

INVESTIGATION OF MICROSOMAL DRUG METABOLISM

The small vesicles formed from the endoplasmatic reticulum during homogenisation called microsomes contain an electron transport system. The components of this system are: NADPH-cytochrom P₄₅₀ reductase, cytochrom P₄₅₀; NADH-cytochrom b₅ reductase and cytochrom b₅. The electrons are transferred from NADPH towards the cytochrom P₄₅₀.

Microsomes can be prepared from a wide variety of tissues. The amount of the proteins of the microsomal electron transport chain and the ratio of its components depend on the source of the preparation. The proteins of the microsomal electron transport system can be induced by xenobiotic compounds. A high amount of electron transport proteins is characteristic for microsomes prepared from liver, intestines, skin, lung and from the adrenal gland. The amount of xenobiotic inducible proteins is high in preparations derived from intestines, lung and skin, but it is the highest in liver microsomes. The different components of the electron transport chain are induced to a different extent; in our case only the NADPH-cytochrom P₄₅₀ reductase and the cytochrom P₄₅₀ are induced. The rate of induction is also different: while the former protein is induced twofold, the induction of the cytochrom P₄₅₀ is fivefold. The microsomal electron transport chain also has a role in the metabolism of endogenous compounds in the liver and in the adrenal gland (e.g. fatty acid desaturation, steroid hydroxylation). On the other hand, the enzymes of the microsomal electron transport chain isolated from the liver, skin, intestines and from lung also take part in the detoxication of xenobiotic compounds. Dealkylation and/or hydroxylation are the reactions catalysed by cytochrom P₄₅₀ in a monooxygenase type reaction and the necessary electrons are provided by NADH or NADPH. The role of the microsomal electron transport system in the process of detoxification explains why the rate of induction is highest in liver and why the cytochrom P₄₅₀ is induced to the greatest extent by xenobiotics. In general we can say that substrates of the cytochrom P₄₅₀ enzyme are usually the inducers of the protein also. Certain drugs (phenobarbiturates) are very strong inducers of the system.

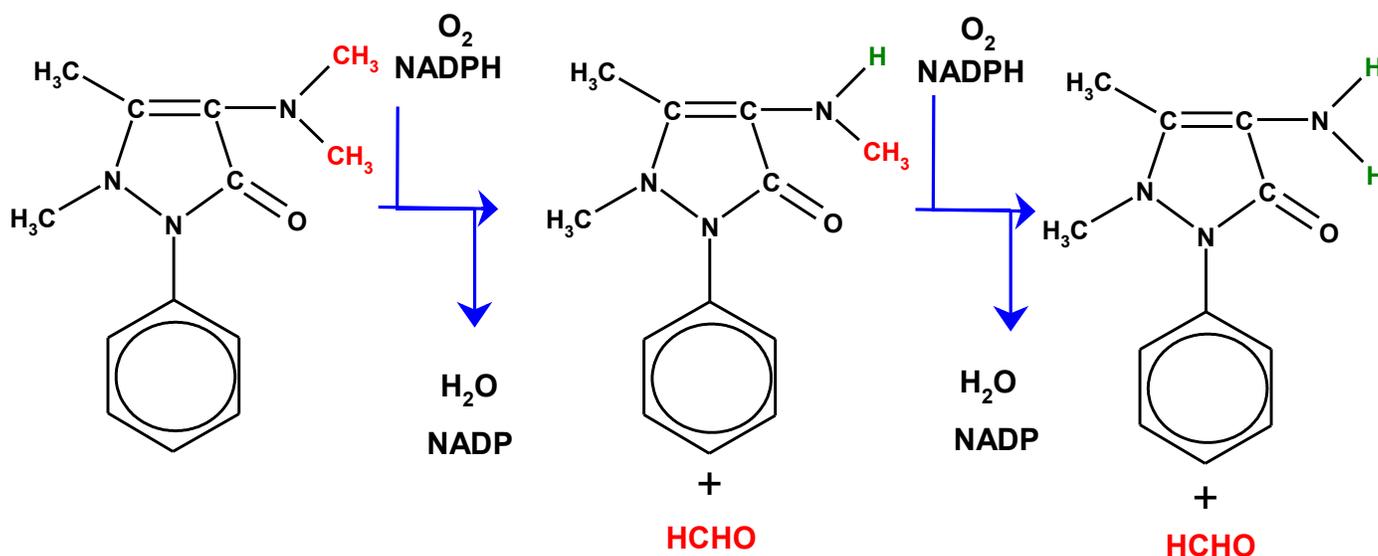
In the laboratory practice the N-demethylase activity of microsomes, prepared from the livers of control and of phenobarbiturate treated rats, will be measured.

Theory of the assay:

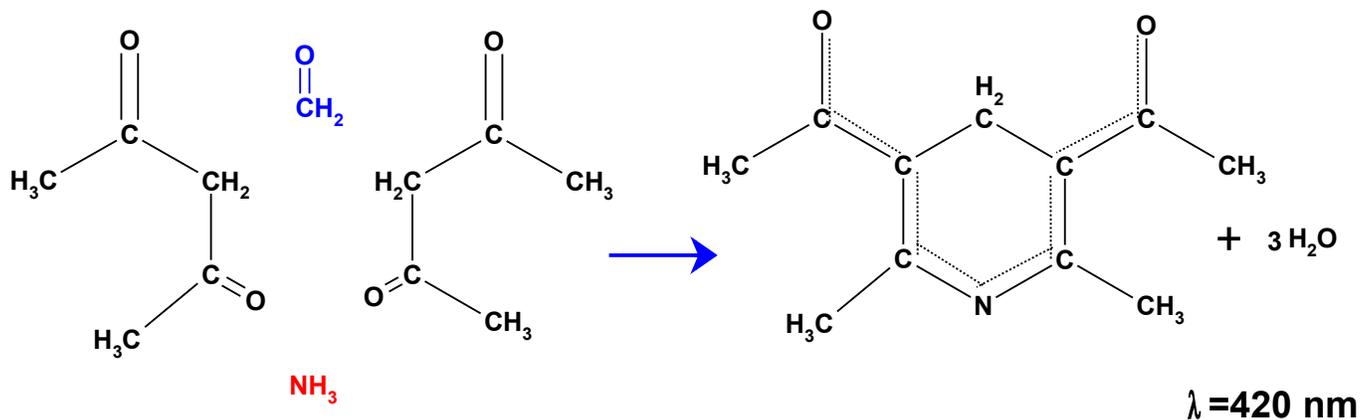
The chemical reactions catalysed by the microsomal electron transport chain have the same in vitro reaction mechanism as in vivo. The in vivo assay contains three main components: the microsomes, NADPH and the substrate. The microsome preparation is isolated from rat liver by differential centrifugation. First the livers are removed from the decapitated animals and are homogenised. The homogenate is centrifuged first with 13 000g to sediment the nuclei and the mitochondria. The supernatant is further pelleted (105 000g) to obtain the microsomes. To obtain higher enzyme activities, the microsomal system of the animals can be induced for 3-4 days before the experiment by adding phenobarbiturate (0.2%) into the drinking water of the animals. NADPH (3 mM) serves as an

electron source for the cytochrom P₄₅₀ system. The level of NADPH can be kept constant by using a coupled NADPH generating system in the reaction mixture. For this purpose glucose-6-phosphate dehydrogenase and glucose-6-phosphate are added to the system. Because glucose-6-phosphate dehydrogenase can be found in the postmitochondrial supernatant of the rat liver homogenate, during our experiments postmitochondrial supernatant will be used instead of isolated microsomes and of glucose-6-phosphate dehydrogenase. Many different chemicals can be used as substrates (e.g. ethylmorphine, amphetamine, aniline e.t.c).

In our test aminopyrine will be used, a compound which is demethylated according to the following scheme.



In the reaction, formaldehyde is formed and the concentration of formaldehyde will be determined. Using the Nash colour reagent, formaldehyde will form a coloured compound reacting with two molecules of acetylaceton and with one molecule of ammonia. In the reaction 3,5-bisacetyl-1,4-dihydrolutidine will be formed and its concentration will be determined spectrophotometrically at 412 nm.



Besides the compound discussed above the reaction mixture has to contain MgCl_2 and sodium-pyrophosphate. While MgCl_2 will reduce the rate of unwanted lipid peroxydation, Na-pyrophosphate inhibits the NADPH destructing pyrophosphatase activity of the microsome preparation.

Solutions used:

1. 0.05 M phosphate buffer, pH 7.5
2. postmitochondrial supernatant (30 mg protein/ml)
3. 0.001 M NADP
4. 0.01 M glucose-6-phosphate
5. 0.1 M aminopyrine
6. 1 M MgCl_2
7. 40 % TCA
8. Nash reagent:
1.5 M acetic acid
2 M ammonium acetate
acetylacetone
9. 1.32 mM formaldehyde

The stock solutions are prepared as follows:

1. 2x concentrated reaction buffer consisting of 680 ml of phosphate buffer, 10 ml of NADP, 100 ml of glucose-6-phosphate, 200 ml of Na-pyrophosphate and 10 ml of MgCl_2 .
2. Nash reagent containing 500 ml of ammonium acetate, 100 ml of acetic acid 6 ml of acetylacetone and 394 ml of distilled water.

Experiment:

Pipet the following compounds into two Erlenmeyer flasks (25 ml of volume):

- 2.65 ml of 0.1 M phosphate buffer, pH 7.5
- 3.5 ml 2x concentrated reaction buffer

Add 0.5 ml of postmitochondrial supernatant of control animals to one Erlenmeyer flasks and 0.5 ml of postmitochondrial supernatant of animals induced by phenobarbiturate to the other Erlenmeyer flask:

The flasks are preincubated 3 minutes at 37°C. The reactions are started by adding 0,35 ml of aminopyrine solutions to the flasks. At 0, 2.5, 5.0, 10, 15 and 20 minutes of incubation 1 ml aliquots are withdrawn into Eppendorf tubes containing 0.1 ml of TCA. The samples are centrifuged and the amount of formed formaldehyde is determined from the supernatants.

Determination of formaldehyde:

The calibration curve is constructed as follows: measure 0.00, 0.02, 0.04, 0.06, 0.08 and 0.10 ml of formaldehyde into numbered test tubes and fill up the samples to 1 ml with phosphate buffer. Also pipet 1 ml of the supernatants into test tubes. Add 1 ml of Nash reagent to each tube and incubate the tubes at 60°C for 15 minutes. The absorbances of the samples are read at 412 nm. Use the 0 minute point as blank. At the construction of the calibration curve use the tube which does not contain formaldehyde a blank. Construct a calibration curve for formaldehyde (absorbance vs. the amount of formaldehyde added). Using the calibration curve, calculate the amount of formaldehyde formed in the experimental points (nm formaldehyde/mg protein). Plot those values vs. the time of incubation.