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Bile pigments
Porphyrins

General Medicine

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1 Bile pigments

Bile pigments are compounds that contain **four pyrrole rings in linear arrangement**, and originate from degradation of heme. They encompass **bilirubin**, **urobilinogen**, **stercobilinogen**, and their oxidation products **urobilin** and **stercobilin**.

1.1 Origin of bile pigments

Majority of bilirubin originates from **degradation of hemoglobin** released from red blood cells (75%); the rest comes from catabolism of other hemoproteins (cytochromes, catalase, peroxidase, myoglobin, etc.)

There are two mechanisms of hemoglobin breakdown, according to where degradation of erythrocytes is localized:

- extravascular – outside the blood vessels in the reticuloendothelial system or tissue macrophages
- intravascular – inside the blood vessels

The extravascular breakdown of red blood cells takes place in the macrophages of spleen, liver, and bone marrow. Under normal condition more than 90 % of hemoglobin is degraded in this way.

Bilirubin formation proceeds in several steps:

- Removal of iron and globin from the hemoglobin molecule together with opening of the porphyrin ring between pyrrole I and II and release of carbon monoxide gives rise to a linear tetrapyrrole, **green** pigment **biliverdin**.
- Reduction of the central methenyl bridge between pyrrole III and IV in the biliverdin molecule by action of **biliverdin reductase** produces **yellow bilirubin**.

This form of bilirubin is denoted as **unconjugated bilirubin**. It is **insoluble in water**, and rather easily passes biomembranes and enters the cells, where it is strongly toxic. That is why bilirubin, following release from its site of origin, is transported in circulation **bound to albumin**. 100 ml of plasma contains about 25 mg of bilirubin tightly bound to a high affinity site on albumin. The bond is non-covalent and other substances compete with bilirubin for this binding (fatty acids, lipophilic drugs and others).

With blood bilirubin enters the liver where it is imported into hepatocytes and **conjugated with glucuronic acid**, producing bilirubin monoglucosiduronates and (mostly) bilirubin diglucosiduronates. Esters with glucose and xylose are formed as well, to some extent. All these forms are collectively designated as **conjugated bilirubin**. Conjugation renders bilirubin **soluble in water.** Normally only traces of conjugated bilirubin are found in blood.

In cases of persistent elevation of conjugated bilirubin in plasma yet another form, called **delta bilirubin** appears. It represents portion of conjugated bilirubin that underwent a non-enzymatic **reaction with albumin** or other plasmatic proteins generating a covalent peptidic bond (propionyl of the bilirubin side chain reacts with a free NH_2 group of lysine on albumin). Level of delta bilirubin is a measure of previous lasting of conjugated hyperbilirubinemia. The complex bilirubin-protein does not pass normal glomerular membrane, that is why conjugated bilirubin is absent from urine in these patients.

Conjugated bilirubin enters bile ducts and with bile is excreted into duodenum. In the intestine, bacterial enzyme **β-glucuronidase** removes the glucuronic acid; and bilirubin is reduced to

- **urobilinogen** (colorless)
- **stercobilinogen** (colorless)

Amount of urobilinogen formed in the body is proportional to the amount of bilirubin that enters the intestine. Most of the urobilinogen is excreted in stool; small part, however, is absorbed and through **enterohepatic circulation** it reaches the liver, where it is again excreted to the bile. Trace amount of urobilinogen can be excreted to urine. Analytical differentiation between urobilinogen and stercobilinogen is difficult and without clinical significance.

The unabsorbed urobilinogens and stercobilinogens in the large intestine spontaneously oxidize to

- **urobilin** (brown)
- **stercobilin** (brown)

They are excreted in stool.

1.2 Bilirubin in serum

Almost all tests for bilirubin are based on the **azo coupling reaction** of bilirubin with some **diazonium salt. Diazotized sulfanilic acid**, produced by reacting sulfanilic acid with sodium nitrite in excess of hydrochloric acid (diazo reagent)[1], is used most often – **the Van den Bergh diazoreaction**. In the presence of diazotized sulfanilic acid bilirubin splits to yield two molecules of azobilirubin (Fig. 1). The azobilirubin behaves as an acid-base indicator with several color transitions: in weakly acidic medium it is red, whereas in strong alkali it has blue color.

Conjugated bilirubin reacts with diazotized sulfanilic acid directly and rapidly – **direct Van den Bergh reaction**. This fraction of bilirubin is therefore called **"direct" bilirubin**.

Unconjugated bilirubin, which is poorly soluble in water and non-covalently bound to albumin, reacts with diazo reagent much more slowly and the color develops only after addition of an **accelerator** – **indirect Van den Bergh reaction**. As the accelerator, alcohols (methanol, ethanol) or other substances (sodium benzoate, caffeine, urea) are used. The accelerator facilitates reaction by releasing bilirubin from bond to albumin and disrupts intramolecular hydrogen bridges, rendering bilirubin more soluble. Fraction of bilirubin that requires an accelerator to give the reaction is called **"indirect" bilirubin**.

In the presence of accelerator all forms of bilirubin react; hence, in this way **total bilirubin** is estimated. The actual amount of unconjugated (indirect) bilirubin can be calculated as a difference between total and direct bilirubin (Fig. 2).

Photometric estimation of total bilirubin is best performed in alkaline medium, where it is the most sensitive, and interference of other compounds in serum is minimal. The basic pH is provided by strongly alkaline tartrate. Nowadays, the recommended approach for routine estimation is the **Jendrassik-Grof modification**, utilizing caffeine and sodium benzoate as the accelerator.

[1] The Ehrlich diazo reagent: Diazo I: sulfanilic acid in HCl, diazo II: sodium nitrite

Fig.1: Reaction of bilirubin with diazotized sulfanilic acid (according to: Kaplan L.A, Pesce A.J.: Clinical Chemistry. Theory, Analysis, Correlation. 3rd edition. Mosby 1996, p. 525)

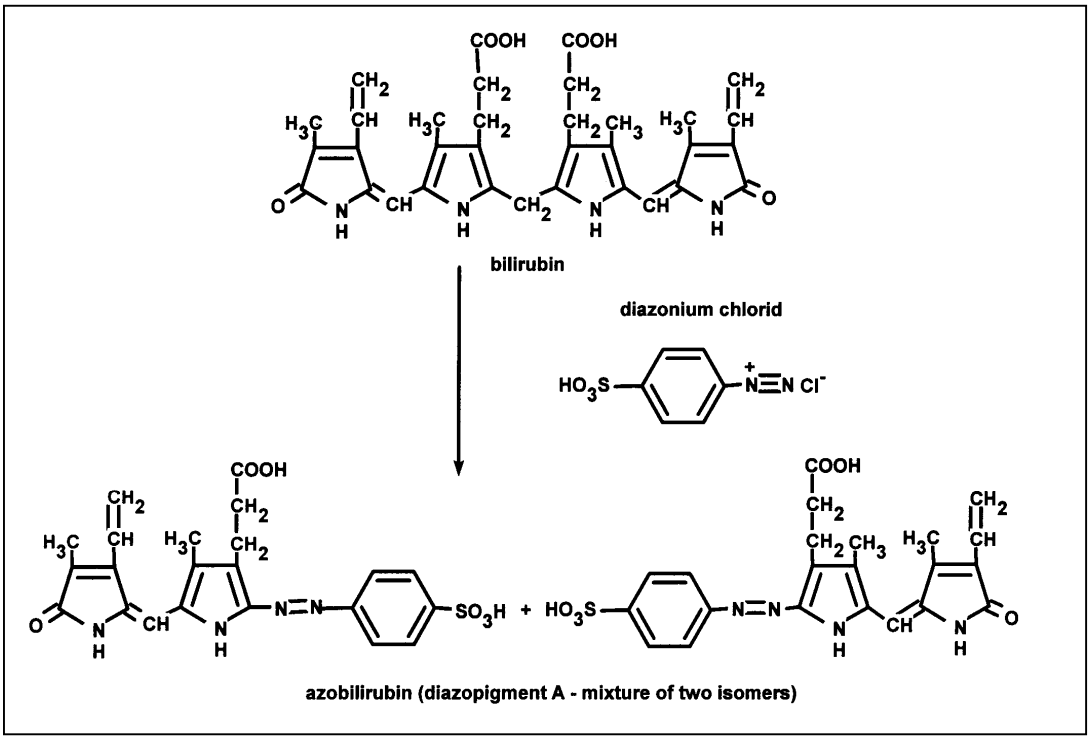
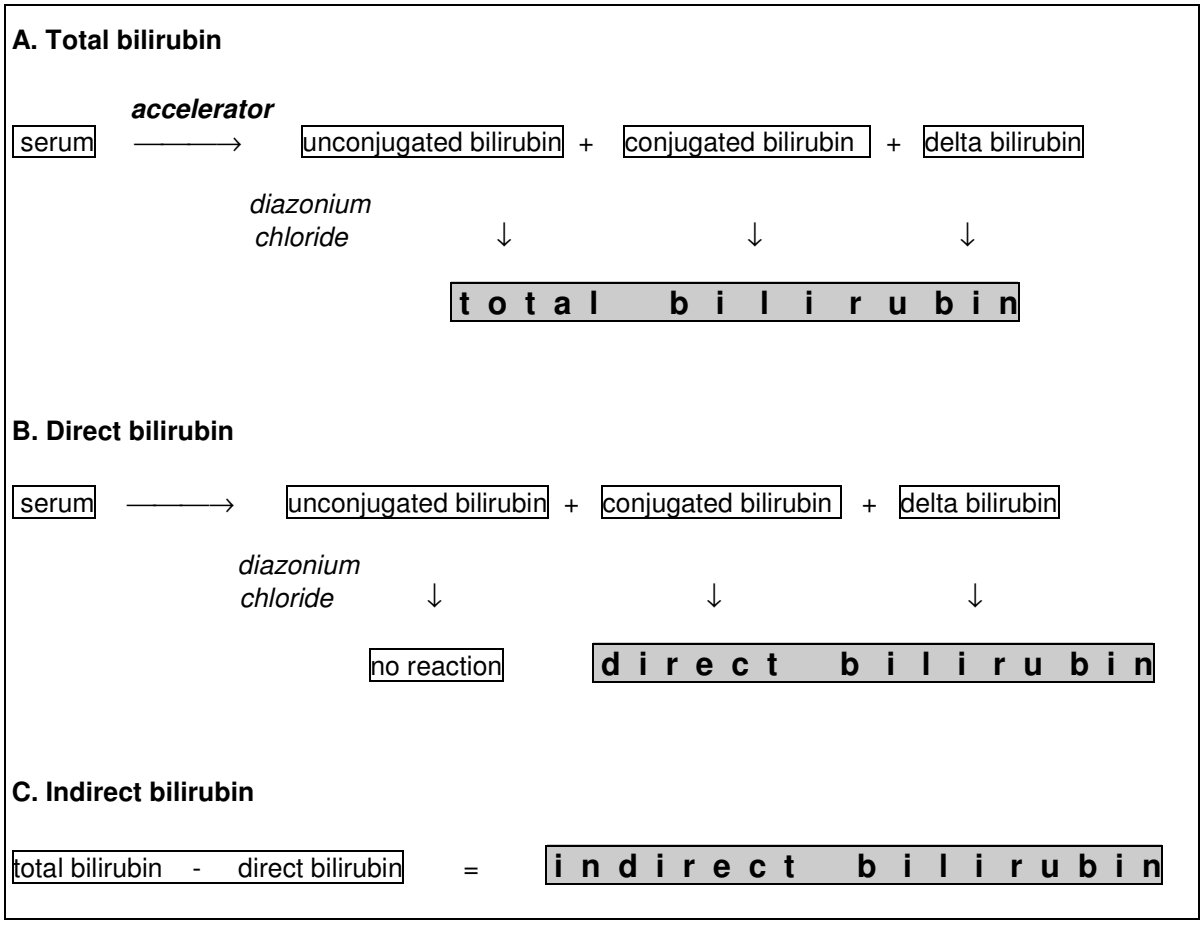


Fig.2: Estimation of total, direct and indirect bilirubin (according to: Calbreath D.F.: Clinical Chemistry. A Fundamentals Textbook. W.B. Saunders Company 1992, str. 228)



For measurement of total bilirubin also a **direct spectrophotometry** can be used, since absorbance of serum at 454 nm is proportional to concentration of bilirubin. It is employed for instance in the estimation of bilirubin in amniotic fluid, cerebrospinal fluid, or blood of the newborn in monitoring of newborn jaundice. **Transcutaneous icterometry** is yet another way how to roughly estimate bilirubin, being used in neonatology for examination of newborn icterus. A manual device, 'bilirubinometer' is put on the front of the newborn for measurement.

Bilirubin is strongly photosensitive, therefore, samples must be **kept in the dark** and processed as soon as possible.

Reference values:

fS-Total bilirubin: up to 17 µmol/l

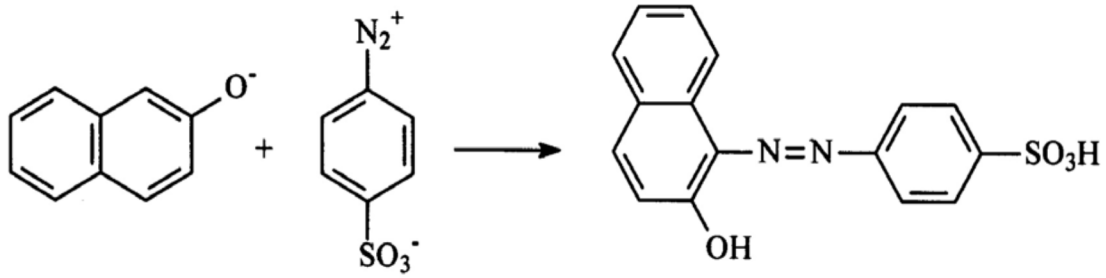
fS-Direct bilirubin: up to 5 µmol/l

Azo coupling reactions:

Arene diazonium salts originate from reactions of aromatic amines with nitrous acid. Their general structure is **Ar_1-$N^+\equiv N$** (Ar … arene, aromatic compound).

They further react (couple) with an aromatic activated hydroxyl- or amino- compound (Ar_2) in acidic medium, yielding a colored product **Ar_1-N=N-Ar_2**. A conjugated system of π-electrons of variable length arises, which absorbs the electromagnetic radiation in the visible range. A wide array of vividly colored substances can be made in this way. They have found usage not only in spectrophotometric assays in biochemistry (bile pigments), but also in industrial production of dyes (the azo dyes).

For example, the coupling of diazonium salt of sulfanilic acid (4-aminobenzene sulfonic acid) with β-naphthol gives a red-orange stain Orange II:



β-naphthol + diazonium salt of sulfanilic acid → Orange II (azo dye)

1.3 Bile pigments in urine

1.3.1 Bilirubin in urine

Only **conjugated bilirubin** can ever be found in urine. Unconjugated bilirubin is bound to albumin and does not pass to glomerular filtrate. Under normal condition urine is practically free of bilirubin. The tests for bilirubin in urine become positive when serum levels of conjugated bilirubin reach 30-34 μmol/l (renal threshold for bilirubin). Presence of higher amount of bilirubin gives urine a **dark-brown color**.

In order to test urine for bilirubin, nowadays **diagnostic strips** are used. The test is based on the **azo coupling reaction**: conjugated bilirubin reacts with a **stabilized diazonium salt** in the strip reaction zone and a pink or red dye develops. In case of simultaneous presence of high concentration of urobilinogen the color turns to orange; in this case re-evaluation 2 minutes after sample application is recommended. False low or even negative results can be caused by high amount of ascorbic acid (vitamin C) in the urine sample. Also, the urine samples must be protected from direct sunlight, which would cause oxidation of bilirubin and again lead to false low or negative result.

It is also possible to use other, classical tests for bilirubin that are based on **oxidation to green biliverdin** (Gmelin's test with nitric acid, Rosin's test with iodine) or blue bilicyanine.

1.3.2 Urobilinogen in urine

Urobilinogen is a normal product of bacterial degradation of bilirubin that is to some extent physiologically excreted in urine. Concentration 17 μmol/l is considered as the upper limit of its physiological urinary excretion.

Increased amount of urobilinogen in urine is a sensitive marker of an overload of the liver functional capacity – the hepatic parenchyma fails to take up majority of urobilinogens absorbed in the intestine. This condition occurs for instance in over-production of bilirubin (pre-hepatic icterus), or in the icterus caused by a primary damage to the hepatocyte (hepatocellular icterus).

On the other hand, **total absence of urobilinogen** from urine accompanies a **complete blockade of biliary ducts**, or failure of bile production due to a very severe liver damage. Another cause may be an **absence of intestinal microflora** that is necessary for reduction of bilirubin. The latter condition comes physiologically in the newborn; or it can result from antibiotic therapy.

If biliary tract is obstructed the stool is whittish-grey due to absence of all bile pigments and due to high content of undigested fat. In contrast, in the case of missing bacterial flora, bilirubin is excreted in the stool either unchanged, or oxidized to biliverdin.

Urobilinogen in urine is demonstrated again with **diagnostic strips**; the test is based on the **coupling reaction with diazonium salt**. The indication zone turns pink or red in the presence of urobilinogen. A weak pink color corresponds to physiologic excretion of urobilinogen. Simultaneous presence of bilirubin changes the color to yellow, further turning to blue or green in one minute.

As a rough preliminary test, the classical reaction with the **Ehrlich aldehyde reagent (4-dimethylaminobenzaldehyde in HCl)** can be used as well. Urine sample is mixed with the reagent and heated; a red condensation product of 4-dimethylaminobenzaldehyde with the

central methenyl bridge of urobilinogen is formed. The test is rather unspecific since porphobilinogen, porphyrins, indol, scatol, and some drugs give the reaction as well.

Tests for urobilinogen must be performed with fresh and cooled urine. During standing, especially on the sunlight, urobilinogens in the sample oxidize to urobilins. Every healthy urine should give a red color upon heating with the Ehrlich reagent, indicating the physiological, trace amounts of urobilinogen.

1.4 Hyperbilirubinemia and icterus

Examination of bile pigments in serum and urine provides valuable information on the intensity of breakdown of red blood cells, as well as on the function of the liver and biliary tract.

If concentration of serum bilirubin exceeds **17 µmol/l**, we talk about **hyperbilirubinemia**. It can in principle arise either from overproduction of bilirubin, or from defects in its metabolism and secretion. Bilirubin passes from blood stream to the tissues and if bilirubinemia exceeds about 35 µmol/l, it brings about a **yellow color of the eye white and the skin** – condition known as **jaundice, icterus**. Because bilirubin is degraded by light, the yellowish color is first visible only on mucosal membranes and on the parts of sclera covered by eyelids (subicterus). Color of skin in moderate hyperbilirubinemias need not be explicitly yellow; rather it can resemble a sun tan.

We can distinguish three main types of hyperbilirubinemias:

- **unconjugated: pre-hepatic (hemolytic) icterus**
  - increased bilirubin production, e.g. hemolytic anemia
  - decreased uptake and/or conjugation in the liver, e.g. jaundice of the newborn, some inborn errors such as Gilbert's disease, Crigler-Najjar's syndrome
- **conjugated: post-hepatic (obstructive) icterus**
  - biliary obstruction, e.g. due to cholelithiasis (stones in gall bladder or biliary ducts) or tumors of biliary tract or pancreas
  - impairment of the secretion of conjugated bilirubin, e.g. some inborn errors such as Dubin-Johnson's and Rotor's syndromes
- **mixed: hepatocellular (hepatic) icterus**
  - e.g. viral hepatitis, toxic liver damage

Table 1: Characteristic findings of the examination of bile pigments in serum and urine in different kinds of icterus:

Type of icterus	*Finding in serum*	*Bilirubin in urine*	*Urobilinogen in urine*	*Color of urine*	*Color of stool*
Pre-hepatic	Indirect bilirubin increased	Negative	Positive (higher than physiological)	Normal	Dark
Post-hepatic	Direct bilirubin increased[2]	Positive	Negative (in complete obstruction)	Dark	Decolorized (grayish)
Hepatocellular	Both direct and indirect bilirubin increased	Positive	Positive (higher than physiological)	Dark	Light

2 Fluorescence of hematoporphyrin

Disorders of heme biosynthesis in which the precursors of heme (porphyrins) accumulate in tissues and subsequently excreted in urine are called porphyrias. They can be caused by certain enzyme deficiencies or even by toxic effects of heavy metals (lead) or polyhalogenated hydrocarbons.

For demonstration of the porphyrins elevated in urine or blood during the porphyrias, their fluorescence in UV light can be used.

Action of concentrated sulfuric acid on hemoglobin produces hematoporphyrin. The hemoglobin loses both the globin and ferrous cation; in addition, the vinyl groups of heme are hydrated. The resulting product displays a wine-red fluorescence under UV lamp. The conversion of heme to hematoporphyrin is employed in ultra-sensitive tests for blood in stool.

Total porphyrins in urine can be semiquantitatively estimated by simple recording of the sample absorption spectrum. The wavelengths at which maxims are observed depend on the presence and concentrations of all contributing porphyrins in the given sample.

[2] Long lasting post-hepatic icterus secondarily damages function of hepatocytes. The laboratory findings then correspond to a combination of post-hepatic and hepatocellular icterus.