of potassium chloride (KCl) (14.92g/l). To 25cm³ of this solution add the volume of 0.2M HCl shown in the table and make up to 100cm³ with water.

pH at 25°C	Volume of 0.2M HCl added (cm ³)
1.0	67.0
1.1	52.8
1.2	42.5
1.3	33.6
1.4	26.6
1.5	20.7
1.6	16.2
1.7	13.0
1.8	10.2
1.9	8.1
2.0	6.5
2.1	5.1
2.2	3.9

Bower & Bates, J. Res. natn. Bur. Stand. 55, 197 (1955)

0.1M Phosphate buffer – pH range 5.8 –8.0

Prepare 0.2M solutions of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (71.64g/l) and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (31.21g/l)

Mix the volumes shown in the table and make the total volume up to 100cm³ or dissolve the masses indicated in water and make up to 100cm³

pH at 25 °C	$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$		$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	
	Volume of 0.2M solution in 100cm ³ (cm ³)	Mass in 100cm ³ (g)	Volume of 0.2M solution in 100cm ³ (cm ³)	Mass in 100cm ³ (g)
5.8	4.0	0.29	46.0	1.44
6.0	6.15	0.44	43.85	1.37
6.2	9.25	0.66	40.75	1.27
6.4	13.25	0.95	36.75	1.15
6.6	18.75	1.34	31.25	0.98
6.8	24.5	1.76	25.5	0.80
7.0	30.5	2.19	19.5	0.61
7.2	36.0	2.58	14.0	0.44
7.4	40.5	2.90	9.5	0.30
7.6	43.5	3.12	6.5	0.20
7.8	45.75	3.28	4.25	0.13
8.0	47.35	3.39	2.65	0.08

Gomori, after Sørensen, Methods in Enzymology 1, 143 (1955)