Determination of the Michaelis constant with Cobra4

(Item No.: P4120360)

Curricular Relevance

Area of Expertise: Biology
Education Level: University
Topic: Biochemistry
Subtopic: Biochemistry
Experiment: Determination of the Michaelis constant with Cobra4

Difficulty	Preparation Time	Execution Time	Recommended Group Size
Intermediate	10 Minutes	40 Minutes	2 Students

Additional Requirements:

- Android tablet or iPad
- PHYWE measure App

Experiment Variations:

- with Computer with USB port, Windows

Keywords:

Michaelis constant, Enzymatic hydrolysis of urea, Conductivity measurement, Bodenstein principle, Enzyme-substrate complex, Lineweaver-Burk plot

Information for teachers

Principle

The enzymatic hydrolysis of urea in an aquous solution yields carbon dioxide and ammonia. The ions of these compounds increase the conductivity of the solution. The speed of urea hydrolysis, which is caused by the urease enzyme, can be determined via conductivity measurements at different substrate concentration levels. The resulting values can then be used for the calculation of the Michaelis constant.

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Fig.1: Experiment setup

Equipment

Experiment with Cobra4 Wireless/USB-Link with android tablet or iPad

Position No.	Material	Order No.	Quantity
1	Cobra4 Sensor-Unit Conductivity+	12632-00	1
2	Conductivity temperature probe Pt1000	13701-01	1
3	Magnetic stirrer without heating, 3 ltr., 230 V	35761-99	1
4	Magnetic stirring bar 30 mm, cylindrical	46299-02	1
5	Separator for magnetic bars	35680-03	1
6	Compact Balance, OHAUS TA 302, 300 g / 0.01 g	49241-93	1
7	Retort stand, h = 750 mm	37694-00	1
8	Boss head	02043-00	1
9	Universal clamp	37715-00	1
10	Beaker, high, BORO 3.3, 100 ml	46026-00	6
11	Beaker, 250 ml, low form, plastic	36013-01	1
12	Erlenmeyer flask 100 ml, narrow neck, PN 19	36418-00	6
13	Rubber stopper, d=22/17 mm, without hole	39255-00	6
14	Volumetric pipette, 20 ml	36579-00	1
15	Volumetric pipette, 50 ml	36581-00	1
16	Pipettor	36592-00	1
17	Micro-l syringe, 100 micro-l	02606-00	1
18	Microspoon, steel	33393-00	1
19	Wash bottle, plastic, 500 ml	33931-00	1
20	Urea, 250 g	30086-25	1
21	Urease soln.in 50% glycerol,10ml	31924-03	1
22	Water, distilled 5 l	31246-81	1
23	Cobra4 Wireless/USB-Link incl. USB cable	12601-10	1
24	USB charger for Cobra4 Mobile-Link 2 and Wireless/USB-Link	07932-99	1
Additional material			
	Android tablet or iPad		
	PHYWE measure App		
	Paper towels		

Android iPad

Experiment with Cobra4 Wireless/USB-Link and PC

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Position No.	Material	Order No.	Quantity
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	Paper towels		

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Task

Calculate the Michaelis constant after determining the required parameters experimentally.

Fig.1: Experiment setup

Determination of the Michaelis constant with Cobra4 (Item No.: P4120360)

Task and equipment

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Additional material			
	Android tablet or iPad		
	PHYWE measure App		
	Paper towels		

Android

iPad

Setup and procedure

Setup

Preparation

The experiment requires several solutions with different urea concentration levels. These solutions must be prepared fresh prior to the execution of the experiment:

- 0.4% urea solution (urea stock solution): Weigh 0.40 g of urea into a 100 ml Erlenmeyer flask and dissolve it in 99.6 g of distilled water.
- 0.2% urea solution: Transfer 50 ml of the 0.4% urea solution with the 50 ml volumetric pipette into a 100 ml Erlenmeyer flask and add 50 ml of distilled water.
- 0.1% urea solution: Transfer 50 ml of the 0.2% urea solution with the 50 ml volumetric pipette into a 100 ml Erlenmeyer flask and add 50 ml of distilled water.
- 0.05% urea solution: Transfer 50 ml of the 0.1% urea solution with the 50 ml volumetric pipette into a 100 ml Erlenmeyer flask and add 50 ml of distilled water.
- 0.025% urea solution: Transfer 50 ml of the 0.05% urea solution with the 50 ml volumetric pipette into a 100 ml Erlenmeyer flask and add 50 ml of distilled water.
- 0.0125% urea solution: Transfer 50 ml of the 0.025% urea solution with the 50 ml volumetric pipette into a 100 ml Erlenmeyer flask and add 50 ml of distilled water.

Note: The urease solution should be stored in a refrigerator!

- Set up the equipment as shown in Fig. 1.
- Fasten the universal clamp with the bosshead to the support rod of the retort stand.
- Secure the conductivity sensor in place with the universal clamp.
- Connect the Cobra4 Sensor-Unit "Conductivity+" with the Wireless/USB-link and switch it on .

  Connect your tablet via WiFi with the Wireless/USB-link (maximum range 50m).
  Open the PHYWE measure App and select the **Sensor** "Conductivity+".

Fig. 2: Select the sensor "Conductivity+"

- The electrical conductance will be measured.

Fig. 3: Measurement of electrical conductance

Procedure

- Transfer 40 ml of the 0.0125% urea solution (the lowest concentration first) in two steps with the 20 ml volumetric pipette into a 100 ml beaker and add a magnetic stirring bar.
- Place the beaker on the magnetic stirrer and lower the conductivity sensor into the solution.
- Set the stirrer to a medium stirring speed (*Caution*: The magnetic stirring bar must never hit the conductivity sensor!).
- Add 50 µl of the urease solution with the microlitre syringe and start the measurement without any further delay by clicking the start button ( ).
- The time course of the reaction can be observed on screen.
- After the measurement (stop button:), save the data for further processing.
- Repeat the measurement with all of the six prepared urea solutions (in order of concentration increase).
- Prior to performing the individual measurements, take the beaker off the magnetic stirrer and remove the magnetic stirring bar from the solution with the aid of the separator.
- Rinse the magnetic stirring bar thoroughly with distilled water, dry it with a paper towel, and put it into the next solution.
- The conductivity sensor must also be thoroughly rinsed with distilled water after every experiment.

Theory and evaluation

Figure 3 shows the example of a conductivity-time diagram.

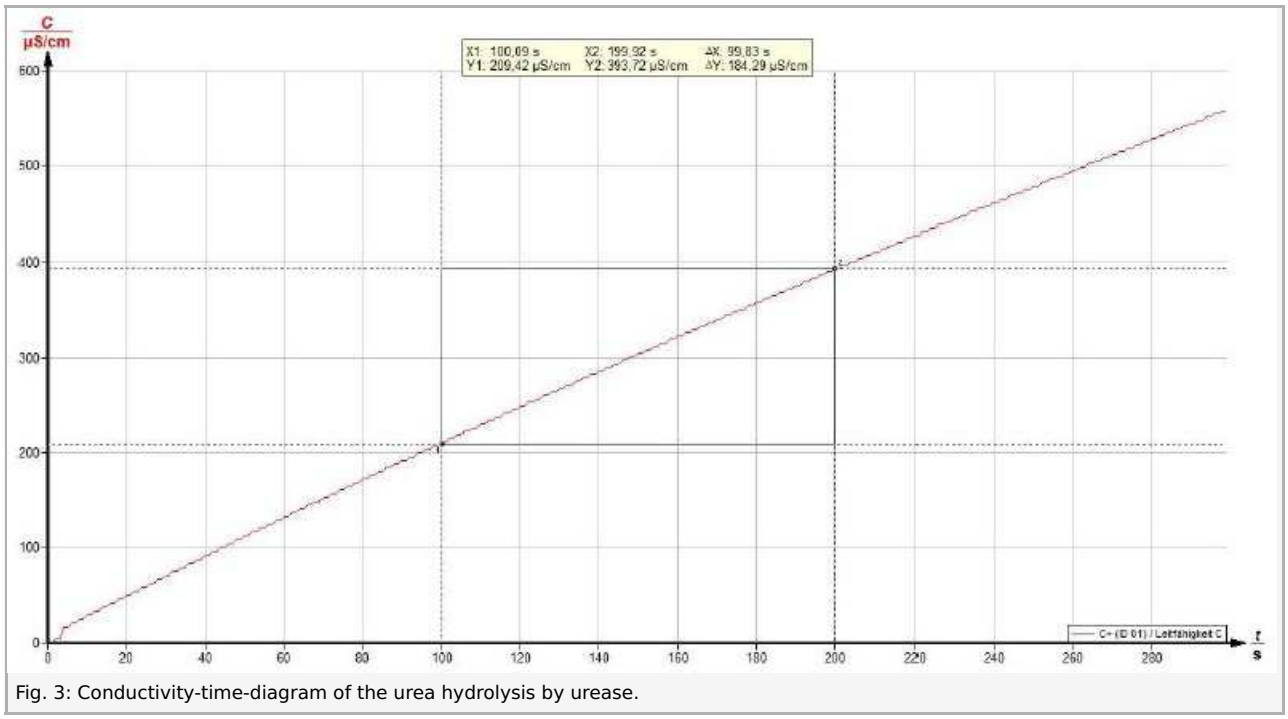

Fig. 3: Conductivity-time-diagram of the urea hydrolysis by urease.

In order to determine the Michaelis constant, determine the conductivity values at 100 s and 200 s as well as their difference ΔY for all of the six measurements ( ).

The mechanism of enzyme-catalysed reactions in accordance with Michaelis-Menten is based on an enzyme-substrate complex ES, which is formed by the enzyme E and the substrate S in a preceding balance reaction and then decomposes to a product P and the unchanged enzyme E:

$$\mathrm{E} + \mathrm{S} \underset{k_1'}{\overset{k_1}{\rightleftarrows}} \mathrm{ES} \xrightarrow{k_2} \mathrm{P} + \mathrm{E} \quad (1)$$

k_1, k_1', k_2 reaction rate constants of the elementary reactions

In accordance with Bodenstein's principle, the time change of the concentration of ES ≈ 0

$$\frac{dc_{\mathrm{ES}}}{dt} = k_1 c_{\mathrm{E}} c_{\mathrm{S}} - k_1' c_{\mathrm{ES}} - k_2 c_{\mathrm{ES}} \approx 0 \quad (2)$$

Solving this equation, with regard to the concentration of ES, leads to:

$$c_{\mathrm{ES}} = \frac{k_1 c_{\mathrm{E}} c_{\mathrm{S}}}{k_1' + k_2} \quad (3)$$

The free substrate concentration c_{S} can be equated with the total concentration of S , since only a small quantity of the enzyme

is added. The total concentration of E, $c_{\mathrm{E},0}$, equals the sum of the concentration of the free enzyme c_{E} and of the enzyme-substrate-complex, c_{ES} :

$$c_{\mathrm{E},\,0} = c_{\mathrm{E}} + c_{\mathrm{ES}} \quad (4)$$

Solving the equation and inserting equation (4) into equation (3) leads to:

$$c_{\mathrm{ES}} = \frac{k_1 \cdot (c_{\mathrm{E},\,0} - c_{\mathrm{ES}}) c_{\mathrm{S}}}{k_1' + k_2} \quad (5)$$

Solving the equation for c_{ES} results in:

$$c_{\mathrm{ES}} = \frac{k_1 \cdot c_{\mathrm{E},\,0} \cdot c_{\mathrm{S}}}{k_1' + k_2 + k_1 c_{\mathrm{S}}} \quad (6)$$

For the production formation, the time law is:

$$\frac{dc_{\mathrm{P}}}{dt} = k_2 c_{\mathrm{ES}} \quad (7)$$

If one replaces c_{ES} with the term from equation (6), the result is:

$$\frac{dc_{\mathrm{p}}}{dt} = \frac{k_1\, k_2 c_{\mathrm{E},\,0} c_{\mathrm{S}}}{k_1' + k_2 + k_1 c_{\mathrm{S}}} \quad (8)$$

The quotient

$$\frac{k_1' + k_2}{k_1} = K_{\mathrm{M}} \quad (9)$$

is combined in the Michaelis constant K_{M} . As a result, the time law is:

$$\frac{dc_{\mathrm{p}}}{dt} = \frac{k_2\, c_{\mathrm{E},\,0}\, c_{\mathrm{S}}}{K_{\mathrm{M}} + c_{\mathrm{S}}} \quad (10)$$

This means that the enzymolysis rate linearly depends on the enzyme concentration. The influence of the substrate concentration, however, is more complicated. In the case of $c_{\mathrm{S}} > K_{\mathrm{M}}$, the equation (10) simplifies to

$$\frac{dc_{\mathrm{p}}}{dt} = k_2\, c_{\mathrm{E},\,0} \quad (11)$$

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In this case, the reaction is a reaction of zero order with regard to S and the maximum enzymolysis rate is $k_2 c_{\mathrm{E},\,0}$. If $c_{\mathrm{S}} = K_{\mathrm{M}}$, then half the maximum rate is reached. The Michaelis constant K_M , therefore, is equal to the substrate concentration at which the reaction proceeds at half the maximum velocity.

If only little substrate is available, i.e. if $c_{\mathrm{S}} > K_{\mathrm{M}}$, the result is

$$\frac{dc_{\mathrm{p}}}{dt} = \frac{k_2}{K_{\mathrm{M}}} \cdot c_{\mathrm{E},\,0} c_{\mathrm{S}} \quad (12)$$

This means that the formation rate of P is of first order with regard to E and S.

For the evaluation, the average enzymolysis rates between 100 s and 200 s after the start are determined. For this purpose, the difference of the conductivity values after 100 and 200 seconds must be formed (ΔY) and divided by 100 s. These velocities (in µS cm $^{-1}$ s $^{-1}$) are plotted against the urea concentration (in mmol l $^{-1}$) (see Fig. 4). The substrate concentration c_{S} (in mmol/l) results from the following formula:

$$c_{\mathrm{S}} = (W10000)/M \quad (13)$$

where:

W = concentration of the urea solution in %

M = molar mass of urea = 60.06 g/mol

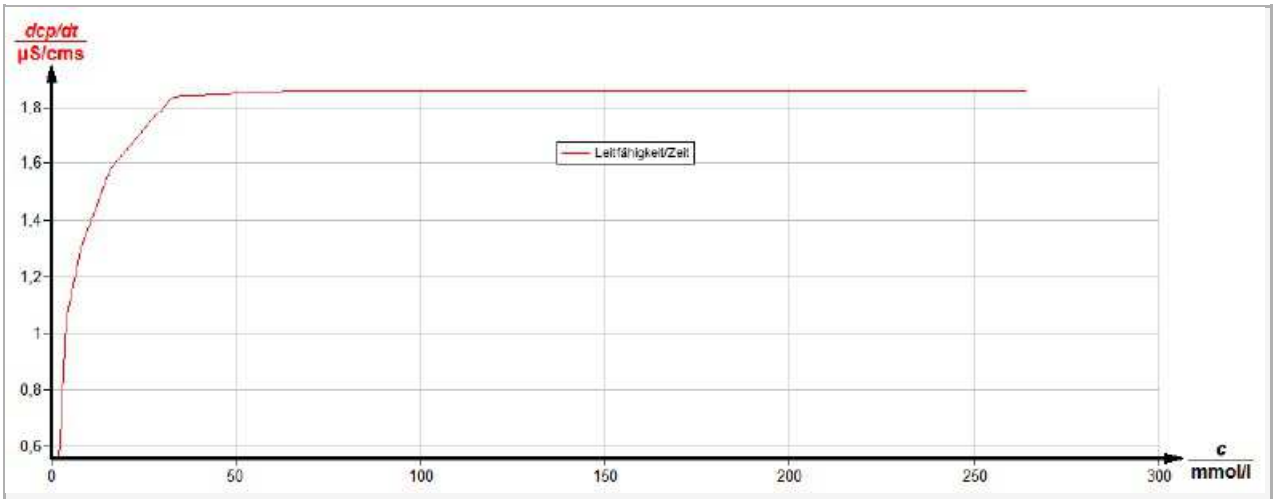

Fig. 4: Dependence of the concentration on the enzymolysis rate.

Since it is very difficult to directly read off a concentration that corresponds to the the half-maximum velocity, i.e. K_{M} , one uses the Lineweaver-Burk plot.

With the reaction velocity $v = dc_{\mathrm{p}} / dt$ and in a reciprocal representation, equation (10) leads to:

$$\frac{1}{v} = \frac{1}{k_2} + \frac{K_{\mathrm{M}}}{k_2} \cdot \frac{1}{c_{\mathrm{S}}} \quad (14)$$

In plotting 1/v against $1/c_S$ (see Fig.5), it thereby leads to k_2^{-1} as the ordinate intercept ($1/c_S = 0$) and K_M/k_2 as the slope of a straight line. First, one determines the ordinate intercept and the slope of the straight line. Then, this slope is divided by the ordinate intercept in order to obtain the Michaelis constant. The calculation results in the value of 4.86 x 10 $^{-3}$ mol l $^{-1}$ for the Michaelis constant of urease.

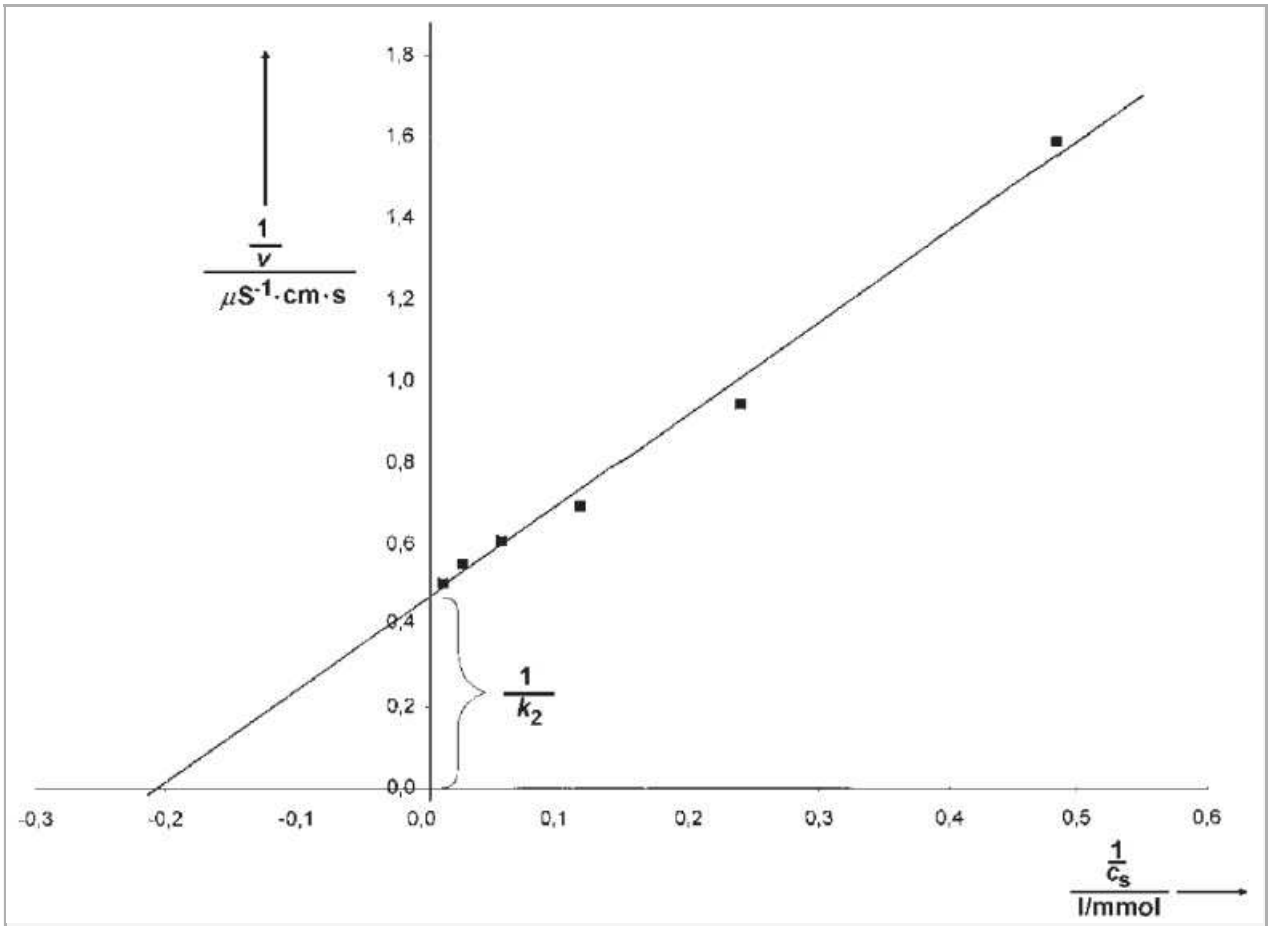

Fig. 5: Lineweaver-Burk plot.

A small K_M value signifies that the enzyme has a high affinity towards its substrate. Urease was the first enzyme that could be produced in crystalline form (Sumner, 1926). Unlike the allosteric enzymes, urease is a part of the “normal” enzymes that fulfil the Michaelis-Menten mechanism.

Task and equipment

Task

Calculate the Michaelis constant after determining the required parameters experimentally.

Fig.1: Experiment setup

Equipment

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Additional material			
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Setup and procedure

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The experiment requires several solutions with different urea concentration levels. These solutions must be prepared fresh prior to the execution of the experiment:

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- 0.1% urea solution: Transfer 50 ml of the 0.2% urea solution with the 50 ml volumetric pipette into a 100 ml Erlenmeyer flask and add 50 ml of distilled water.
- 0.05% urea solution: Transfer 50 ml of the 0.1% urea solution with the 50 ml volumetric pipette into a 100 ml Erlenmeyer flask and add 50 ml of distilled water.
- 0.025% urea solution: Transfer 50 ml of the 0.05% urea solution with the 50 ml volumetric pipette into a 100 ml Erlenmeyer flask and add 50 ml of distilled water.
- 0.0125% urea solution: Transfer 50 ml of the 0.025% urea solution with the 50 ml volumetric pipette into a 100 ml Erlenmeyer flask and add 50 ml of distilled water.

Note: The urease solution should be stored in a refrigerator!

- Set up the equipment as shown in Fig. 1 and 2.
  Connect the Cobra4 Sensor-Unit "Conductivity+" with the Wireless/USB-link.

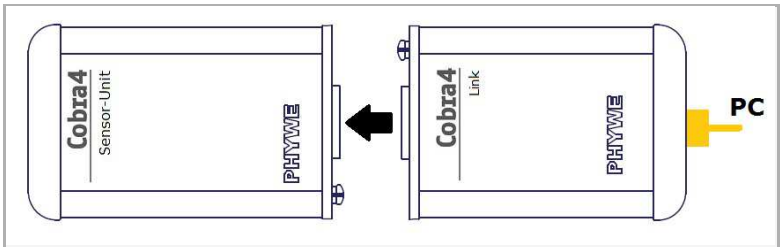

Fig. 2: Cobra4 Sensor-Unit with Cobra4 Link in USB mode

- Fasten the universal clamp with the bosshead to the support rod of the retort stand.
- Secure the conductivity sensor in place with the universal clamp.
- Switch on the Wireless/USB-link .
  Connect your PC with the Wireless/USB-link (via WiFi or via cabel in USB-mode).
  Start PHYWE measureLAB and switch on the Cobra4 Wireless-Link . The sensor is detected automatically.
  Select the Cobra4 Sensor-Unit "Conductivity+" in PHYWE measureLAB . The electrical conductance will be measured.

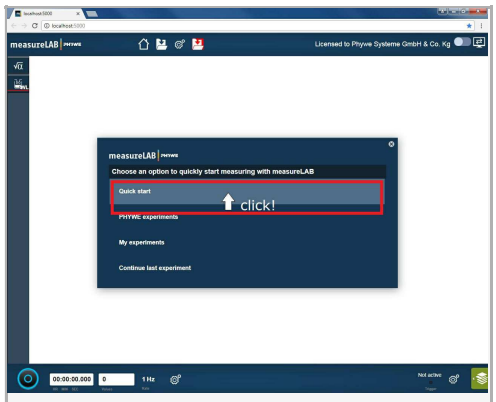

Fig. 3

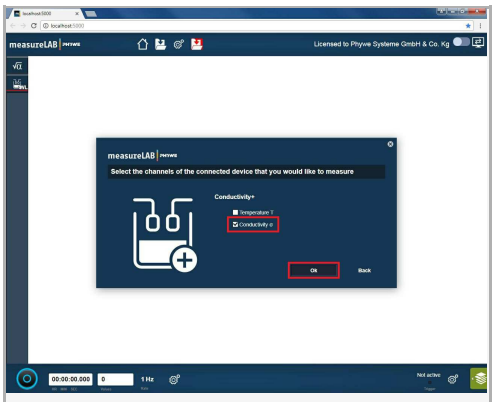

Fig. 4

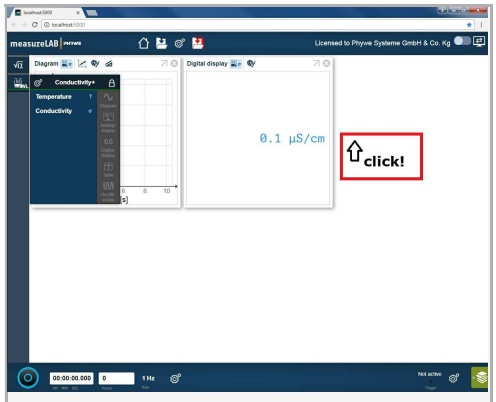

Fig. 5

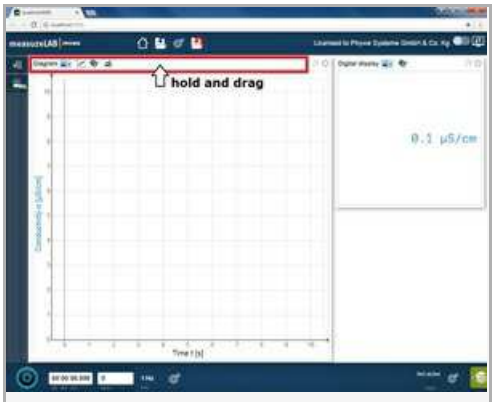

Fig. 6

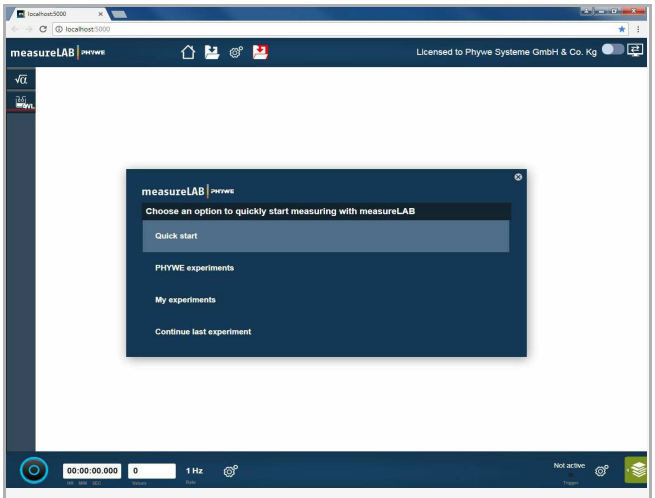

Fig. 2: Select the sensor "Conductivity+"

Procedure

- Transfer 40 ml of the 0.0125% urea solution (the lowest concentration first) in two steps with the 20 ml volumetric pipette into a 100 ml beaker and add a magnetic stirring bar.
- Place the beaker on the magnetic stirrer and lower the conductivity sensor into the solution.
- Set the stirrer to a medium stirring speed (*Caution*: The magnetic stirring bar must never hit the conductivity sensor!).
- Add 50 µl of the urease solution with the microlitre syringe and start the measurement without any further delay by clicking the start button ( ).
- The time course of the reaction can be observed on screen.
- After the measurement (stop button:), save the data for further processing.
- Repeat the measurement with all of the six prepared urea solutions (in order of concentration increase).
- Prior to performing the individual measurements, take the beaker off the magnetic stirrer and remove the magnetic stirring bar from the solution with the aid of the separator.
- Rinse the magnetic stirring bar thoroughly with distilled water, dry it with a paper towel, and put it into the next solution.
- The conductivity sensor must also be thoroughly rinsed with distilled water after every experiment.

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Theory and evaluation

Figure 3 shows the example of a conductivity-time diagram.

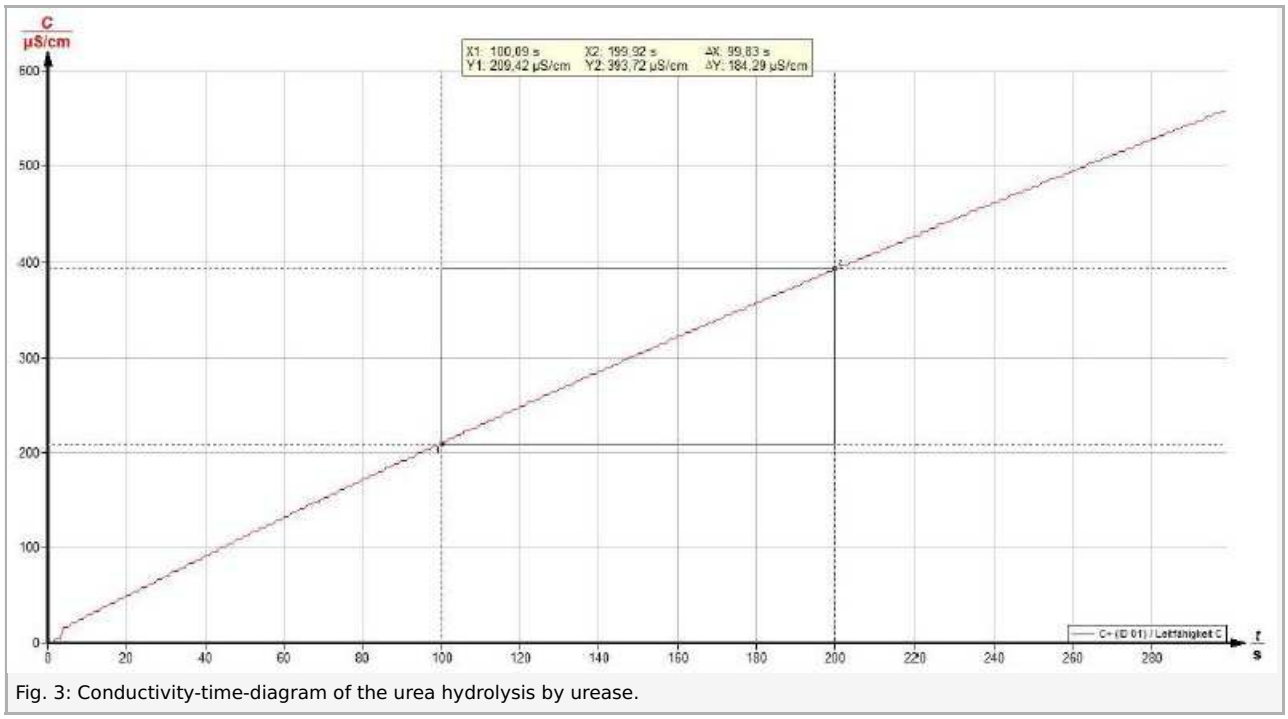

Fig. 3: Conductivity-time-diagram of the urea hydrolysis by urease.

In order to determine the Michaelis constant, determine the conductivity values at 100 s and 200 s as well as their difference ΔY for all of the six measurements ( ).

The mechanism of enzyme-catalysed reactions in accordance with Michaelis-Menten is based on an enzyme-substrate complex ES, which is formed by the enzyme E and the substrate S in a preceding balance reaction and then decomposes to a product P and the unchanged enzyme E:

$$\mathrm{E} + \mathrm{S} \underset{k_1'}{\overset{\kappa_1}{\rightleftarrows}} \mathrm{ES} \xrightarrow{\mathrm{k_2}} \mathrm{P} + \mathrm{E} \quad (1)$$

k_1, k_1', k_2 reaction rate constants of the elementary reactions

In accordance with Bodenstein's principle, the time change of the concentration of ES ≈ 0

$$\frac{dc_{\mathrm{ES}}}{dt} = k_1 c_{\mathrm{E}} c_{\mathrm{S}} - k_1' c_{\mathrm{ES}} - k_2 c_{\mathrm{ES}} \approx 0 \quad (2)$$

Solving this equation, with regard to the concentration of ES, leads to:

$$c_{\mathrm{ES}} = \frac{k_1 c_{\mathrm{E}} c_{\mathrm{S}}}{k_1' + k_2} \quad (3)$$

The free substrate concentration c_{S} can be equated with the total concentration of S , since only a small quantity of the enzyme

is added. The total concentration of E, $c_{\mathrm{E},0}$, equals the sum of the concentration of the free enzyme c_{E} and of the enzyme-substrate-complex, c_{ES} :

$$c_{\mathrm{E},\,0} = c_{\mathrm{E}} + c_{\mathrm{ES}} \quad (4)$$

Solving the equation and inserting equation (4) into equation (3) leads to:

$$c_{\mathrm{ES}} = \frac{k_1 \cdot (c_{\mathrm{E},\,0} - c_{\mathrm{ES}}) c_{\mathrm{S}}}{k_1' + k_2} \quad (5)$$

Solving the equation for c_{ES} results in:

$$c_{\mathrm{ES}} = \frac{k_1 \cdot c_{\mathrm{E},\,0} \cdot c_{\mathrm{S}}}{k_1' + k_2 + k_1 c_{\mathrm{S}}} \quad (6)$$

For the production formation, the time law is:

$$\frac{dc_{\mathrm{P}}}{dt} = k_2 c_{\mathrm{ES}} \quad (7)$$

If one replaces c_{ES} with the term from equation (6), the result is:

$$\frac{dc_{\mathrm{p}}}{dt} = \frac{k_1\, k_2 c_{\mathrm{E},\,0} c_{\mathrm{S}}}{k_1' + k_2 + k_1 c_{\mathrm{S}}} \quad (8)$$

The quotient

$$\frac{k_1' + k_2}{k_1} = K_{\mathrm{M}} \quad (9)$$

is combined in the Michaelis constant K_{M} . As a result, the time law is:

$$\frac{dc_{\mathrm{p}}}{dt} = \frac{k_2\, c_{\mathrm{E},\,0}\, c_{\mathrm{S}}}{K_{\mathrm{M}} + c_{\mathrm{S}}} \quad (10)$$

This means that the enzymolysis rate linearly depends on the enzyme concentration. The influence of the substrate concentration, however, is more complicated. In the case of $c_{\mathrm{S}} > K_{\mathrm{M}}$, the equation (10) simplifies to

$$\frac{dc_{\mathrm{p}}}{dt} = k_2\, c_{\mathrm{E},\,0} \quad (11)$$

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In this case, the reaction is a reaction of zero order with regard to S and the maximum enzymolysis rate is $k_2 c_{\mathrm{E},\,0}$. If $c_{\mathrm{S}} = K_{\mathrm{M}}$, then half the maximum rate is reached. The Michaelis constant K_M , therefore, is equal to the substrate concentration at which the reaction proceeds at half the maximum velocity.

If only little substrate is available, i.e. if $c_{\mathrm{S}} > K_{\mathrm{M}}$, the result is

$$\frac{dc_{\mathrm{p}}}{dt} = \frac{k_2}{K_{\mathrm{M}}} \cdot c_{\mathrm{E},\,0} c_{\mathrm{S}} \quad (12)$$

This means that the formation rate of P is of first order with regard to E and S.

For the evaluation, the average enzymolysis rates between 100 s and 200 s after the start are determined. For this purpose, the difference of the conductivity values after 100 and 200 seconds must be formed (ΔY) and divided by 100 s. These velocities (in µS cm $^{-1}$ s $^{-1}$) are plotted against the urea concentration (in mmol l $^{-1}$) (see Fig. 4). The substrate concentration c_{S} (in mmol/l) results from the following formula:

$$c_{\mathrm{S}} = (W10000)/M \quad (13)$$

where:

W = concentration of the urea solution in %

M = molar mass of urea = 60.06 g/mol

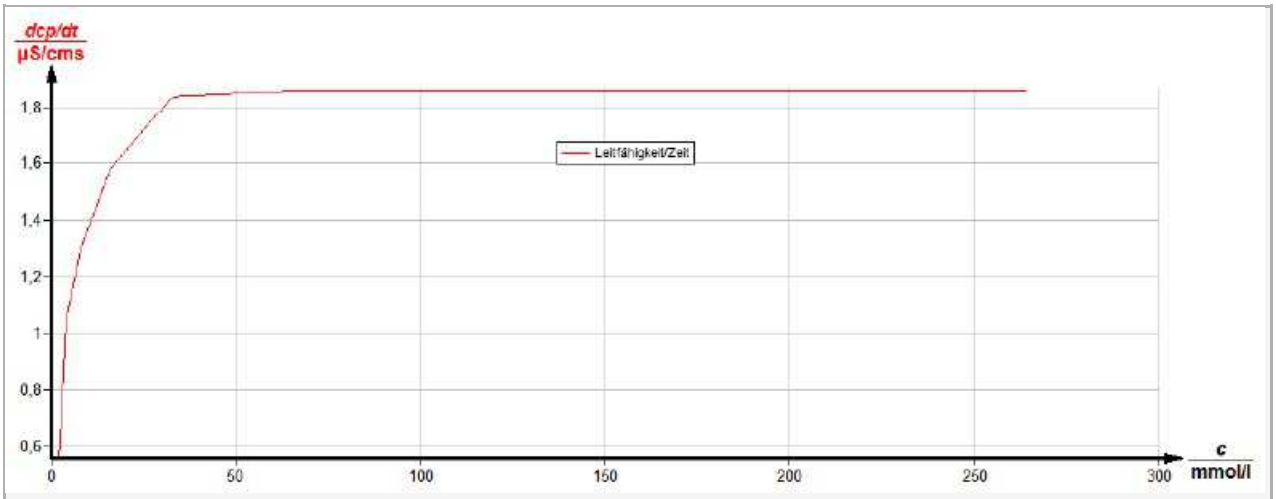

Fig. 4: Dependence of the concentration on the enzymolysis rate.

Since it is very difficult to directly read off a concentration that corresponds to the the half-maximum velocity, i.e. K_{M} , one uses the Lineweaver-Burk plot.

With the reaction velocity $v = dc_{\mathrm{p}} / dt$ and in a reciprocal representation, equation (10) leads to:

$$\frac{1}{v} = \frac{1}{k_2} + \frac{K_{\mathrm{M}}}{k_2} \cdot \frac{1}{c_{\mathrm{S}}} \quad (14)$$

In plotting 1/v against $1/c_S$ (see Fig.5), it thereby leads to k_2^{-1} as the ordinate intercept ($1/c_S = 0$) and K_M/k_2 as the slope of a straight line. First, one determines the ordinate intercept and the slope of the straight line. Then, this slope is divided by the ordinate intercept in order to obtain the Michaelis constant. The calculation results in the value of 4.86 x 10 $^{-3}$ mol l $^{-1}$ for the Michaelis constant of urease.

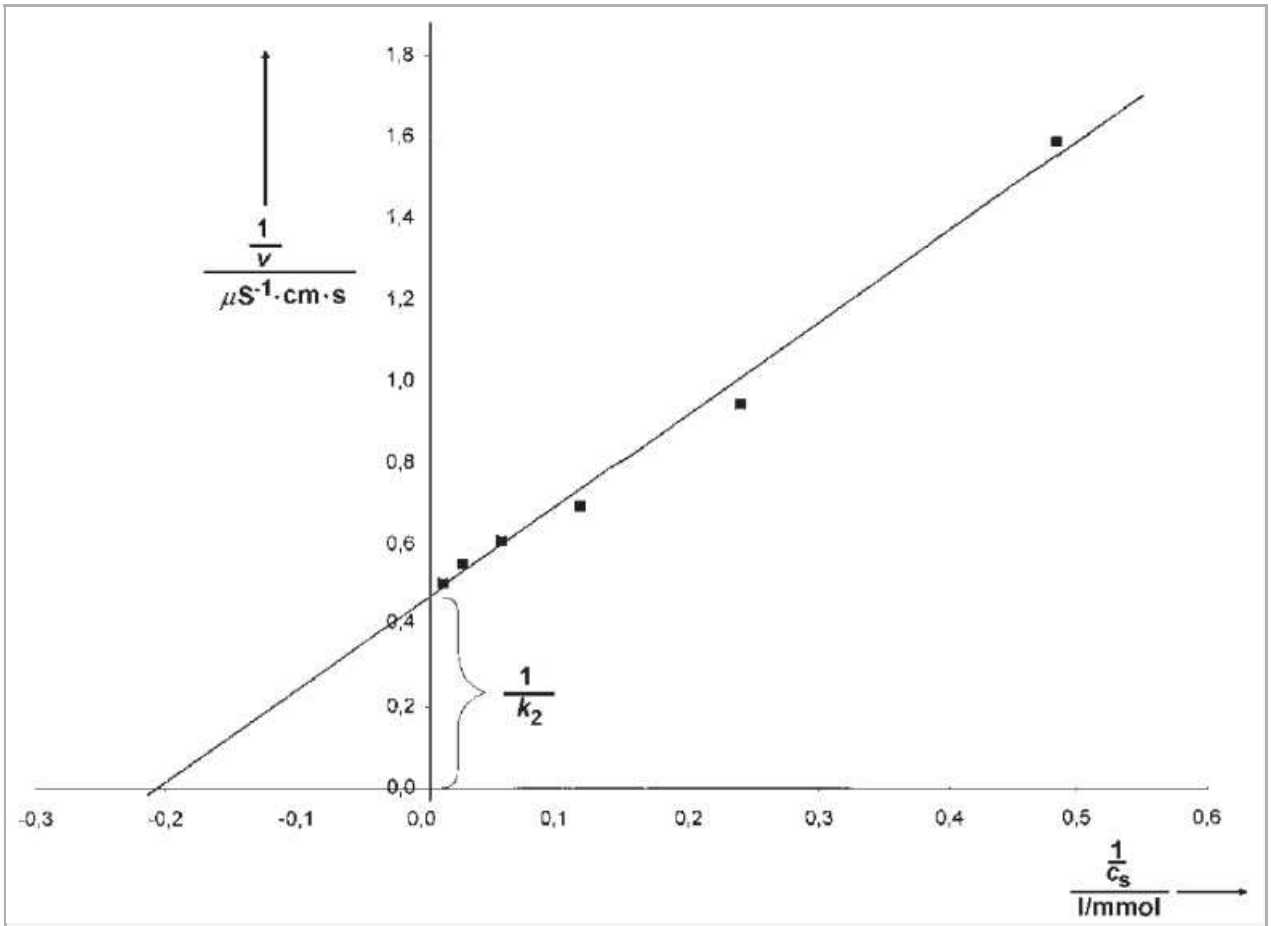

Fig. 5: Lineweaver-Burk plot.

A small K_M value signifies that the enzyme has a high affinity towards its substrate. Urease was the first enzyme that could be produced in crystalline form (Sumner, 1926). Unlike the allosteric enzymes, urease is a part of the “normal” enzymes that fulfil the Michaelis-Menten mechanism.