HOW TEMPERATURE EFFECTS THE MOVEMENT OF PIGMENT THROUGH CELL MEMBRANES

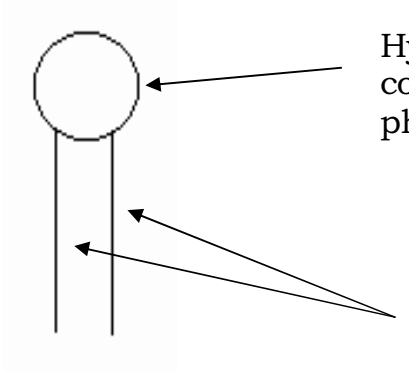
Abstract

The experiment below displays the effects of temperature on the pigment in uncooked beetroot cells. The pigment in beetroot cells lies within the cell vacuole and is called anthocyanin, each vacuole is surrounded by a tonoplast membrane and outside it, the cytoplasm is surrounded by the plasma membrane, therefore the foundation of this experiment lies with the temperature at which the membranes will rupture and therefore leak the pigment. To do this a series of uncooked beetroot cylinders will be exposed to different temperatures and then to distilled water at room temperature (24°C). The colour of the distilled water is the variable here which will show us, using a colorimeter what temperature the membranes splits using the transmission of the water (light passing directly through and the absorbency (light getting absorbed by the anthocyanin molecules).

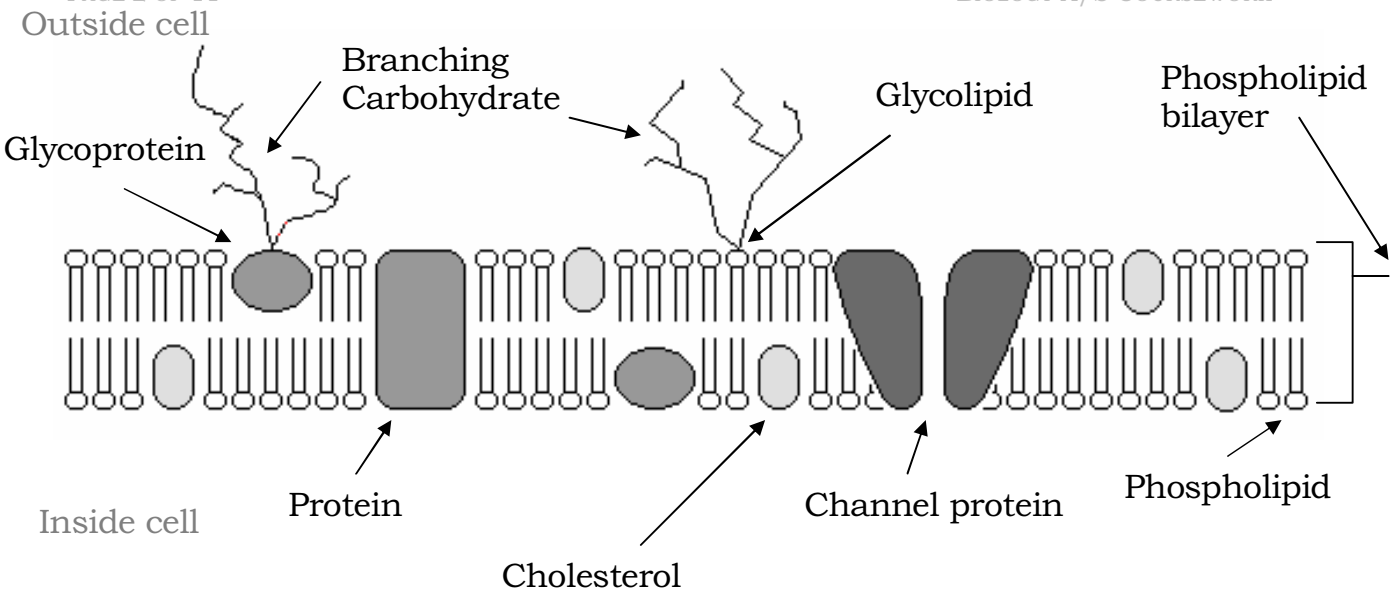
Introduction

Within the cells of a beetroot plant, a pigment is held within the vacuole of a beetroot cell, this pigment gives the beetroot its red/purple colour. If a cell is damaged or ruptured in a beetroot and the cell surface membrane ruptures, the pigment 'drains' from the cells like a dye. It is this distinction that can be employed to test which conditions may affect the integrity of the cell surface membrane. The pigments are actually betalain pigments, named after the red beetroot (beta vulgaris) it breaks down at about 60°C. They replace anthocyanins in plants. Unlike anthocyanins, betalains are not pH indicators, i.e. they do not change colour when the pH is lowered. Beetroot pigments are unstable at high temperatures, but the chemistry depends on a number of variables. Including the pH and composition of the solution, oxygen concentration and how long the solution is heated. However for the cell membranes an increase in temperature weakens the structure, just as the decrease in temperature decreases membrane fluidity until death, the increase in temperature does likewise until the membrane ruptures by the phospholipids breaking down to produce holes in the membrane, this is what will release the pigment.

A single phospholipid:



Phospholipids are a special type of lipid. Each molecule has the unusual property of having one end which is soluble in water and another that is not. This is due to one of the three fatty acid molecules being replaced by a phosphate group, which is polar and can therefore dissolve in water (hydrophilic) whereas the fatty acid tails can not (hydrophobic).



Above is a diagram of the "fluid mosaic model"[1], it shows the double layer of the phospholipid molecules. The individual phospholipid molecules move about by diffusion within their own monolayer. The phospholipid tails point inwards, facing each other and forming a non-polar hydrophobic interior. The phospholipid heads face the aqueous medium that surrounds the membrane. Some of the phospholipid tails are saturated and some are unsaturated, unsaturated is a when a fatty acid tail has one or more carbon double bonds involved in the structure reducing the melting point and so increasing the fluidity, this is called the thermal-fluidity of the membrane. The more unsaturated they are, the more fluid the membrane. This is because the unsaturated fatty acid tails are bent and therefore fit together more loosely. As temperature decreases membranes become less fluid and vice versa for the opposite effect.

Phospholipids form the bilayer, which is the basic structure of the membranes. Because their tails are non-polar, it is difficult for polar molecules, or ions, to pass through them, so they act as a barrier to most water-soluble substances. The cholesterol molecules, like the phospholipids, have a hydrophilic head and hydrophobic tails, so they fit neatly between the phospholipid molecules, they help to regulate the fluidity of the membrane, preventing it from becoming too fluid or too rigid. Cholesterol is also important for the mechanical stability of membranes, as without it membranes would quickly break and the cell would burst open. This is expected to happen as the temperature increases due to more heat making the membranes more fluid and causing the cell membrane to eventually burst. Glycolipids and glycoproteins also contribute towards the strength of the membrane structure, they do this by forming hydrogen bonds with the water molecules on the carbohydrate chains that project out into the watery fluids surrounding the cell. This helps to stabilise the membrane structure, like the cholesterol and phospholipids, glycolipids and glycoproteins break down quicker at higher temperatures. The proteins within the bilayer also

[1] Biology 1 – Cell membranes and transport

have a structural role, as they contribute their own rigid structure to the composition of the membrane. However at high temperatures a process pertaining to a change in the structure of a protein from regular to irregular arrangement of the polypeptide chains, causes a physical disfigurement called denaturing. Once a protein is denatured it can no longer be used by the body as it lacks its necessary structure and as the protein function is dependent upon this, it becomes useless. This malformation in the protein also increases the fluidity of the membrane as the protein is rejected by the cell when denatured and so creates gaps in the membrane structure allowing anthocyanin to diffuse out.

"The fluidity of a lipid bilayer is affected by temperature, fatty acid composition and cholesterol content. At low temperature the hydrocarbon tails of bilayer lipids can pack closely together to form an ordered arrangement or gel like state which is fairly rigid. As temperature is increased, the lipid molecules vibrate more rapidly, causing the bilayer to melt into a more disordered arrangement or liquid state which is more fluid. The temperature at which the lipid bilayer melts is called the transition temperature."[2]

Anthocyanin will move out of the beetroot cells by diffusion; "this is the net movement of molecules from a region of high concentration to a region of low concentration. The movement follows a concentration gradient. It happens because of the natural kinetic energy possessed by molecules or ions, which makes them move about at random. As a result of diffusion, molecules tend to reach an equilibrium situation where they are evenly spread within a given volume of space."[3] By heating the molecules they gain more kinetic energy than they would at low temperatures this means they move around faster and thus diffusion takes place quicker. Therefore an increase in the temperature will cause a larger loss of anthocyanin, as the molecules will be moving around faster due to the increase in available energy.

<u>Pilot Study</u>

Before this experiment a pre-test was carried out to find out the approximate temperature at which the membranes fracture and the beetroots leaked pigment, it was carried out using the same procedure described later but at a wider range of temperatures, the results are shown below:

Temperature (ºC)	20	30	40	50	60	70	80	90
Transmission (%)	99.00	99.00	98.00	98.00	93.00	50.00	27.00	9.00
Absorbency (%)	0.03	0.03	0.05	0.05	0.10	0.30	0.55	1.20

This shows that the transition temperature here is in the range of 60ºC-70ºC, therefore the actual experiment will be focused more on that temperature range to find a more accurate temperature.

[2] Biological membranes – Membrane fluidity

[3] Biology 1 – Cell membranes and transport

Aim

To study the effects that temperature has on the movement of pigment through beetroot membranes, to do this, the temperature at which the membranes in the beetroot split and leak the red pigment, anthocyanin, will be the time investigated.

Hypothesis

Based on the data gathered from the pre-test a stable hypothesis can be justified, this is that the increase in temperature will damage the beetroot membranes and cause them to rupture, leaking anthocyanin.

Null Hypothesis

The null hypothesis can therefore be said to be that the increase in temperature will not affect the beetroots cell membranes. The above hypotheses will be used with the statistical data later in the report.

Prediction

My prediction is based on my scientific knowledge and understanding of cell membranes and what effect temperature has on them. I anticipate that the increase in temperature will have little or no effect on the membranes up until approximately 60°C. Beyond this temperature the membrane will start to experience a change in fluidity and stability as the lipids that make up the membrane begin to lose their rigid structure and weaken, eventually leading to perforations along the membrane thus leaking the red pigment anthocyanin by diffusion, as the high concentration of the pigment inside the cell seeks an equilibrium with the low concentrations outside. As the temperature increases from this the membrane will continue to breakdown rapidly.

Variables

The majority of my analysis will be on temperature as this is my dependant variable, consequently the experiment will be repeated using a range of temperatures to find which is the temperature at which the membranes begin to break down. The variables for the amount of anthocyanin released on break down of the beetroot cell walls, will be the transmission and absorbency of the water samples taken at each break in the temperature range, these will be the independent variables.

Other variables that will remain constant throughout the experiment by means of control must also be accounted for, these will be the control variables, most are easily regulated however some are not so easily contained. One of these variables is the beetroot dimensions, it is kept constant as the cross-sectional area will remain the same due to the same cork borer being used and the length will be cut to exactly four centimetres. The beetroots are also from the same batch of beetroots, as a difference in packs or producers or possibly the country of where the beetroots are grown could have a small effect on their anthocyanin concentration or the strength of the cell membranes. Other variables are the volumes of water that will be kept constant throughout the experiment, these

include the 10cm³ of distilled water put into each of the eight test tubes and the 200cm³ of distilled water the beetroot cylinders are placed into to be heated each time, also the time in which the beetroots are washed to begin with will be the same. The heated distilled water that the beetroot cylinders are put into will only be in the water for one minute otherwise it will effect how much the membranes are damaged, and then they are only given half an hour in the 10cm³ distilled water to make sure that they all are allowed the same amount of time to let the pigment diffuse out. The water is also another variable which we have controlled as regular tap water has certain impurities and a marginally lower pH than pure water which, if the water was slightly more acidic, could have a small effect on the results, therefore for all the areas of the experiment, distilled water is used as it has a neutral pH of 7. Distilled water also accounts for the water potential (ψ) as tap water will have impurities and will have a lower water potential than distilled, if this were the case during the experiment the rate of diffusion may have been effected.

The above are all control variables however there is one variable that, although slightly controlled we do not have full power over, this is the constancy of the temperature. Although it is the dependant variable it is also the one which we have limited control over, this is because as we heat the distilled water to the desired temperature the water automatically begins to cool therefore a human judgement has to be reached upon when to put the beetroot cylinder into the heated water. If the beetroot is put into the water a few degrees above the desired temperature then as it cools the membranes will begin to fracture at the right temperature or as close to there as possible. Water baths would have been preferred for the experiment however we did not have access to those appliances. The room temperature must also be considered as an independent variable as at the beginning of the experiment the room temperature was recorded as 20.1°C, and at the end of the experiment, due to the increase in heat from the Bunsen's in the laboratory and also the increase in body temperature of the people in the room due to an increase in work, increased this figure to 22.3°C. This means that the water in the test tubes was of a higher temperature, possibly having an effect on anthocyanin leakage and membrane rupture.

Method

A cork borer will be used to cut eight beetroot cylinders each, four centimetres in length. These will then be washed overnight in running tap water and transferred to a beaker of distilled water. Using a Bunsen burner on a mat, a tripod and gauze, 200cm³ of distilled water will be heated to 80°C in a 250cm³ beaker. Whilst the water is heating, using a syringe 10cm³ of distilled water (at room temperature) will be added to each of the ten test tubes in the test tube rack. The test tubes will be labelled according to the desired temperature. Then each beetroot cylinder will be placed; one after another, in the beaker of distilled water at the desired temperature, starting with 80°C, this time will be noted. After four minutes the cylinder is transferred to the test tube of cold distilled water marked with the correct label. The time will again be noted.

As the distilled water is cooled the experiment will be carried out a further seven times for temperatures at 5°C intervals from 80°C, down. The cylinder is then left in the test tube of cold distilled water for exactly half an hour. After thirty minutes have elapsed the test tube will be shaken and the beetroot cylinder removed. The distilled water with the anthocyanin pigment will then be transferred to cuvettes for each temperature. Using a colorimeter the absorbency and transmission of the distilled water with the pigment will be recorded. This is done by taking a sample of the water with the leaked pigment into a cuvette, the cuvettes have four sides two of which are frosted to allow for grip and accidental smudging. They will then be placed within the colorimeter once it has been zeroed with distilled water, and put to the desired setting, a light is then shone through a filter and then through the sample, the light that leaves the cuvette is read, the colour of filter used for the experiment will be yellow. The reading given is either; transmission which is how much light goes through the sample, or absorbency, which is how much is reflected or absorbed. This data will then be recorded and two sets of repeat results will be made to gain an average and provide evidence to exclude anonymous results, from here an evaluation can be drawn.

<u>Apparatus</u>

Beaker (250cm^3) – To contain the distilled water at different temperatures
Bunsen burner, mat, tripod and gauze – To allow for the heating of the distilled water
Ceramic tile – To act as a suitable cutting surface for the beetroots
Colorimeter which takes cuvettes – To measure the transmission and absorbency of the samples of anthocyanin/water
Cork borer – To cut beetroot cylinders to a precise size
Cuvettes x 8 – To hold the samples of anthocyanin/water
Forceps – To remove the cylinders of beetroot from the distilled water into the test tubes
Graduated syringe (10cm^3) – To measure exactly 10cm^3 of distilled water
Measuring cylinder (200cm^3) – To measure exactly 200cm^3 of distilled water
Scalpel – To cut the beetroot into its desired size
Stopwatch – To record the time the cylinders are left in the beaker and test tubes
Test tube rack – To hold the test tubes
Test tubes x 8 – To contain the cylinders and distilled water for heating
Thermometer (-20°C to 110°C) – To measure the changes in temperature
Uncooked beetroots – To be the subject matter used for explaining how temperature effects the movement of pigment through cell membranes. It is uncooked to retain the anthocyanin pigment

Safety

For the purpose of this experiment the only real hazard is the Bunsen flame, therefore the only safety clothing that should be worn is safety goggles and an apron to protect from the Bunsen flame, there are also fire extinguishers located in the lab. The apron also protects from the red pigment, anthocyanin, which stains material. The only other potential hazards in this test are the cork borer and the scalpel as they can damage the skin if not used correctly, however the position of the first aid kit was known in case of an emergency.

Results

1st Set

Temperature (°C)	45	50	55	60	65	70	75	80
Transmission (%)	98.00	98.00	97.00	95.00	52.00	25.00	10.00	10.00
Absorbency (%)	0.03	0.03	0.05	0.10	0.31	0.42	0.93	0.93

2nd Set

Temperature (°C)	45	50	55	60	65	70	75	80
Transmission (%)	98.00	97.00	96.00	94.00	54.00	28.00	18.00	11.00
Absorbency (%)	0.03	0.05	0.08	0.13	0.29	0.40	0.82	0.90

3rd Set

Temperature (°C)	45	50	55	60	65	70	75	80
Transmission (%)	97.00	97.00	96.00	93.00	53.00	26.00	20.00	11.00
Absorbency (%)	0.05	0.05	0.08	0.15	0.30	0.38	0.86	0.90

Average

Temperature (°C)	45	50	55	60	65	70	75	80
Transmission (%)	97.67	97.33	96.33	94.00	53.00	26.33	19.00	10.67
Absorbency (%)	0.04	0.04	0.07	0.13	0.29	0.40	0.84	0.91

The transmission and absorbency for the average of 75°C is the average found from the second set of results', as the first results' data for 75°C seemed like an anomalous result as it was significantly out of the expected range for this temperature, so the repeated data was used to rectify the problem, reasons for this incorrect figure is figure explained in the evaluation.

A mathematical calculation can be carried out on this experiment to see if there is a correlation between the temperature and one of the readings in the results; transmission or absorbency. If there is then it is possible to accept or reject our original hypothesis. For this calculation I will be using transmission, as it is an easier figure to use being larger, the average data will be applied to the calculation to get a more accurate result. The hypotheses remain the same from the main experiment found earlier in the report.

Spearman's Rank Correlation Co-efficient

Column Number (n)	1 Temperature (°C)	2 Rank 1 (r1)	3 Transmission	4 Rank 2 (r2)	5 Diff. (r1 – r2)	6 Diff.² (d²)
1	80	1	10.67	8	-7	49
2	75	2	19.00	7	-5	25
3	70	3	26.33	6	-3	9
4	65	4	53.00	5	-1	1
5	60	5	94.00	4	1	1
6	55	6	96.33	3	3	9
7	50	7	97.33	2	5	25
8	45	8	97.67	1	7	49
					Σd^2 =	168

$$R = 1 - \frac{6 \times \Sigma d^2}{n(n^2-1)}$$

$$R = 1 - \frac{6 \times 168}{8(63)}$$

$$R = 1 - \frac{1008}{504}$$

$R = 1 - 2$

$R = \underline{-1}$

Where: Σ = The sum of
n = number
d = difference
R = Spearman's rank

When this figure is matched against the Spearman's data table it gives this figure the description of having a strong negative correlation, this is between the temperature, at which the beetroots are exposed to, and the transmission of a sample of water with the corresponding temperature containing the beetroot. The percentage significance level used was actually accurate enough to get to a 1% significance level as this was a figure of very high significance.

Conclusion

The above result supports my original hypothesis and so gives a 99% possibility to negate my null hypothesis. This shows that there is a very strong negative relationship between temperature and transmission, which the graph of the average results also shows and further supports the original hypothesis, that the increase in temperature will damage the beetroot membranes and cause them to rupture, leaking anthocyanin.

Analysis

Further examination of the results and experiment allows for the results to be broken down into more specified areas. A cell membranes main function is one of structure and containment, and in this case the strength of this structure in relation to temperature changes and the containment of the pigment

anthocyanin. The structure of the membranes is made up of a phospholipid bilayer, each phospholipid consists of a hydrophobic tail and a hydrophilic head. The head is a phosphate group while the tails are strands of fatty acids, these fatty acid tails may have a double bond within their hydrocarbon chain making them unsaturated, if there is more than one double bond, the fatty acid is described as polyunsaturated; if there is only one it is monounsaturated. The more double bonds present the more like a liquid the lipid becomes, as it is has a higher fluidity. This means that it is easier to break them and so it is easier to melt these tails than a saturated tail. Therefore when the results showed a slight increase in absorbency (55°C) which was not the actual transition temperature, it could have been these phospholipids breaking and releasing the anthocyanin. The major increase was due to the other lipids breaking down at higher temperatures and further releasing the rest of the pigment.

"Short chains will interact less with one another than will long chains, hence a lower temperature is needed to melt the bilayer containing them. Double bonds put bends in the hydrocarbon tails, making it more difficult for the phospholipids to pack together, and bilayer fluidity is thus increased. Bilayer fluidity is influenced by cholesterol. Cholesterol fits in between the bilayer phospholipids with its hydroxyl group close to the phospholipid heads, and its hydrophobic ring and side chain buried within the fatty acid chains of the membrane interior. The rigid steroid ring interacts with the neighbouring regions of the lipids tails and stiffens them, making the membranes less fluid."[5]
The lipids all have an optimum temperature at which they remain stable (transition temperature), this was just above 60°C and once this temperature had been breached the lipids simply melted. The increase in temperature caused an increase in kinetic energy as expected, it is the this change in kinetic energy that caused the fluidity to, in turn, increase. Causing weaknesses to form in the membrane, and as the kinetic energy was further multiplied this weakened it additionally leading to perforations along the membrane structure causing a tilt in the diffusion gradient, enabling the increased leakage of anthocyanin.

The presence of the glycolipids, glycoproteins and membrane proteins would have also had an effect on the results; as they all cease to function at higher temperatures and as they all give structural advantages to the membrane their loss would weaken the structure significantly. The glycolipids and glycoproteins would break down and so the hydrogen bonds formed with the external solution would be gone, with this the proteins would denature at a certain optimum temperature and become useless. Thus, allowing greater possibilities for the diffusion of anthocyanin out of the beetroot cell.

<u>Discussion:</u>
The results above show that at 60°C the membranes begin to break down, as there is a considerable increase in absorbency in comparison to the amount at 55°C. At 65°C the change is extremely noticeable, the first significant change is at 55°C however as explained above, this could be due to the weaker lipids breaking down first, therefore we can primarily assume that between 60°C and

[5] Biological membranes – Membrane fluidity

65ºC is the temperature at which the membranes within beetroot cells begin to seriously rupture and leak the red pigment anthocyanin.

Thus my prediction was correct in the sense that the change in absorbency and transmission before the beetroot membranes split was fairly small, and that the transition temperature was about 60ºC. Looking back at the graph it clearly shows at what temperature the membranes rupture, as at the beginning of the graph, it shows an almost straight vertical line and then the gradient of the graph decreases rapidly with transmission at 59ºC, this is a more accurate reading. This relates to when the lipids inside the cell surface membrane begin to breakdown producing openings in the membrane through which the red pigment, anthocyanin leaks.

Evaluation

The experiment used had a suitable and reliable procedure and as a result there were very few anomalous results, however there was one serious anomaly, the first set of results give the transmission and absorbency for a beetroot cylinder at 75ºC that was equal to that of the data at 80ºC. Although not a major problem it did cause some confusion, it was later rectified by the introduction of the second set of results which offered a much more realistic piece of data for this temperature. There were also certain limitations which made this experiment a little harder than it could have been, for example keeping the temperature at a steady rate was incredibly difficult as the water kept on cooling, an answer to this problem would be the use of water baths at the temperatures required this would have meant that the temperature error measurement would have been minimal and focusing on an exact temperature would have been made possible. This would have given better results and a completely reliable and accurate answer to the problem at hand.

The problem with the Bunsen burners as the temperature regulators, was that if we were to put the beetroot, already in the distilled water, over the flame for a period of time for the minute required, the temperature would have changed significantly and so disrupted the results accordingly. Another problem lay with the beetroots themselves, this was that the beetroots used were very small and so getting larger cylinders of the beetroot was very difficult, therefore we had to endeavour with smaller pieces, if larger beetroots were used and larger cylinders created, the experiment would have been made fairer as we could have had a larger view over the actions of the beetroot's membranes during heating.

The validity of the data can be questioned as the temperature fluctuations during the test were certainly the biggest limiting factor and so had a substantial effect upon the evaluation. Objections made are to the limitations of available apparatus that would have helped to create a better environment for the beetroots and thus giving a much more accurate result. Moreover the experiment was sufficient as it gave the answer to the problem, this was that the transition temperature for this relationship was 59ºC where the cell surface membranes of the beetroot cells seriously began to rupture and so leaked, from

its vacuole, the red pigment anthocyanin, there was a good relationship between the pre-test, the actual experiments and the scientific research done on the topic and so we can safely presume that it is correct.

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