Faculty of Medicine, University of Debrecen
Department of Biochemistry and Molecular Biology

BIOCHEMISTRY PRACTICE

GLYCOLYTIC ENZYMES

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TABLE OF CONTENTS

ABREVIATIONS

$(NH_4)_2SO_4$	ammonium sulfate
ADP	adenosine diphosphate
ALDO	aldolase
ATP	adenosine triphosphate
BPB	bromophenol blue
DHAP	dihydroxyacetone phosphate
DNPH	dinitrophenyl hydrazine
F-1,6-BiP	fructose 1,6-bisphosphate
FDG	2-^{18}F-2-deoxyglucose
G3P	glyceraldehyde 3-phosphate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HCl	hydrogen chloride
LDH	lactate dehydrogenase
NAD^+	nicotinamide adenine dinucleotid
NADH	reduced form of nicotinamide adenine dinucleotid
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NBT	nitro blue tetrazolium chloride
PET	positron emission tomography
Pi	inorganic phosphate
pI	isoelectric point
PMS	phenazine methosulfate
TCA	Trichloro-acetate (trichloro-acetic acid)
U/L	unit/liter
UV	ultra violet light

GLYCOLYTIC ENZYMES

1. Theoratical background

1.1. Glycolysis

Glycolysis is the metabolic pathway that converts glucose $C_6H_{12}O_6$, into lactate (anaerobic glycolysis) or pyruvate (aerobic glycolysis). The free energy released in this process is used to form the high-energy compounds ATP (adenosine triphosphate) and NADH (reduced nicotinamide adenine dinucleotide). Glycolysis occurs with variations, in nearly all organisms. ***Aerobic glycolysis*** of glucose to pyruvate, requires two equivalents of ATP to activate the process, with the subsequent production of four equivalents of ATP and two equivalents of NADH. Thus, conversion of one mole of glucose to two moles of pyruvate is accompanied by the net production of two moles each of ATP and NADH.

Glucose + 2 ADP + 2 NAD^+ + 2 P_i ➔ **2 Pyruvate** + 2 ATP + 2 NADH + 2 H^+

The NADH generated during glycolysis is used to fuel mitochondrial ATP synthesis via oxidative phosphorylation, producing either two or three equivalents of ATP depending upon whether the glycerol phosphate shuttle or the malate-aspartate shuttle is used to transport the electrons from cytoplasmic NADH into the mitochondria.

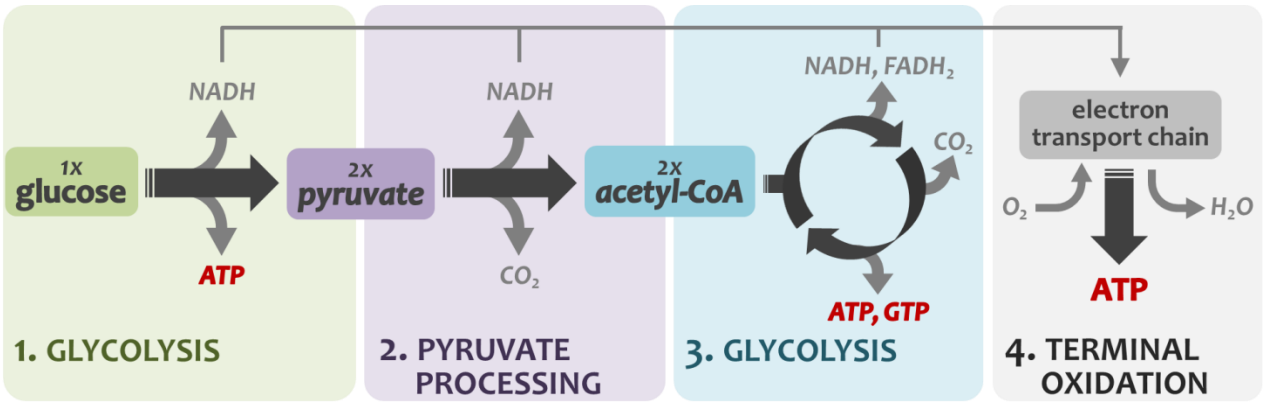
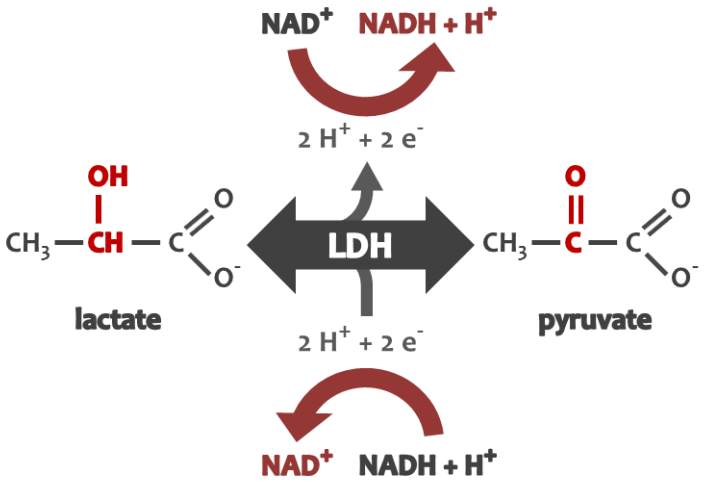


Figure 1. Integration of glucose catabolism

Complete oxidation of the 2 moles of pyruvate, through the TCA cycle, yields an additional 30 moles of ATP; the total yield, therefore being either 36 or 38 moles of ATP from the complete oxidation of 1 mole of glucose to CO_2 and H_2O.



Under anaerobic conditions and in erythrocytes, pyruvate is converted to lactate by the enzyme lactate dehydrogenase (LDH), and the lactate is transported out of the cell into the circulation. The conversion of pyruvate to lactate provides the cell with a mechanism for the oxidation of NADH to NAD^+.

Figure 2. Lactate dehydrogenase (LDH) reaction

The conversion of pyruvate to lactate provides the cell with a mechanism for the oxidation of NADH to NAD^+ which occurs during the LDH catalyzed reaction.

Glucose + 2 ADP + 2 Pi ••••➤ **2 Lactate** + **2 ATP** + 2 H_2O

Aerobic glycolysis generates substantially more ATP per mole of glucose oxidized than does anaerobic glycolysis. The utility of anaerobic glycolysis to a muscle cell when it needs large amounts of energy, stems from the fact that the rate of ATP production from glycolysis is approximately 100x faster than from oxidative phosphorylation. During exertion muscle cells do not need to energize anabolic reaction pathways. The requirement is to generate the maximum amount of ATP, for muscle contraction, in the shortest time frame. This is why muscle cells derive almost all of the ATP consumed during exertion from anaerobic glycolysis.

1.2. Glycolyic enzymes

The entire glycolytic pathway occurs in the cytosol of the cells and requires a determined sequence of ten enzyme-catalyzed reactions. Many of them may be catalyzed by several isoenzymes.

In biochemistry, ***isozymes (or isoenzymes)*** *are isoforms (closely related variants) of enzymes. Isozymes are enzymes that differ in amino acid sequence but catalyze the same chemical reaction. These enzymes usually display different kinetic parameters (e.g. different* K_M *and* V_{max} *values), intracellular localizaton, tissue or cell specific expression and different regulatory properties. The existence of isozymes permits the fine-tuning of metabolism to meet the particular needs of a given tissue or developmental stage. Amino acid substitutions that change the electric charge of the enzyme are simple to identify*

In point of diagostic role there are two important glycolytic izozyme families: aldolase and LDH family.

1.2.1. Aldolase (ALDO)

Aldolase catalyses the hydrolysis of fructose 1,6-bisphosphate (F-1,6-BiP) into two 3-carbon products: dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P). The aldolase reaction proceeds readily in the reverse direction, being utilized for both glycolysis and gluconeogenesis. Three aldolase isozymes (A, B, and C), encoded by three different genes, are differentially expressed during development.

- ***aldolase A (ALDOA)*** is found in the developing embryo and is produced in even greater amounts in adult muscle. Aldolase A expression is repressed in adult liver, kidney, and intestine and similar to aldolase C levels in brain and other nervous tissue.
- ***aldolase B (ALDOB)*** is preferentially expressed in the liver, kidney, and enterocytes;
- ***aldolase C (ALDOC)*** is highly expressed in the brain (specifically in the hippocampus and Purkinje cells).

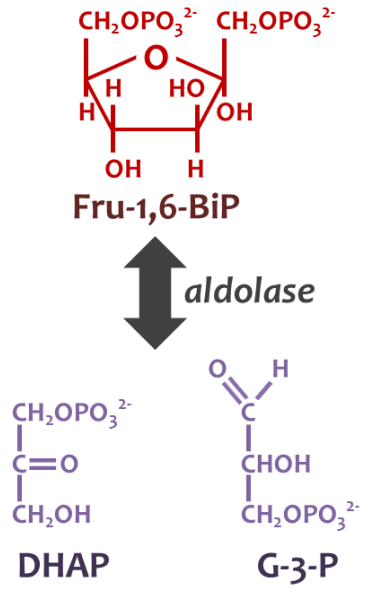


Figure 3. Aldolase rection

Aldolases A and C are mainly involved in glycolysis, while aldolase B is involved in both glycolysis and gluconeogenesis.

1.2.2. Lactate dehydrogenase (LDH)

Mammalian cells contain two distinct types of LDH subunits, termed M (muscle type) and H (heart type) Combinations of these different subunits generate five LDH isozymes with different characteristics.

The H type subunit predominates in aerobic tissues such as heart muscle (as H_4 tetramer) while the M type of subunit predominates in anaerobic tissues such as skeletal muscle (M_4 tetramer).

H_4 LDH has a low K_M for pyruvate and also is inhibited by high levels of pyruvate. The M_4 LDH enzyme has a high K_M for pyruvate and is not inhibited by pyruvate. This suggests that the H-type LDH is utilized for oxidizing lactate to pyruvate and the M-type the reverse.

	LDH1	LDH2	LDH3	LDH4	LDH5
subunit composition	H_4	MH_3	M_2H_2	M_3H	M_4
liver	-	*	*	*	***
cardiac muscle	***	**	*	*	*
skeletal muscle	*	*	*	*	***
brain	**	**	**	**	*
WBCs	*	*	***	**	*
lung	*	**	**	**	*
kidney	***	***	*	*	-
RBCs	***	***	**	*	*

Figure 4. LDH variants and their tissue distribution

RBC: red blood cell; leuko.: leukocyte

The other variants contain both types of subunits. Usually LDH-2 is the predominant form in the serum. A LDH-1 level higher than the LDH-2 level (a "flipped pattern") suggests myocardial infarction (damage to heart tissues releases heart LDH, which is rich in LDH-1, into the bloodstream). The use of this phenomenon to diagnose infarction has been largely superseded by the use of Troponin I or T measurement.

M and H protein subunits encoded by different genes:

- The M subunit is encoded by LDHA (chromosome 11p15.4)
- The H subunit is encoded by LDHB (chromosome 12p12.2-p12.1)
- A third isoform, LDHC (or LDHX), is expressed only in the testis; its gene is likely a duplicate of LDHA and is also located on the eleventh chromosome (11p15.5-p15.3)

1.3. Determination of enzyme activities of glycolytic enzymes

The activity of each glycolytic enzyme can easily be measured using a variety of different methods. In this practical we'll use two different procedures: a colorimetric method to quantify aldolase activity and the Warburg optical test to quantify the LDH activity.

1.3.1. Colorimetric determination of aldolase enzymatic activity

These assays are based on the quantitative determination of either a colored substrate or product. The assays rely on the quantification of either a colored substrate consumed or colored product produced. Since the vast majority of natural enzyme substrates and products are colorless (e.g. they do not absorb visible light) either a colored substrate analogue is used (if the enzyme accepts the modified molecule as its substrate) or the product of the enzymatic reaction is first converted to a colored derivative.

Aldolase activity measurement is based on the quantification of triose phosphates which form hydrazones with hydrazine.

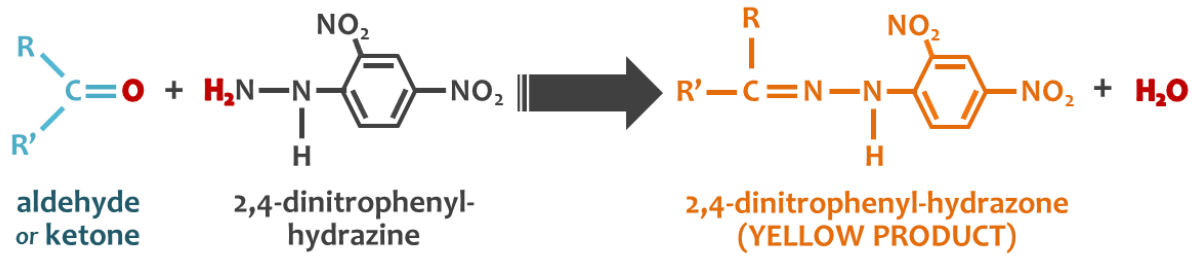


Figure 5. Hydrazine/hydrazone conversion

Since G3P hydrazone or DHAP hydrazone are not substrates of aldolase the equilibrium of the aldolase reaction is shifted toward the formation of triose phosphates.

To prevent the further conversion of G3P before it forms a hydrazone by glyceraldehyde-3-phosphate dehydrogenase (GAPDH), monoiodoacetate is used, which inactivates the latter enzyme also present in the sample. The enzymatic reaction is terminated by acidification of the solution, which irreversibly inactivates aldolase.

Triose phosphate hydrazones are converted to triose hydrazones by alkalification of the sample by the addition of NaOH. Under basic pH the phosphate group becomes unstable. In the next step triose hydrazones are reacted with dinitrophenyl hydrazine, to form a dinitrophenyl hydrazone derivative of the triose molecules. These compounds are colored, which is enhanced by alkalifying the solution with NaOH. (The phosphate group needs to be removed because the presence of the phosphorylation on the triose molecules would decrease the molar absorption coefficient of the colored dinitrophenyl hydrazone derivatives of the trioses making the assay much less sensitive.)

1.3.2. Warburg optical test for LDH activity measurement

One of the easiest ways to measure the activity of glycolytic enzymes is the, named after the brilliant Nobel-laureate biochemist Otto Heinrich Warburg. The Warburg optical test is a method in which the activity of an enzyme is determined by the photometric quantification of the change in $NAD(P)^+$/NAD(P)H ratios.

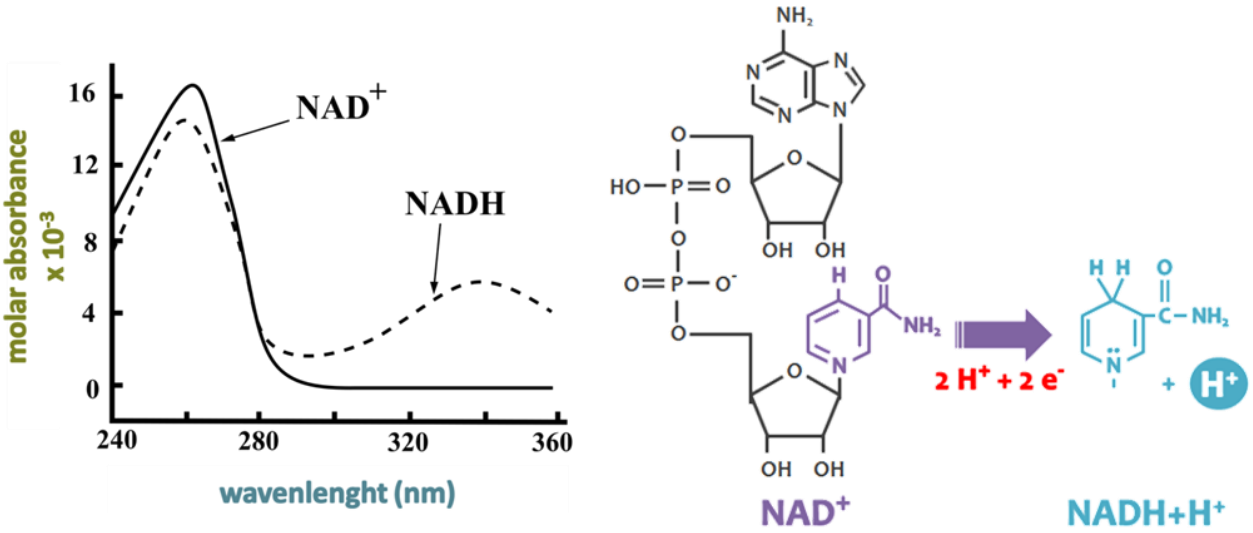


Figure 6. NAD^+/NADH

This is made possible by the different absorption spectra of the reduced and oxidized cofactors. The reduced form of the cofactor strongly absorbs at around 340 nm while the oxidized form

shows hardly any absorbance at the same wavelength. Since the measurement is carried out at the wavelength of around 340 nm, the test is often referred as the UV-test.

Activities of enzymes that catalyze oxidoreductive reactions utilizing NAD or NADP as a cofactor can be measured directly. This is called the *simple Warburg optical test*. For example in this practical the LDH activity will be directly measured by following the formation of NADH upon addition of lactate to the serum sample.

Yet enzymatic reactions that do not involve the oxidation or reduction of these cofactors can often be quantified with this method. The trick is to couple the enzymatic reaction to an indicator reaction, which does involve the oxidation or reduction of the NAD/NADH or NADP/NAPH cofactors. This is called the *coupled Warburg optical test*.

1.4. Clinical relevance of measuring glycolytic enzyme activities

Blood plasma is a complex mixture of ions, small molecules and macromolecules. The levels of each of these compounds may change in pathological circumstances, making the appropriate compounds ideal markers of the state of health or disease. Most enzymes are active within the cell - like all of the glycolytic enzymes -; therefore under non-pathological conditions only very low levels of these enzymes are found in the plasma. Cell and tissue injuries or infections often lead to the necrotic death of cells, in the process of which cellular proteins are released into the circulation. The increased activity of intracellular enzymes in serum is therefore a good indicator of cell, tissue or organ damage.

1.4.1. Aldolase

Normal range: 1.0 to 7.5 U/l. There is a slight difference between men and women. Normal value ranges may vary slightly among different laboratories.

A higher than normal level may be due to:

- Damage to skeletal muscles
- Heart attack
- Muscle disease (e.g. muscular dystrophy, polymyositis)
- Swelling and inflammation of the liver (hepatitis)
- Liver, pancreatic, or prostate cancer
- Viral infection called mononucleosis

The aldolase activity monitoring was used to follow up the pathological development of progressive muscular dystrophy. As the disease progresses more and more muscle undergoes dystrophy and as a result more and more aldolase is released to the blood. Since aldolase-B is expressed predominantly by the liver measurement of the aldolase-B activity has a great diagnostic value in recognizing hepatitis.

Genetic abnormalities: Aldolase A deficiency has been associated with myopathy and hemolytic anemia, while mutations leading to defects in aldolase B result in a condition called hereditary fructose intolerance.

1.4.2. LDH

LDH is most often measured to check for tissue damage.

Normal range: 105 - 333 U/L, which may vary slightly among different laboratories.

Drugs that can increase LDH measurements include anesthetics, Aspirin, Clofibrate, narcotics, alcohol etc.

If the LDH level is raised, the measurement of LDH isoenzymes helps determine the location of any tissue damage (all of the isoforms can be measured in the blood).

A higher-than-normal level may indicate:

- Blood flow deficiency (ischemia)
- Heart attack
- Hemolytic anemia
- Infectious mononucleosis
- Liver disease (e.g. hepatitis)
- Low blood pressure
- Muscle injury
- Muscle weakness and loss of muscle tissue (muscular dystrophy)
- Some cancers (e.g. Lymphoma)
- Pancreatitis, kidney disease
- Stroke
- Tissue death (e.g. intestinal and pulmonary infarction)

Genetic abnormalities: LDH deficiency is classified into H-subunit deficiency and M-subunit deficiency. Mutations of the M subunit have been linked to the rare disease exertional myoglobinuria, and mutations of the H subunit have been described but do not appear to lead to disease (to date, four cases of complete deficiency of LDH H-subunit have been detected).

In addition, abnormal electrophoretic patterns of serum LDH isoenzymes and additional bands may correlate with cancers found in humans.

In oncology, the ***Warburg effect*** is the observation that most cancer cells predominantly produce energy by a high rate of anaerobic glycolysis followed by lactic acid production in the cytosol, rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in mitochondria as in most normal cells. Malignant, rapidly growing tumor cells typically have glycolytic rates up to 200 times higher than those of their normal tissues of origin; this occurs even if oxygen is plentiful.

Metabolic reprogramming in cancer is controlled largely by oncogenic activation of signal transduction pathways and transcription factors.

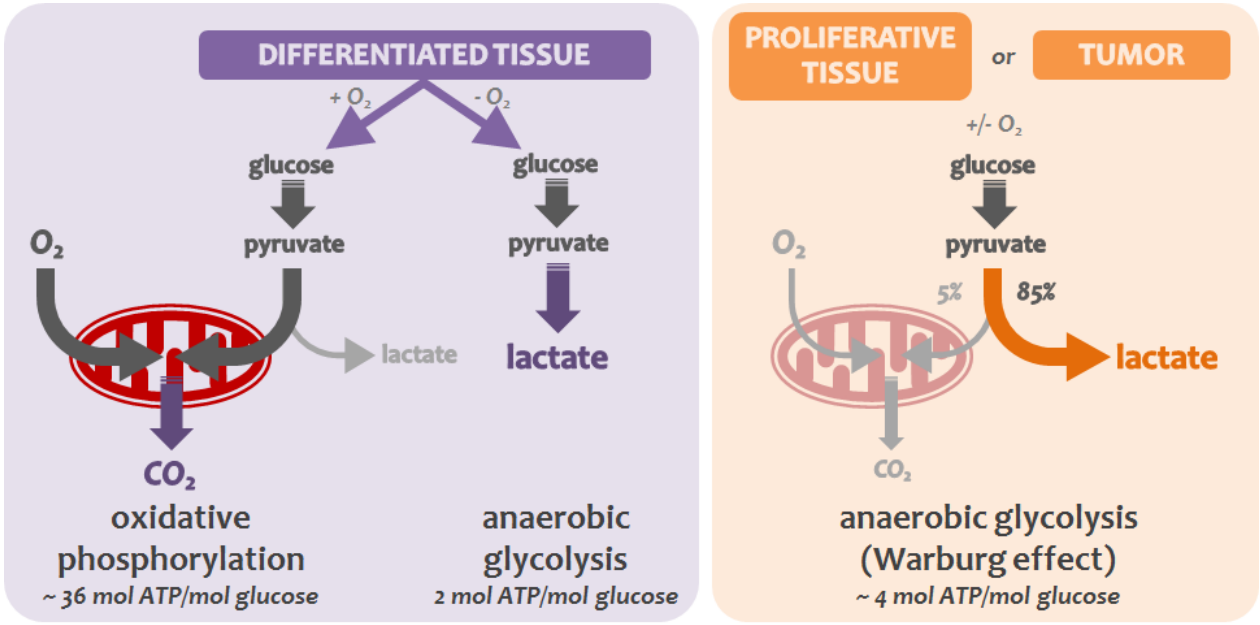


Figure 7. Warburg effect

Rapidly proliferating and cancer cells switch their metabolism to intensive glucose uptake and lactate production, while differentiated cells favor oxidative phosphorylation and anaerobic glycolysis.

The Warburg effect has important medical applications as high anaerobic glycolysis by malignant tumors is used clinically to diagnose and monitor treatment responses of cancers by imaging uptake of 2-18F-2-deoxyglucose (FDG) (a radioactive modified hexokinase substrate) with positron emission tomography (PET, figure 8).

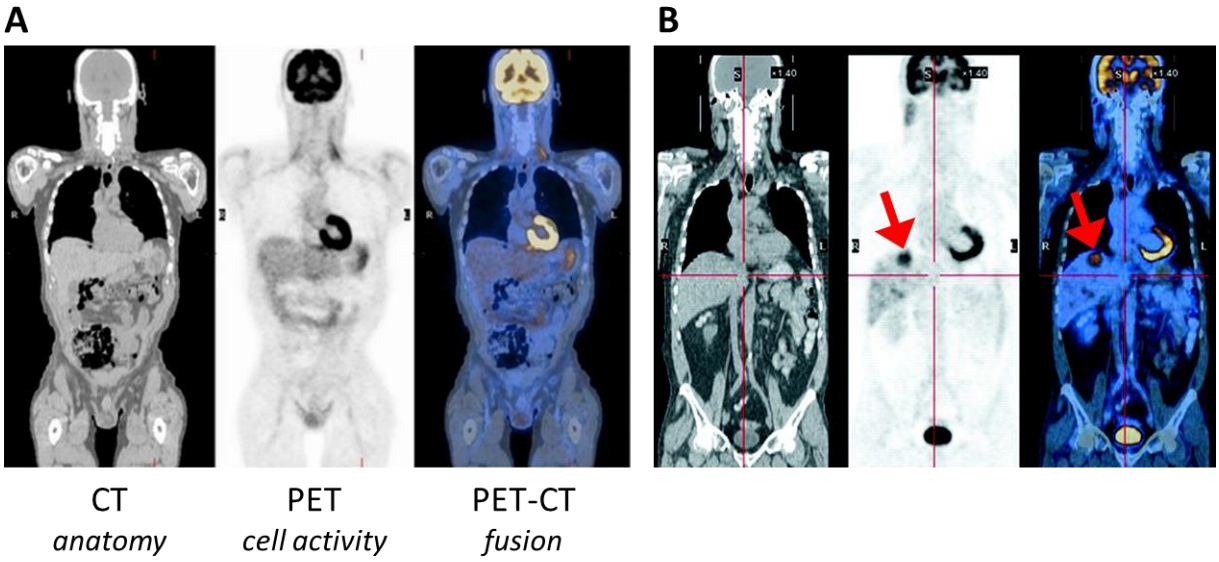


Figure 8. CT, PET, PET-CT imaging

CT: computed tomography, PET: Positron emission tomography

A normal; B liver metastasis (arrows)