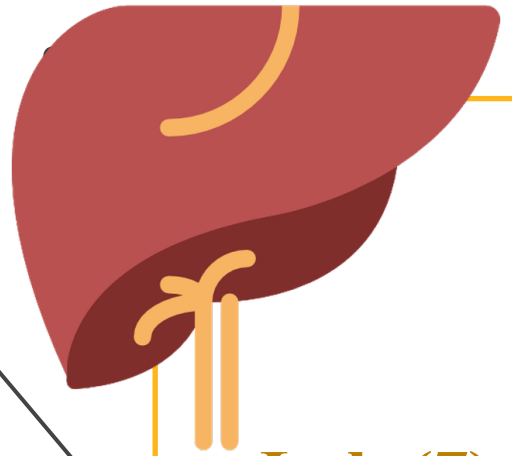


Blood Biochemistry BCH 471[Practical]

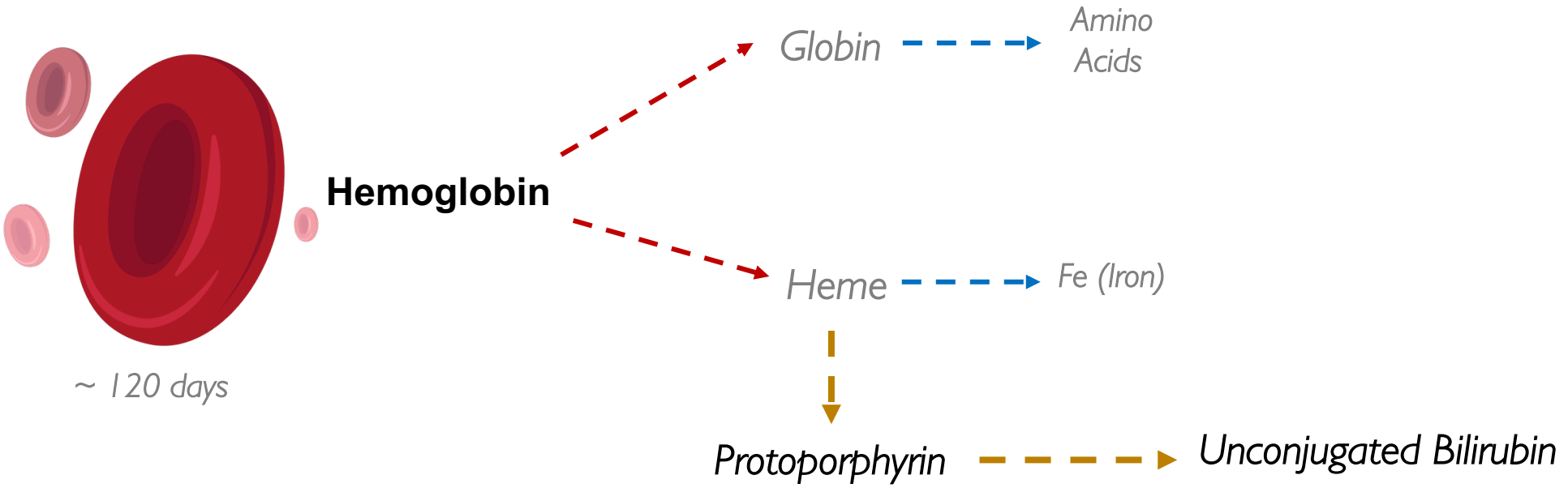
Lab (7) Estimation of Serum Bilirubin (Total & Direct)

Objective

- To estimate the amount of bilirubin in serum.

Bilirubin

- It is the **yellow** breakdown product of normal heme catabolism.
- Heme is formed from **hemoglobin**, a principal component of red blood cells.
- Bilirubin is excreted in bile, and its levels are elevated in certain diseases.
- It is responsible for the yellow color of bruises and the yellow discoloration in **jaundice.**

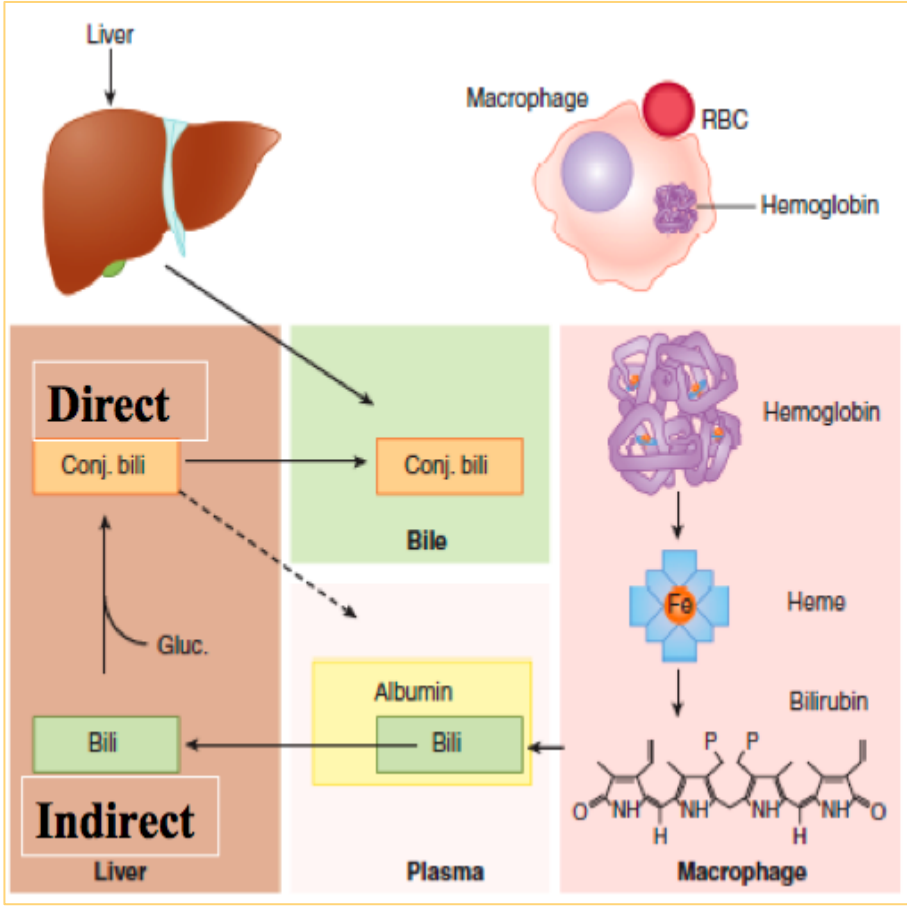


Types of Bilirubin

- **Direct bilirubin:** Conjugated with glucuronic acid, water soluble.
- **Indirect bilirubin:** unconjugated, water insoluble.
- **Total bilirubin:** sum of the direct and indirect of bilirubin.

Notes:

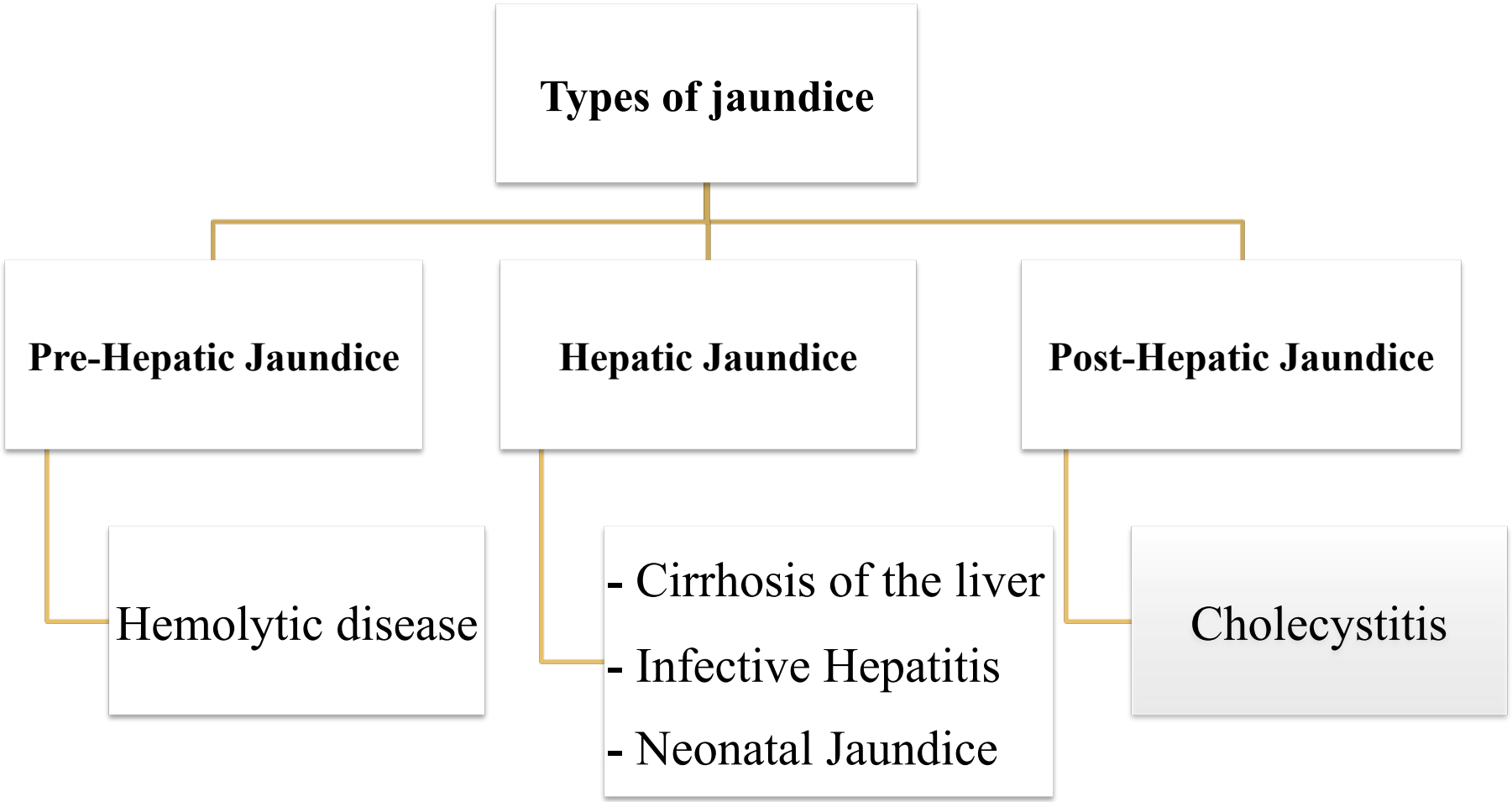
1. **About 200 mg** per day of unconjugated bilirubin are transported to the liver.
2. Disturbances in the powers of conjugated and/or excretion of the liver of this yellow compound will lead to raised levels in serum.



Bilirubin and Jaundice

- Above about 2 mg/dl in the blood, leads to disease called **Jaundice.**
- Jaundice is caused by a **build-up of bilirubin (yellow color)** in the blood and tissues of the body.
- Jaundice is the **discoloration** of skin and sclera of the eye caused by high concentration of bilirubin.

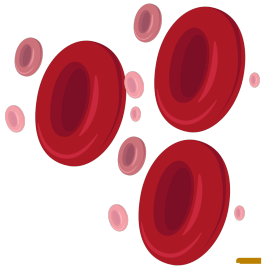
Causes of Jaundice



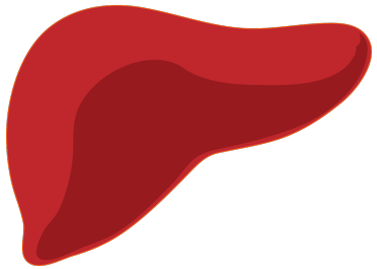
Pre-Hepatic Jaundice

Hemolytic disease (excess hemolysis)

- The production of un-conjugated bilirubin may **exceed the conjugating capacity of the liver.**
- Direct bilirubin in the upper normal range or just a little elevated.
- The serum levels of indirect (and of total) bilirubin **will be raised.**
- The other liver function tests will usually give **normal results.**



Excess Hemolysis



Indirect bilirubin ► increased

Direct bilirubin ► Slightly increased

Total bilirubin ► increased

↑↑↑ UCB + ↑CB = ↑↑↑ TB

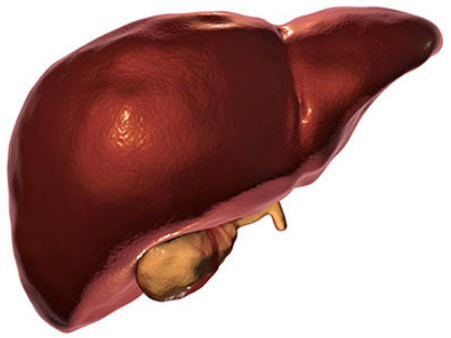
- ↑ Unconjugated bilirubin (in blood)
- Upper normal range conjugated bilirubin (released to bile duct)

Hepatic Jaundice

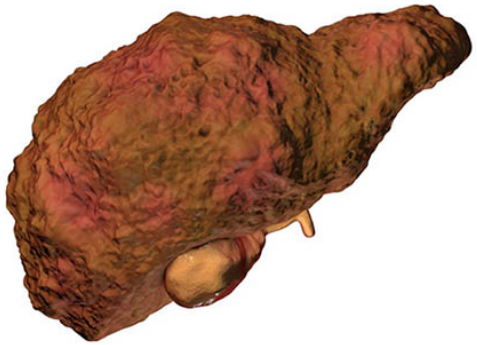
1- Cirrhosis (in the absence of infection)

- Destruction of liver cells will lead to a reduced conjugating capacity.
- **Raised** serum level of indirect (and of total) bilirubin.
- **Low** level of direct bilirubin.
- An **abnormally high release**, into the blood, of the enzymes: AST, ALT and ALP.
- Synthesizing power of liver will be **diminished** and hence low levels of total protein, albumin and cholesterol.

↑↑↑ UCB + ↓CB = ↑↑↑ TB



Healthy liver

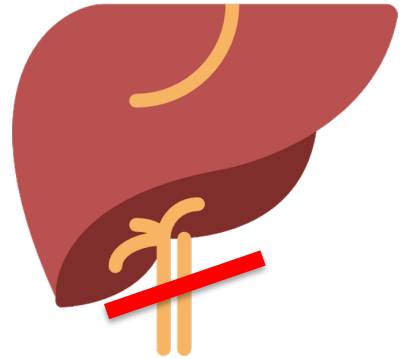


Cirrhotic liver

Hepatic Jaundice

2- Hepatitis (in the presence of infection)

- The conjugative capacity of the liver is approximately normal, but there is the inability to transport the conjugated bilirubin from the liver cells to the biliary system, and it will be regurgitated back into the blood.
- The serum level of **unconjugated** bilirubin will be **normal**.
- **Conjugated** (and total) bilirubin will be **raised**.
- Synthesizing power is diminished leading to **low** serum levels of proteins but the **raising** of antibodies to infection usually leads to raised total proteins level.



- Normal unconjugated bilirubin (in blood)
- ↑ conjugated bilirubin (in blood)

Hepatic Jaundice

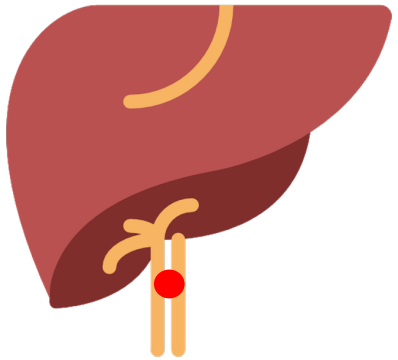
3- Neonatal Jaundice

- Conjugating enzymes in the liver are often **absent at birth.**
- **Raised** serum level of indirect (and total) bilirubin is to be expected.
- **Low** level of direct bilirubin.
- The other liver functions are **normal**.
- The indirect bilirubin level will rise for the first few days after birth until the conjugating enzymes begin to synthesize.
- If the conjugation process is delayed and the serum level of indirect bilirubin rises towards **20 mg/dl**
- Can be treated by **Phototherapy** or an exchange **blood transfusion.**
- Deposition of the insoluble unconjugated bilirubin into basal ganglia of the brain leads to **permanent Brain Damage.**

Post-hepatic Jaundice

Cholecystitis

- The bile duct is **blocked**.
- The indirect bilirubin level is **normal** but conjugated bilirubin is regurgitated into the blood and excreted into the urine (**raised conjugated and total bilirubin**).
- Enzymes will be regurgitated into the blood giving **raised** levels.
- The other liver function tests are **normal**.



- Normal unconjugated bilirubin (in blood)
- ↑ conjugated bilirubin (in blood)

Practical Part 💉

Principle

- Bilirubin in serum is coupled with **diazotized sulfanilic acid** to form **azobilirubin**.
- The water soluble conjugated bilirubin (direct bilirubin) reacts easily with reagents such as **diazotized sulfanilic acid.**
- while the water insoluble unconjugated bilirubin (indirect bilirubin) requires a solubilizing reagent, such as **Caffeine**, in order to react with the **diazotized sulphanilic acid.**
- In this experiment, the direct bilirubin is estimated in the absence of the solubilizing agent and then further bilirubin estimation in the presence of the solubilizing agent will give the **total bilirubin level.**
- The indirect or unconjugated bilirubin is then found by difference.

💡 **Pause and Think why direct/indirect bilirubin are called so?**

Conjugated bilirubin (direct bilirubin) + diazotized sulfanilic acid → azobilirubin

Unconjugated bilirubin (indirect bilirubin) + diazotized sulfanilic acid $\xrightarrow{\text{Caffeine}}$ **azobilirubin**

Method

- Label 4 tubes as **TT** (total test), **TB** (total Blank), **DT** (direct test), **DB** (direct Blank).

Solutions	Direct Bilirubin		Total Bilirubin	
	TB	DT	TB	TT
Solution 1 (sulfanilic acid + HCl	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Solution 2 (Sodium nitrate)	--	0.05 ml	--	0.05 ml
Solution 3 (Caffeine + Sodium benzoate)	--	--	1 ml	1 ml
NaCl solution 0.9%	2 ml	2 ml	--	--
Sample	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Mix, let stand for 5 min. at 20-25ºC. Read absorbance of test against blank (A_{DB}) **for direct only at 546 nm.** **For total** stand for 30 min at 20-25oC.				
For total bilirubin Solution 4 (NaOH + tartarate)	--	--	1 ml	1 ml
Mix and let stand for 15 min and read the absorbance **at 578 nm against blank** (A_{TB}).				

Pause and Think why we used NaCl solution 0.9%

Calculations

- **Concentration of direct bilirubin** = (abs. DT) x 14.4 = mg /dl

Normal range: Up to 0.25 mg/dl

- **Concentration of total bilirubin** = (abs. TT) x 10.8 = mg /dl

Normal range: Up to 1 mg/dl

- **Concentration of indirect bilirubin** = Conc. of total bilirubin – Conc. of direct bilirubin= mg /dl

Normal range: 0.1-0.4 mg/dl