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A. Pentose phosphate pathway. Brewer test

The pentose phosphate pathway (also called the phosphogluconate pathway and the hexose monophosphate shunt) is a metabolic pathway parallel to glycolysis.

The two major pathways for the catabolism of glucose have little in common. Although glucose 6-phosphate is common to both pathways, the pentose phosphate pathway is markedly different from glycolysis. Oxidation utilizes NADP rather than NAD, and CO2, which is not produced in glycolysis, is a characteristic product. No ATP is generated in the pentose phosphate pathway, whereas ATP is a major product of glycolysis.

The pentose phosphate pathway serves as an alternative glucose oxidizing pathway for the generation of NADPH that is required for reductive biosynthetic reactions such as those of cholesterol biosynthesis, bile acid synthesis, steroid hormone biosynthesis, and fatty acid synthesis. The pentose phosphate pathway can also function as an anabolic pathway that utilizes the six carbons of glucose to generate five carbon sugars, particularly ribose-5-phosphate (R5P) that is required for purine and pyrimidine nucleotide biosynthesis. The pentose phosphate pathway can, under certain conditions, completely oxidize glucose to $CO_2$and water.

Tissues with active pentose phosphate pathways are:

Tissue	**Function**
Adrenal gland	Steroid synthesis
Liver	Fatty acids and cholesterol synthesis
Testes	Steroid synthesis
Adipose tissue	Fatty acids synthesis
Ovary	Steroid synthesis
Mammary gland	Fatty acids synthesis
Red blood cells	Maintenance of reduced glutathione

The pathway is especially important in red blood cells (erythrocytes).

Enzymes that function primarily in the reductive direction utilize the $NADP^+$/NADPH co-factor pair as their co-factors as opposed to oxidative enzymes that utilize the NAD^+/NADH co-factor pair. The reactions of fatty acid biosynthesis and steroid biosynthesis utilize large amounts of NADPH. As a consequence, cells of the liver, adipose tissue, adrenal cortex, testis and lactating mammary gland have high levels of the PPP enzymes. In fact 30% of the oxidation of glucose in the liver occurs via the PPP. Additionally, erythrocytes utilize the reactions of the PPP to generate large amounts of NADPH used in the reduction of glutathione (see below). The conversion of ribonucleotides to deoxyribonucleotides (through the action of ribonucleotide reductase) requires NADPH as the electron source, therefore, any rapidly proliferating cell needs large quantities of NADPH.

Although the PPP operates in all cells, with high levels of expression in the above indicated tissues, the highest levels of PPP enzymes (in particular glucose 6-phosphate dehydrogenase) are found in neutrophils and macrophages. These leukocytes are the phagocytic cells of the immune system and they utilize NADPH to generate superoxide radicals from molecular oxygen in a reaction catalyzed by the NADPH oxidase complex. Superoxide anion, in turn, serves to generate other reactive oxygen species (ROS) that kill the phagocytized microorganisms. Following exposure to bacteria and other foreign substances there is a dramatic increase in O_2 consumption by phagocytes. This phenomenon is referred to as the oxidative burst or respiratory burst.

There are two distinct phases in the pathway. The first is the oxidative phase, in which NADPH is generated, and the second is the non-oxidative synthesis of 5-carbon sugars.

The primary results of the pathway are:

- The generation of reducing equivalents, in the form of NADPH, used in reductive biosynthesis reactions within cells (e.g. fatty acid synthesis, cholesterol).
- Production of ribose 5-phosphate (R5P), used in the synthesis of nucleotides and nucleic acids.
- Production of erythrose 4-phosphate (E4P) used in the synthesis of aromatic amino acids.

One of the uses of NADPH in the cell is to prevent oxidative stress. It reduces glutathione via glutathione reductase, which converts reactive H_2O_2 into H_2O by glutathione peroxidase. If absent, the H_2O_2 would be converted to hydroxyl free radicals by Fenton chemistry, which can attack the cell. Erythrocytes, for example, generate a large amount of NADPH through the pentose phosphate pathway to use in the reduction of glutathione. Hydrogen peroxide is also generated for phagocytes in a process often referred to as a respiratory burst.

Regulation

Glucose-6-phosphate dehydrogenase is the rate-controlling enzyme of this pathway. It is allosterically stimulated by $NADP^+$ and strongly inhibited by NADPH. The ratio of NADPH:$NADP^+$ is normally about 100:1 in liver cytosol. This makes the cytosol a highly-reducing environment. An NADPH-utilizing pathway forms $NADP^+$, which stimulates Glucose-6-phosphate dehydrogenase to produce more NADPH. This step is also inhibited by acetyl CoA.

Erythrocytes

The predominant pathways of carbohydrate metabolism in the red blood cell (RBC) are glycolysis, the PPP and 2,3-bisphosphoglycerate (2,3-BPG) metabolism (refer to discussion of hemoglobin for review of the synthesis and role role of 2,3-BPG). Glycolysis provides ATP for membrane ion pumps and NADH for re-oxidation of methemoglobin. The PPP supplies the RBC with NADPH to maintain the reduced state of glutathione. The inability to maintain reduced glutathione in RBCs leads to increased accumulation of peroxides, predominantly H_2O_2, that in turn results in a weakening of the cell wall as a result of membrane lipid peroxidation resulting in concomitant hemolysis. Accumulation of H_2O_2 also leads to increased rates of cysteine sulfhydryl oxidation in hemoglobin resulting in the formation of cross-linked complexes of denatured hemoglobin. Glutathione removes peroxides from membrane lipids and serves as a substrate for H_2O_2 reduction to H_2O via the action of glutathione peroxidase. The PPP in erythrocytes is the only pathway for these cells to produce NADPH, therefore, any defect in the production of NADPH, such as due to deficiencies in glucose-6-phosphate dehydrogenase, will have profound effects on erythrocyte survival.

Deficiency in the level of activity of glucose-6-phosphate dehydrogenase (G6PDH) is the basis of favism, primaquine (an anti-malarial drug) sensitivity and some other drug-sensitive hemolytic anemias, anemia and jaundice in the newborn. In addition, G6PDH deficiencies are associated with resistance to the malarial parasite, *Plasmodium falciparum*, among individuals of Mediterranean and African descent. The basis for this resistance is the weakening of the red cell membrane (the erythrocyte is the host cell for the parasite) such that it cannot sustain the parasitic life cycle long enough for productive growth.

Brewer test

Principle

Brewer test is a test for hemoglobin reduction in vitro, in terms of integrity of the pentose phosphate pathway. Hemoglobin found in erythrocytes is oxidized by sodium nitrite to methemoglobin. This process is reversible in the presence of methylene blue (if the pentose phosphate pathway is functioning in physiological conditions). If an enzymatic defect exists at the level of the pentose phosphate pathway, hemoglobin transformation will be irreversible.

Reagents

1. sodium nitrite 0.18m in 0.28M glucose
2. methylene blue 0.0004M in 0.9% NaCl
3. heparin 50 UI / 1ml blood

Procedure

Pipette reagents into three test tubes as follows:

Reagent	**T1**	**T2**	**T3**
heparin blood	2.00 ml	2.00 ml	2.00 ml
sodium nitrite	-	0.100 ml	0.100 ml
Methylene blue	-	-	0.100 ml

Mix the solutions in each test tube and incubate the mixture for 3 hours at 37^0C.

Results and discussions

The **test tube no 1**: contains only heparin blood so it is the reference tube for hemoglobin - **red color**

The **test tube no 2**: contains methemoglobin - **brown color**

The **test tube no 3**: contains the sample and this would be:

- red color similar with tube no. 1 (when the pentose phosphate pathway works and produces NADPH + H)
- brown color similar with tube no. 2 (when an enzymatic defect at the pentose phosphate pathway level exists and NADH + H^+ production is altered).

B. URIC ACID DETERMINATION

Introduction

Uric acid in the body originates from the catabolism of nucleic acids obtained from the diet or from the breakdown of cellular material in the body. In fact, uric acid is the end-product of purine catabolism in humans and primates. Purines (such as adenine and guanine) are components of nucleotides, which are the building blocks of the nucleic acids such as RNA and DNA. Other animals have the final product allantoin, due to the presence of a special enzyme, uricase, which can disrupt the hexagonal heterocycle of uric acid.

Hyperuricemia (elevated blood uric acid level) is caused by a variety of diseases or conditions. These include an increased synthesis of uric acid or a decreased excretion of uric acid. ***Gout*** is a condition resulting from overproduction or underexcretion of uric acid. It is typified by the deposition of urate crystals in the joints and tissues which cause inflammation.

Renal retention is associated with renal failure and toxicity caused by lead and alcohol. It may also be caused by the administration of salicylates and diuretics.

Hyperuricemia also appears in leukemia, myeloma, Hodgkin lymphoma, infectious mononucleose, psoriasis, Pb and Hg intoxications, extended burnings, etc.

Hypouricemia (decreased blood uric acid level) is not common. It may occur from severe hepatocellular disease or impaired renal tubular reabsorption of uric acid, in xanthine oxidase deffect. Occasionally, it is a result of treatment for hyperuricemia.

Experimental part

Uric acid determination in plasma or serum

Uric acid can be determined by the phosphotungstic acid method or by an enzymatic method using uricase.

1. Enzymatic method

Principle

Uric acid is oxidized to allantoin and hydrogen peroxide in the presence of water and oxigen according to the formula:

$$\text{uric acid} + 2H_2O + O_2 \xrightarrow{\text{uricase}} \text{allantoin} + H_2O_2 + CO_2$$

This reaction is monitored by two methods:

- One is based in the decrease in absorption at 293 nm given by uric acid.
- In the other, the hydrogen peroxide that is produced is utilised in a coupled reaction catalysed by peroxidase to produce a colour change in a redox indicator such as o-dianisidine, o-toluidine, methylbenzothiazolinone hydrazone, or dichlorohydroxybenxene.

$$2H_2O_2 + \text{3,5-dichloro-2-hydroxybenxenesulphonic acid} + \text{4-aminophenazone} \xrightarrow{\textit{peroxidase}} \text{N-(4'-antipyril)-3-chloro-5-sulphonate-p-benzochinonimin}$$

Reagents

1. Buffer solution: phosphate buffer, pH 7 - 50 mM; 3,5-dichloro-2-hydroxybenzen-esulfonic acid 4 mM.
2. Enzymatic reagent: 4-aminophenazone - 0.3 mM; peroxidase - 1000 U/litre; uricase - 200 U/litre all in the buffer solution (1). The stability of the solution is 21 days at 2-8°C and 5 days at 15-25°C, at dark.
3. Standard solution of uric acid: 10 mg%

Procedure

Pipette the reagents as follows:

Reagents (µl)	**Sample**	**Standard**	**Blank**
Serum	20	-	-
Standard uric acid	-	20	-
Enzymatic reagent	1000	1000	1000

Mix the tubes and incubate them 15 minutes at 20 - 25°C. Read the sample and standard absorbencies at 520 nm, within 30 minutes, zeroing the spectrophotometer with the blank.

Calculation

$$\textbf{mg uric acid \%} = \frac{E_{sample}}{E_{sstandard}} \times 10$$

2. Colorimetric method

Principle

It is a method based on the reductive character of uric acid. Uric acid reduces phosphotungstic acid, in alkaline medium, forming a blue coloured complex. The colour intensity is directly proportional to the uric acid concentration in the analysed sample. Ascorbic acid interferes with the reaction but it is eliminated by the alkaline pH (10-11) of the buffer.

Reagents

1. Deproteinisation solution: anhydrous sodium sulphate - 150g; 0.1N sulphuric acid - 1000ml
2. Phosphotungstic reagent: sodium tungstate - 50g; 85% phosphoric acid - 40ml; distilled water - 400 ml. The mixture is boiled at reflux for 4 hours. If the solution is green, 1-2 drops of bromine are added and the solution is boiled again for a few minutes. After cooling, the solution is diluted to 500 ml with distilled water.
3. Glycine buffer solution, pH = 12.5: Glycine - 3g and 0.1N NaOH - 100 ml.
4. Stock standard solution of uric acid - 100 mg%: 100 mg uric acid and 50 mg lithium carbonate are dissolved in 60 ml of bidistilled water at 60°C. The solution is cooled and 2.5 ml of formaldehyde 40% and 0.3 ml 0f acetic acid are added, than the solution is diluted at 100 ml with bidistilled water.
5. Work standard solution of uric acid - 5 mg%; is obtained diluting the stock standard solution.
6. Serum or plasma may be used. If plasma is used, the blood should be collected in EDTA and not with heparin.

Procedure

1. Serum deproteinisation

Introduce 0.5 ml of serum in a test tube; add 4.5 ml of deproteinisation solution and boile the mixture 15 minutes on the water bath, shaking from time to time. Filter the warm solution on a dried filter in another test tube.

2. Colour reaction

Pipetted the reagents in the test tubes as follows:

Reagents, ml	**Sample**	**Standard**	**Blank**
Deproteinized filtrate	2.0	-	-
Deproteinisation solution	-	1.8	1.8
Work standard	-	0.2	-
Distilled water	-	-	0.2
Buffer solution	0.5	0.5	0.5
Mix and let for 5 minutes at room temperature, than add:			
Phosphotungstic reagent	0.1	0.1	0.1

Shake the solutions and let them at room temperature for 15 minutes. Read the sample and standard absorbencies at 710 nm, zeroing the spectrophotometer with the blank.

Calculation

$$\text{mg uric acid \%} = \frac{E_{sample}}{E_{standard}} \times 5$$

Normal Values

Adults: 1 - 7 mg/100 ml serum

Children: 1 - 3 mg/100 ml serum