EXPERIMENT 13

ENZYMES OF BIOTRANSFORMATION

Degradation and elimination of lipophylic xenobiotics occur in two steps of biotransformation. In order to increase the water solubility e.g. the aromatic compounds are hydroxylated or other reactive, more hydrophylic residues are formed (Phase I reactions). Further substituents (glucuronic acid, glycin, sulfate) can be connected to these new hydroxyl groups increasing the solubility and thus fasten the excretion from the body (Phase II reactions).

Phase I

The cytochrome P450 system of Phase I participates in:

a) inactivation or activation of drugs

b) conversion of compounds producing reactive molecules

c) some steps of the steroid synthesis

d) metabolism of fatty acids and some of their derivatives

The name cytochrome P450 derives from the spectral features of the molecule. Reducing an endoplasmic reticulum preparation (microsome fraction) or a homogenate prepared from inner mitochondrial membrane with Na-dithionite ($Na_2S_2O_4$), then introducing carbon monoxide (CO) in the suspension after binding the carbon monoxide a pigment is formed which has absorption maximum at 450 nm.

The cytochrome P450 heme proteins can be found in all mammalian cell types except the erythrocytes and muscle cells. Their substrate specificity is broad, they catalyze the oxidation of various compounds. Among their substrates there are compounds synthesized in the body, including steroids, fatty acids, prostaglandins, leukotrienes, and compounds like medicines, food additives or industrial by-products entering the body with food, injection, and breathing or through the skin.

The cytochrome P450s can be divided into gene families according to their amino acid sequence similarities (isoenzymes). Today the number of the known isoenzymes is about 150.

The general form of the cytochrome P450 catalyzed reaction:

$$\text{Substrate—H} + O_2 + \text{NADPH} + H^+ \rightarrow \text{Substrate—OH} + \text{NADP}^+ + H_2O$$

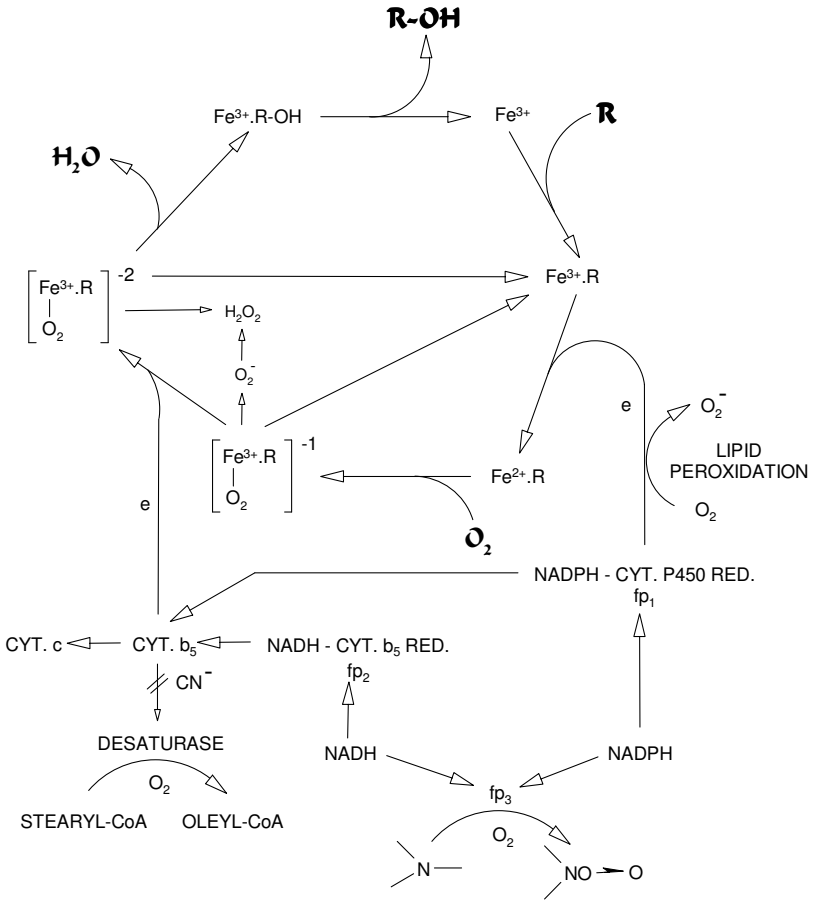
The source of the molecular oxygen is the air. At the end of the reaction one of the oxygen atoms can be found in the hydroxyl group of the modified substrate and the other one is present in the produced water. The reaction is called monooxygenation and the cytochrome P450 isoenzymes are also known as monooxygenases.

In the cytochrome P450 protein there is a heme protoporphyrine IX prosthetic group. The heme - protein complex has a substrate binding and a molecular oxygen binding site. The iron atom is bound to the four pyrrole nitrogens of the porphyrine ring. One of the two axial ligands binds a cysteine sulfhydryl group of the protein chain. The substrate binds to the enzyme containing oxidized iron (Fe^{3+}). During the reaction the valence of the iron atom changes between the Fe^{3+} and Fe^{2+} states.

On the practice we use aniline as a substrate. During the oxygenation an OH group is formed in para position to the amino group and 4-aminophenol is produced. In alkaline solution the 4-aminophenol forms a blue colored complex with the added phenol.

The monooxygenation reaction uses NADPH as a reductant (hydrogen donor). The NADPH is extremely expensive, that is why we use it in the lowest possible concentration, but we enable the

regeneration of it. The regeneration is made by the glucose-6-phosphate - glucose-6-phosphate dehydrogenase system.



Scheme of the cytochrome P 450 monooxygenase reaction steps

Phase II

In the second phase of biotransformation (detoxication) a major role is played by glucuronyl-transferase. Glucuronyl-transferase transfers glucuronic acid from UDP-glucuronic acid to a xenobiotic made ready in the first phase. A glycosidic bond is formed. The hydroxyl residues of glucuronic acid and the dissociable carboxyl residues render the so arising molecule highly water soluble. The pKa value of the conjugate is less than 4, thus the molecule is in the completely dissociated form under physiological circumstances. In contrast to lipophylic substances it doesn't get reabsorbed in the kidney after it has been filtered through the glomerulus.

UDP-glucuronate + acceptor = UDP + acceptor-β-d-glucuronide

At times there are more than one hydroxyl group to be glucuronylated on the molecule undergoing detoxication. Then the molecular weight of the product is over 400-500 Da. Such a big molecule cannot be excreted in the kidney and the detoxicated xenobiotic is rather discharged into the bile. Glucuronic acid is then cleaved off from some of the glucuronylated metabolite by the intestinal glucuronidase.

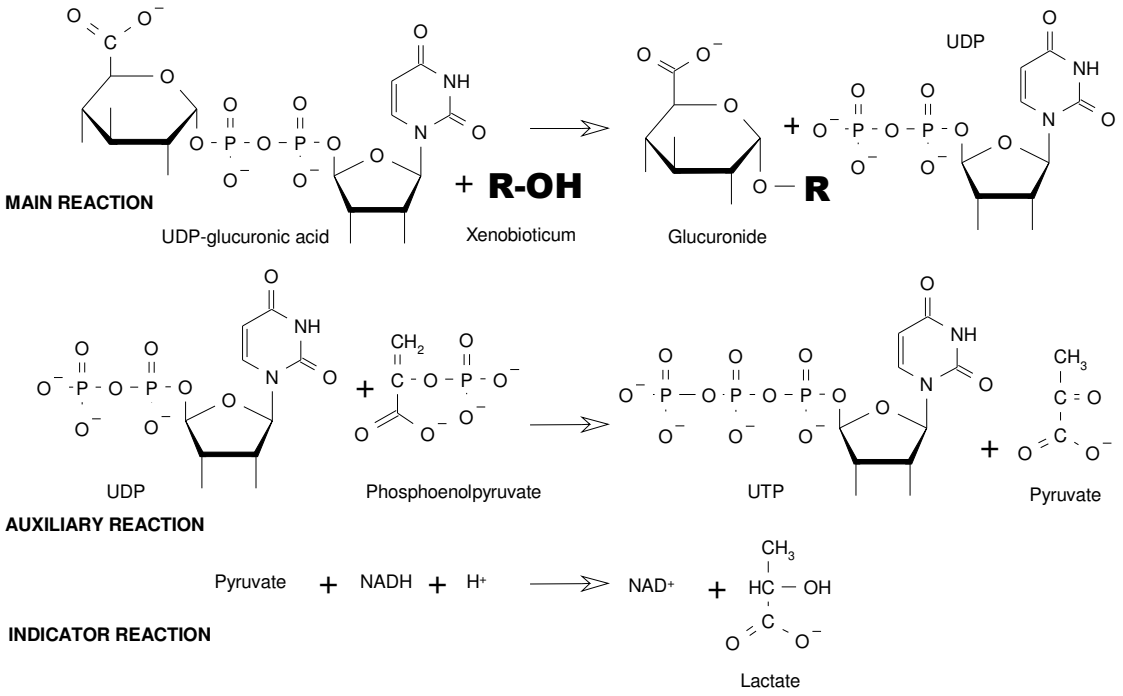
The metabolite - thereby becoming lipophylic - is reabsorbed, glucuronylated and excreted in bile again. This process is called enterohepatic circulation.

The enzyme occurs mainly in the liver (in the endoplasmatic reticulum) in the close proximity of cytochrome P450 allowing for an easy interconnection between the two phases of biotransformation. The

enzyme shows a low order of specificity for its substrate which can be phenols, alcohols, amines and also fatty acids.

In addition to glucuronidation sulfation, methylation, acetylation, conjugation with glycin or glutathione has a role in detoxication.

In our experiment this will be the measured reaction. The second line shows the helper reaction, whereas the indicator reaction is depicted in the third line. The one molecule UDP generated in the UDP-glucuronyl-transferase (main) reaction is converted into UTP by pyruvate-kinase (helper reaction). Next, NADH and the pyruvate formed of phoshpoenolpyruvate are converted into lactate and NAD by lactate-dehydrogenase in the indicator reaction. A drop in the absorption in the 340-365 nm range signals the oxidation of NADH.



Phases of the UDP-glucuronyl-transferase activity measurement with the aid of coupled reactions in the Warburg optical test

1. Study of aniline hydroxylase activity

Materials, Equipment

Substrate solution (KCl 187.5 mmol/l, aniline.HCl 6.2 mmol/l, albumin 0.025 %, glucose-6-P.Na 6.25 mmol/l, dithiothreitol 0.125 mmol/l, G-6-P dehydrogenase 1.25 U/ml)

Trichloroacetic acid solution (15.0 %)

Microsome fraction: Liver of two 150 - 200 g rats, fasted overnight is homogenized in ten volumes (according to the weight of the liver) of ice-cold 50 mmol/l HEPES, 250 mmol/l sucrose buffer (pH 7.5) (10-12 g liver in 100 - 120 ml buffer). The homogenate is centrifuged for 20 minutes with 9000 g, with cooling. The supernatant is further centrifuged with 100 000 g for 45 minutes (at +4 °C). The precipitate is resuspended in one volume of buffer (the same volume as the weight of the liver). At this step, the protein content of the preparate is about 20 mg/ml. The microsome suspension is stored at -70 °C.

$NADPH.Na_4$ solution (10.0 mmol/l)

Sodium carbonate solution (10.0 %)

Alkaline phenol solution (2.0 %)

Standard solution (4-Aminophenol.HCl 0.5 mmol/l)
Photometer, micro-cuvettes, 37 °C water bath, centrifuge, centrifuge tubes, test tubes, pipettes

Procedure

Standard curve for 4-aminophenol

Prepare the following mixtures:

	1.	2.	3.	4.	Blank
Distilled water (ml)	0.95	0.90	0.85	0.80	1.00
4-Aminophenol (ml)	0.05	0.10	0.15	0.20	—
TCA (ml)	0.25	0.25	0.25	0.25	0.25
Measure into new test tubes (ml)	0.75	0.75	0.75	0.75	0.75
Sodium carbonate (ml)	0.25	0.25	0.25	0.25	0.25
Alkaline phenol (ml)	0.75	0.75	0.75	0.75	0.75

Let stand the test tubes for 30 minutes at room temperature then measure the absorbance at 620 nm.

Measurement of aniline hydroxylase activity

Prepare the following reaction mixtures:

	SAMPLE	BLANK
SUBSTRATE SOLUTION (ml)	0.80	0.80
TCA solution (ml)	-	0.25
Microsome suspension (ml)	0.10	0.10
NADPH.Na_4 solution (ml)	0.10	0.10
Incubation with frequent shaking at 37°C, for 30 minutes		
TCA solution (ml)	0.25	-
Centrifuge with about 2000 g for 10 minutes		
from the TCA supernatant (ml)	0.75	0.75
Sodium carbonate solution (ml)	0.25	0.25
Alkaline phenol solution (ml)	0.75	0.75

Let stand the test tubes for 30 minutes at room temperature then measure the absorbance at 620 nm.

From the standard curve calculate the molar extinction coefficient of 4-Aminophenol (Mw.: 145.6 g/mol). With the help of the standard curve and the protein content of the microsome fraction calculate the specific activity (U / g protein) of the microsome fraction. The protein content of the microsome fraction is 17.5 mg / ml.

2. Measurement of UDP-glucuronyl-transferase activity

Materials, Equipment

Microsome fraction: The livers of two 150-200 g rats that had been fasting overnight were homogenized with an Elvehjem-Potter glass-teflon homogenizer in 0.15 M KCl ten times the weight of the organs (say 10-12 g liver in 100-120 ml buffer). The homogenate was spinned at 9000 g for 20 minutes at 4 °C. The supernatant was taken and spinned at 100 000 g for another 45 minutes at 4 °C. The sediment was then suspended with the homogenizer in 0.15 M KCl that was again ten times the weight of the livers. Protein was measured in the suspension. At this point, the protein concentration is around 15-20 mg/ml. The microsome suspension was stored at -70 °C until use.

Solution A ($MgCl_2.6H_2O$ 8.25 mM, Phosphoenolpyruvate.3CHA 0.35 mM, UDP-glucuronic-acid.Na_3 2.5 mM, Pyruvate-kinase 8.3 U/ml, Lactate-dehydrogenase 1.04 U/ml)

Substrate solution: 3-methyl-2-nitrophenol (3 mM)

NADH solution (1.5 mM)

2.5 % TRITON-X100 solution

125 mM HEPES buffer (pH 7.2)

Photometer, cuvettes, pipettes, test tubes

Activity measurement

The glucuronyl transferase enzyme is embedded into the lipid bilayer of the microsome. In order to disrupt the lipid bilayer we add the non-ionic detergent TRITON-X 100 to the suspension.

	SAMPLE
Microsome suspension (ml)	0.5
2.5 % TRITON-X100 solution (ml)	0.1
HEPES buffer, pH 7.2 (ml)	0.4

Incubate the mixture at 37 °C for 5 minutes and dilute it to 5 ml with adding 4 ml 125 mM HEPES buffer (pH 7.2). For the measurement use this TRITON-activated diluted sample!

	SAMPLE
Solution A (ml)	0.6
Substrate solution (ml)	0.1
NADH solution (ml)	0.2
Activated microsome suspension (ml)	0.1

The measurement of activity is done at room temperature. The solutions must be pipetted into the cuvette in the order given. Read the absorbance at 365 nm promptly and in one-minute intervals up to 5 minutes.

Use the average of the one-minute measurements (ΔA_{365}/min) to calculate the specific activity of the microsome preparation. Take 17.5 mg/ml for the usual concentration of microsome protein!

specific activity (nmol/min/mg) = 30303 × (ΔA_{365}/min) / mg protein

$$30303 = \frac{V \times 10^9 \times h}{\varepsilon \times d \times \upsilon}$$

V total volume of the reaction mixture (1.0 ml)

10^9 factor to switch from mol to nmol

ε molar extinction coefficient of NADH (at 365 nm, it is 3300 $mol^{-1}.\ell.cm^{-1}$)

d length of the path of light through the cuvette (1 cm)
v volume of sample added (0.1 ml)
h dilution of the sample (10 ×)

The kinetics of the glucuronidation of aromatic compounds containing phenolic hydroxyls depends upon the degree of dissociation in the -OH group as well. 3-methyl-2-nitrophenol is the most susceptible to glucuronidation among many phenol-derivatives. The pKa of the phenolic -OH residue is 6.9, so at physiological pH 75 % of the molecules are in the dissociated form.

Kinetic constants of a few phenol-derivatives:

Substrate	K_m mM	V_{max} µmol / min / mg prot.
Phenol	1.80	0.03
4-Chlorophenol	0.16	0.07
4-Methylphenol	0.27	0.06
4-Methoxyphenol	1.70	0.06
3-Methyl-2-nitrophenol	0.08	0.18

Questions

Explain the role of the reagents of the substrate solution in the aniline hydroxylase reaction: HEPES buffer, albumin, KCl, G-6-P.Na, G-6-P-dehydrogenase, dithiothreitol, aniline.
What part of the cell is the microsome?
What other enzymes do microsomes contain that count in biotransformation?
What is the unit of enzyme activity?
What are volume-activity and specific activity?
Using the table with the Vmax and K_m values give the substrate with the highest K_{cat}? What does the K_{cat} show?
What is the difference between the kinetic and the end-point measurement of activity?