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# **CLINICAL CHEMISTRY AND ENZYMOLOGY**

## **Laboratory Manual**



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## **PRINCIPLES OF SECURITY IN A BIOCHEMICAL LABORATORY**

1. Only practicing students, as specified by the schedule, are allowed to be present at the practical classes. No visitors are admitted. When a student needs to leave the room, he or she should inform a laboratory instructor.
2. Lab coats are obligatory. Combustible scarfs, long hair are not recommended.
3. Only prescribed activities are allowed in a laboratory. Eating, drinking, and smoking is not allowed. Laboratory vessels could be used only for their proper purposes.
4. Manipulation of irritable, etching, and toxic stuffs should be performed under special precautions only. The usage of security pipettes, burettes, safety goggles, fume chambres, etc., is obligatory.
5. The manipulation rules with the laboratory burner should be respected. The burner must be placed in a safe distance from combustibles and explosives (e.g. ether, petrol alcohols, etc.)
6. All centrifugation procedures should be controlled by laboratory assistants or teachers in charge. The cuvettes should be carefully balanced. Similarly, electric apparatuses (electrophoresis) should be switched on only by a skilled person, and manipulation with complementary electric equipment is forbidden.
7. Pouring out the solvents immiscible with water down the sink is not allowed. They should be placed in the waste container provided. Limited amounts of solvent miscible with water (up to 0.5 l) should be poured after dilution 1:10, acids and hydroxides after dilution 1:30. Acids are to be diluted by pouring an acid into water, not vice versa.
8. A breaking of laboratory glass should be reported to a laboratory assistant. Potsherds should be collected in a special litter.
9. Any accident should be immediately announced to a teacher in charge. After contact with a harmful liquid, contaminated body parts (mouth, skin, eyes) should be immediately washed

off with a sufficient amount of tap water. Before pipetting, notice if the capillary tip of the pipette is intact!

10. Notice the location of fire extinguishers, extinguishing clothes, and emergency exits! Even the simplest experiment can be hazardous if care is not taken. Always think carefully about what you are doing in the laboratory, so that accidents can be avoided.

## CLINICAL EXAMINATION OF URINE I

### ***Introduction***

The chief function of the kidneys is to maintain the constant state of the body's internal environment by regulating the volume and composition of the cellular fluids. Kidneys remove from the body unwanted substances, i.e. metabolic waste (e.g. urea), drugs and toxic agents while simultaneously retaining other, essential substances (e.g. glucose, amino acids). Formation of ammonia by the renal tubular epithelium and regulation of excretion of certain anions and cations play an important part in the regulation of the acid-base equilibrium.

The basic functional unit of the kidney is called a nephron. It is composed of a glomerulus, with its associated afferent and efferent arterioles, and a renal tubule.

The *glomeruli*, mostly situated in the cortex, consist of a bundle of 20-40 capillary loops invaginating Bowman's capsule, which is the beginning of the renal tubule. The plasma that traverses the glomerular capillaries is filtered by the glomerular membrane. Especially the basement membrane provides a sieving function for retention of plasma proteins and blood cells.

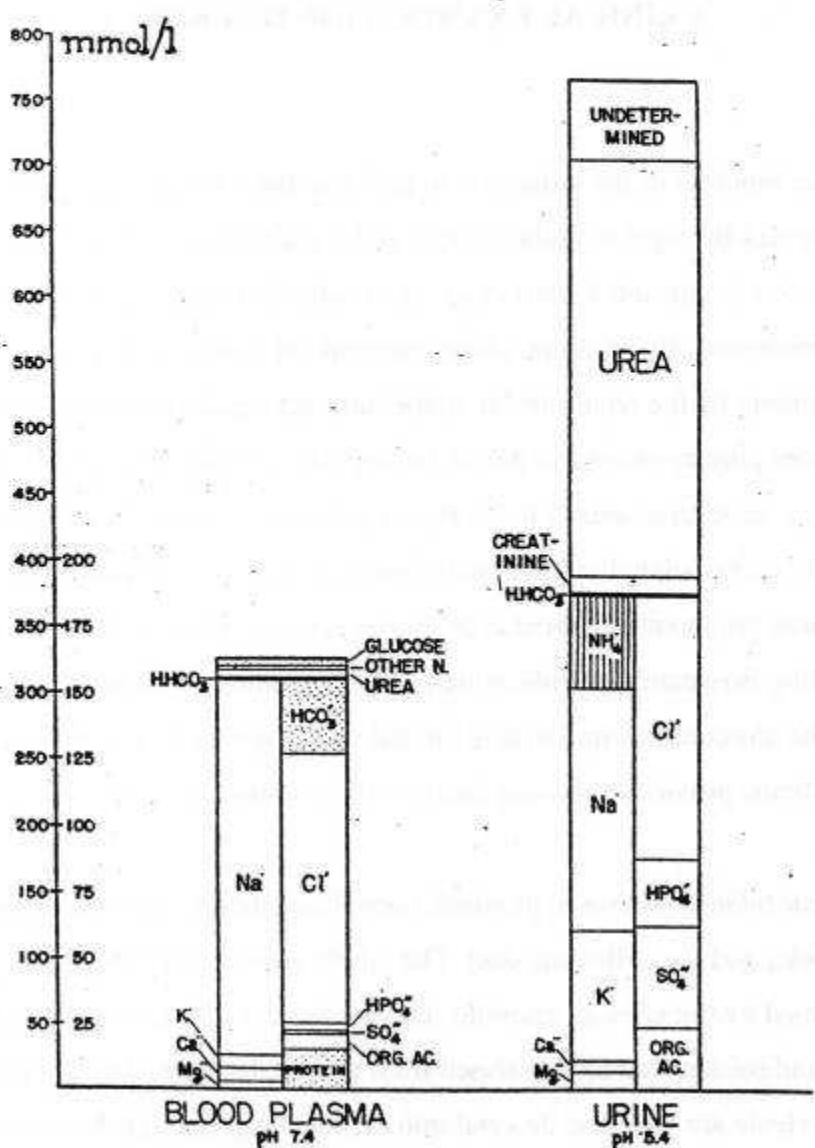
The *renal tubule* consists of proximal convoluted tubule, the loop of Henle, the distal convoluted tubule, and the collecting duct. The tubule is surrounded by the peritubular capillaries, formed by the efferent arteriole. This meshwork of microvessels functions to remove water and solutes that have diffused from the renal tubules. In man, about one-seventh of the loops of Henle are long and descend into the renal papillae. These structures are important in the countercurrent system, by which the kidneys concentrate urine. The proportions of long loops of Henle is much higher and the renal papillae into which they extend are longer in desert animals which have adapted for water conservation.

More details of the structure and function of the kidney may be found in the corresponding textbooks (see e.g. *The Textbook of Physiology*).

### **1. Physical examination of urine**

#### **a) Naked-eye appearances**

Urochrome is the chief pigment and is responsible for the amber colour of normal urine. In addition, normal urine contains traces of urobilin and other pigments. Urobilinogen is colourless, contained also in traces. Irrespective of pathological conditions, urine may be pale owing to intake of large volumes of fluid or deeper in colour (more orange) owing to copious sweating or to reduction of fluid intake (= "concentrated").



Substances which may make urine depart from normal amber:

*red* - blood, hemoglobin, myoglobin, beets

*port-wine* - porphyrin

*brownish-black* - melanin (oxidation of melanogen), alkaptoneuria (oxidation of homogentisic acid)

*brown* - bilirubin, methemoglobin

*orange* - small amount of bilirubin

*greenish* - biliverdin (oxidation product of bilirubin)

*deep yellow* - riboflavin, tetracycline antibiotics, certain chemotherapeutic

*white* - chyluria

Under naked-eye appearance will be noted also whether urine is clear or cloudy, whether there is any deposit visible (see the examination of urinary sediments). As a rule phosphates are deposited when the urine is alkaline and urates or uric acid when it is acid. Brownish urates may be then dissolved in KOH, phosphates in acetic acid. The deposits mentioned in a cooled specimen are in most cases of no significance. On the other hand turbidity caused by the massive occurrence of white blood cells microorganisms is always pathological.

b) *Odour*

There may be a characteristic odour, as, for example, of ammonia due to decomposition by bacteria (increased pH), or unpleasant odour of phenylacetic acid in phenylketonuria.

c) *Volume*

An average urine output ranges from 1 - 1.5 l/day. *Oliguria* is said to be present when the urine amount is less than 400 ml/day, *anuria* less than about 100 ml/day. When the urine output exceeds 2.5 l/day, *polyuria* is present.

d) *Density*

The specific gravity may be taken with an urinometer. It ranges from about 1.003 to 1.035.

e) *pH-Value*

The limits for pH encountered in urine range from 4.7 to 8.0, an average being about 6.0. Owing to bacterial decomposition an acid urine may become alkaline in a very short time, e.g. in 2-4 hours at room temperature. The amount of NaOH required to titrate urine to plasma acidity is called the *titratable acidity* (25-70 mmol/l on average).

## EXCERCISE 1

In the routine examination of urine for pathological constituents fresh unpreserved urine is analyzed. For quantitative studies, it is necessary to use a preservative. Toluene is the most satisfactory by minimizing the bacterial action.

- Collect urine in a clean vessel provided. Record colour, odour, and specific gravity with urinometer. For the gravimetric measurement, urine should be placed into a cylinder. The urine density is read at the bottom of the meniscus.
- Determine the reaction to pH-indicator strip. If acid, titrate 10 ml of urine diluted with water with 0.1 M NaOH in the presence of phenolphthalein. The volume  $\times$  10 gives the titratable acidity in mmol/l.

(c) Cloudy urine specimens examine as follows. If the pH is acid try to dissolve the brownish urate deposit by the addition of KOH. White oxalate deposit may be dissolved in HCl. Phosphates (neutral or alkaline urine) are soluble in acetic acid. A pus deposit forms in the presence of 10% KOH a viscous gelatinous matter, a bacterial deposit remains unchanged.

## 2. Proteinuria

Normal urine contains a small amount of protein insufficient to give positive reactions to the usual chemical tests. The normal daily amount is about 150 mg consisting of up to 40 mg of albumin, rather less globulin, and Tamm-Horsfall mucoprotein. An increase is important in the diagnosis of renal disease, and especially of the early stages of pyelonephritis.

Proteinuria may be due in part to an increased permeability of the glomerulus, to impaired reabsorption of normal amounts of protein from the glomerular filtrate or to tubular excretion. Distribution of proteins in urine in pathological proteinuria varies with the primary disease; the more glomeruli are seriously affected the larger are the pores in the basement membrane which thereby allow high molecular weight protein to pass through. If the pore sizes remain small the proteinuria is *selective*. Using special methods (e.g. immunoassay) permit classification of proteinuria as selective or non-selective. The clearance ratio of IgG/transferrin may be used. In minimal change glomerular nephritis in children there is usually selective proteinuria and a good prognosis.

Orthostatic or postural proteinuria, common in adolescents and young adults, is due to venous vasoconstriction caused by a lordotic erect posture (3% of otherwise normal adolescents). A transitory proteinuria can also follow exercise or common pyrexial illnesses.

Overflow proteinuria occurs with low molecular weight proteins, e.g. in Bence-Jones proteinuria of myeloma. Bence-Jones proteins are the light chains of the myeloma Ig and are precipitated at 45-55°C and redissolve at 95-100°C. Bence-Jones protein is now usually detected by gel electrophoresis of urine. Proteins originating from the renal tract include Tamm-Horsfall mucoprotein which forms hyaline casts in normal urine, IgA associated with renal inflammatory disease such as pyelonephritis, and urinary enzymes.

## EXERCISE 2

For a routine qualitative *analysis of proteins* in urine the following tests are made:

a) *Sulfosalicylic acid test*

Filter the urine, if cloudy, and, if alkaline, make it acid by adding dilute acetic acid.

To 0.5-1.0 ml of urine in a test tube, add about 5 drops of 20% sulfosalicylic acid. A white precipitate will be produced if protein is present. This is a very sensitive test.

Semiquantitative estimation:

hardly visible cloud	50-100 mg/l (+ -)
discrete transparent cloud	100-250 mg/l (+)
homogenous opaque cloud	0.5-1.0 g/l (++)
milky flocculating deposit	2 - 5 g/l (+++)
courdy precipitate	5 and more g/l (++++)

b) *Heller's ring test*

Place 1-2 ml of concentrated nitric acid in a test tube, incline the tube, and pour a little clear urine down the side of the tube in a manner that will produce a stratification. If protein is present, a white ring will appear at the junction of the two liquids. In the presence of bilirubin, a green ring instead of white ring is formed.

c) *Heat coagulation*

The urine must be clear and acid. If cloudy, filter. To 1 ml of urine in a test tube add about 0.2 ml of acetate buffer pH 4.6 and heat to boiling. A turbidity is due to protein, sometimes to phosphates, if present in greater amounts.

d) *Diagnostic test strip for semiquantitative determination of protein and pH in urine (Albuphan)*

Reagent area for protein is impregnated with a mixture of acidic buffer and a special indicator changing its colour in the presence of protein from yellow through green to blue. The pH area contains a mixed acid-base indicator with colour changes from orange through yellow and green to blue within pH range 5-9. At pH >8 the urine specimens may give falsely positive results in the absence of protein.

Immerse the strip briefly in urine specimen to moisten both the test areas and remove immediately. Tap edge against the side to remove excess urine. Compare colour of the pH area immediately with colour chart at the label. Allow the reaction for protein to continue for 1 min and read in a similar manner. Colour blocks correspond to the concentrations 0.3 - 10 g/l of protein.

e) *Exton quantitative determination of total protein in urine*

Proteins in a known volume of urine are precipitated and stained in the presence of sulfosalicylic acid and bromphenol blue.



To 1 ml of clear urine in a small flask add 9 ml of 0.9% NaCl and 5 ml of Exton's reagent. Mix and allow to stand for 10 min. Determine yellow absorbance of the suspension at 610 nm in 1 cm photometric cell. Read the concentration from the calibration diagram.

### 3. Determination of sugar in urine

Reabsorption of glucose increases with rising plasma concentration, but there is a maximum rate for this process. Thus if the load of glucose filtered by the glomeruli exceeds the maximum rate of glucose reabsorption ( $T_m\text{Glc}$ , 27-33 umol/s) then *glycosuria* occurs. Some subjects have individual nephrones with a low  $T_m\text{Glc}$  and excrete glucose in the urine at normal plasma levels and are said to have *renal glycosuria*. In general,  $T_m$  values decrease with age. The glucose plasma level corresponding to the  $T_m$  in healthy young individuals is approximately 9 mmol/l.

In diabetic coma, when the concentration of glucose in the plasma is very high, the concentration of glucose in urine will depend on the maximum osmotic work of which the kidneys are capable, as well as on other substances requiring excretion, i.e. there will be no direct relationship to the concentration in the blood.

*Fructosuria* is uncommon. Alimentary fructosuria is occasionally due to ingestion of fructose or fruits.

*Lactosuria* is common in nursing mothers and in infants on a milk diet for a long time.

*Pentosuria* is a rare inborn error of metabolism, alimentary pentosuria is occasionally encountered in the "fruit season".

*Galactosuria* is one of the symptoms of congenital galactosaemia.

Most sugars are reducing substances and as such may be detected in urine. However, many other substances reduce to some extent cupric solutions: uric acid, glucuronic acid, creatinine, tetracyclines, ascorbic acid, gentisic acid, etc.. As a working rule, if reduction is considerable, and there are symptoms typical for diabetes, it may safely be assumed that the reducing substance is glucose. In all other cases additional tests should be performed.

There are several enzyme strip tests for glucose which differ slightly in their sensitivity and susceptibility to interfering substances. The strips are impregnated with glucose oxidase, a vegetable peroxidase and a chromogen. When the strip is dipped in urine containing glucose and withdrawn the glucose in the minute amount of urine absorbed on the strip is oxidized by atmospheric oxygen in the presence of oxidase. Gluconic acid and hydrogen peroxide are formed, and this last product subsequently reacts with the chromogen in the presence of the

peroxidase to produce a colour. The reaction is inhibited by very high concentration of ascorbic acid, such as may occur in urine after the ingestion of therapeutic doses of the vitamin.

In infancy and childhood the use of the enzyme tests as a routine screening test might lead to a failure to recognize congenital galactosemia, for the adequate treatment of which early diagnosis is essential.

Lactosuria, galactosuria, pentosuria and fructosuria may be distinguished by paper chromatography.

### EXCERCISE 3

#### a) *Benedict's test*

Cupric sulfate in alkaline solution is reduced to cuprous oxide by boiling with reducing agents.

Place in a clean test tube 4 drops of deproteinized urine and add about 1.0 ml of Benedict's reagent (cupric sulfate, sodium carbonate, sodium citrate). Boil carefully for two minutes. Remove to a test tube rack and allow to cool.

Semiquantitative estimation:

solution unchanged	neg.
green without precipitate	5-20 mmol/l + -
green with precipitate	25-30 mmol/l +
olive-green	50-60 mmol/l ++
orange	70-90 mmol/l +++
reddish-brown precipitate	100 and more +++++

#### b) *Fehling's test*

As in Benedict's test, cupric sulfate is reduced to cuprous oxide. The copper solution, however, is made up with stronger alkali, and small quantities of sugar are more likely to be caramelized. When testing with both reagents, a large amount of protein may cause trouble: decomposition products may reduce the cupric solution if boiling is prolonged. The difficulty is easily overcome by coagulating proteins and filtering.

Mix equal volumes of Fehling I (Cull sulphate) and Fehling II (NaOH, NaK-tartarate). If boiled, the mixture must not change its appearance. To 1-2 ml of the reagent in a test tube add an equal volume of urine and boil for two minutes. A green colour without precipitate is of no significance.

c) *Nylander test*

Bismuth subnitrate in alkaline solution is reduced by glucose and other reducing sugars to metallic bismuth on heating.

To 1 ml of urine in a test tube add 1 ml of the reagent and boil for 2 minutes. In the presence of reducing substances the solution turns grayish yellow, ev. brown to black colour of reduced bismuth.

d) *Diagnostic test strips (Glukophan)*

Besides the own test area for glucose (upper, bright yellow) it has an auxiliary test area (lower, pale yellow) indicating the presence of strongly reducing substances which diminish the reactivity for glucose.

The detection is based on glucose oxidase reaction (see text above) in the course of which a colour change from yellow to green occurs. Indication of the presence of reducing substances (mainly ascorbic acid and gentisic acid) is based on reduction of pale yellow phosphomolybdate to molybdenum blue.

Dip the strip briefly in urine specimen to moisten completely both areas and remove immediately; tap edge of the strip against the vessel wall to remove excess urine. First 30 seconds after wetting read the test for reducing substances, in about a minute read the glucose test and compare with colour blocks on the chart for glucose concentration ranging from 0.3 g/l to several g/l. If the reducing power is above block "2", the correct reading of glucose is unreliable.

e) *Quantitative estimation of glucose in urine by caramelisation*

To 0.1 ml of urine in two test tubes add 2.5 ml of 5%  $\text{Na}_2\text{CO}_3$ , mix, and place one of the test tubes in a boiling water bath for 10 min. The second test tube will serve as a blank (may be common!). After cooling estimate the absorbance in 1 cm cell at 520 nm. Read the concentration in a calibration diagram.

f) *Seliwanoff's test for fructose*

Place into a test tube 0.5-1.0 ml of resorcinol in HCl, add about 2 ml of urine. Bring to the boil. Fructose is decomposed and forms with resorcinol a red compound.

g) *Bial's test for pentoses*

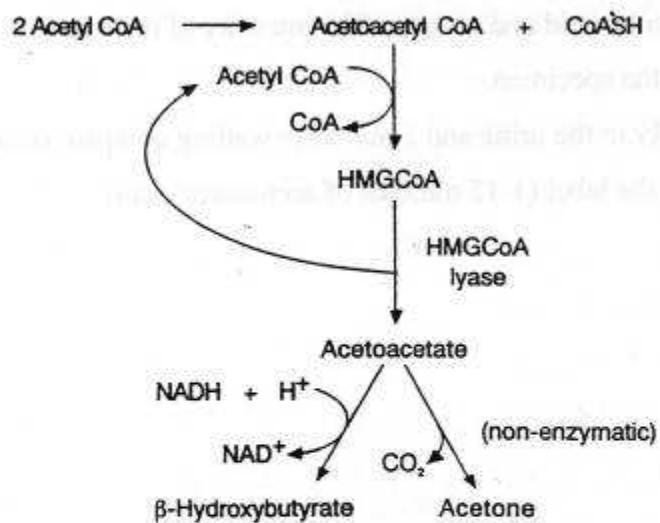
The reagent is prepared by dissolving 15 mg of orcinol (m-dihydroxytoluene) in 5 ml of 30% HCl and adding 2-3 drops of 1%  $\text{FeCl}_3$ . To the reagent add 0.5 ml of urine and bring

just to the boil. Allow the mixture to stand for 5-20 min. Under these conditions pentoses yield a green colour.

#### 4. Ketonuria

The fatty acids are degraded in the liver into acetyl CoA which, if sufficient oxaloacetic acid is available from carbohydrate oxidation, is oxidized in the tricarboxylic acid cycle. If not, or if fatty acid degradation is excessive, acetyl-CoA is converted into acetoacetyl-CoA or synthetized into cholesterol via *beta-hydroxy-beta-methyl-glutaryl-CoA*. The latter may be converted into *acetoacetic acid*, which, with the reduction product *beta-hydroxybutyric acid* and its decarboxylation product *acetone*, form the ketone bodies. Acetoacetic acid can be utilized by the tissues, especially the muscles, for the production of energy, but if it is formed by the liver at a rate greater than the muscles can utilize it, then ketone bodies accumulate in increased quantities in the blood, ketosis develops and ketone bodies appear in urine.

The routine tests are for acetoacetic acid and acetone, using nitroprusside. Beta-hydroxybutyric acid, a major part of ketone bodies, is therefore not detected.



## EXERCISE 4

### a) *Lestrade's test*

Place onto filter paper a small amount of Lestrade's reagent (a powder consisting of nitroprusside, ammonium sulfate, and sodium carbonate). Add a drop of urine and wait about one minute. In the presence of acetone a purple-violet colour develops.

### b) *Legal's test*

Several crystals of sodium nitroprusside dissolve in a small amount of water, add one drop of NaOH, and a small amount of urine. A red colour, intensifying after addition of acetic acid, appears in the presence of acetone.

### c) *Gerhard's test for acetoacetic acid*

Ferric chloride yields a Bordeaux-red with acetoacetic acid, but no colour with acetone.

To about 5 ml of urine in a centrifuge tube add several drops of  $\text{FeCl}_3$ . Remove by centrifugation or filtration the precipitate. If supernatant (filtrate) is red, acetoacetic acid or a drug (salicylate, tetracycline) is present. To distinguish a reaction due to a drug, boil another portion of untreated urine for several minutes - acetoacetic acid is decomposed, the test will be negative.

### d) *Diagnostic test strip for determination of ketones (Ketophan)*

Test area contains mixture of alkaline buffer with sodium nitroprusside which gives a violet colour with acetoacetic acid and acetone. The intensity of the colour is proportional to the content of ketones in the specimen.

Dip the strip briefly in the urine and 1 min after wetting compare colour of the test area with colour chart on the label (1-15 mmoles of acetoacetic acid).

## CLINICAL EXAMINATION OF URINE II

### 1. Glomerular and tubular functions, clearances

Glomerular filtration is the initial step in urine formation. The glomeruli filter about 180 l of fluid per day although only about 1.5 l is passed as urine, due to tubular reabsorption. The filtrate is an ultrafiltrate, i.e. it has the composition of plasma except that it is almost free of protein. Ultrafiltration across the glomerular capillary is determined by imbalance between transcapillary hydraulic and colloid osmotic pressure gradients. As blood flows along the glomerular capillary, the protein concentration rises because of the protein-free nature of the ultrafiltrate and oncotic pressure increases. On the other hand, net ultrafiltration pressure decreases at the efferent end of the capillary and, as a result of both factors, net filtration ceases. The *glomerular filtration rate* (GFR) refers to the volume of glomerular filtrate formed per unit of time and is related to body size, or, more accurately, the surface area and is normally about  $2 \text{ ml/s} (120 \text{ ml/min})$  per  $1.73 \text{ m}^2$  body surface area.

As a measure of GFR, a *clearance* of a substance which is freely filtered and neither secreted nor absorbed by the renal tubules, can be used. Renal clearance of a given substance is the ratio of the renal excretion rate of the substance to its concentration in the blood plasma. In other words, it is the volume of plasma which would be completely cleared of the substance per unit of time. It expresses the efficiency with which the plasma is cleared of the substance.

If volume of urine excreted per sec (min) =  $V \text{ ml}$ , and if concentration of substance in urine =  $U \text{ mmol/ml}$ , then quantity of substance excreted per s (min) =  $UV \text{ mmol}$  (*renal excretion rate*). If concentration of substance in plasma =  $P \text{ mmol/ml}$ , volume of plasma providing UV mmol of the substance is its *clearance*,  $C$ :

$$C = \frac{U \cdot V}{P}$$

As a measure of GFR, a clearance of inulin, endogenous creatinine,  $^{51}\text{Cr}$ -EDTA, radioactive cobalamine and mannitol can be used. Inulin, a fructo-polysaccharide that does not occur naturally in the body, should be administered intravenously. Hence, in clinical practice it is more common to determine the clearance of creatinine, although a small amount of creatinine is secreted in humans and, therefore, the clearance might be slightly higher than GFR.

Creatinine clearance declines with age, and so does also the GFR. Measurement of *plasma creatinine* itself may be also used as a day-to-day indication of changes in GFR, mainly in

patients with renal failure. Plasma creatinine roughly doubles for every 50% fall in GFR. However, using serum creatinine concentration alone as a measure of the net GFR can lead to serious errors, especially in elderly individuals.

The clearances of many substances are much greater than the GFR because *tubular excretion* results in a greater clearance of plasma than can be caused by glomerular filtration alone. p-Aminohippuric acid (PAH) in low plasma concentrations has an extremely high plasma clearance; the renal clearance is nearly complete and PAH concentration in renal venous plasma may be taken to be zero. Accordingly, the  $C_{PAH}$  actually measures the effective *renal plasma flow* (ERPF, about 10 ml/s or 600 ml/min).

From  $C_{IN}$  and  $C_{PAH}$ , a *filtration fraction (FF)* can be calculated:  $FF = C_{IN}/C_{PAH}$ . FF is a measure of the fraction of the entering plasma volume that is removed through the glomeruli as filtrate (about 20% of the entering plasma volume).

When  $C_x$  is less than  $C_{IN}$ , excretion is by filtration and *reabsorption*. In this case, the mass of the substance excreted in the urine is less than the mass of the substance filtered during that time.

#### APPROXIMATE CLEARANCE VALUES

glucose	0.0 (unless the threshold concentration is reached)
$Na^+$	0.01 ml/s
$K^+$	0.15 ml/s
osmotically active solutes	0.05 ml/s
urea	1.25 ml/s (75 ml/min)

The most clinical useful *tubular function tests* include the urinary sodium (UNa), fractional excretion of sodium (FENa), urinary concentrating and diluting ability, and urinary acidification ability.

#### *Urinary concentration and dilution ability:*

Normal serum osmolality is about 290 mmol/kg. Urine of this osmolality is called *isotonic*. If water excretion increases, the urine becomes *hypotonic* (<290 mmol/kg), if water intake is restricted, urinary osmolality rises (*hypertonic urine*), reflecting to body need to conserve water. If urine osmolality fails to increase, a water regulatory defect should be

suspected. Similarly, as serum tonicity falls (dilution), the appropriate renal response is a water diuresis. If the appropriate response does not occur, a defect in water regulation is suggested.

*Renal failure*, in which the end products of metabolism accumulate in excess in the body fluids, merges with the late stages of *renal insufficiency*, in which, under limited intake of protein in the diet, the concentrations of the end-products are maintained within normal limits. The concentrations of urea, creatinine, uric acid and also inorganic phosphate and potassium in the plasma are raised and the accumulation of acidic products reduces plasma bicarbonate and leads eventually to metabolic acidosis.

*Acute renal failure*, associated with both impairment of glomerular filtration and ischaemic damage to the tubules, is a common complication of many primary medical and surgical conditions, and even today has a high mortality. In chronic renal failure, as the number of functional nephrons falls there is an increased amount of urea excreted per nephron, causing an osmotic diuresis. Patients become salt depleted, the loss of renal parenchyma reduces renal NH<sub>3</sub> synthesis and aggravates the metabolic acidosis. A complex of signs, symptoms, and biochemical abnormalities in advanced chronic renal failure is referred to as *uremic syndrome*.

## EXCERCISE 1

### *Creatinine clearance*

Traditionally, creatinine clearance is calculated from 24-h urine collection, although shorter collection times may be appropriate in certain clinical situations. However, difficulty in emptying the bladder fully at the start or end of the collection introduces greater error in short timed collections.

For the clearance calculation, plasma and urine creatinine concentrations, and volume of urine per sec (min) should be determined. The chemical estimation is based on Jaffe's test, i.e. on the photometric measurement of the orange coloured reaction product of creatinine with alkaline picrate.

### SOLUTIONS (Lachema Creatinine Test Kit):

- (1) Serum precipitaiton agent trichloroacetic acid 1.22 mol/l (Caution, caustic!);
- (2) Picric acid 0.04 mol/l (Caution, poison!)
- (3) NaOH 0.75 mol/l (Caution, caustic!);

(4) Creatinine standard 177 umol/l (prepared by dilution of the concentrated stock solution with albumin solution).

Analyzed samples: 1. Fresh urine (collected by students)

diluted 100x (i.e. 1 ml urine + 99 ml  
of distilled water);

2. Blood serum;

	<i>serum</i>	<i>creatinine standard</i>	<i>urine</i>	<i>blank</i>
<i>serum (ml)</i>	0.5	-	-	-
<i>creatinine standard (ml)</i>	-	0.5	-	-
<i>diluted urine (ml)</i>	-	-	0.5	-
<i>distilled water (ml)</i>	1.0	1.0	0.25	0.75
<i>trichloroacetic acid (ml)</i>	0.5	0.5	0.25	0.25
Mix properly each test tube and allow to stand for 5 min, centrifuge 10 min at 3000 r.p.m., take supernatant fluid.				
<i>supernatant (ml)</i>	1.0	1.0	-	-
<i>picric acid (ml)</i>	0.5	0.5	0.5	0.5
<i>NaOH (ml)</i>	0.5	0.5	0.5	0.5
Mix properly, allow to stand for 20 min, then measure in 1 cm cells against the blank at 505 nm.				

$$\text{Serum creatinine (umol/l)} = \frac{A_{\text{serum}}}{A_{\text{standard}}} \times 177$$

$$\text{Urine creatinine (umol/l)} = 50 \times 177 \frac{A_{\text{dil. urine}}}{A_{\text{standard}}}$$

$$\text{Clearance}_{\text{creat}} = \frac{U \cdot V}{P}$$

Note: Normal level of creatinine (serum) ranges from 62-124 umol/l.

V (urine): if not estimated calculate from 24-h output 1.5 l

## 2. Bilirubine and its derivatives in urine

Urine is tested for bile pigments on account of its colour, or of the colour of the patient's skin or conjunctiva (icterus, jaundice), or as a part of a routine examination. Normally there is no bilirubin in urine, or possibly the merest traces. In general terms bilirubinuria signifies disease of the liver or obstruction of the bile passages. Tests for bilirubin depend on oxidation to green (biliverdin) or blue (bilicyanin) pigments, or formation of azobilirubin.

In the intestine bilirubin is reduced to a mixture of colourless chromogens referred as "*faecal urobilinogen*". These chromogens are later dehydrogenated to give orange-red pigments, the so called "*faecal urobilin*". Some faecal urobilinogen is reabsorbed and via portal circulation passes to the liver, where it is normally either metabolized or reexcreted in the bile. In liver disease the reabsorbed urobilinogen is diverted to the blood circulation and, as threshold concentrations for kidney excretion equals zero, urobilinogen is found in urine. Urobilinogen gives Ehrlich's reaction with p-dimethylaminobenzaldehyde in the presence of HCl. Urine is examined for urobilin on account of its colour, or during routine examination, or because liver damage or excessive hemolysis is diagnosed. Normally urine contain traces of urobilin, and these quantities do not affect the colour. When there is an excess of the pigment urine is more orange than usual. Tests for the pigment depend on the characteristic absorption spectrum or the fluorescence of the zinc-urobilin compound.

## EXERCISE 2

### (A) Estimation of bilirubin in urine

#### a) Naumann's test

- (1) To about 3 ml of urine add a pinch of talc. Mix and filter.

(2) Unfold the filter paper and spread on another dry filter paper. Allow 1 drop of Fouchet's reagent (trichloroacetic acid and  $\text{FeCl}_3$  solution) to fall on the precipitate. A blue bilicyanin colour shows that bilirubin is present. This test is more sensitive than the ring tests.

b) *Hamarsten's test*

To about 0.5 ml of the mixture of nitric and hydrochloric acids (1:19) add 2 ml of ethanol and a few drops of urine. In the presence of bilirubin, a green coloured biliverdin will appear.

c) *Gmelin's test*

Carefully overlay 1 ml of urine upon an equal quantity of concentrated nitric acid in a test tube. Upon agitating very gently, a play of colours will appear at the junction of the two liquids, varying from violet to green, if bile is present.

d) *Rosin's test*

A small amount of urine in a test tube overlay with 1% alcoholic solution of iodine. In the presence of bilirubin, a green colour of biliverdin at the junction will appear.

e) *Diagnostic test strips (Biliphan, Ictophan)*

The test areas of the strips consist of cellulose impregnated with diazo-reagent and with a strongly acidic buffer. In the presence of bilirubin, a purple coloured azodye will appear. The same reaction is employed in the asbestos-cellulose diagnostic tablets.

**(B) Estimation of urobilinogen and urobilin in urine**

a) *Ehrlich's aldehyde test*

Urobilinogen, as well as porfobilinogen, stercobilinogen, give colour reaction with Ehrlich's aldehyde reagent (para-dimethylaminobenzaldehyde in HCl).

Add 1 ml of Ehrlich's aldehyde reagent to equal volume of fresh urine (cool off, if warm) and allow to stand for 3 min; a red colour suggests "*Ehrlich positive substances*" are present.

Note: Urobilinogen can be extracted into chloroform, and its coloured product does not disappear after adding saturated sodium acetate solution.

b) *Diagnostic strips for urobilinogen in urine*

The presence of urobilinogen in urine is indicated by a faint pink shade on the test area impregnated with diazo reactant in strongly acid buffer. Thus, the test indicates even the physiological excretion of urobilinogen (3-4 mg/l). The differentiation between "normal" and elevated amounts is possible according to the intensity of the red colour. Read the result 1 min

after removal of the strip by comparing with colour charts on the label. Ignore colours developing after 3 minutes.

c) *Schlesinger's test for urobilin in urine*

To about 5 ml of urine in a test tube add an equal amount of well mixed Schlesinger's reagent (suspension of zinc-acetate in conc. ethanol), mix and filter. Examine the filtrate by transmitted light. A green fluorescence is due to a compound of zinc with urobilin, eventually stercobilin.

Note: The product can be extracted with chloroform.

**(C) Estimation of bile salts in urine**

Dissolve a pinch of sucrose in a small amount of urine. Hold the test tube at an angle and carefully pour down the side 1-2 ml of concentrated sulphuric acid (Caution, caustic!). A red ring indicates the presence of bile acids. On shaking the tube gently the whole layer of urine turns red. The tube should be placed under running water during shaking.

### 3. Hematuria

The definition of *hematuria* encompasses the presence of gross blood in the urine on the one hand to as few as three erythrocytes per microscopic field in a urine sediment.

Hematuria may be extrarenal or intrarenal. *Extrarenal* injury involves urinary ways (pelvis, ureter, bladder, prostate), *intrarenal* causes include e.g. injuries of glomeruli (inflammation) and malignancies (Grawitz's tumor). *Myoglobin* released following muscle injury gives a red-brown colour to the urine. Following intravascular hemolysis, free *hemoglobin* may be filtered and excreted, giving a distinctly red colour to the urine. Both pigments cause standard reagents and dip sticks to give a positive reaction for hemoglobin. The colour reactions are catalyzed by the hem iron.

### EXERCISE 3

#### *Estimation of blood and hemoglobin in urine*

a) *Heitz-Boyer test*

- (1) To 1-2 ml of urine in a test tube add an equal amount of Heitz-Boyer reagent (reduced phenolphthalein by zinc in alkaline solution) and mix properly.
- (2) Over the tube content layer 3% solution of hydrogen peroxide. A lilac ring indicates blood or hemoglobin.

b) *Pyramidone ring test*

- (1) Acidify 2-3 ml of urine with a few drops of glacial acetic acid. Add 5 drops of 3% solution of hydrogen peroxide.
- (2) Carefully superimpose an equal volume of 5% solution of pyramidone (Amidopyrin) in concentrated ethanol. If blood is present a pink colour appears at the junction.

c) *Diagnostic test strips for blood and hemoglobin in urine*

*(Hemophan)*

Hemophan test area is impregnated with stabilized org nic hydroperoxide, an acidic buffer, and a chromogen which is oxidized in the presence of hemoglobin by hydroperoxide to a blue product. Intact erythrocytes exhibit on the white background of test area blue points to spots. Read 30 s after the test strip was dipped into a urine specimen, ignore any colour developed after 3 min.

## CLINICAL EXAMINATION OF URINE III

### 1. Examination of urinary sediment

This examination remains one of the most useful non-invasive clinical tests. It complements the physical and chemical examination of the urine.

Deposits may be classified under three headings, each beginning with "C": *Cells*, *Casts*, and *Crystals and amorphous chemical deposits*. In the first group are included white blood corpuscles (WBC), red blood corpuscles (RBC), epithelial cells, bacteria, yeast cells, etc.. Casts are commonly of three types, i.e. hyaline, granular and cellular.

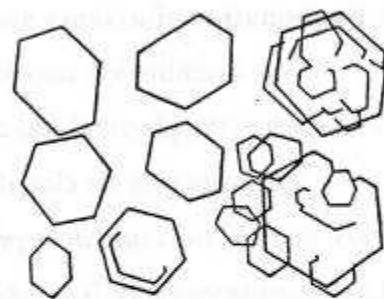
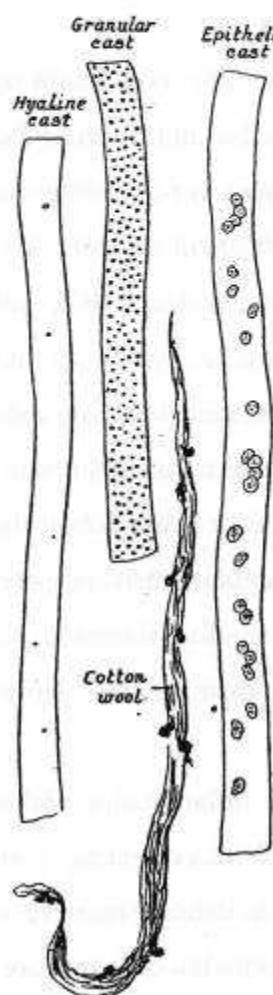
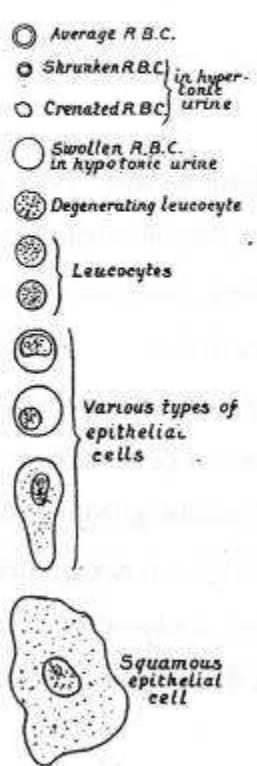
The presence of *erythrocytes* indicate bleeding somewhere in the urinary system, whereas the absence of RBC in a specimen that diagnostic strips tested positive for blood suggests the presence of myoglobinuria or hemoglobinuria. RBCs resulting from glomerular bleeding are often fragmented and irregular when compared with RBCs of nonglomerular origin. In hypotonic urines RBCs are smaller, shrunken, sometimes crenated; in dilute urine they swell and then may burst leaving their "ghosts". Normally, RBC are recognized by the biconcave shape and pink colour.

*Leukocytes* in the urine signify inflammation within the kidney or along the urinary tract. Although the actual number of WBCs correlates poorly with the degree of inflammation, clumps of WBCs tend to indicate more severe inflammation. It is important to distinguish WBCs from the tubular epithelial cells that are approximately of the same size. The most convenient differentiation method is the peroxidase staining specific for WBCs.

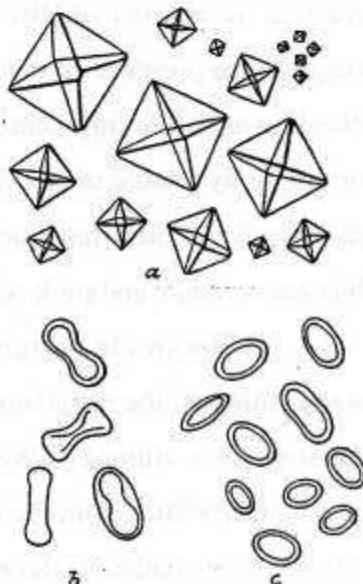
*Epithelial cells* may originate from any part of urinary tract. Epithelial cells from the distal parts of urinary ways are the most striking objects being well contoured, large polygonal cells with a distinct nucleus. A limited number of large epithelial cells is physiological. However, the appearance of the small round tubular epithelial cells accompanies degenerative tubular processes.

The presence of *bacteria* in a properly collected urine sample is abnormal and is termed *bacteriuria*. Bacteriuria associated with WBCs is strongly suggestive of infection, and a urine culture is usually indicated. Other identifiable organisms include yeast forms and *Trichomonas*, a protozoan.

Urinary *casts* are formed by the precipitation of mucoproteins, primarily Tamm-Horsfall protein, in the renal tubules. These casts may contain only mucoprotein matrix (*hyaline casts*), or they trap RBCs, WBCs, or renal tubular epithelial cells present in the

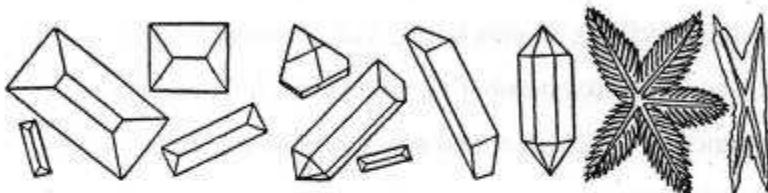


Hexagons of cystine.

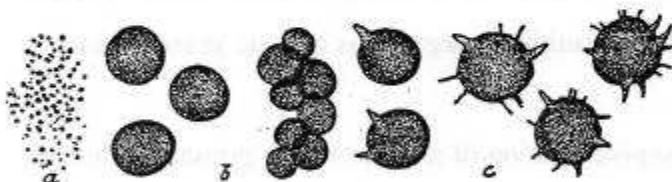


Calcium oxalate crystals.

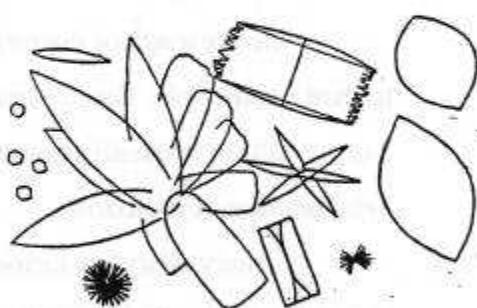
- (a) Octahedra, or "envelope" crystals.
- (b) Dumb-bells.
- (c) Biscuit forms.



Triple phosphate crystals (ammonium magnesium phosphate).



Urate deposits. (a) Amorphous urates. (b) Pigmented spheres of ammonium urate (alkaline urine). (c) "Thorn-apple" crystals of urates of ammonium or sodium.



Different forms of uric acid.

lumen (*cellular casts*). Clinically, RBC casts are generally diagnostic of glomerular bleeding and indicate the presence of glomerulonephritis. Large numbers of cellular casts indicate the presence of inflammation either within the glomerulus or around the tubules (interstitial nephritis). With time, cellular elements within casts degenerate, losing their identifiable characteristics and leaving *granular casts*. *Waxy casts* may represent further degradation of this cellular material to a homogenous appearance. Red cell casts may be degenerated into *hemoglobin casts*. The significance of degenerated casts is the same as that of their parent cast.

*Crystals* can be a normal finding, although they may occur with increased frequency in renal stoneformers. Only *cystine crystals* are always abnormal and indicative of disease. Cystine crystals have a flat, hexagonal shape and indicate the presence of cystinuria. Clinically a deposit of *uric acid* or *urates* is most often caused by cooling urine in vitro, and only occurs at acid reaction (ammonium urate excepted). Uric acid crystallises in many forms (barrels, plates, prisms, needles, etc.). Urates are often amorphous, but sodium urate and ammonium urate may be crystalline (thorn-apple crystals). Like uric acid, urates are usually pigmented, and redissolve on warming, or in excess of alkali. Crystals of ammonium magnesium (triple) *phosphate* are often seen in urinary deposits. Morphologically the commonest form of *calcium oxalate* is the flattened octahedron or "envelope" crystal, though it may appear in the shape of a biscuit or dumb-bell.

## EXERCISE 1

### ***Microscopic examination of urinary sediment***

Urinary sediment is prepared by centrifugation of the fresh morning urine. It can be prepared either as native sediment or as stained sediment which is especially suitable for less experienced examining person. The most convenient is the *Sternheimer's supravital cytodiagnostic staining method for urinary sediment*, known as "*The Finnish modification*".

This staining provides specific colours to cellular elements. In epithelial cells and in leukocytes the nucleus appears dark blue and the cytoplasm pink. Red blood cells appear pink or grey-pink. Hyaline casts appear bright blue and cellular casts show the colours typical for the cells.

#### EQUIPMENT:

Microscope, specimen chambers, centrifuge, centrifugation tubes, balance

Staining kit "Staining of Urinary Sediment" ("Finnish Modification") that contains two solutions: 50 ml of Alcian blue ("National fast blue") and 50 ml of Pyronin B red. The two reagents are mixed 1:1 forming the "*working solution*" that is added as one drop to the sediment.

Urine jars, pipettes, pH indicators

*The procedure directs you to perform both type of sediment, stained and native. Of course, you can prepare only one type of sediment. As already mentioned, it is much easier for less experienced person to read the stained sediment under the microscope.*

*Procedure:*

1. Collect your own urine into a clean jar. Check the pH, it should be below 7 and mostly it is. At higher pH the staining components may form a precipitate but usually without effect on the stained elements.
2. Mark two clean conical centrifugation tubes with the number of your laboratory bench. Mix the urine and transfer 5 ml into each of the two centrifugation tubes. One is for native and the other one for stained sediment. The technician will place the tubes into the centrifuge and will perform the centrifugation at 2000 rpm for 5 minutes.
3. Immediately after centrifugation pour out carefully the content of tubes leaving in about 0.5 ml of supernatant. Add 1 drop of working solution from the staining kit and shake thoroughly to mix fully the sediment and the staining solution. When necessary, use the pipette to achieve total dispersion. Leave 5 – 10 minutes to complete the staining.
4. Meanwhile prepare the native sediment. Shake well the sediment with remnants of supernatant and transfer one drop onto the microscopic glass or into the plastic chamber and observe under microscope.
5. Transfer also one drop of stained sediment onto a microscopic glass or into a plastic chamber and observe under microscope. For a full evaluation there must be seen 20 observation fields at the magnification of 400.
6. Apart of your own urine which is not expected to contain pathological elements observe the supplied pathological specimens.
7. Also, carefully observe the pathological urinary sediment photographs available on the computer.

Any sediment should be evaluated with respect to:

- Cells (WBC, RBC, epithelial cells)
- Casts (hyaline, granular, epithelial, RBC, waxy)
- Crystals (calcium oxalate, phosphates, uric acid, urates, cystin, etc.)
- Other (parasites, bacteria, fungi, yeast cells, foreign bodies)

The following number of cells (casts) per field of vision at 200x magnification is admissible:

RBC	0 - 3
WBC	0 - 4
Non-hyaline casts	none
Gross epithel. cells	0 - 5

At least 20 fields should be evaluated.

#### *A quantitative approach:*

*Hamburger's sediment* is an examination of 3-hour specimen of urine (usually collected from 7 to 10 a.m.). The urine volume should range from 30 - 250 ml. The number of elements is evaluated in a counting chamber and expressed as excretion counts per second.

The physiological ranges:

RBC	up to 33/s
WBC	up to 67/s
hyaline casts	up to 1/s

The outdated *Addis'sediment* was referred to the time period of 12 or 24 hours (1 million of WBC or RBC/h).

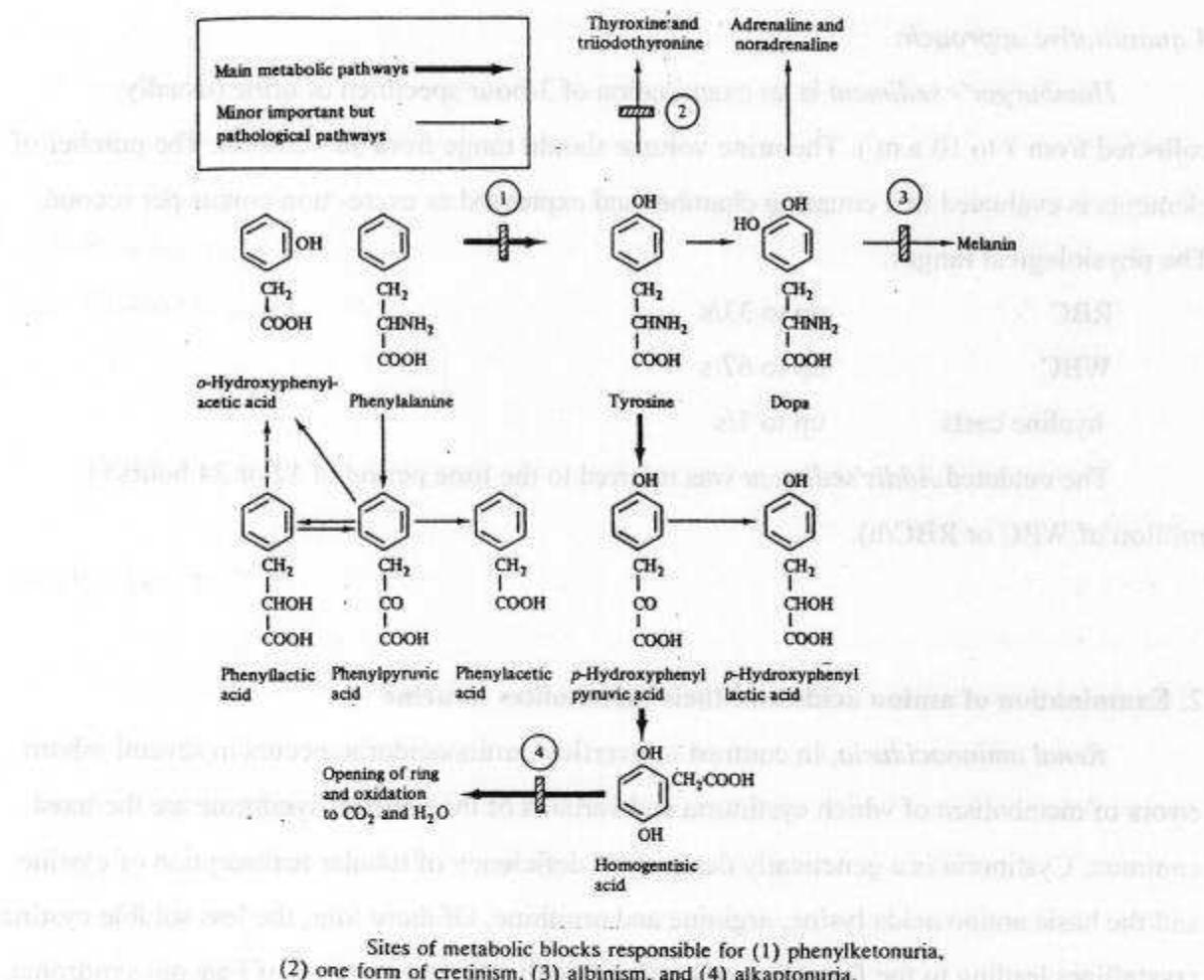
## **2. Examination of amino acids and their metabolites in urine**

*Renal aminoaciduria*, in contrast to overflow aminoaciduria, occurs in several inborn errors of metabolism of which cystinuria and variants of the Fanconi syndrome are the most common. Cystinuria is a genetically determined deficiency of tubular reabsorption of cystine and the basic amino acids lysine, arginine and ornithine. Of these four, the less soluble cystine crystallizes leading to the formation of cystine calculi. Of the two types of Fanconi syndrome, the more severe syndrome in infants is accompanied with cystine deposits in the body fluids, non-specific aminoaciduria, glycosuria and vitamin-D-resistant rickets.

In some inheritable biochemical disorders as well as in liver disease there is a production of amino acids in excess which exceeds the normal reabsorptive capacity of the renal tubules, leading to an *overflow aminoaciduria* (e.g. homocystinuria, cystathionuria).

There are many inborn errors of metabolism of the branched chain amino acids, leucine, valine and isoleucine. *Maple syrup urine disease* is due to deficiency or abnormality of the common oxidative decarboxylase, leading to the urinary excretion of increased amounts of the three amino acids and their ketoacids.

In *alkaptonuria* and *albinism*, there are blocks in the metabolism of phenylalanine and tyrosine respectively. In the former, the metabolism of these amino acids is blocked at the homogentisic acid stage; this acid is therefore excreted. The urine darkens on standing and is strongly reducing.



In *phenylketonuria (PKU)* the mental disturbance is related to the excretion of phenylpyruvic acid and o-hydroxy- phenylacetic acid together with phenylalanine, phenyllactic acid and phenylacetic acid. Of these, *phenylpyruvic acid* is detected by the ferric

chloride test but is insufficiently reliable for screening (for the screening of the newborns, the microbiological Guthrie test is used). The patients have an inherited deficiency of the enzyme phenylalanine hydroxylase (or a cofactor) responsible for forming tyrosine from phenylalanine which is present in the plasma in increased concentrations. These patients are now effectively treated with low-phenylalanine diets started as early in their lives as possible.

In circumstances in which *acute porphyria* (an inborn error of the porphyrine metabolism) is suspected, i.e. in surgical emergencies, nervous and psychiatric conditions in which the diagnosis is obscure, a test for *porphobilinogen* should be carried out. This is identical with the Ehrlich aldehyde test for urobilinogen. However, the coloured product cannot be extracted with amyl alcohol.

## EXCERCISE 2

### a) A qualitative estimation of cystine in urine

*Principle:* Cystine is reduced by alkaline cyanide to cysteine which gives a magenta colour with nitroprusside.

*Procedure:* Place a small amount of the powder reagent (sodium nitroprusside, ammonium sulphate, sodium carbonate, sodium cyanide - Caution, poison!) onto a glass or porcelain base. Add a drop of urine. The colour reaction of cystine appears immediately, in contrast to the late reaction of acetone which is, however, more constant.

### b) Test for phenylketonuria

*Principle:* Urine in phenylketonuria (PKU) containing phenylpyruvate gives with ferric chloride in acid a well-marked green colour.

*Procedure:* To about 2 ml of urine in a test tube add 2 drops of 10% HCl and several drops of 10% FeCl<sub>3</sub>. In the presence of phenylpyruvate a deep green colour appears.

*Note:* For this purpose also specific diagnostic dipsticks are available.

### c) A qualitative test for tyrosin

Tyrosin reacts with Millon's reagent (mercury in fuming HNO<sub>3</sub>) upon red colour formation.

## 3. Examination of enzymes and hormones in urine

In acute pancreatitis, an "acute abdomen" condition, there is a considerable increase in the serum *amylase* concentration. The *urinary amylase* is also increased shortly after that in the blood. Parotitis, usually due to mumps, causes increased levels as well.

Amylase hydrolyses starch, yielding in sequence erythrodextrin, achroodextrin and maltose. In Wohlgemuth's test digestion is regarded as complete when no starch is left, which is estimated by naked eye. The photometric estimation employs an insoluble starch-like substrate which bears chromogenic groups. Following incubation, the amount of soluble coloured material is estimated as absorbance of the supernatant.

More than 30 steroidal compounds, including precursors and unchanged adrenal steroids, reduction products and metabolites, are excreted in the urine, either free or conjugated with glucuronic or sulphuric acids. About 1% of *cortisol* is excreted by the kidneys as free cortisol. The excretion rises in Cushing's syndrome. *17-Oxosteroids (17-ketosteroids)* in the urine consist of a complex mixture of steroid compounds originating from both adrenals and gonads and are characterized by an *oxo (keto)*-group in the 17 position. The normal urinary excretion varies from 24 - 87 umol/24 h and from 14 - 66 umol/24 h in adult males and females, respectively.

A catecholamine-secreting tumor of the adrenal medulla (*pheochromocytoma*) is characterized by the intermittent secretion of adrenaline and noradrenaline and their metabolites. The chemical estimation of the metabolite 4-hydroxy- 3-methoxy mandelic acid (*vanillyl mandelic acid*) in urine is useful as a screening test.

### EXERCISE 3

#### a) Estimation of alpha-amylase in urine (and serum) by Wohlgemuth's test

- (1) Into a series of ten test tubes pipette 1 ml of 0.9% NaCl with the exception of the first test tube.
- (2) Into tube No 1 and 2 place 1 ml of urine. Mix the content of tube 2 and take over 1 ml to the third test tube. Mix, measure off again 1 ml and in this way continue the dilution of urine in the following test tubes. The last 1 ml volume from tube No 10 should be discarded.
- (3) To each test tube add 2 ml of the buffered 0.1% starch solution. Allow incubate in a water bath at 45°C for 15 min.
- (4) Cool test tubes and add into each tube 3 drops of iodine solution. Examine the coloration of the content. Record the number of the last test tube which gives no blue colour with iodine.
- (5) Calculate the activity in Wohlgemuth units corresponding the volume (in ml) of 0.1% starch digested by 1 ml of urine under conditions described. For example, if 1/4 ml of urine (tube 3) digested 2.0 ml of starch, the enzymic activity equals  $2 \times 4 = 8$  U. The Wohlgemuth units correspond very roughly to ukat/l.

b) *Estimation of alpha-amylase in urine (and serum) by the SPOFA Test*

*Procedure:*

1. Pipette 0.1 ml of the urine and 1.0 ml of distilled water into a conical centrifugation test-tube. Temperate for 5 minutes at 37° C on the water bath.
2. Add 1 tablet of SPOFA TEST with a forceps and incubate for 15 minutes at 37° C without shaking!
3. To terminate the reaction add 4 ml of the STOP solution and let to stay 5 minutes at room temperature.
4. Centrifuge 5 minutes at 1 700 rpm.
5. Measure the absorbance of the clear supernatant at 620 nm against water as blank.
6. Read the activity of alpha-amylase from the calibration table.

#### **4. Examination of drugs in urine**

##### **Nitrites in urine**

The finding of *nitrites* in urine gives information about the bacterial contamination of the urinary tract. In most people, urine contains at least traces of nitrates from digested food and water. In the presence of common bacteria (Escherichia coli, Proteus, Klebsiella, Salmonella, etc.) nitrates are reduced to nitrites. A diagnostic strip test reveals as little as 1.0 mg NO<sub>2</sub><sup>-</sup> per liter urine. To increase the nitrate concentration, patients are recommended to eat vegetables before collecting the urine.

##### *A proof of nitrites in urine with diagnostic test strips*

*Principle:* White area, impregnated with naftylamine and sul-fanilic acid, gives in the presence of nitrite a pink to carmine coloured azo-dye.

*Procedure:* Dip the strip briefly in urine and remove immediately. Read the colour 1 min later. Owing to the fact that nitrite may be formed in urine only as a product of action of some bacteria, the red colour indicates an unambiguous proof of the significant bacteriuria. On the other hand, a negative result does not exclude the presence of bacteria in urine.

## CLINICAL EXAMINATION OF BLOOD SERUM I

### 1. Examination of blood plasma proteins

When blood is allowed to clot, several plasma proteins contribute in forming the matrix of the clot. The resulting solution, lacking fibrinogen, fibrin, and several clotting factors, is known as *serum*. Most clinical chemical determinations are made on serum rather than plasma.

Normal plasma contains as much as 62 - 82 g/l of protein. Of this, 52-60% is *albumin*, the rest is composed of a mixture of *globulins*. Most plasma proteins are synthesized in the liver. However, the gamma-globulins are synthesized by a class of lymphocytes known as plasma cells. More than 80% of the total hepatocyte proteosynthesis is directed for export into plasma. Therefore one of the hallmarks of severe liver disease is an abnormality, usually a decrease, in one or more of the plasma proteins.

The principal functions of plasma proteins are the generation of an intravascular colloid osmotic pressure (albumin), specific and nonspecific transport, defence (circulating antibodies), coagulation and fibrinolysis, and the availability of certain enzymes and/or their precursors.

Serum proteins are usually separated by zone electrophoresis on media such as cellulose acetate into five main arbitrary classes in the order of mobility. Most clinical analyses are carried out with a barbital buffer of pH about 8.6. At this pH all normal serum proteins are negatively charged and migrate toward the anode of the electrophoresis cell: the highest negative charge and thus the highest anodal mobility shows albumin ( $pI = 4.8$ ), the gamma-globulins are the least negatively charged. The quantification of the individual classes of plasma proteins following electrophoresis involves staining and densitometric scanning.

Individual proteins may be measured accurately and specifically by various *immunotechniques*. In the Mancini radial immunodiffusion, serum diffuses from a cup cut into agar gel containing a specific antiserum, and the radius of the resulting precipitation zone is proportional to the protein concentrations. In electroimmunoassay, after separation by simple elecrophoresis, a second elfo causes the separated proteins to pass into a sheet of gel containing anti-whole-human serum antiserum. The proteins are precipitated with their specific antibodies, and the area under each protein peak is directly proportional to the concentration of that protein. These (and other) techniques can be used to estimate any of the 13 most abundant proteins as well as the other 20 or so important plasma proteins whose concentration is too low to detected by zone electrophoresis.

Major plasma proteins			%
Albumin			53-65
Globulins	Alpha <sub>1</sub>	$\alpha_1$ -antitrypsin orosomucoid $\alpha_1$ -lipoprotein transcortin (CBG)	2-4
	Alpha <sub>2</sub>	$\alpha_2$ -macroglobulin haptoglobins ceruloplasmin	8-13
	Beta	transferrin $\beta$ -lipoprotein C-reactive protein $C_3$ -complement (fibrinogen)	9-16
	Gamma	IgG      80% IgA      12% IgM      7% IgD      < 1% IgE      < 1%	12-19

In front of the albumin fraction migrates a small, by zonal electrophoresis not detectable fraction of *prealbumin*. Prealbumin takes part in the thyroxine transport and its estimation is useful especially in the determination of protein malnutrition; being a protein rich in the essential amino acid tryptophan and with a short half-life, its concentration rapidly falls in malnutrition and rises when an adequate diet is given.

The most abundant protein in plasma is *albumin*. Mature human albumin consists of one polypeptide chain of 585 amino acids and MW of about 69,000. Albumin is initially synthesized as a prealbumin in the liver. Therefore, the synthesis of albumin is depressed particularly in diseases of the liver. The plasma of patients with liver disease often shows a decrease in the ratio of albumin to globulins (A/G ratio). The plasma of certain humans lacks albumin (*analbuminemia*). Subjects with analbuminemia shows only moderate edema, due to the compensatory increase in globulins. Because of the relatively low molecular weight, high concentration, and the binding capacity for inorganic ions, albumin is responsible for 75-80%

of the *osmotic pressure* of human plasma. Many metabolites, such as free fatty acids and bilirubin, are poorly soluble in water. Albumin fulfills the function of a *carrier* to enhance the solubility of these substances. Albumin binds also poorly soluble drugs such as aspirin, digoxin, the coumarin anticoagulants, and barbiturates so that they are efficiently carried through the bloodstream. About 50% of the calcium in the plasma exists as a complex with albumin (see the chapter on plasma ions).

$\alpha_1$ -*Antitrypsin* comprises 80-90% of the  $\alpha_1$ -globulin. Its function is protective through anti-proteolytic activity. It inhibits trypsin, elastase, and certain other serine proteases by forming complexes with them. Deficiency is inherited as a recessive character and presents either as neonatal hepatitis or as emphysema (degenerative affection of alveoli) in early adult life.

Acid  $\alpha_1$ -glycoprotein with a high sugar moiety (38%) is called *orosomucoid*. Its concentration increases in inflammation.

$\alpha_1$ -*Lipoprotein* corresponds to the high-density lipoprotein (HDL) of the blood plasma.

The main plasma glucocorticoid binding protein is called *transcortin* or *corticosteroid-binding globulin (CBG)*.

In the first globulin fraction may be involved also so called *alpha-fetoprotein (AFP)*. AFP is normally synthesized almost exclusively by the fetal liver. After birth the level falls and raised levels are found with many malignant conditions, especially hepatoma and malignant teratoma, as well as viral hepatitis. In obstetrics, raised levels are found in the amniotic fluid of fetuses with neural tube and other defects.

$\alpha_2$ -*Macroglobulin*, a high-molecular-weight (MW~725,000) plasma protein, is another protein that plays an important role in the body's defence against excessive action of proteases. It shows also a certain antithrombin activity. Macroglobulin concentration is increased in nephrotic syndrome.

*Haptoglobins* are  $\alpha_2$ -globulins capable of binding free hemoglobin and may play an important role in the degradation of hemoglobin. Free Hb passes through the glomerulus, where-as the Hb-Hp complex is too large to pass through. The function of haptoglobin thus appears to be to prevent loss of free Hb and its valuable iron. The half-life of the Hb-Hp complexes is much shorter than that of Hp alone, and as a consequence, low levels of Hp are found in patients with hemolytic anemias. On the other hand, haptoglobin is an acute phase protein, and its plasma level is elevated in inflammations.

*Ceruloplasmin* carries 90% of the copper present in plasma, the remaining 10% is accounted for by albumin. It has a blue colour because of its copper content. Ceruloplasmin bears a special relationship to Wilson's disease, a condition of copper toxicosis.

*Transferrin* plays a central role in the body's metabolism of iron because it transports iron in the circulation to sites where iron is required, e.g. from the gut to the bone marrow and other organs. Free iron is toxic, but association with transferrin diminishes its potential toxicity. The concentration of transferrin in plasma is about 3g/l. This amount can bind approximately 3 mg of iron per liter, so that this represents the *total iron-binding capacity* of plasma. However, the protein is normally only one-third saturated with iron. In iron-deficiency anemia, the protein is even less saturated with iron, whereas in conditions of storage of excess iron in the body (e.g. hemochromatosis) the saturation with iron is much greater than one-third.

$\beta$ -*Lipoprotein* corresponds to the plasma low density lipoproteins (LDL). They transport free and esterified cholesterol with a little triglyceride. This fraction contains the majority of the total plasma cholesterol.

*C-reactive protein* is a serum protein which can react with a somatic C-polysaccharide of the pneumococcus. Its concentration increases early in acute inflammation and reflects activity.

The *complement system* is involved in foreign cell lysis and inflammation. It has nine main factors but C3 and C4, which make up 95% of the total, are the two proteins most easily measured immunochemically. Low levels of complement are of more importance, indicating increased consumption or decreased synthesis. It is an acute phase marker.

A rise in *fibrinogen* (in the plasma only) is a sensitive indicator of connective tissue disorders such as rheumatoid arthritis.

The  $\gamma$ -globulin fraction contains the *immunoglobulins (Ig)*, which on electrophoresis normally separate as a diffusely stained band. Intensely stained, narrow, localized bands observed on electrophoresis are called *paraproteins* and are signs of a *monoclonal gammopathy*. The immunoglobulins are a mixture of proteins all of which possess antibody activity. There are five main classes of immunoglobulins: IgG, IgM, IgA, IgD and IgE. These all have similar molecular weight, except IgM, which is made up of five subunits and IgA, which is normally a dimer. The Ig monomers possess the common basic structure consisting of two heavy chains and two light chains covalently linked by disulphide bonds. The light chains have a molecular weight of about 22,000, the molecular weight of heavy chains ranges from 50,000 to 75,000. Immunoglobulins are synthesized by plasma cells

in the lymphoid tissue and bone marrow. According to the clonal selection theory, a given plasma cell synthesizes Ig of a single molecular type only. Tumors of Ig-synthesizing cells are called *plasmocytomas (myelomas)*. The paraproteins they produce are seen on electrophoresis of plasma proteins. Sometimes, light or heavy chains are produced only. Then they may be detected also in urine, e.g. as Bence Jones protein consisting of intact light chains. Paraproteins are found in serum on screening, by electrophoresis, the precise biochemical identification is carried out by immunotechniques. The incidence rises markedly with age (1% over 50, 3% over 70 years of age). No malignancy can be found in about 25% of the patients with paraproteinemia (*benign gammopathy*).

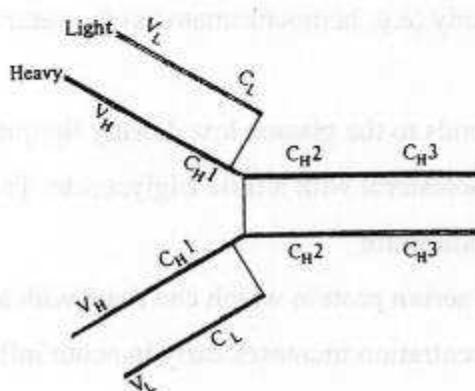


Diagram of IgG molecule made up of two heavy and two light chains.  $V_H$  and  $V_L$  = N-terminal variable sequence of heavy and light chains.  $C_H1-3$  and  $C_L$  = C-terminal constant sequence of heavy and light chains.

#### *Assessment of plasma protein examination*

The concentration of plasma *albumin* varies much more than does that of globulin. Albumin is affected much more readily by the protein intake, whereas globulin is regenerated more quickly after hemorrhage. The generalization may be made that the albumin fraction is rarely increased above normal and the globulin rarely decreased below normal. An increase in the concentration of albumin may occur in dehydration. In such conditions the globulin and albumin fractions will be proportionally increased. Diminution in plasma albumin occurs in the following conditions:

- (1) Loss of albumin (proteinuria in nephritis and nephrosis, ascites, extensive burns, hemorrhage, etc.);
- (2) Inadequate supply;

- (3) Impaired synthesis (cirrhosis);
- (4) Excessive protein catabolism (diabetes mellitus);
- (5) Plasma dilution.

Plasma proteins whose concentration alters following trauma, surgery, tissue necrosis (myocardial infarction) or inflammation are known as *acute phase reactants*. These are: haptoglobins, orosomucoid, alpha<sub>1</sub>-antitrypsin, C-reactive protein, complement and fibrinogen. Note that they are present in the fastest globulin fractions and rise in the conditions mentioned above.

#### DYSPROTEINEMIAS (simplified)

Condition	Total P	A	alpha <sub>1</sub>	alpha <sub>2</sub>	beta	gama
hypoproteinemic	↓ ↓	↓↓	N, ↑	N ↑	↓	↓, N
nephrotic	↓ ↓	↓↓		↑↑		
hepatic	↓, N	↓↓	↓	↓	↓	↑
acute inflam.		↓	↑	↑		N
chronic inflam.		↓	↑	↑		↑
hyper-gamma	↑	↓				↑
analbuminemia		↓↓↓				
agammaglobulin.						↓↓↓

X-chromosome-linked *hypogammaglobulinemia* is due to lack of B lymphocytes. Without special precautions, affected babies die from infection with pyogenic bacteria. Unlike in Ig-synthesizing tumors, in chronic inflammation the overall increase in IgG fractions involving various types of antibody molecules is found. This condition is called the *polyclonal gammopathy*.

#### EXERCISE 1

##### a) Estimation of total plasma protein

*Principle:* Proteins and peptides, similarly to biuret, react with cupric ions in alkaline solutions to form a violet complex suitable for the photometric determination.

*Reagents:* Biuret reagent (mix 1.5g CuSO<sub>4</sub>.5H<sub>2</sub>O and 6g of NaK-tartarate with 500ml of distilled water and 300ml of 2.5M NaOH and under shaking dissolve in this mixture 1g of KI; fill up to 1 l with dist. water);  
 Solution of NaCl (0.9g NaCl/100ml);  
 Protein standard.

*Procedure:*

- (1) Pipette into three common test tubes:

	sample	standard	blank
sample	0.1	-	-
standard	-	0.1	-
NaCl solution	-	-	0.1
biuret reagent	5.0	5.0	5.0

- (2) Mix properly and allow to stand for 30 min.  
 (3) Read immediately the absorbances of a sample and standard at 546 nm (520-570 nm) against the blank.  
 (4) Calculate the total protein concentration:

$$\text{Total protein (g/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \cdot c_{\text{standard}}$$

b) *Estimation of plasma albumin*

*Principle:* Bromocresol purple (5,5'-dibromo-o-cresolsulfon-phthalein) yields with albumin in the presence of detergents in acid a blue-green complex suitable for the photometric determination.

*Solutions:* Reagent solution (dissolve 1 tablet of BCP in 60 ml of acetate buffer 0.1 mol/l pH 5.2 with Brij 35, 0.35g/l);  
 Albumin standard (30-50g/l).

*Procedure:*

(1) Pipette into three common tubes:

	sample	standard	blank
sample (serum)	0.010	-	-
standard	-	0.010	-
dist. water	-	-	0.010
reagent	1.5	1.5	1.5

(2) Mix and allow to stand for 10 min at the room temperature.

(3) Read the absorbance of the sample and standard against the blank at 600-605 nm.

Calculate the concentration of albumin:

$$\text{Albumin (g/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \cdot c_{\text{standard}}$$

(4) Calculate the A/G coefficient:

$$A/G = \frac{c_{\text{albumin}}}{c_{\text{total prot.}} - c_{\text{alb.}}}$$

## 2. Serum turbidity tests

The liver plays an important part in normal protein metabolism. Its chief function in this connection appears to be the deamination of amino acids, urea formation, and the formation of many important proteins. *Fibrinogen* is formed entirely in the liver and remarkable variations may be observed in the fibrinogen content of blood plasma in liver disease. In the presence of severe liver damage the fibrinogen may fall to extremely low levels. *Prothrombin*, a factor essential for normal coagulation, is formed in the liver as well. For the maintenance of a normal plasma concentration, adequate hepatocellular function is necessary, along with the adequate vitamin K supply.

The liver plays also a major role in the formation of plasma *albumin*. The diminution in serum protein in hepatic disease occurs chiefly if not entirely in the albumin fraction. In some instances, particularly in acute forms of liver disease, serum albumin may be only moderately reduced and the serum *globulin* increased. This increase is observed much more

commonly in hepatocellular disorders than in obstructive jaundice, and in some cases, especially in cirrhosis, may be so great as to more than counterbalance the albumin deficit, the total serum protein concentration being actually increased.

Several simple tests were developed to establish the dysproportion between albumin and globulin fraction concentrations, respectively. They make use of the imbalance in the colloidal stability of the serum proteins, caused by the relative increase of globulins with respect to the albumin level. Certain concentrations of mercuric, zinc or calcium salts, or of thymol, added to serum may turn the mixture turbid. These tests were regarded as "*liver function tests*" because of the role of the liver in the metabolism of proteins. Of them, the *Thymol Turbidity Test (TTT)* is still used for its simplicity and reliability. It should be stated, however, that the estimation of the *A/G coefficient* has a similar value.

## EXERCISE 2

### *Thymol turbidity test*

- (1) To 0.1 ml of serum in a test tube add 6.0 ml of thymol reagent (thymol in a veronal buffer, pH 7.8), mix and allow to stand at a laboratory temperature for 30 min.
- (2) Read absorbance of a turbid solution against the thymol reagent and estimate the result in TTT units from the calibration diagram. ( w.l. 660 nm )

*Evaluation:* Normal values do not exceed 4 units. Higher values accompany especially acute hepatitis. The turbidity is thought to be caused by the complex containing gamma-globulins and beta-lipoproteins.

### **3. Serum mucoproteins and beta-lipoproteins**

Serum glycoproteins containing more than 4% glycosamines are called *mucoproteins*. Physical properties of mucoproteins allow their detection as perchloric acid non-precipitable fraction of the blood serum. The increase of this fraction is found in acute inflammation conditions (some components belong to the "acute phase reactants") and in a cancer. Low values accompany liver damage. Physiological concentration ranges from 16.7 - 23.7 mg of tyrosine /l. This rather unusual amount expression corresponds to the calibration used in the protein estimation.

*Beta-lipoproteins* are easily precipitated from the blood serum in the presence of calcium ions and heparin. This property is employed in the quantitative estimation of this fraction or, in a modified form, for the distribution of the lipoprotein cholesterol. Beta-

lipoproteins contain majority of the plasma cholesterol and, therefore, the increase in this value is in most cases proportional to the degree of hypercholesterolemia.

### EXERCISE 3

#### a) *Estimation of serum mucoproteins*

*Principle:* Perchloric acid precipitates all plasmatic proteins with exception of mucoproteins. These are in turn precipitated by phosphotungstic acid, the precipitate dissolved and reducing amino acids (tyrosine) estimated with phenol (Folin) reagent. The amount of tyrosine is proportional to the absorbance of a blue complex.

*Procedure:*

- (1) To 1.0 ml of serum in a test tube add under shaking 4.0 ml of perchloric acid (0.75 mol/l) by drops.
- (2) Let stand for 10 min, filter (or centrifuge), and pipette 2.0 ml of filtrate (supernatant) into conical centrifuge test tube.
- (3) Add 0.2 ml of phosphotungstic acid (5% in HCl), mix and allow to stay for 10 min.
- (4) Centrifuge at 3000 rpm for 10 min and decant carefully the entire supernatant.
- (5) Add to the sediment 1.3 ml of sodium carbonate solution (1/5 saturated) and dissolve completely the mucoprotein. Add 0.2 ml of phenol (Folin) reagent, mix, and allow to stand 1/2 h at room temperature.
- (6) Read the absorbance in 1 cm cuvette against blank (1.6 ml of sodium carbonate and 0.25 ml of Folin reagent) at 610 nm. Read the concentration in the calibration graph as mg of protein tyrosine/l.

#### b) *Estimation of beta-lipoproteins in serum*

*Principle:* Concentration of serum beta-lipoproteins is proportional to the serum turbidity in the presence of calcium ions and heparin.

*Procedure:*

- (1) Put into two test tubes 2.0 ml of  $\text{CaCl}_2$  solution, respectively.
- (2) To both test tubes add 0.2 ml of serum, mix by drawing up and expelling the liquid by the pipette.
- (3) To one of the test tubes add 0.04 ml of heparin solution, mix and, 4 min later, measure the absorbance against the second test tube at 720 nm in 1 cm cell.
- (4) Calculate the concentration:

$$\text{beta-lipoproteins (g/l)} = A_{\text{sample}} \times 10.7$$

Normal range: 4.0 - 6.0 g/l

#### **4. Transudate and exudate**

Traditionally, abnormal accumulations of fluid in the subcutaneous tissues and serous cavities (pleural, abdominal) are divided into *transudative* and *exudative*. Transudative fluids are supposed to have their pathogenesis in increased hydrostatic pressure in the venous beds with or without diminished serum oncotic pressure or increased pressure in lymphatics, whereas exudative fluids represent inflammatory or neoplastic causes that damage and disrupt capillaries and lymphatics. The traditional tools for making the division are the use of appearance, the protein concentration, and specific gravity. A fluid that is produced by transudation is usually clear and straw-coloured, with a low protein and specific gravity. There is no fibrinogen and no clot formation. Since inflammation is absent, the cellular content of such a fluid is low. On the other hand, exudates have a high specific gravity that corresponds to the concentration of protein and to solutes, both of which approach the levels observed in the plasma. Exudates often clot after removal since they contain fibrinogen. Defibrination may occur in body cavities, however, and not all exudates will clot. Cellular elements are more frequent and may include inflammatory cells or red blood cells. In addition, the glucose content of inflammatory exudates, unlike of transudates, is relatively low due to the catabolism of glucose by the action of bacteria and cells present in the fluid. Due to the presence of acid metabolites, pH of exudates is significantly lower than pH of transudates.

#### **EXERCISE 4**

##### *Discrimination of transudates from exudates*

###### a) *Rivalta's test*

Drop the pathological fluid into 0.1% acetic acid. In the presence of exudate, a white cloud is formed.

###### b) *Gangi's test*

Layer diluted HCl onto the fluid to be examined in a test tube. A white ring on the junction confirms the presence of an exudate.

###### c) *Acidity test*

Dilute 10 ml of a pathological fluid up to 50 ml with distilled water and add 1-2 drops of phenolphthalein. Titrate against NaOH standard 0.1 mol/l. Volumes below 0.4 ml are characteristic for transudate, higher volumes are found in exudates.

## CLINICAL EXAMINATION OF BLOOD SERUM II

### 1. Blood glucose and disorders of carbohydrate metabolism

Relative constancy of the *blood glucose concentration* (3.3 - 5.6 mmol/l) depends upon the precisely adjusted balance of glucose entering the blood and glucose leaving the blood. The input of glucose is derived from dietary carbohydrate, hepatic glycogenolysis and gluconeogenesis from protein. On the other hand, glucose is removed from the body by oxidation to carbon dioxide and water, conversion to fatty acids in adipose tissues, conversion to muscle glycogen or, if blood glucose is high, by renal excretion.

*Insulin* is the only hormone capable of lowering or preventing excessive rise in blood glucose, for it increases carbohydrate utilization by all the metabolic pathways. Insulin primarily increases cell permeability to glucose and most, if not all, its other metabolic effects are secondary to this. In muscle, insulin enhances glycogen formation, in adipose tissue, insulin promotes the synthesis of fatty acids from glucose. Insulin also increases hepatic glycogen formation, increases protein synthesis especially in muscle. Hence, insulin has been termed the hormone of nutrient storage.

In contrast to insulin, *glucagon* causes glycogenolysis, lipolysis, ketogenesis and gluconeogenesis. *Growth hormone, adrenaline, glucogenic steroids* and, indirectly, *ACTH* all tend to increase or to prevent excessive fall in blood glucose. Growth hormone causes increased protein synthesis and antagonizes insulin (e.g. depresses the glucose uptake). Adrenaline mobilizes liver glycogen by the activation of phosphorylase and stimulates the secretion of ACTH, which in turn enhances the secretion of glucogenic steroids which increase gluconeogenesis.

Clinical *diabetes mellitus* is broadly classified into two types: (1) *Type I, juvenile, insulin-dependent*; and (2) *Type II, maturity onset, not insulin-dependent*, often controlled by diet but may require oral hypoglycaemic agents. Type I diabetes is a chronic autoimmune disease associated with selective islet beta-cell destruction. More than 95% of individuals express the "diabetogenic" HLA allele DR 3/4. Virtually all patients with type II diabetes have some degree of insulin resistance due to the defects at the level of the insulin receptor and at several postreceptor steps involved in insulin action.

Metabolic abnormalities in diabetes include hyperglycaemia, glycosuria, polyuria and dehydration, hyperlipidaemia, ketonemia and ketonuria, acidosis, changes in plasma electrolytes, and sometimes azotaemia. *Hyperglycaemia* is due to an absolute or relative insufficiency of insulin, causing deficient utilization of carbohydrate, glycogenolysis and

gluconeogenesis. *Glucosuria* depends on the concentration of glucose in the plasma, glucose  $T_m$ , GFR, and other factors. Glycosuria causes an osmotic diuresis and the resulting *polyuria* results in *dehydration* in which the water loss is shared by the entire body water. Associated sodium deficiency leads to water loss in the extracellular fluid. The decreased utilization of glucose increases the utilization of triglyceride with formation of glycerol and *non-esterified fatty acids (NEFA)*. The NEFA are utilized by the tissues for energy production or are utilized in the liver to triglycerides. These, together with cholesterol synthesized in the liver from acetyl-CoA, result in *hyperlipoproteinemia*. The fatty acids are degraded into acetyl-CoA which, if not enough oxaloacetic acid is available, is converted into acetoacetyl CoA or synthesized into cholesterol via beta-hydroxy-beta-methylglutaryl CoA. The latter may produce acetoacetic acid, which, with the reduction product beta-hydroxybutyric acid and its decarboxylation product acetone, form the *ketone bodies*. Acetoacetic acid can be utilized by the tissues, especially by the muscles, for the production of energy, but if it is formed at a rate greater than the muscle can utilize it, then ketone bodies accumulate in the blood and appear in the urine. In the initial stages of diabetic ketosis, the associated fall in bicarbonate (*metabolic acidosis*) is compensated by a fall in  $P_{CO_2}$ , in later stages, however, the fall in plasma bicarbonate becomes so extreme that the  $P_{CO_2}$  fails to decrease proportionately and compensate sufficiently the acidosis.

Elderly diabetic patients are subject to the *late complications of diabetes*: macrovascular disease (heart infarction, peripheral vascular disease, stroke); microangiopathy (retinopathy, diabetic glomerulosclerosis); neuropathy. Several disorders may share a common pathogenesis. Schwann cells, glomerulus, and possibly retinal capillaries contain aldose reductase which reduces glucose to sorbitol. It was shown that sorbitol accumulation may damage these tissues by causing them to swell.

The estimation of *blood glucose* is one of the most important laboratory tests. The most precise determinations include enzymatic tests, e.g. the combination of glucose oxidase and peroxidase reactions. Many patients with diabetes control their treatment by semiquantitative urine tests, eventually use simple devices for the measurement of glucose in capillary blood.

In the diagnosis of diabetes, single determinations of blood sugar sometimes give borderline results difficult to interpret. A *glucose tolerance test* must then be performed. Because of the impairment of glucose tolerance occurring during fasting the patient must be given a full carbohydrate diet for 7 days before test. After fasting overnight, blood and urine

specimens are analysed for glucose before and usually 2 hours after the administration of 75g glucose in 250-350 ml of water. Persons with a normal glucose tolerance have a fasting blood glucose below 6-7 mmol/l, which does not rise during the test above 10 mmol/l and returns to normal within 2 h. Impaired glucose tolerance is present when the fasting blood glucose is about 7 mmol/l and rises to a level above 10 (glycosuria!) and the values between 8-11 mmol/l are observed 2 hours after the glucose uptake. In diabetes, both the fasting and final levels are higher. Impaired glucose tolerance is observed not only in diabetes mellitus but also in hyperthyroidism, severe liver disease, Cushing's syndrome, and many other conditions.

In patients with impaired glucose tolerance or mild diabetes it is sometimes useful to obtain *serum insulin* levels. The presence of normal or elevated serum insulin with a peak response to oral glucose indicates islet cells reserve and possible normalization of blood glucose with diet restriction.

In the biosynthesis of insulin, a pro-insulin consisting of two subunits of insulin linked by a *connecting peptide (C-peptide)* is cleaved for secretion. Whereas insulin may be degraded in the liver, C-peptide remains unimpaired and thus provides a measure for the pancreatic insulin production. Insulin level is normally 0.5-0.8 ug/l, C-peptide level is as much as 0.9-3.5 ug/l.

An important tool in monitoring diabetic control is the blood concentration of *glycosylated hemoglobin*. HbA glycosylated on the N-terminal valine of the beta-chain by a nonenzymic reaction is proportional in amount to the mean blood glucose concentration over the preceding 2-3 months (normal value 3.5-7.0 umol fructose/g Hb). Thus, measurements of glycosylated hemoglobin provide an objective measure of average glycemia during the preceding 6-12 weeks. It was shown that in uncontrolled diabetics high concentrations of glycosylated Hb will diminish delivery of oxygen to the tissues because of the block of conformational changes.

*Hypoglycaemia* is the clinical state due to a low plasma glucose, usually less than 2.2 mmol/l, but the severity of symptoms may be related more to the rate of fall than to the actual glucose concentration. Irreversible coma may quickly develop if the condition is not effectively treated caused by the impairment of the normal CNS metabolism.

*Galactosemia* is due to deficiency of the gene directing the synthesis of the enzyme galactose-1-phosphate uridyl transferase concerned with the conversion of galactose to glucose. Children with the disease fail to thrive and the accumulation of galactose and the sugar alcohol galactitol causes mental retardation, liver damage, cataracts, and other defects.

Normal growth and development can occur if a galactose-free regimen is started early enough. Early diagnosis is therefore essential.

*Glycogenesis* involves glycogen storage diseases, a number of different clinical forms according to which of the enzymes responsible for degrading glycogen is deficient. Gierke's disease (hepatomegaly, failure to thrive, hypoglycaemia, ketosis) is due to a deficiency of glucose-6-phosphate. Diagnosis depends upon glycogen and enzyme estimations of various tissues.)

## EXERCISE 1

### **Blood glucose estimation**

**Principle:** Glucose oxidase catalyses oxidation of glucose to hydrogen peroxide and gluconate. In the presence of peroxidase, hydrogen peroxide reacts with the chromogen (e.g. 3-methylphenol and 4-aminophenazone) to form coloured product.

**Reagents:** glucose standard (usually 10 mmol/l);  
deproteinization solution (5% trichloroacetic acid, TCA);  
reagent (solution of enzymes mixed with buffered chromogen )

#### *Procedure without deproteinization:*

##### (1) Pipette:

	sample	standard	blank
sample/standard	0.02	0.02	-
distilled water	-	-	0.02
reagent	2.0	2.0	2.0

(3) Incubate for 15 min at 37°C or 30 min at room temperature. Read the absorbances of the sample and standard against the blank within 30 min after incubation (498 nm).

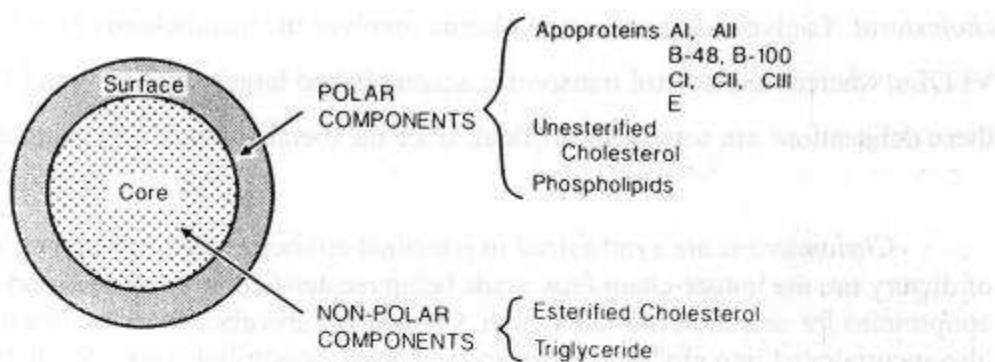
(4) Calculate the concentration according to

$$\text{glucose (mmol/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \cdot c_{\text{standard}}$$

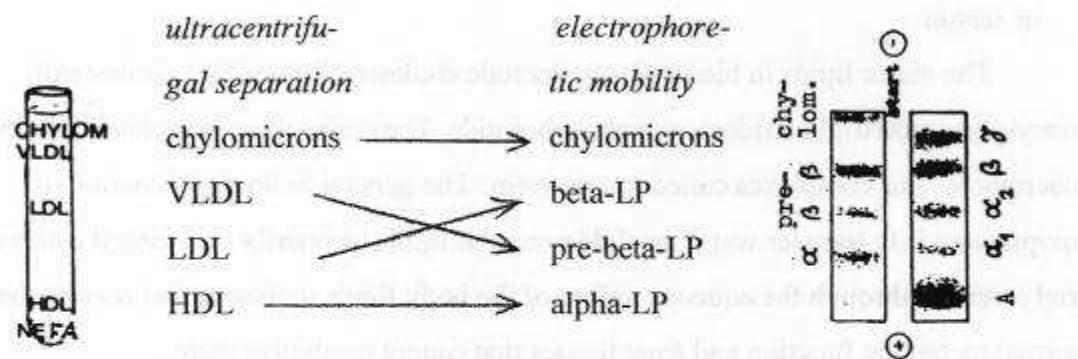
## 2. Lipid metabolism and its disorders, examination of cholesterol and triacylglycerols in serum

The major lipids in blood plasma include cholesterol, esterified cholesterol, triacylglycerols (triglycerides), and phospholipids. They exist there associated with proteins in macromolecular complexes called *lipoproteins*. The general biological function of lipoproteins is to transfer water insoluble unpolar lipids (primarily cholestryl esters and triglycerides) through the aqueous milieu of the body fluids *to* tissues that require them for normal metabolic function and *from* tissues that cannot catabolize them.

Lipoproteins are globular or spherical particles that contain polar components (phospholipids, unesterified cholesterol, and proteins) on the surface in contact with body fluids, and a core of nonpolar lipid, including cholestryl esters and triglycerides (see the figure). The proteins of lipoproteins (LP) are called *apolipoproteins (apoproteins)*; they are required for several important functions, including the synthesis of LP, the activation of enzymes important in LP metabolism, and the interaction of LP with cell surface receptors that promote the cellular uptake of LP.



The plasma lipoproteins are divided into five major families, including *chylomicrons*, *very low density LP (VLDLs)*, *intermediate density LP (IDLs)*, *low-density LP (LDLs)*, and *high-density LP (HDLs)*. Each family contains a spectrum of particles with similar metabolic functions, and the families are differentiated from one another by certain physicochemical properties, such as their hydrated density range (ultracentrifugation) or their migration during electrophoresis:



The density of a lipoprotein is determined largely by the ratio of protein to lipid in the particle. The least-dense LPs contain the least amount of protein as a percent of their composition; as the protein content in a LP family increases relative to lipid, the hydrated density also increases. The least-dense LPs are also the largest and contain the greatest amount of triglyceride (chylomicrons, VLDLs). In contrast, HDL particles, the most dense of the LPs, are the smallest in size and contain more phospholipid per particle than the LPs of lower density.

The constituents of lipoproteins of most clinical interest are *triglycerides* and *cholesterol*. Triglyceride transport in plasma involves the metabolism of chylomicrons and VLDLs, whereas cholesterol transport is accomplished largely by LDLs and HDLs. However, these delineations are somewhat artificial, since the metabolism of LPs is highly interrelated.

*Chylomicrons* are synthesized in intestinal epithelial cells in response to the ingestion of dietary fat, the longer-chain fatty acids being reesterified to glycerides and combined to apoproteins for secretion into the lymph. Cholesterol absorbed from the intestinal lumen is also incorporated into chylomicrons and although it contributes only 5% to the mass of the particle, CHMs represent a major route by which dietary cholesterol is taken into the body. Chylomicrons enter the plasma whenever a meal containing fat is consumed, and they are removed from the plasma with a half-life of about 30 min. After entering plasma they acquire additional apoproteins, especially CII, from HDL. Apo CII activates *lipoprotein lipase* anchored to capillary endothelial cells to hydrolyze the core triglyceride to free fatty acids (NEFA, FFA). NEFAs are taken into tissues for oxidation (e.g. muscle) or storage for future use (adipose tissue). After most of the CHM-triglyceride core is hydrolyzed, the particle dissociates from lipoprotein lipase as a *chylomicron remnant*. The remnant particles are rapidly removed from the circulation in the liver by a receptor that binds with Apo E on the remnant particle surface.

*VLDLs* are somewhat smaller in size and of higher density than the chylomicrons. They are produced in the liver and secreted as triglyceride-rich particles containing many apoproteins, among them Apo B and the Apo C, partly transferred from HDLs. VLDLs interact with lipoprotein lipase in capillaries, where the triglyceride core is hydrolyzed to yield NEFAs, principally for adipose tissue and muscle.

LP class	CHYL	VLDL	LDL	HDL
Elfo mobility	start	pre-beta	beta	alpha
Size (nm)	10 <sup>2</sup> -10 <sup>4</sup>	30-70	15-70	7.5-10
Composition:	%	%	%	%
PROTEINS	1	10	22	50
PHOSPHOLIPIDS	4	15	23	30
CHOLESTEROL	5	15	45	18
TRIGLYCERIDES	90	60	10	2
Apolipo-proteins	Apo A Apo B Apo C I-III Apo E		Apo B	Apo A I Apo A II Apo D Apo E

When VLDL remnants are released from lipoprotein lipase, they are termed *intermediate-density LPs (IDLs)*. IDL particles have two major metabolic fates: they may be taken up by the LDL receptor on the liver or they may be converted to *LDLs*, the cholesterol-rich particles that supply CHOL to cells via the LDL receptor pathway or by receptor-independent mechanisms (one third of the total LDL). LDL receptors are found in most tissues throughout the body, however, more than 60% are located in the liver.

Cells require *cholesterol* for normal function and they obtain it from two major sources: biosynthesis from acetyl-CoA and uptake of lipoprotein-cholesterol from the interstitial fluid. Cells must also be able to rid themselves of excess cholesterol, since they lack the enzymes to break apart the sterol nucleus. Cells will take up CHOL from LDL via the LDL receptor. The interaction with the receptor is mediated by Apo B-100. Depending on the tissue, CHOL can be used for new membrane formation, bile acid formation (liver), steroid hormone production (ovary, testis, adrenal). When adequate amounts of CHOL are entering the cell via the LDL receptor pathway, cells suppress their endogenous synthesis as reflected by the decreased activity of *HMG-CoA reductase*, the rate-limiting enzyme in cholesterol biosynthesis. Cells also decrease the number of LDL receptors active on the cell surface and they increase the activity of *acyl-CoA: cholesterol acyltransferase* to esterify any excess cholesterol for storage as cholesteryl ester droplets. The removal of CHOL is mediated by the disk-shaped "nascent" HDL particles (produced in the liver, intestine, and perhaps other tissues), which acquire unesterified CHOL when they come in contact with cell membranes. This activity is enhanced by *lecithin-cholesterol acyltransferase (LCAT)*, an enzyme produced in liver and active in plasma. LCAT esterifies the CHOL on the surface of the disks, obtaining

the fatty acid from the number 2 carbon of lecithin (facilitated by Apo AI). The nonpolar CHOL esters then migrate to the core of the disk, evolving into spheric particles. There are indications that the liver directly takes up HDL through the agency of a specific HDL receptor.

The tissue cholesterol initially acquired by nascent HDL particles may eventually be transferred as cholesteryl ester to triglyceride-rich LP during their catabolism. If this transfer is to chylomicron remnants, the transferred cholesteryl esters are returned to the liver. If the transfer is to VLDL remnants and IDLs, the CHOL esters may return to the liver as IDLs, or they may appear in LDLs. Thus, "reverse cholesterol transport" does not often involve a direct route from peripheral tissues to the liver, but depends on the repeated transfer of cholesteryl esters among lipoproteins before final excretion occurs through the liver.

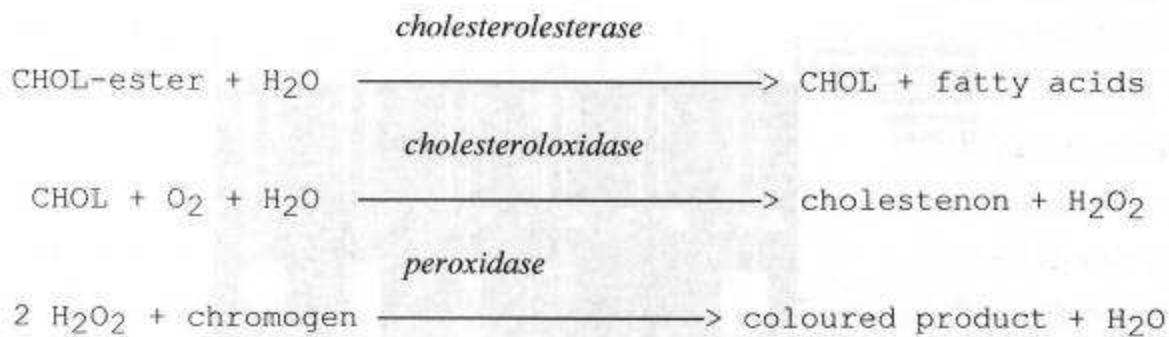
*Lipoprotein (a)* is another lipoprotein found to a variable extent in human plasma. This LP is important because its presence and concentration are linked to increased risk for coronary heart disease, independent of the other LP. LP (a) is comparable in size to LDL, but it is somewhat more dense and exhibits pre-beta migration on electrophoresis. It is sometimes called *sinking pre-beta LP* to distinguish it from VLDL. The major lipid is CHOL ester, but the protein content is unusual in that it consists of Apo B-100 linked by disulfide bonds to a plasminogenlike protein. LP (a) is detectable by sensitive immunoassay techniques.

Plasma lipids show a unimodal skewed distribution in the normal population, so there is no clear-cut definition of a normal upper limit. *Reference fasting ranges* of the population vary according to the geographic and racial distribution in that population. The local "normal range" is shown in the following table:

<i>CHOLESTEROL</i>	4.14 - 6.22 mmol/l
<i>LDL-cholesterol</i>	4.0 - 4.5 mmol/l
<i>HDL-cholesterol</i>	males: 1.29 mmol/l females: 1.55 mmol/l
<i>TRIACYLGLYCEROLS</i>	males (30-40yrs): 0.34-2.36 mmol/l females ( " ) : 0.34-1.44 mmol/l
<i>PHOSPHOLIPIDS</i>	1.75 - 3.00 g/l
<i>NEFA</i>	0.1 - 0.6 mmol/l

All of the risk factors established thus far, lipid disorders play a key role in the pathogenesis of atherosclerotic vascular diseases, especially of coronary heart disease. It is widely accepted today that a serum cholesterol level of about 5.2 mmol/l represents the cutoff value beyond which the risk increases progressively. However, the atherogenic significance of the total cholesterol must be viewed judiciously. There is evidence that LDLs, as the carrier of ca. 70% of the total cholesterol, are the most potent atherogenic lipoproteins. Patients with low LDL cholesterol have a high life expectancy. In contrast to LDL, an increase of the second CHOL-rich class - the HDL - is not associated with risk. It has become apparent that there is an inverse relation between coronary heart disease and the HDL (or HDL cholesterol) concentration. This would seem to indicate that a raised HDL may even be protective against the disease.

Several enzymic methods currently exist for the assay of *CHOLESTEROL*, one of which, the CHOD-PAP method, has become established worldwide. The method is based on three enzyme catalyzed steps:



In most countries the precipitation technique employing phosphotungstic acid/Mg<sup>2+</sup> is the most commonly used for determination of HDL-cholesterol. After centrifugation, HDL fraction remains in the supernatant. Here, cholesterol is estimated by the current methods. LDL-cholesterol may be calculated from the value of the total CHOL, TG, and HDL-cholesterol, or may be estimated by a suitable precipitation method.

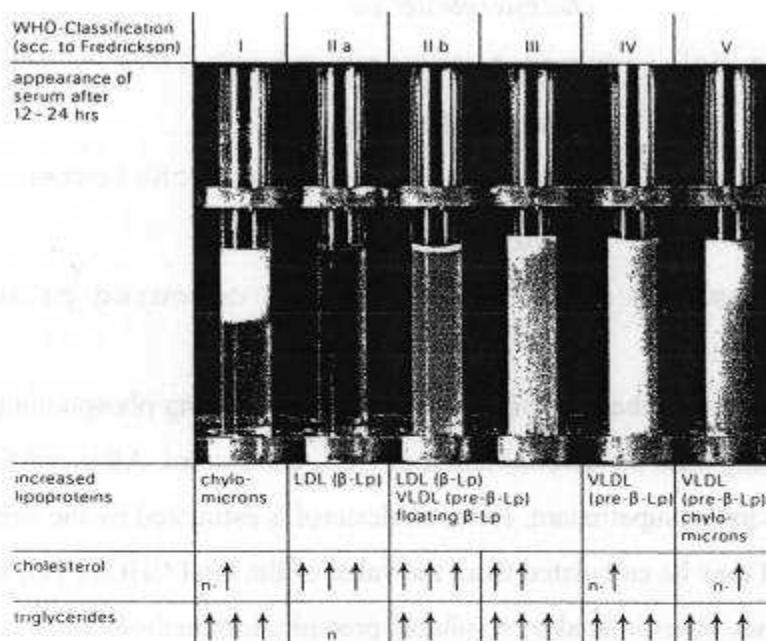
Determination of the *TRIACYLGLYCEROLE* (triglyceride, TG) concentration has become a basic routine test as many patients with coronary heart disease exhibit significantly elevated TG levels. Determination of the TG concentration is performed mainly by the enzymic methods (e.g. by the combined action of lipase, glycerokinase, and glycerolphosphateoxidase), or by the determination of glycerol as a coloured product following saponification of the specimen.

*Hyperlipoproteinemas* occur as primary, mostly familial inborn defects, or secondary to hypothyroidism, renal or hepatobiliary disease, alcoholism, diabetes, gout, oestrogen or corticosteroid therapy. An international classification was proposed by Fredrickson. Routine typing is based on measurement of *fasting serum* cholesterol and triglyceride, with inspection of the serum after 18 hours at 4°C.

*Type I* is a rare disease, chylomicronemia is due to deficiency of extrahepatic LP lipase, or Apo C-II defect.

*Type IIa* represents familial hypercholesterolemia. The serum is clear, elfo analysis shows a dense beta-LP band. This disorder commonly presents after a myocardial infarct. The disease may be due to a defect in the cellular uptake of CHOL from LDL or from failure in regulation of CHOL biosynthesis. Lovastatin, an HMG-CoA reductase inhibitor, is used in the medical treatment. *Type IIb* shows the tendency for VLDL to be elevated in addition.

*Type III* is caused by the deficiency in remnant clearance by the liver due to abnormality in Apo E. IDLs are shown in electrophoresis as a "broad beta".



*Type IV*, familial hypertriglyceridemia, is a common condition, associated with overproduction of VLDL, but CHOL and LDL are normal. It occurs in patients with ischaemic heart disease and/or peripheral vascular disease.

*Type V* is accompanied by elevated chylomicrons and VLDL, but this is the least common hyperlipoproteinemia.

There is a growing interest in the quantitative determination of *APOLIPOPROTEINS* in serum. In clinical research the determination serves mainly for the identification of apolipoproteinopathies and for the detection of patients with risk of cardiovascular disease. Monospecific antibodies for determination of *Apo A1*, *AII*, *B*, and *Apo (a)* are available.

## EXCERCISE 2

### a) *Plasma cholesterol estimation*

*Principle:* Cholesterol and its esters in the presence of cholesterol esterase and cholesterol oxidase produce stoichiometric amount of hydrogen peroxide which, under the action of peroxidase, turns 4-aminoantipyrin and p-hydroxybenzoic acid into coloured quinonimine.

*Reagents:*

Enzyme + chromogen solution buffered with phosphate buffer

pH 6.7;

Cholesterol standard;

*Procedure:*

(1) Pipette (ml):

	sample	standard	blank
sample	0.015	-	-
standard	-	0.015	-
enzyme reagent	1.5	1.5	1.5

(2) Mix and incubate 10 min at 37°C.

(3) Read the absorbance against the blank in 1 cm cuvette at 510 nm.

(4) Calculate the plasma concentration:

$$\text{Cholesterol (mmol/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times C_{\text{standard}}$$

b) *Plasma triacylglycerol estimation*

*Principle:* Triacylglycerols are saponified to glycerol by potassium hydroxide. The glycerol is oxidized in the presence of iodate to formaldehyde which forms with acetyl acetone and ammonium ions yellow 3,5-diacetyl-1,4-dihydrolutidine.

*Reagents:*

Standard solution of trioleine (3.39 mmol/l);

Acetyl acetone in 20% isopropanol (Reagent 2);

Oxidizing solution (Reagent 3);

Potassium hydroxide 1 mol/l (Reagent 4)

Isopropanol (Reagent 5)

Adsorbent (provided with measuring vessel)

*Procedure:*

- (1) Pipette into conical test tubes (ml):

	sample	standard	blank
serum	0.1	-	-
standard (Reag. 1)	-	0.1	-
dist. water	-	-	-
isopropanol (R.5)	4.0	4.0	4.0

*To remove phospholipids*

- (2) Add to all tubes 0.4 g (a full measuring vessel) of adsorbent (Reagent 6), stopper the tubes and shake for about 10 min. Remove stoppers and centrifuge for 5 min at 3000 rpm.

- (3) Pipette into dry test tubes:

supernatant	2.0	2.0	2.0
KOH (Reagent 4)	0.5	0.5	0.5

- (4) Mix, stopper test tubes and incubate for 10 min at 60°C. Cool under running water, add into all test tubes 0.5 ml of oxidizing solution (Reagent 3) and let stand for 10 min at room temperature.

- (5) Pipette into all test tubes 0.5 ml of acetyl acetone (Reagent 2), mix and incubate for 30 min at 60°C.

- (6) Cool under running water and read the absorbance of a sample and standard against the blank at 405-420 nm in 1 cm cuvettes.

(7) Calculate the concentration:

$$\text{Triacylglycerols (mmol/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 3.39$$

### 3. Estimation of non-esterified acids in blood serum

The decreased utilization of glucose for energy production by the tissues, as it was seen in diabetes, increases the utilization of triglycerides, which are hydrolyzed in adipose tissues with the formation of glycerol and fatty acids. These are released as *non-esterified*, or *free fatty acids (NEFA, FFA)* into the blood. Free fatty acids in blood have a half-life of less than eight minutes and are noncovalently bound to proteins such as albumin. Most plasma free fatty acids are taken up by skeletal muscle and myocardium and used for energy. If the needs of muscles is exceeded, the hepatocytes takes up a progressively larger portion of the NEFA which is partly oxidized, partly esterified to form triglycerides. During unrestrained lipolysis, such as during severe insulin deficiency, excess FFA is diverted to triglyceride formation for storage ("fatty liver") and export (hypertriglyceridemia). On the other hand, the excess of acetyl CoA is diverted into formation of ketone bodies. Therefore, it is common to observe a fatty liver, increased plasma VLDL triglyceride, and ketoacidosis in the presence of poorly controlled diabetes or starvation.

Non-esterified fatty acids in serum are estimated to complete the clinical examination if the mobilization of adipose tissue is suspected. For this purpose, enzymic methods can be used or, following extraction, NEFAs are determined as cupric soaps.

#### EXERCISE 3

##### *Non-esterified acids (NEFA) estimation*

*Principle:* Free fatty acids contained in a chloroform extract of serum yield in the presence of Cu<sup>++</sup> ions cupric soaps. Copper bound in the soap is determined as a coloured complex with diethyldithiocarbamate (*cupral*).

##### *Reagents:*

Copper reagent (cupric nitrate, triethanolamine, acetic acid);

Chloroform;

Cupral reagent (sodium diethyldithiocarbamate in sec. buta-nol);

Standard solution of palmitic acid in chloroform (0.6 mmol/l);

*Procedure:*

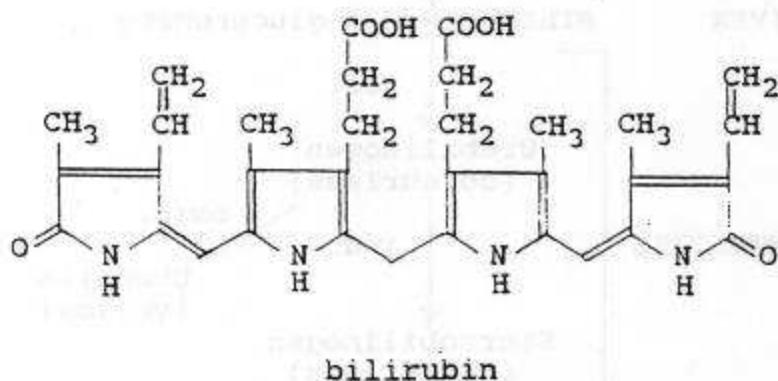
- (1) Into two centrifuge conical tubes measure up 5.0 ml of chloroform. To one of them add 0.5 ml of serum, to the second tube pipette 0.5 ml of palmitic acid standard.
- (2) To both tubes add 2.5 ml of copper reagent, stopper, and shake for 2 min.
- (3) Centrifuge unstoppered tubes for 3 min at 3000 rpm to separate the lower, chloroform layer containing NEFAs, from the upper, water layer. Denatured proteins are found in the interphase.
- (4) Draw up 3 ml of the lower chloroform layer by a dry injection needle. Transfer the liquid into dry test tubes.
- (5) To the chloroform extract add 0.5 ml of cupral reagent. The solution turns brown according to the concentration of NEFA. It should not be turbid. Allow to stand for 10 min.
- (6) Read the absorbance in 1 cm cells at 470 nm against a blank consisting of 3.0 ml of chloroform and 0.5 ml of cupral reagent. Do not wash the cuvette with water!
- (7) Calculate the concentration:

$$\text{NEFA (mmol/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 0.6$$

## CLINICAL EXAMINATION OF BLOOD SERUM III

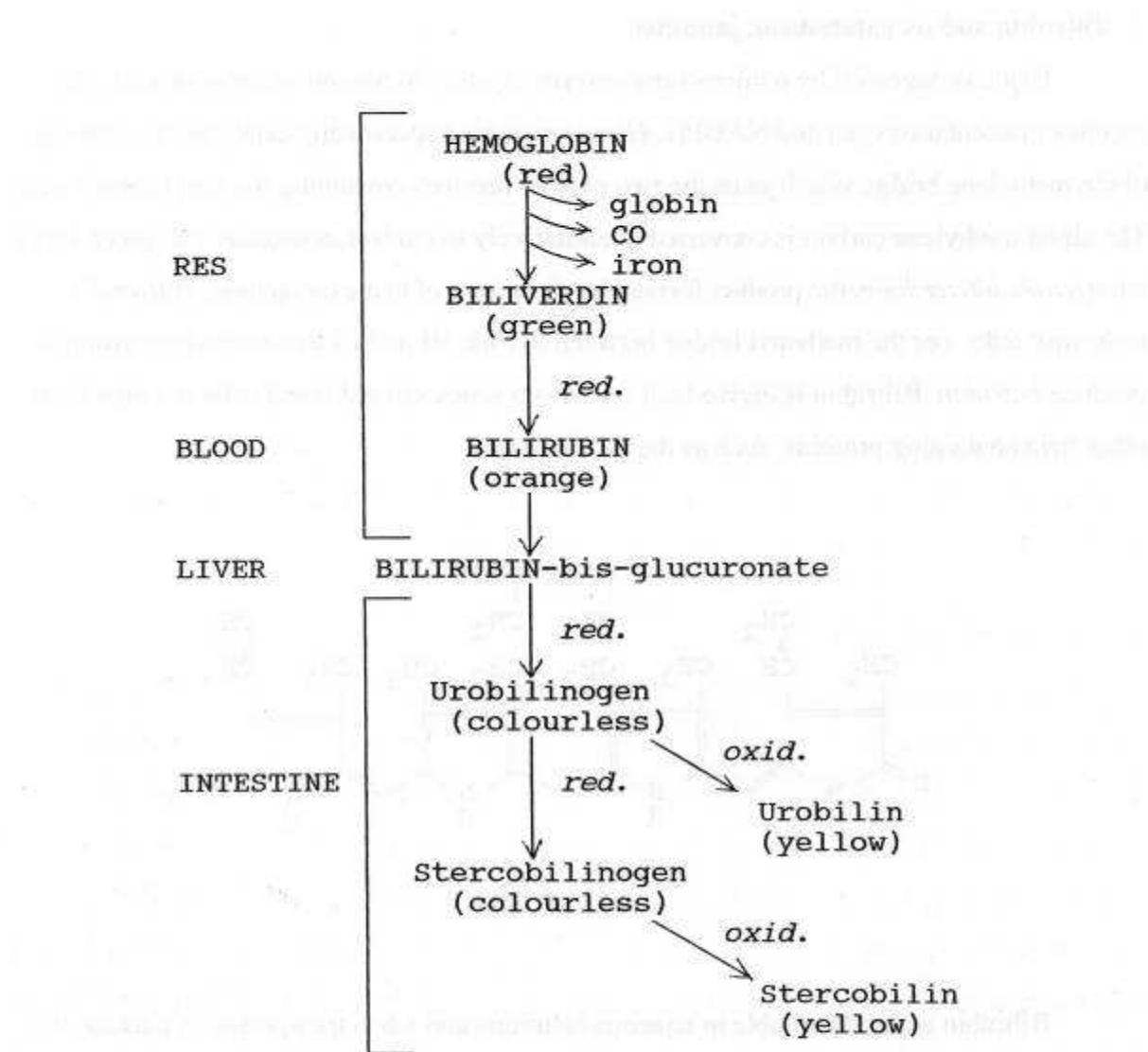
### 1. Bilirubin and its catabolism, jaundice

Heme is degraded by a microsomal enzyme system in reticuloendothelial cells that requires molecular oxygen and NADPH. *Heme oxygenase* specifically catalyzes the cleavage of the methylene bridge which joins the two pyrrole residues containing the vinyl substituents. The alpha-methylene carbon is converted quantitatively to *carbon monoxide*. The green linear tetrapyrrole *biliverdin* is the product formed by the action of heme oxygenase. *Biliverdin reductase* reduces the methenyl bridge between pyrrole III and IV to a methylene group to produce *bilirubin*. Bilirubin is derived not only from senescent red blood cells but also from other hem containing proteins, such as the cytochromes.



Bilirubin is poorly soluble in aqueous solutions and when transported in plasma, it is bound to serum albumin. Albumin contains one high-affinity binding site and another with lesser affinity. The weak affinity of the second site does not allow it to serve effectively in the transport of bilirubin in extreme concentrations (neonatal hemolysis). A number of drugs compete with bilirubin for binding sites and can displace bilirubin from albumin. Bilirubin on serum albumin is rapidly cleared in the liver by a carrier-mediated system at the sinusoidal surface. This transport system has a large capacity. Once in the hepatocyte, bilirubin is bound to a cytosolic protein, *ligandin*. The liver increases the water solubility of bilirubin by the conjugation with glucuronic acid to form a *bilirubin diglucuronide*. Uridine diphosphoglucuronate serves as a glucuronate donor, and the reaction is catalyzed by *UDP-glucuronyltransferase*. This enzymatic activity can be induced by certain drugs, e.g. by

phenobarbital. Secretion of conjugated bilirubin into the bile is mediated by an active transport, which is rate-limiting for the entire hepatic bilirubin metabolism.



Bilirubin diglucuronide is poorly absorbed by the intestinal mucosa. The glucuronide residues are released in the terminal ileum and large intestine by bacterial hydrolases; the released free bilirubin is reduced to colorless linear tetrapyrroles known as *urobilinogens* and *stercobilinogens*. *Urobilinogens* and *stercobilinogens* can be oxidized to coloured products known as *urobilins* and *stercobilins*. A small fraction of colourless products can be reabsorbed by the terminal ileum and large intestine to be removed by hepatic cells and reexcreted in bile (enterohepatic cycle). When *urobilinogens* and other products are reabsorbed in large amounts in certain disease states, they are excreted in urine.

Meconium contains biliverdin, and stools of very young infants generally contain unaltered bilirubin. With development of the bacterial flora bilirubin is more and more reduced to urobilin and stercobilin, unless intestinal contents are hurried through so rapidly that this reduction is incomplete. The reduction is impaired also when the bacterial intestinal flora is reduced by the use of antibiotics.

The normal serum *bilirubin concentration* is up to 22.2 umol/l, of it the conjugated form should not exceed 4.3 umol/l. This concentration represents a balance between the rate of bilirubin production and the hepatic clearance of bilirubin. *Hyperbilirubinemia* results when bilirubin formation exceeds hepatic clearance, as in hemolytic states, or when elimination is impaired due to a liver abnormality (i.e. defective uptake, conjugation, or biliary excretion). This is frequently manifest as *jaundice (icterus)*, which is the visible accumulation of excess bilirubin in the skin, mucous membranes, or sclera, imparting a distinct yellow discoloration to these tissues. Jaundice is usually evident when serum bilirubin level reaches about 35 umol/l.

*Classification of jaundice (hyperbilirubinemia):*

(1) *Unconjugated hyperbilirubinemias* may or may not be associated with excessive hemolysis of red blood cells; in either case the excess bilirubin in the blood plasma is unconjugated. In hemolytic jaundice there is an excessive rate of breakdown of RBC and of their hemoglobin, so unconjugated bilirubin passes into the blood faster than it may be removed by the liver. Jaundice can sometimes occur as a hereditary abnormality (the Crigler-Najjar syndrome or Gilbert syndrome) without evidence of excessive hemolysis. A common feature of both disorders is impaired or absent conjugation of bilirubin by virtue of decreased hepatic bilirubin UDP-glucuronyl transferase activity. Transient unconjugated hyperbilirubinemia of the newborn is relatively common disorder which develops in the first week of life. Delayed functional maturity of glucuronyltransferase, along with a severalfold increase in bilirubin production from the degradation of fetal hemoglobin, appears to be the major cause of physiological jaundice. Physiological jaundice must be differentiated from the hemolytic disease due to maternal-fetal blood group incompatibility. Bilirubin encephalopathy ("kernicterus") is the most feared complication of hyperbilirubinemia in the newborn. The laboratory findings are characteristic: unconjugated bilirubin in the blood, the stools are very dark (urobilin, stercobilin), in excessive hemolysis urobilin and stercobilin are found also in the urine.

(2) *Hepatocellular (hepatic) jaundice* may be eg. due to hepatitis or due to drugs and toxins which cause diffuse hepatocellular injury. In hepatic jaundice the blood plasma contains both conjugated and unconjugated bilirubin. The increase in conjugated form is explained by the impaired transport into the bile which is the rate-limiting step in the overall transhepatic transport of bilirubin. The laboratory findings are relatively rich: both forms of bilirubin are present in the blood, the plasma activities of many enzymes are increased (ALT, AST, LDH, gamma-GT), imbalance in plasma proteins causes the increase in TTT, excretory liver tests are positive. In urine, bilirubin and increased amounts of urobilinogen and urobilin are found. When the jaundice is severe, Ubg is found in urine only in early stages of the condition and during recovery. The stools may be pale or clay-coloured.

#### *LABORATORY FINDINGS IN JAUNDICE*

		unconjugated	hepatic	obstructive
URINE	bilirubin	-	+	+
	urobilinogen	+	+	-
	urobilin	+	+	-
	bile acids	-	+	+
SERUM	conjugated Bi	-	+	+
	unconjugated Bi	+	+	-
	TTT	-	+	-
	ALP	-	-	+
	ALT, AST, LDH	-	+	-
	funct. tests	-	+	-

(3) *Obstructive jaundice* is due to the obstruction of biliary passages from gall stones, tumours or due to intrahepatic cholestasis. The distension of the biliary passages was believed to rupture the biliary canaliculi within the liver with regurgitation of bile in the venous sinusoids. There is more likely an abnormality of transport of conjugated bilirubin to the biliary canaliculi; conjugated bilirubin, therefore, passes into the blood. In posthepatic jaundice less pigment than normal reaches the gut and feces become less pigmented (clay-

coloured). Bilirubin is always present in the urine, along with the bile acids, and urobilin is absent. A high percentage of cases of obstructive jaundice show markedly increased alkaline phosphatase.

A quantitative assay for bilirubin was introduced by *Van den Bergh* by application of Ehrlich's test for bilirubin in urine. This reaction is based on the coupling of diazotized sulfanilic acid (Ehrlich's *diazo* reagent) and bilirubin to produce a reddish-purple azo compound in acid solutions. In alkali, azobilirubin is blue. In the clinical setting conjugated bilirubin is expressed as *direct bilirubin* because it can be coupled readily with diazonium salts. Unconjugated bilirubin is bound to albumin and will not react until it is released by the addition of an organic solvent such as ethanol. This reaction is indirect Van den Bergh reaction and nonconjugated bilirubin is therefore called *indirect bilirubin*. Extreme concentrations of bilirubin in some cases of neonatal hyperbilirubinemia are estimated by a direct photometry of serum bilirubin at two wavelengths.

## EXCERCISE 1

### *Determination of the total and conjugated bilirubin*

**Principle:** Bilirubin forms with diazotized sulfanilic acid an azo-dye suitable for the photometric determination. The total bilirubin (conjugated and unconjugated) reacts in the presence of the accelerator, the direct (conjugated) bilirubin reacts without the accelerator.

**Reagents:**

- (1) Sulfanilic acid in HCl;
- (2) Potassium-sodium tartarate, NaOH;
- (3) Accelerator: caffeine in sodium benzoate;
- (4) Sodium nitrite solution;

### *Procedure:*

#### **Total bilirubin**

Pipette (ml)	sample	blank 1	blank 2
reagent 1	0.2	0.2	0.2
reagent 4	1 drop	1 drop	-
reagent 3	1.0	1.0	1.0
physiol. solution	-	0.2	-
serum	0.2	-	0.2

Mix and after 10 min pipette

reagent 2	1.0	1.0	1.0
Mix and within 60 min read the absorbances of a sample and blank 2 against the blank 1 at 580 nm, cuvette 1 cm			

### Conjugated bilirubin

Pipette (ml)	sample	blank 1	blank 2
reagent 1	0.2	0.2	0.2
reagent 4	1 drop	1 drop	-
physiol. solution	1.0	1.2	1.0
serum	0.2	-	0.2
Mix, allow to stand for exactly 5 min and read the absorbance of a sample and blank 2 against the blank 1 at 510 nm, cuvette 1 cm.			

#### Calculation:

Read the bilirubin content from the calibration curves using the difference  $A_{\text{sample}} - A_{\text{blank}}$ . The blank 2 compensates the natural colour of the serum. If it is not used, the bilirubin concentrations are higher by about 5  $\mu\text{mol/l}$ .

#### Normal range:

Total bilirubin: < 22.2  $\mu\text{mol/l}$

Conjugated bilirubin: < 4.3  $\mu\text{mol/l}$

## 2. Nonprotein nitrogenous compounds of the blood, estimation of blood urea and blood uric acid

The nonprotein nitrogen of the blood, that portion of the nitrogenous substances not precipitated by the usual protein precipitants, includes especially urea, uric acid, amino acids, creatine, creatinine, and ammonia. From a metabolic standpoint, the nonprotein nitrogenous constituents of the blood are usually of greater interest than the plasma proteins since they represent products of the intermediary metabolism. The total NPN concentration, although measurable, is of a little diagnostic value, and it is substituted by the measurement of individual component concentrations.

*Urea* is in man the chief end-product of protein catabolism. It is produced in the liver following transamination of alpha-amino nitrogen, oxidative deamination of glutamate, and reactions of urea cycle. Urea is an extremely diffusible substance and, as such, exists in all body fluids in practically the same concentration. As it is eliminated almost exclusively by the kidneys, its concentration in the blood is influenced markedly by the renal function. However, blood urea concentration corresponds also to the urea production, which is influenced by the protein intake, increased or decreased catabolic rate, and a liver disease. Blood urea ranges from 2.5 to 8.3 mmol/l.

urea		2.5 - 8.3 mmol/l
alpha-amino nitrogen		3.6 - 4.8 mmol/l
uric acid	men	200 - 420 umol/l
	women	140 - 340 umol/l
creatinine		62 - 124 umol/l
ammonia		12 - 50 umol/l

*Creatinine* (creatine anhydride) is formed in muscle from creatine phosphate by irreversible, nonenzymatic dehydration and loss of phosphate. Its production is proportional to a patient's body mass and therefore relatively stable over time. Creatinine is excreted solely by the kidneys and its excretion is primarily determined by the glomerular filtration rate. Plasma creatinine roughly doubles for every 50 per cent fall in glomerular filtration rate. Normal range is given as 62 - 124 umol/l. Generally, in renal failure the rate of excretion of nitrogenous nonprotein components is much less than their rate of formation and there is a rapid increase in their concentration in the blood, i.e. there is *azotemia*.

*Uric acid* is the end product of purine metabolism in humans. The average person eliminates approximately 4.4 mmol of uric acid each day. About two thirds of the urate is excreted in the urine, and one third is excreted in the intestinal secretions, where bacteria degrade uric acid to allantoin and carbon dioxide by a process of intestinal uricolysis. An elevated serum urate concentration beyond the upper limits (420 umol/l in men, 340 umol/l in women) is called *hyperuricemia*. There are two major causes of hyperuricemia in humans: increased production of uric acid and decreased renal excretion of uric acid. Hyperuricemia needs to be distinguished from *gout*. Although only a minority of hyperuricemic patients ever

become gouty, all patient with gout have hyperuricemia at some stage in their clinical course. Gout is further manifest by recurrent attacks of a characteristic type of acute arthritis, in which crystals of monosodium urate are demonstrable in leukocytes of synovial fluid, by deposits of urate around the joints of the extremities, and often by the renal damage.

One of the final products of protein-amino acid degradation in most organs is *ammonia*,  $NH_3$ . Ammonia is also absorbed from the lumen of the colon, as a product of bacterial deamination of protein from the diet, sloughed epithelial cells, and from the action of bacterial urease on luminal urea. This potentially toxic compound can be detoxicated by two routes. The first is a preliminary step and involves transfer of  $NH_3$  to ketoacids (such as 2-oxoglutarate) to form amino acids, or to glutamate to form glutamine. The kidney can excrete ammonia from glutamine to acidify urine. However, the final detoxification for most of the  $NH_3$  occurs in hepatocyte through synthesis of urea. In fulminant *hepatic failure*, blood ammonia increases beyond the limit of 50 umol/l because of hepatocyte death and loss of urea cycle enzymes. In chronic liver disease, shunting of portal-venous  $NH_3$  to the systemic circulation is the major factor for increased blood  $NH_3$ , although decreased urea synthetic capacity may be a contributing factor. Relatively rare inherited deficiencies of the urea cycle, responsible for retarded mental development, are of interest in that they are all associated with hyperammonemia.

## EXERCISE 2

### a) Estimation of urea in blood serum

*Principle:* Urea forms in the presence of diacetylmonooxime, thiosemicarbazide, and ferric ions in acid solutions a red complex suitable for photometric determination.

#### *Reagents:*

1. Urea standard 16.65 mmol/l
2. Reagent containing diacetylmonooxime, thiosemicarbazide, and a ferric salt in sulphuric acid (Caution, poison!)

*Procedure:*

Pipette (ml)	sample	standard	blank
serum (dil. urine)	0.01	-	-
standard (reag.1)	-	0.01	-
dist. water	-	-	0.01
reagent (2)	2.00	2.00	2.00
Mix, cover test tubes with aluminum foil, and put the test tubes into the boiling water bath for exactly 10 min. Cool test tubes in running water and within 15min read the absorbance against blank at 525 nm.			

*Calculation:*

$$\text{urea (mmol/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 16.65$$

b) *Estimation of uric acid in serum*

*Principle:* Serum proteins are precipitated by uranyl acetate. Uric acid reduces phosphotungstate to a blue product containing tungsten<sup>V+</sup>, which is determined photometrically.

*Reagents:*

1. Sodium carbonate, saturated water solution;
2. Phosphotungstic reagent;
3. Deproteinizing solution containing uranyl acetate;

*Procedure:*

- (1) Dilute 0.5 ml of serum with 3 ml water and add 1.0 ml of deproteinizing solution (uranyl acetate) into the centrifuge conical tube. Mix the content and allow to stand for 10 min.
- (2) Centrifuge 10 min at 3000 rpm. To 2 ml of a clear supernatant add 0.1 ml of phosphotungstic reagent and 1.0 ml of saturated sodium carbonate solution.
- (3) Let stand for 10 min. Read the absorbance against water in 1 cm cuvette at 660 nm. Read the concentration from the calibration diagram.

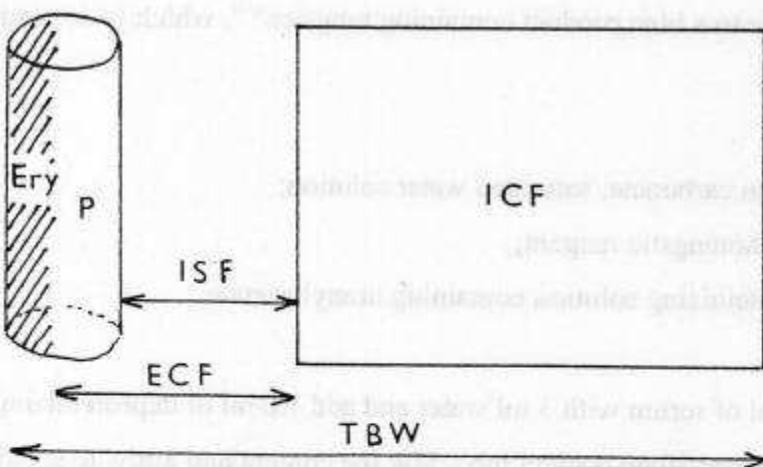
## CLINICAL EXAMINATION OF BLOOD IV

### 1. Body fluid balance and its disturbances

The *total body water (TBW)* amounts to between 55-70% of the body mass (weight), depending on the age, sex, and mass of the adipose tissue. In adults it constitutes about 60% of the total body mass (weight).

Part of this body-water is within the cells - the *intracellular fluid (ICF)*; and part is outside the cells - the *extracellular fluid (ECF)*; the latter includes the water of circulating *plasma (P)*. Extracellular fluid not including plasma is referred to as *interstitial fluid (ISF)*. The synovial fluids of joints, gastrointestinal, peritoneal, pleural, cerebrospinal and ocular fluids and lymph are also fractions of the extracellular fluids but are discontinuous with the interstitial fluid and plasma (*transcellular fluid*). Transcellular fluid occupies in most humans about 2.5% of the total body weight. However, it may be substantially increased under pathological conditions.

ECT represents about 20% of the total body mass, of which plasma volume constitutes 5% and ISF 15% of the total body mass, respectively. ICF occupies approximately 40% of total body mass.



Although the nature of the ions and molecules is different in each compartment, the osmotic pressure is uniform throughout the body fluids, except where local activities are responsible for osmotic gradients (e.g. in the renal medulla, throughout the length of the capillaries). The chief cation of plasma and ISF is sodium, with small, but important, amounts of potassium, calcium and magnesium. In intracellular fluid the chief cation is potassium. Chloride and bicarbonate are the main anions of ISF and plasma, but the latter also contains protein which is in low concentration in the interstitial fluid. Chloride and bicarbonate are low in the ICF, the

principal anions of which are proteins, organic phosphates and organic acids. The concentration of potassium within the cell is 20-30 times that in the ECF, and the concentration of sodium in the ECF is much higher than in the ICF. The ATP required to maintain this relative exclusion of sodium from within the cells and of potassium from the plasma is derived from the metabolism of glucose. If oxidative phosphorylation is inhibited, potassium immediately diffuses out from, and sodium into, the cell.

The distribution of fluid in the three main fluid compartments depends on the quantities and concentrations of non-diffusible compounds and ions (or effectively non-diffusible ions) in these compartments. Thus, the absolute volumes of the intracellular and extracellular compartments depend respectively on the amounts of potassium and sodium in the body. The water contents of both compartments are regulated so that the osmolar concentrations are 275-295 mmol/kg of water. Osmolality may be estimated by freezing point depression using an osmometer but a rough estimate of osmolality (in mmol/kg) is given by adding the concentrations, in mmoles, of the main constituents:

$$\text{Osmolality (mmol/kg)} = 2([\text{Na}^+] + [\text{K}^+]) + 5$$

Under conditions of glucose/urea increase:

$$\text{Osmolality (mmol/kg)} = 2[\text{Na}^+] + [\text{Glucose}] + [\text{Urea}]$$

In the intracellular fluid the main cation is potassium, the concentration of which is maintained such that with the polyvalent anions, organic phosphate and protein, the osmolality is maintained equal to that of the extracellular fluid. Thus, the quantity of potassium in the body determines the volume of the ICT. During prolonged loss of gastrointestinal secretions by vomiting and/or diarrhoea, by obstruction or by malabsorption, the extracellular fluid volume will fall and potassium may pass out of the cell and be excreted, leading to a fall in intracellular fluid volume as well.

The total body water is normally maintained fairly constant by an adjustment of the total output of fluid to balance the intake of fluid. Since the loss of fluid by extrarenal routes usually varies relatively little, the volume of urine is the main variable concerned with balancing fluid output with total fluid intake. The inevitable water loss amounts to approximately 1 l per day and takes place in the expired air and in the perspiration. In temperate climates the amount of salt lost in the sensible perspiration, which is hypotonic in comparison with the body fluids, is quite small, and for the practical purposes the inevitable water loss is one of pure water only. In tropical or other conditions of high environmental temperature this inevitable water loss may

be even higher, and may be accompanied by some loss of salt in the sensible perspiration. The inevitable water loss per kg of body weight is greater in the infant than that of the adult.

A normal diet entails the ingestion of about 800 ml of water as moisture of food. The metabolic oxidation of the hydrogen of foodstuffs makes available about 350 ml of water. Even when the caloric value of the food intake is reduced, this source of water will still be available by the oxidation of the hydrogen of the constituents of the tissues.

Volume abnormalities are expressed by clinical terms *dehydration* and *overhydration*. Dehydration may be caused most frequently by the simple water loss (*hyperosmotic dehydration*) or by the loss of water accompanied by the loss of  $\text{Na}^+$  (*isotonic dehydration*). Remember, that ECF volume is determined by the substance amount of  $\text{Na}^+$  in the body, not by the  $\text{Na}^+$  concentration in the blood serum.

In isoosmotic dehydration, isoosmotic fluid is lost from the plasma and then is repleted from the interstitial space (hemorrhage, exudation through burned skin, gastroin-testinal fluid loss in vomiting, diarrhoea, drainage). The volume of the ECF is reduced with no change in osmolality. To control these conditions, along with the fluid intake and output, the body weight should be recorded.

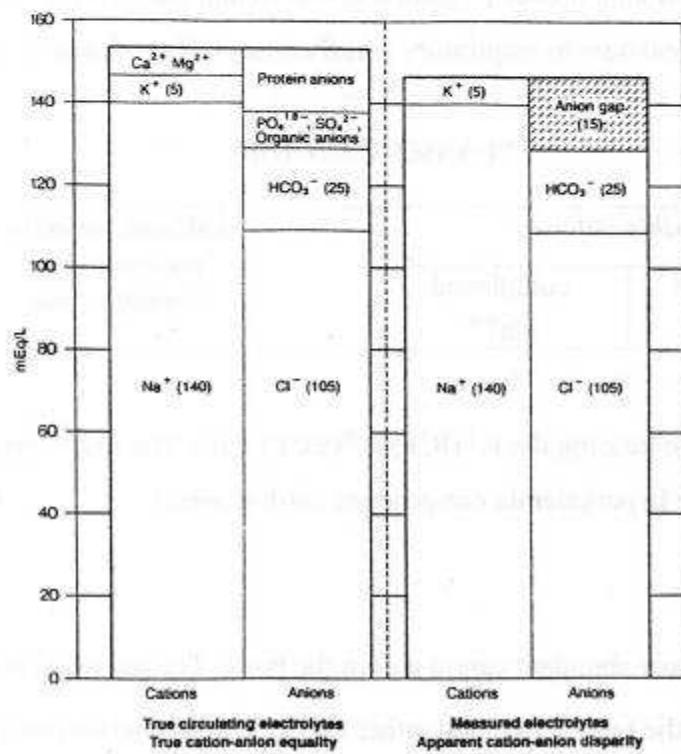
In the hyperosmotic dehydration, fluid is lost from the plasma (decreased intake, excessive evaporation from the skin and breath), which becomes hyperosmotic, causing a fluid shift from the ISF to the plasma and, in turn, a fluid shift from the ICF to the ECF compartments. Finally, both the ECF and ICF volumes decrease, and the osmolality of both major fluid compartments is increased. The concentration of hemoglobin and plasma protein is increased as well.

Volume expansion states are represented by the isoosmotic, hyperosmotic, and hypoosmotic overhydration. As in the dehydration, the fluid shift occurs just in the hyperosmotic and hypoosmotic conditions.

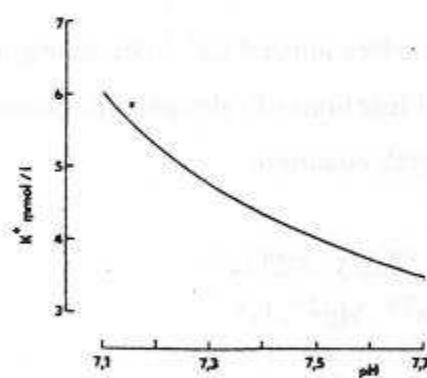
## 2. Ionic composition of the blood plasma

Ions constitute about 95% of the solutes in the body fluids. The sum of the concentrations (rather in mEq/l than in mmol/l) of the *cations* equals the sum of the concentrations of the *anions* in each compartment, making the fluid in each compartment electrically neutral. Physicians rely on the changes in electrolyte concentrations in the ECF compartment, particularly in the plasma, in the diagnosis and treatment of patients with fluid or electrolyte imbalances.

As stated above, the monovalent cations  $\text{Na}^+$  and  $\text{K}^+$  are the predominant cations of the ECF and ICF compartments, respectively. The divalent cations  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  exist in body fluids in relatively low concentrations. Almost all of the body  $\text{Ca}^{2+}$  (in bone) and most of the body  $\text{Mg}^{2+}$  (in bone and cells) is nonexchangeable. After  $\text{K}^+$ ,  $\text{Mg}^{2+}$  is the main cation of the ICF. After  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  is the main cation of the ECF.



Plasmatic concentration of *potassium ions* ranges from 3.8 - 5.2 mmol/l. Acidosis predisposes to *hyperkalemia* secondary to cellular redistribution:  $\text{K}^+$  is substituted with  $\text{H}^+$  ions in e.g.  $\text{HPO}_4^{2-}$  salts. Similarly, hyperkalemia is caused by increased catabolism of binding macromolecules in the cell (proteins, glycogen).



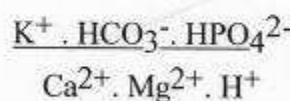
*Hypokalemia* results from a shift of K<sup>+</sup> into the intracellular compartment (e.g. alkalosis, insulin treatment) or from potassium losses that decrease both extracellular and intracellular K<sup>+</sup> (renal, gastrointestinal fluid loss).

The functional impact of K<sup>+</sup> depletion results mainly from an increased ratio of ICF to ECF K<sup>+</sup> concentration, which increases the threshold for initiation and impairs termination of the action potential in excitable tissues (it results in ventricular tachycardia, fibrillation, digitalis intoxication, muscular weakness to respiratory insufficiency). Hyperkalemia alters the function

PLASMA CALCIUM		
<i>diffusible calcium</i>		calcium bound to protein (45%) ( <i>nondiffusible</i> )
ionized Ca <sup>++</sup> (free)	complexed Ca <sup>++</sup>	

of excitable tissues by decreasing the K<sup>+</sup>(ICF)/K<sup>+</sup>(ECF) ratio. The major organ system affected is again the heart: severe hyperkalemia can produce cardiac arrest.

*Calcium* is the most abundant cation within the body. The majority, however, is present in bone and only 1% of the total is in blood, other ECFs, and various soft tissues. The Ca<sup>++</sup> in the ECF is critical for a variety of functions and is maintained within a remarkably narrow range (2.25-2.75 mmol/l). Less than one half of the circulating calcium occurs as free ions. The remainder is largely bound to serum proteins(45%), but a certain portion is complexed with phosphates, citrate, and hydrogen carbonate. Albumin is the principal Ca-binding protein, therefore, changes in the plasma albumin concentration may cause alteration in the total serum concentration of calcium. The distribution between free (ionized) Ca<sup>++</sup> and complexed or protein-bound ion is pH -dependent with the free ionized Ca<sup>++</sup> decreasing as the pH increases. The decrease may impair the physiological functions of calcium ions, mainly the neuromuscular activity which is proportional to the *Gyorgyi's equation*:



The lack of free calcium ions may result in tetany.

Technically, sodium and potassium ions are estimated by the emission flame spectrophotometry, calcium and magnesium by atomic absorption spectrophotometry. Calcium may be determined by a suitable colour or complexometric reaction as well. The ionized fraction of calcium in blood may be estimated by means of the ionic selective electrode.

The chief anions of the body fluids are chlorides, bicarbonates, phosphates, organic ions, and polyvalent proteins. Organic phosphates, proteins, and organic ions are predominant anions in the ICF.  $\text{Cl}^-$  and  $\text{HCO}_3^-$  are the predominant anions in the ECF.

Chlorides may be examined by various precipitation or colour reactions, or by ionic selective electrodes. The estimation of proteins is based on various colour reactions, hydrogencarbonates are determined during the examination of acid-base balance (see below). The remaining anions (residual anions) are not determined routinely.

The ionic profile of normal serum is depicted in the above diagrams. The law of electroneutrality states that the number of positive charges ("mEq") in any solution must equal the number of negative charges. If every ion present in serum were measured, the concentration of cations would equal the concentration of anions if these concentrations were expressed in mEq/l. Routine serum electrolyte determinations measure essentially all cations but only a fraction of the anions. This apparent disparity between the total cation concentration and the total anion concentration is termed the *anion gap*. It is a virtual measurement and does not represent any specific ionic constituent. The anion gap, which has a normal value of 12-18 mmol/l, reflects the concentration of: plasma proteins, inorganic phosphates, sulfate, ions of organic acids (e.g. lactic, beta-hydroxybutyric, and acetoacetic acids). When the anion gap is increased, unmeasured anions fill this gap. Most of these anions usually are the products of metabolic processes that generate  $\text{H}^+$ .

Determination of anion gap:

$$\text{AG} = ([\text{Na}^+] + [\text{K}^+]) - ([\text{HCO}_3^-] + [\text{Cl}^-])$$

### 3. Acid-base balance

Control of blood pH is important because changes in blood pH cause changes in intracellular pH, which in turn may profoundly alter metabolism. Protein conformation is affected by pH, as is enzyme activity. In addition, the equilibria of important reactions that

consume or generate H<sup>+</sup> ions, such as any of the oxidation-reduction reactions involving pyridine nucleotides, will be shifted by changes in pH.

The normal arterial plasma pH is 7.40 + 0.04, the pH compatible with life is about 6.8 - 7.8. Intracellular pH is usually lower, in most cells about 7.0 (in RBC 7.2), however, value as low as 6.0 was reported for skeletal muscle.

It is fortunate for both diagnosis and treatment of diseases that the acid-base status of the ICF influences and is influenced by the acid-base status of the blood. Blood is readily available for analysis, and when alteration of body pH becomes necessary, intravenous administration of acidifying or alkalinizing agents is efficacious.

The body produces large amounts of acid in two forms - *carbonic*, in a form of CO<sub>2</sub> anhydride, as a result of oxidative metabolism (*volatile*, can be eliminated by the lungs), and *noncarbonic (nonvolatile)*, derived from diet and intermediary metabolism, e.g. sulphuric acid, phosphoric acid, lactic acid, acetoacetic and beta-hydroxybutyric acid, can be eliminated by the kidney).

The pathways for acid removal include the lungs, kidneys, and to a little extent also the gastrointestinal tract. These organs interact with each other and all other parts of the body by way of the blood. In the course of a day, the equivalent of 20,000-40,000 H<sup>+</sup>mmoles are eliminated via the lungs, 50-100 H<sup>+</sup>mmoles excreted by the kidneys (1/4 as H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, 3/4 as NH<sub>4</sub><sup>+</sup>).

On the other hand, under normal conditions the pH of the blood is not substantially affected by the sudden addition of relatively large quantities of acid or basic compounds. This constancy of reaction is maintained through the operation of the *buffering systems*.

Blood plasma is a mixed buffer system; in the plasma the major buffers are bicarbonate, phosphate, and proteinate buffers. The pH is the same throughout the plasma, so each of these buffer pairs distributes independently according to its own Henderson-Hasselbalch equation:

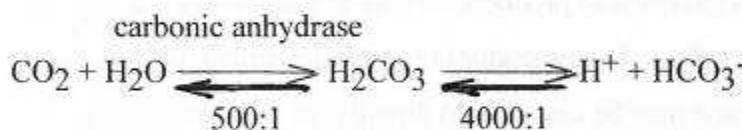
$$\text{pH} = \text{pK}_1 + \log \frac{[\text{HCO}_3^-]}{[\text{CO}_2]} = \text{pK}_2 + \log \frac{[\text{HPO}_4^{2-}]}{[\text{H}_2\text{PO}_4^-]} = \text{pK}_3 + \log \frac{[\text{protein}^-]}{[\text{Hprotein}]}$$

Because each pK is different, the [conjugate base]/[acid] ratio is also different for each buffer pair. Notice, though, if the ratio is known for any given buffer pair, one automatically has information about the others (assuming the pK values are known).

In the RBC, the most essential buffering capacity is available in the Hb system, where reduced Hb (deoxyhemoglobin) is a stronger base than oxy-Hb, i.e. deoxy-Hb has a stronger capacity to combine with  $H^+$ . Bicarbonate buffer system is quantitatively the most important buffer of interstitial fluid (ISF  $[HCO_3^-] = 27 \text{ mmol/l}$ ). However, the major buffer capacity of the body is not in the blood but in the  $H^+$  acceptors found in other tissues, principally in the muscle (organic anions) and in bone ( $CO_3^{2-}$ )

BUFFERING CAPACITY OF THE WHOLE BLOOD (%)			
<i>Erythrocytes:</i>			
$Hb^-/HHb$ and $HbO^-/HHbO$	35		
$HCO_3^-/H_2CO_3$	18		total
$HPO_4^{2-}/H_2PO_4^-$ organic	3		57
inorganic	1		
<i>Blood plasma:</i>			
$HCO_3^-/H_2CO_3$	35		
$Protein^-/H.Protein$	7		total
$HPO_4^{2-}/H_2PO_4^-$ inorganic	1		43

As we have seen, the major buffer of the plasma and ISF is the *bicarbonate buffer system*. It should be noted that the component we consider to be the acid in this buffer system is  $CO_2$ , which is not truly an acid, but an acid anhydride. But it reacts with water to form carbonic acid, which is indeed a typical weak acid:



At equilibrium there are approximately 500 mmol of  $CO_2$  for every 1 mmol of carbonic acid and approximately 4000 mmol of carbonic acid for every 1 mmol of  $H^+$ . Because of the presence of *carbonic anhydrase*, equilibrium between  $CO_2$  and carbonic acid is rapid and constant.

The concentration of a gas in a solution is proportional to the partial pressure of the gas. Thus we measure  $PCO_2$  and multiply it by a *conversion factor* ( $0.03 \text{ mmol/l} \times \text{mmHg}$  or  $0.225 \text{ mmol/l} \times$

kPa) to get the millimolar concentration of dissolved CO<sub>2</sub>. Then the Henderson-Hasselbalch equation for this buffer becomes:

$$\text{pH} = 6.1 + \log \frac{[\text{HCO}_3^-]}{0.03 \times P_{\text{CO}_2}}$$

In a typical buffer, when a strong acid is added, most of the added H<sup>+</sup> combined with the conjugate base. As a result, weak acid increases and conjugate base diminishes. The ratio of both therefore changes and so does the pH (of course much less than if there were no buffer present). Now consider a system in which the excess of weak acid is somehow removed so that while base diminishes, weak acid remains nearly constant. In this case the ratio conj. base/weak acid would change much less and the pH as well. This is what happens with bicarbonate buffer system in the body where the excess of CO<sub>2</sub> is exhaled. In like manner, if strong base is added, it will be neutralized by carbonic acid, CO<sub>2</sub> being replaced by metabolism. The bicarbonate system is thus an *open system* and the denominator in the henderson-Hasselbalch equation may be called a *respiratory component* of the bicarbonate system.

Inability of lungs to excrete adequately load of CO<sub>2</sub> leads to *respiratory acidosis* (RAc) or *respiratory alkalosis* (RAlk). Respiratory acidosis is the result of hypoventilation which occurs in obstructions of the airway, neuromuscular disorders, etc.. Respiratory alkalosis , on the other hand, arises from decreased alveolar PCO. Hyperventilation due to anxiety, CNS injury involving the respiratory centre, salicylate poisoning, fever, and artificial ventilation are the most common causes.

Metabolism normally produces certain amounts of nonvolatile acids (lactic, acetoacetic, beta-HB acids). In some physiological or pathological states these are produced in excess, and cause acidosis of the *metabolic type*. This excess acid production reduces bicarbonate fraction in the Henderson-Hasselbalch equation. Therefore, the numerator is sometimes called the *metabolic component* of the equation. Loss of this base may be caused also directly, in the kidneys (e.g. in renal tubular acidosis), or in the gastrointestinal tract (severe diarrhoea). Mammals do not synthesize alkaline components from neutral starting materials. *Metabolic alkalosis* (MAlk) therefore arises from intake of excess alkali (NaHCO<sub>5</sub>) or loss of acid (prolonged vomiting). A less obvious source of alkali is the salt of any metabolizable organic acid. Sodium lactate is often administered to combat acidosis. Normal metabolism converts it to sodium bicarbonate:

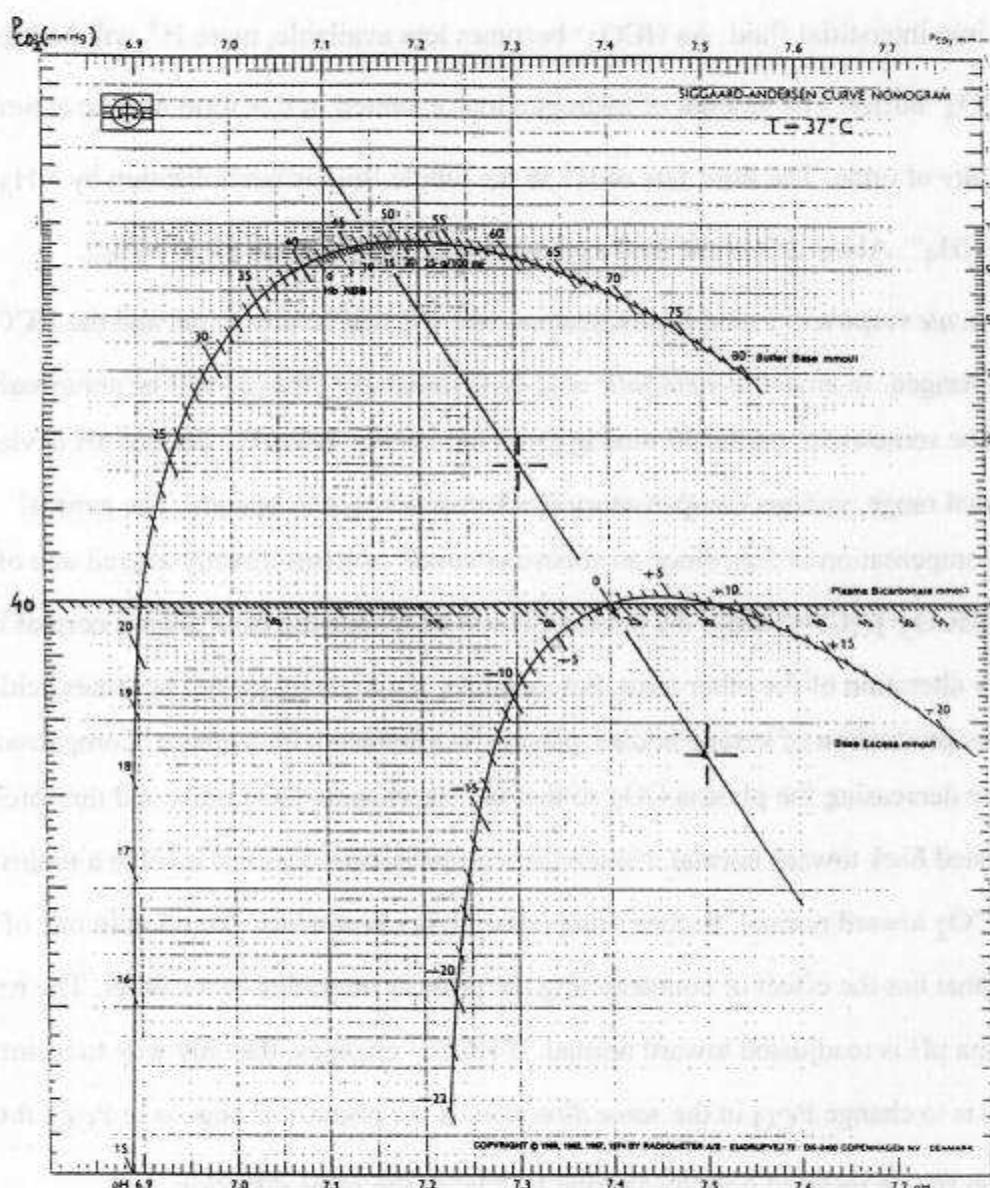


Excess nonvolatile acid and bicarbonate are excreted by the *kidney*. As a result, the pH of the urine varies as a function of this body's need to excrete these materials. This regulation is based on the formation of  $H^+$  and  $HCO_3^-$  from  $CO_2 + H_2O$  within the tubule cells where  $H^+$  is actively secreted into the tubule fluid in exchange for  $Na^+$ . In this way  $NaHCO_3$  is generated.  $H^+$  in the tubule fluid can react with a  $HCO_3^-$  to form  $CO_2 + H_2O$  and as a result to move  $HCO_3^-$  back into interstitial fluid. As  $HCO_3^-$  becomes less available, more  $H^+$  will be taken by  $HPO_4^{2-}/H_2PO_4^-$  buffer. The amount of hydrogen ions excreted in this form may be estimated as *titratable acidity* of urine. The third fate of  $H^+$  in the tubule fluid is neutralization by  $NH_3$  from glutamine to  $NH_4^+$ . About 2/3 of the acid excreted by kidneys is excreted as  $NH_4^+$ .

In an *acute respiratory* acid-base imbalance the pH will be abnormal, and the  $HCO_3^-$  will be nearly unchanged. In an *acute metabolic* acid-base imbalance, the pH will be abnormal and the  $PCO$  will be somewhere on the 40 mmHg (5.3 kPa) isobar. When the plasma pH deviates from the normal range, various compensatory mechanisms begin to operate. The general principle of compensation is that, since an abnormal condition has directly altered one of the terms of the  $[HCO_3^-]/[CO_2]$  ratio, the plasma pH can be readjusted back toward normal by a compensatory alteration of the other term. For example, if a diabetic patient becomes acidotic due to excess production of ketone bodies, plasma bicarbonate will decrease. Compensation would involve decreasing the plasma  $CO_2$  so that the bicarbonate/ $CO_2$  ratio, and therefore the pH, is readjusted back toward normal. Notice that compensation does not involve a return of  $HCO_3^-$  and  $CO_2$  toward normal. Rather, compensation is a secondary alteration in one of these, an alteration that has the effect of counteracting the primary alteration in the other. The result is that the plasma pH is readjusted toward normal. If  $HCO_3^-$  changes, the only way to restore the original ratio is to change  $PCO$  in the *same direction*. If the primary change is in  $PCO$ , the original ratio can be restored only by altering  $HCO_3^-$  in the *same direction*.

#### 4. Examination of acid-base balance

Most of the current methods used to assess acid-base balance have been based upon the use of the Hander-son-Hasselbalch equation which links pH to the molar concentrations of carboxylic acid and bicarbonate in plasma. Thus, for the precise investigation of acid-base balance the three parameters, pH,  $\text{PCO}_2$  and bicarbonate concentrations, must be determined.



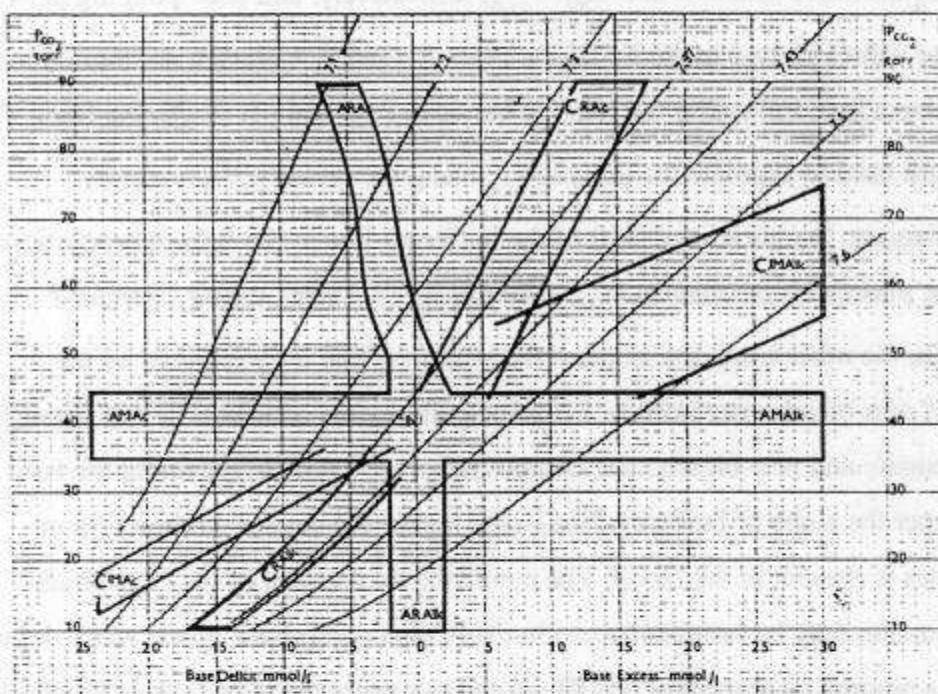
It is customary to measure hydrogen ion in heparinized arterial blood but many modern pH-meters require less than 100  $\mu$ l of blood, and capillary blood is satisfactory in normal subjects. However, if the peripheral circulation is poor, it is technically difficult to collect satisfactory specimens and the capillary blood is more acid than arterial blood by a varying amount. Blood should normally be collected into a heparinized capillary without leaving air bubbles in the capillary. After capping the capillary to prevent leakage, the blood should be sent to the laboratory in an ice-tray. At 4°C there is no significant change in  $[H^+]$  for 4h. The  $P_{CO_2}$  is normally measured by a modified pH electrode using blood collected in the same way as for pH. The missing value in the Henderson-Hasselbalch equation, i.e.  $[HCO_3^-]$ , is in turn calculated from the pH value,  $P_{CO_2}$ , and apparent pK. Unlike in the *direct estimation of  $P_{CO_2}$* , a classical Astrup's method determines this value according to the relation between  $\log P_{CO_2}$  and pH for the fully oxygenated blood samples. To this purpose, two extra blood samples should be *equilibrated* with the  $CO_2/O_2$  mixture, with low and high  $P_{CO_2}$ , respectively, and corresponding pH values measured. These values plotted in the nomogram (see Siggaard-Andersen nomogram in the picture) determine a line, indicating the relation between  $P_{CO_2}$  and pH (*buffering line*). This function is in turn used to estimate the *actual  $P_{CO_2}$*  using the *actual pH* as an entry.

In the diagram, the horizontal shift of the buffering line to the right is proportional to the concentration of plasma bicarbonate at  $P_{CO_2} = 40$  mmHg (5.3 kPa), called "*standard bicarbonate*". On the other hand, the slope of the buffering line is determined by the concentration of non-bicarbonate buffers, i.e. principally by the concentration of hemoglobin. Both the bicarbonate and non-bicarbonate components are summarized under the term "*buffer base*". The steeper the slope of buffering line is, the higher the concentration of non-bicarbonate bases is present in the blood. The metabolic component of the Henderson-Hasselbalch equation may be estimated quantitatively also as the *base excess (BE)*, defined originally as the amount (in mmol) of NaOH needed to restore the pH of whole blood saturated with oxygen in vitro to pH 7.40 at a  $P_{CO_2}$  5.3 kPa at 37°C. For clinical use it is not measured in this way but is read from a nomogram. The "*normal buffer base*" (NBB) may be then calculated by subtracting the BE value from the "*buffer base*" (BB) to extrapolate the situation to the "*basal conditions*", i.e. pH 7.40,  $P_{CO_2}$  40 torr (5.3 kPa). As this value is influenced mainly by the hemoglobin concentration, an extra scale for the blood Hb is provided.

The standard bicarbonate scale corresponds to the bicarbonate concentration at the physiological  $P_{CO_2}$ . If  $P_{CO_2}$  deviates substantially from the normal value, the *actual bicarbonate*

concentration differs from that of "standard bicarbonate" due to the cooperation of non-bicarbonate buffers. It was demonstrated that the body regulatory mechanisms react more on the actual bicarbonate concentration than on the "standard bicarbonate". To estimate the actual  $\text{HCO}_3^-$  it is necessary to draw a new line in slope 45° through the point on the equilibration line (buffering line) corresponding to the actual pH, and read out the value where it intersects the "plasma bicarbonate" line.

At any acid-base examination, a partial pressure of  $\text{O}_2$  should be measured. The undersaturation of the blood with oxygen changes the properties of hemoglobin buffer as HHb is weaker acid than  $\text{HHbO}_2$  and can therefore bind more  $\text{H}^+$  ions.  $\text{P}_{\text{O}_2}$  value allows thus the correction of estimated acid-base parameters which are carried out under fully oxygen saturation.



After the acid-base imbalance has been in effect for a period of time the patient may become *compensated*. The "compensated state" does not necessarily imply that the plasma pH is within the normal range. It is expected on the basis of experience with individuals in an acid-base imbalance that plasma bicarbonate,  $\text{PCO}_2$ , and pH combine in a similar way which is expressed in computing formulas or in *diagrams of compensated states*. In Englis' diagram,  $\text{P}_{\text{CO}_2}$  is plotted against "base excess" (BE) value of the patient. Acute imbalances are distinguished

from compensated states typical for each abnormality. Individual cases may be followed with respect to both the type of the disorder and its temporal development. Points off the limits of the defined states are either in the course of compensation (or decompensation), or they represent combinations of two imbalances. The combinations are more frequent than the "pure" disorders. The combination of AMAc and AMAlk, however, can not be recognized in the diagram.

## EXERCISE 1

### *Examination of acid-base imbalance on a given case*

- 1) Estimate following biochemical values from the Siggaard-Andersen nomogram:  
 $P_{CO_2}$ , BE, BB, NBB, g/l hemoglobin, standard bicarbonate, actual bicarbonate;
- 2) Classify the acid-base imbalance. Evaluate the development of the disorder using data obtained from one person at different time periods. Use the English' diagram to determine the course of compensation.
- 3) Evaluate the ionic composition of the plasma, using given concentrations of ions. Calculate the actual "anion gap".
- 4) Calculate the approximate osmolality using a suitable equation. Summarize the biochemical findings and suggest therapeutic measures, if necessary.

#### *Accessories:*

- pH data (actual, equilibrated blood samples) and biochemical values;
- Siggaard-Andersen nomogram;
- Compensation diagram acc. to Engl.

#### *How to operate the Siggaard-Andersen nomogram?*

- (1) Use the concentrations of carbon dioxide in equilibration gasses to calculate the partial pressures of  $CO_2$  in the oxygen- $CO_2$  mixtures according to the equation:

$$P_{CO_2}(\text{mmHg}) = \frac{(750 - 50) \cdot \%CO_2}{100} \quad \begin{matrix} 50 \text{ mmHg(torr): water} \\ \text{vapour tension} \end{matrix}$$

- (2) Plot in the nomogram the two points corresponding to the two pH values measured after equilibrating the blood with the gass mixtures, with low and high  $CO_2$ , respectively.
- (3) Draw a *buffering line* through these points.

- (4) Determine  $\text{PCO}_2$  (respiratory component) by using the actual pH as an entry.
- (5) Read *base excess* value at the point where the buffer line intersects the Base Excess curve.
- (6) Read the *buffer base* value at the point where the line intersects the Buffer Base curve.
- (7) Calculate the *normal buffer base* by subtracting the found value for base excess of fully oxygenated blood from the found value for buffer base. The Hb concentration corresponding to this "normal buffer base" on the nomogram should not deviate more than by 3g/100 ml from that ascertained by direct determination.
- (8) Read the value of *standard bicarbonate* at the point where the buffering line intersects the Plasma Bicarbonate line.
- (9) Draw a new line (slope 45°) through the point on the buffering line corresponding to the actual pH, and read out the *actual bicarbonate* where it intersects the Plasma Bicarbonate line.

*Note:* In case of reduced  $\text{O}_2$  saturation (less than 90%) a new and more alkaline line (to the right of the original) should be established by applying an alkaline correction on both the "buffer base" and "base excess" scales. The correction is 0.2 mmol/l blood for each gram of deoxygenated hemoglobin contained per 100 ml of blood.

If kPa for the estimation of  $\text{PCO}_2$  is used instead of mmHg (torr), the conversion may be computed as follows:

$$\text{kPa} = 0.1333 \text{ mmHg (torr)}$$

$$\text{mmHg (torr)} = 7.5 \text{ kPa}$$

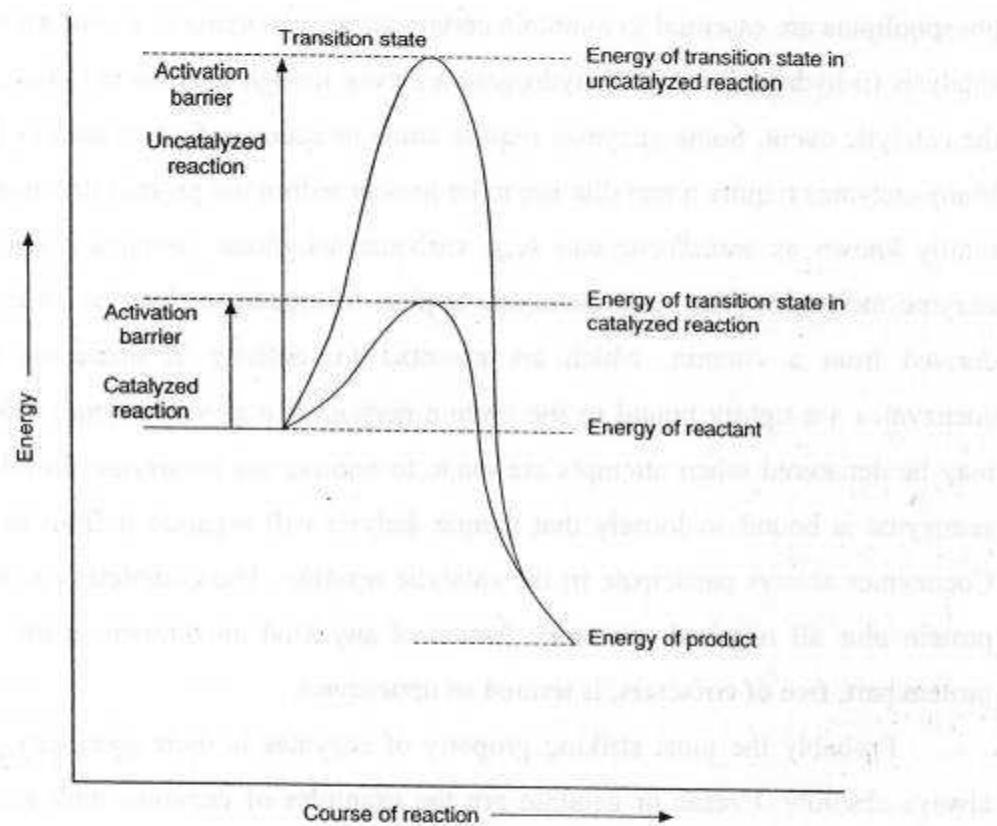
If the range of the Base Excess curve is not great enough, so that the buffering line does not intersect the scale, the *base excess* value can be found from:  $\text{BE} = \text{BB} - \text{NBB}$ , where NBB is found as the *buffer base* corresponding to the Hb concentration in the blood (normally 15 g/100 ml).

*Reference values:*

actual pH	7.40 + 0.04
$\text{PCO}_2$	5.33 kPa (40 torr - mmHg)
base excess (BE)	0.00 + 2.0 mmol/l
buffer base (BB)	48 mmol/l
normal buffer base (NBB)	48 mmol/l
hemoglobin	15 g% (150 g/l)
standard bicarbonate	24 mmol/l
actual bicarbonate	24 mmol/l
oxygen Hb saturation	95-99%

## INTRODUCTION TO ENZYMOLOGY

Virtually all the biologically important reactions are catalysed. This is achieved with the help of specific biocatalysts known as *enzymes*. The enzymes are able to increase the *rate* of reactions that may occur in the particular cell or tissue. It should be noted that enzymes do not change the chemical equilibrium neither total energy input or output of the reaction. *Enzymes increase reaction rates by decreasing the activation barrier of the reaction.*



Almost all known enzymes are *proteins*. The molecular weights of enzymes cover a wide range. For example, the enzyme ribonuclease is relatively small, having a molecular weight approximately 13,700. In contrast, aldolase, an enzyme of glycolysis, has a molecular weight of approximately 156,600. It is composed of four subunits, each with a molecular weight of about 40,000. Pyruvate dehydrogenase, which catalyzes the conversion of pyruvate to acetyl CoA, is a multienzyme complex in which the components are so tightly organized that the entire system can be isolated as a discrete, particulate entity from many tissues. The complex from pig heart has a molecular weight of about  $1 \times 10^7$ ; each complex contains no

fewer than 42 individual molecules, including several important and essential cofactors. The entire structure of the pyruvate dehydrogenase complex is required for catalysis.

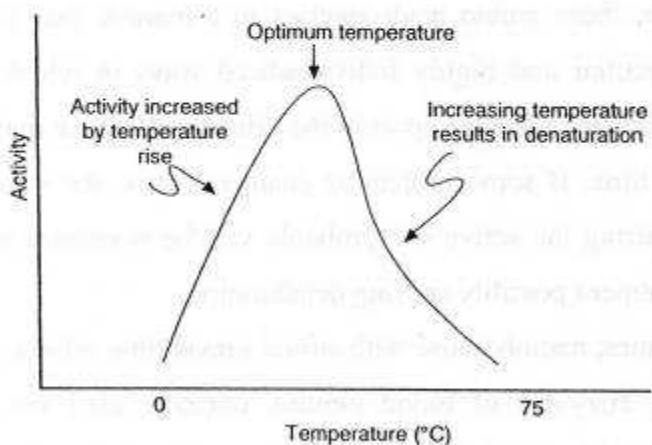
In addition to the protein component, many enzymes require non-protein constituents for their function as catalysts. These accessory moieties are variously termed prosthetic group, cofactor, and coenzyme. The term *prosthetic group* applies to any non-amino acid portion of a protein that confers on that protein some particular property. Prosthetic groups are connected to the protein part either covalently (hem in cytochromes) or non-covalently (hem in hemoglobin). The term *cofactor* is also broadly defined. Small organic molecules such as phospholipids are essential to maintain certain enzyme proteins in a conformation suitable for catalysis ( $\beta$ -hydroxybutarate dehydrogenase), even though they do not directly participate in the catalytic event. Some enzymes require anion or cation cofactors such as the chloride ion. Many enzymes require a metallic ion to be present within the protein structure - they form the family known as *metalloenzymes* (e.g. carbonic anhydrase contains a zinc atom in every enzyme molecule). The term *coenzyme* applies to organic molecules, often but not always derived from a vitamin, which are essential for activity of numerous enzymes. Some coenzymes are tightly bound to the protein portion of a given enzyme; indeed, the enzyme may be denatured when attempts are made to remove the coenzyme. In other instances the coenzyme is bound so loosely that simple dialysis will separate it from its protein partner. Coenzymes always participate in the catalytic reaction. The complete functional complex of protein plus all required accessory factors of any kind are known as the *holoenzyme*; the protein part, free of cofactors, is termed an *apoenzyme*.

Probably the most striking property of enzymes is their *specificity*, even if it is not always absolute. Urease or catalase are the examples of enzymes with absolute specificity toward their substrates, chymotrypsin on the other hand shows a somewhat lesser specificity, it prefers to cleave peptide bonds in which one participant amino acid has an aromatic ring.

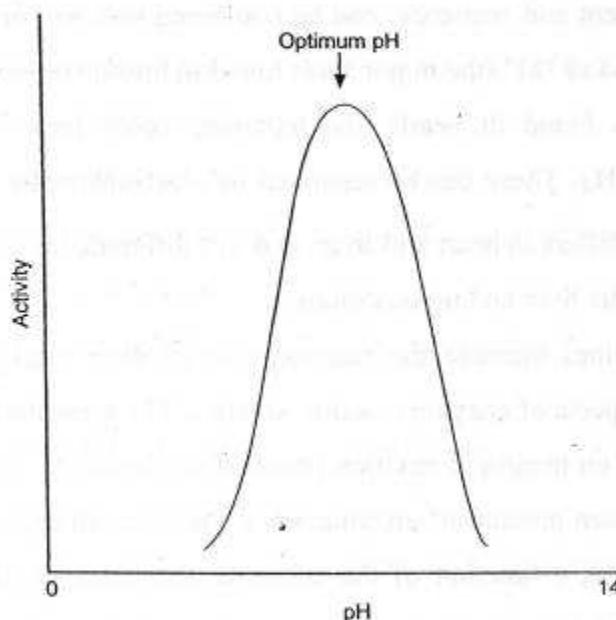
Enzymes isolated from their natural sources can be used *in vitro* to study in detail the reactions they catalyze. Reaction rates may be altered by varying such parameters as pH or temperature, by changing the ionic composition of the medium, or by changing ligands other than the substrate or coenzymes.

Since protein structure determines enzyme activity, anything that disturbs this structure may lead to a change in activity. Denaturation of proteins, which means the spatially random arrangement, can be produced by many agents. These include heat and chemicals that destroy hydrogen bonds in the protein, such as urea at high concentration, detergents such as sodium dodecyl sulfate, and sulphydryl reagents such as mercaptoethanol. Enzymes frequently

show great *thermal sensitivity*. When heated to temperatures greater than 50°C, most but not all enzymes are denatured. High-temperature denaturation is usually irreversible. Even under conditions where denaturation does not occur, most enzymes show an optimum temperature at which activity is maximal. The changes in activity above or below the optimum temperature are not always symmetric.



Enzyme activity is also related to the ionic state of the molecule and especially of the protein part, since the polypeptide chains contain groups that can ionize to a degree that depends on the prevailing pH. As is true of proteins generally, enzymes have an *isoelectric point* at which their net free charge is zero.



The pH of the isoelectric point (pI) as a rule is not the same as the pH at which maximal activity is demonstrated. The *pH optima* shown by enzymes vary widely; pepsin, which exists in the acid environment of the stomach, has a pH optimum at about 1.5, whereas arginase, an enzyme that cleaves the amino acid arginine, has its optimum at 9.7. However, most enzymes have optima that fall between pH 4 and 8. Some enzymes show a wide tolerance for pH changes, but others work well only in a narrow range. If any enzyme

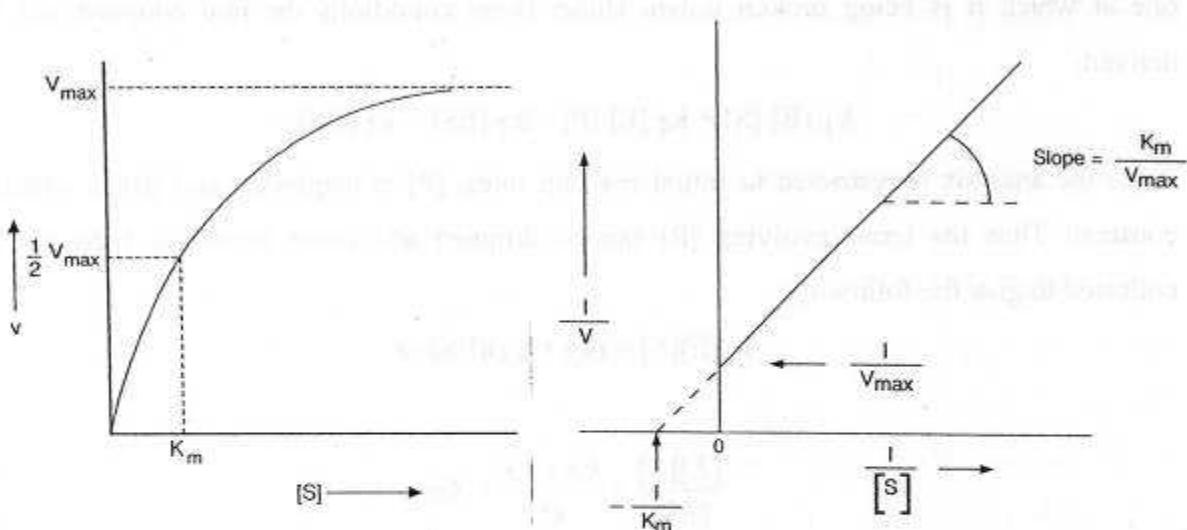
is exposed to extreme values of pH, it is denatured. The sensitivity of enzymes to altered pH is one reason why regulation of body pH is so closely controlled and why changes from normal may involve serious consequences.

Enzymes differ from other proteins in that they possess what has been termed an *active catalytic site*. The active site can be regarded as being composed of a relatively small number of amino acid residues, not necessarily in immediate sequence in terms of primary structure. However, these amino acids interact in a manner that allows catalysis to occur. Because of the peculiar and highly individualized ways in which peptide chains may be folded, amino acids some distance apart in the primary sequence may contribute to the active site. At the same time, if some molecular change occurs, the necessary interaction of the amino acids composing the active site probably will be weakened or lost. This accounts for relatively mild treatment possibly causing denaturation.

Some enzymes, namely those with strong irreversible effects (e.g. proteolytic enzymes of digestive tract, enzymes of blood clotting cascade, etc.) are synthesized as inactive precursors also named *proenzymes* or *zymogens*. Typical activation mechanism is the excision of a peptide fragment followed by a change of conformation and a formation of the active site.

In many species, including humans, different molecular forms of certain enzymes may be isolated from the same or different tissues. The different molecular forms have been termed *isoenzymes*, or *isozymes*. Lactate dehydrogenase (LDH) and malate dehydrogenase have been thoroughly studied as examples of isoenzymes. LDH is composed of four subunits. The two subunit types, differing in amino acid content and sequence, can be combined into tetramers in five ways. If one subunit type is identified as "M" (the major form found in muscle or liver) and the second as "H" (the major form found in heart), the tetramers could have the compositions M<sub>4</sub>, M<sub>3</sub>H, M<sub>2</sub>H<sub>2</sub>, MH<sub>3</sub>, or H<sub>4</sub>. These can be separated by electrophoresis. In humans the content of several isoenzymes differs in heart and liver, and this difference is used in diagnostic differentiation of diseases of the liver and myocardium.

It was mentioned above that enzymes increase the reaction rate by their catalytic action. Let us explain some quantitative aspects of enzyme reaction kinetics. The quantitative analysis of enzyme action depends largely on measured reaction times. If the *initial reaction rate*, defined as the rate observed for a given amount of enzyme when the concentration of product formed is nearly zero, is plotted as a function of the substrate concentration, the results appear similar to those shown below:



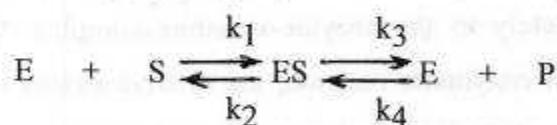
The curve connecting the observed points would be hyperboloid and would asymptotically approach a maximum value, as shown by  $V_{\max}$ . This is the maximum initial velocity that can be obtained without increasing the amount of enzyme.

The hyperbola described by a plot of reaction velocities as a function of substrate concentrations is difficult to use. If reciprocals of the velocities are plotted as a function of the reciprocal substrate concentrations, the hyperbola is converted to a straight line. The double-reciprocal plots are frequently called Lineweaver-Burk plots (see previous page).

If the usual convention is followed, representing concentrations by means of brackets, that is, by letting  $[S]$  stand for the molar concentration of the substrate, and if a few assumptions are made regarding the experimental situation, one can obtain a useful mathematical equation that describes the enzyme kinetics. Assume for the present that:

1. The system involves only a single substrate.
2. The system is at a steady state, that is,  $[ES]$  is a constant and the free enzyme E is in equilibrium with ES.
3. The system is established so that  $[E] < [S]$  on a molar basis.
4. Since the analysis deals with initial reaction rates,  $[S] \gg [P]$  and  $[P]$  is negligible under these conditions.

In this case the reaction mechanism may be formulated as follows:



where  $k_1$ ,  $k_2$ ,  $k_3$ , and  $k_4$  are the respective rate constants. At the steady state the concentration of ES is constant; that is, the rate at which it is being formed is the same as the

rate at which it is being broken down. Under these conditions the rate equation can be derived:

$$k_1 [E] [S] + k_4 [E] [P] = k_2 [ES] + k_3 [ES]$$

Since the analysis is restricted to initial reaction rates,  $[P]$  is negligible and  $[S]$  is virtually constant. Thus the term involving  $[P]$  can be dropped and those involving  $[ES]$  can be collected to give the following:

$$k_1 [E][S] = (k_2 + k_3)[ES] \text{ or}$$

$$\frac{[E][S]}{[ES]} = \frac{k_2 + k_3}{k_1} = K_m$$

The ratio of rate constants,  $(k_2 + k_3)/k_1$ , can be replaced by a single constant,  $K_m$  (*Michaelis constant*).

The *maximum initial velocity* is achieved only when all the enzyme is in the form of the active complex (ES), from which it follows that:

$$V_{\max} = k_3 [E]$$

Under any other conditions, the observed initial velocity will be the following:

$$v = k_3 [ES]$$

The final version of *Michaelis-Menten equation* can be derived:

$$v = \frac{[S]V_{\max}}{[S] + K_m} = \frac{[S]k_3[E]}{[S] + K_m}$$

A significance of  $K_m$  is seen from this equation. When  $K_m$  is equal to  $[S]$ , then  $v = \frac{1}{2} V_{\max}$ . Both,  $K_m$  and  $[S]$  are expressed in the same units, moles per litre. Also, when  $[S] \gg K_m$ ,  $K_m$  can be dropped and  $v = k_3 [E] = V_{\max}$ . On the other hand, if  $[S] \ll K_m$ , then  $v = V_{\max} [S] / K_m$ . These relations should be followed in any laboratory reaction. In designing any assay to measure the amount of an enzyme in blood or other material, it is important to ensure that sufficient substrate is present to saturate the enzyme completely, that is, to convert it completely to the enzyme-substrate complex. On the other hand, when substrate is measured in enzymatic reaction, the relative excess of enzyme is necessary to have the reaction rate be a function of substrate concentration.

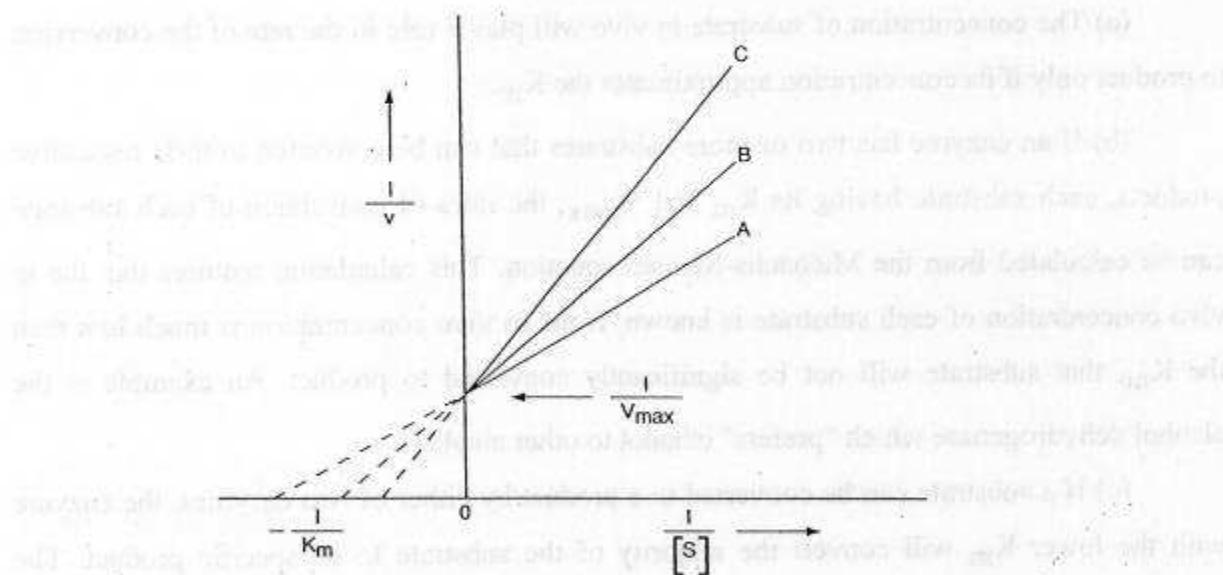
The significance of  $K_m$  in metabolism centres its operational definition as the concentration of substrate at which the initial velocity is half its maximum. From this, some important points can be made:

- (a) The concentration of substrate in vivo will play a role in the rate of the conversion to product only if its concentration approximates the  $K_m$ .
- (b) If an enzyme has two or more substrates that can be converted to their respective products, each substrate having its  $K_m$  and  $V_{max}$ , the rates of conversion of each substrate can be calculated from the Michaelis-Menten equation. This calculation requires that the in vivo concentration of each substrate is known. If the in vivo concentration is much less than the  $K_m$ , that substrate will not be significantly converted to product. An example is the alcohol dehydrogenase which "prefers" ethanol to other alcohols.
- (c) If a substrate can be converted to a product by either of two enzymes, the enzyme with the lower  $K_m$  will convert the majority of the substrate to its specific product. The physiological importance of the reaction can be predicted, using the  $K_m$ ,  $V_{max}$ , and the in vivo concentration of the substrate.

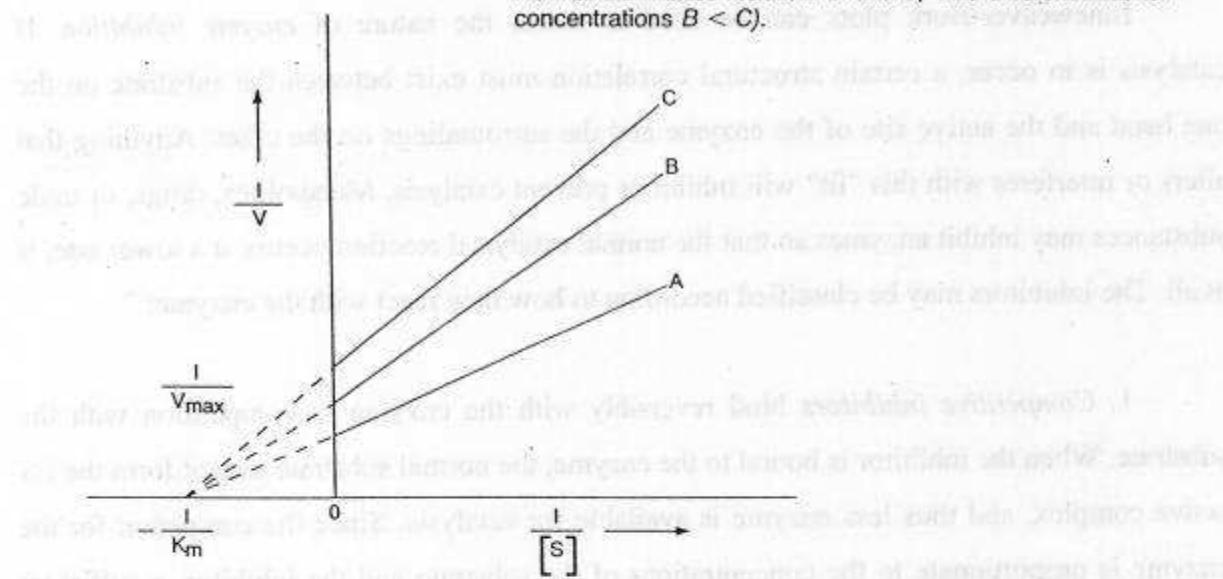
Lineweaver-Burk plots can be used to assess the nature of *enzyme inhibition*. If catalysis is to occur, a certain structural correlation must exist between the substrate on the one hand and the active site of the enzyme and the surroundings on the other. Anything that alters or interferes with this "fit" will inhibit or prevent catalysis. Metabolites, drugs, or toxic substances may inhibit enzymes so that the normal catalyzed reaction occurs at a lower rate, if at all. The inhibitors may be classified according to how they react with the enzyme:

1. *Competitive inhibitors* bind reversibly with the enzyme in competition with the substrate. When the inhibitor is bound to the enzyme, the normal substrate cannot form the ES active complex, and thus less enzyme is available for catalysis. Since the competition for the enzyme is proportionate to the concentrations of the substrate and the inhibitor, a sufficient concentration of the substrate will overwhelm the inhibition, and the  $V_{max}$  will be the same as with no inhibitor present. At concentrations in which substrate and inhibitor are more comparable, the  $K_m$  for the substrate will be reduced.

Competitive inhibition depicted by Lineweaver-Burk plots. A, Normal uninhibited reaction. B and C, Two different inhibitor concentrations ( $B < C$ ).



Noncompetitive inhibition depicted by Lineweaver-Burk plots: A, Normal uninhibited reaction. B and C, Two different inhibitor concentrations  $B < C$ .



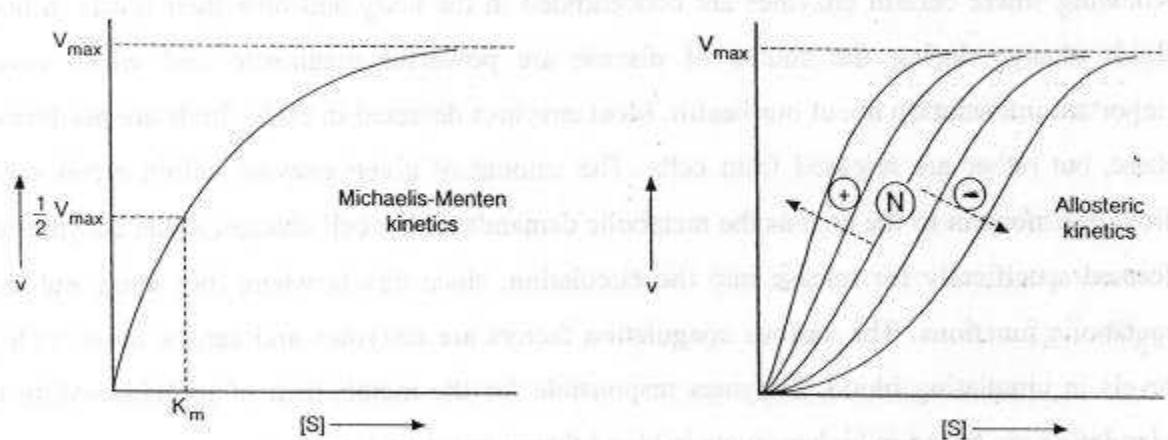
2. *Noncompetitive inhibitors* bind either to the enzyme or the enzyme-substrate complex. In this case the  $V_{max}$  is decreased without a change in the  $K_m$  for the substrate. A high concentration of the substrate will not display the inhibitor.

3. *Uncompetitive inhibitors* bind only to the free enzyme and not to the enzyme-substrate complex. In this case both  $V_{max}$  and  $K_m$  are changed. The Lineweaver-Burk plots show parallel lines at the different inhibitor concentrations.

Some enzymes do not follow the kinetics of the Michaelis-Menten model. A significant group of such enzymes are subject to control by molecules that bind to sites on the enzyme other than the catalytic site. Such molecules, called *effectors*, influence the binding of the substrate to the catalytic site. These enzymes are known as *allosteric enzymes*. Some allosteric enzymes are composed of subunits of identical or closely related peptide chains. The quaternary conformation is modified by the appropriate allosteric effectors. One or more of the functional sites on these enzymes may be *catalytic* (C), whereas one or more other sites may be *regulatory* (R) and not identical with the catalytic or active sites. In some instances R and C sites are on different subunits; in other instances the R and C sites are located on the same subunit. When the reaction velocity of an allosteric enzyme is plotted as a function of substrate concentration, a sigmoid rather than a hyperbolic curve is obtained. One can see that the shapes of the allosteric curves are changed considerably by altering the concentration of either positive or negative effectors. In effect, decreasing the amount of negative effector or increasing the amount of positive effector produces a response equivalent to lowering the  $K_m$  of the substrate. In the most general case allosteric kinetics can be represented by the following equation:

$$v = \frac{[S]^n V_{\max}}{[S]^n + K}$$

where n is a coefficient that represents the interaction of the binding site, K represents a measure of the affinity of substrate for the enzyme, and the other symbols have their previously stated meanings.



A unit of enzymatic activity is the *catal* (cat) which is defined as the moles of substrate transformed per second. Enzyme concentrations are expressed as *cat/l* (*mcat/l*, *μcat/l*, etc.).

### *Clinical applications of enzymes*

Measurement of enzyme activity is a useful monitor of overt disease, of genetic tendencies toward a disease state, and of a patient's response to a particular type of therapy.

A growing number of purified enzymes are becoming commercially available. These can be employed as reagents for the accurate determination of small amounts of such blood constituents as glucose, urea, uric acid, cholesterol, and triacylglycerols. Frequently these methods are more specific and faster than the chemical determinations used previously.

Enzymes may be employed as diagnostic aids in yet another way, which takes advantage of their turnover numbers. Different antibodies are often used to determine specifically many antigens as molecules of interest. Before use, the generated antibody is modified by coupling it to some indicating enzyme with a fairly high turnover number, such as phosphatase. If sample containing specific antigen is added to the modified antibody, a precipitate is formed that can be collected, washed, and dissociated. If a suitable phosphatase substrate is added and the mixture properly incubated, colour is produced proportional to the amount of enzyme. That, in turn, is indicative of the presence of antigen. Various commercial immunoassay kits are now available for this purpose.

Our knowledge of enzyme reactions and the sources of enzymes in tissues allows us to detect changes in body function and assess the presence or absence of specific tissue damage. Knowing where certain enzymes are concentrated in the body and how their levels in body fluids change during the course of disease are powerful diagnostic tool which reveal important information about our health. Most enzymes detected in body fluids are not formed there, but rather are released from cells. The amount of given enzyme within a cell varies from one moment to the next as the metabolic demands on the cell change. Some enzymes are formed specifically for release into the circulation, since this is where they carry out their metabolic functions. The various coagulation factors are enzymes and can be found in high levels in circulating blood. Enzymes responsible for the metabolism of proteins within the circulation are found in higher levels in blood than in most tissues.

On the other hand, the levels of activity of most enzymes in blood or other body fluids are quite low. The body fluid concentration of a given enzyme reflects a variety of processes.

One major factor is the amount of *cellular turnover* - how rapidly the old cells are dying and breaking down. As a cell reaches the end of its life span, it disintegrates and releases its contents into the surrounding tissues. The enzymes within the cell escape, and a certain amount of this material enters the bloodstream, urine, CSF. Since most cells turn over fairly slowly, only low levels of the enzyme are observed in the body fluid under normal circumstances. Once the enzyme enters the body fluid, further changes in concentration take place. In blood (and, to a lesser extent, other fluids) there are a number of proteases, that attack the various enzymes and metabolize them at different rates. The circulating levels of some enzymes are also affected through excretion by the kidney. Proteins with a molecular weight of less than approximately 60,000 are filtered by the kidney and excreted in the urine. One such enzyme is pancreatic amylase which is readily excreted by way of the kidneys and its level in urine is higher than that in blood.

The routine turnover of cells and the accompanying steady level of an enzyme in a body fluid is altered in a striking fashion in many disease states. Instead of a fairly constant concentration of the enzyme, the level may rise markedly over a short time and then subside fairly rapidly to the normal amount for that enzyme. These drastic alterations of the normal enzyme level provide valuable diagnostic clues as to the underlying pathological state in the body. In many disease states, one of the consequences is the rapid destruction of the tissue. Let us the case of *myocardial infarction* as an example. As a result of loss of oxygen, changes in heart beat, decreased blood flow, or other factors, heart tissue begins to break down very quickly. The rate of cell breakdown rises, and greatly increased quantities of certain enzymes are released into the circulation. Later, when the cell turnover returns to normal, metabolism (and excretion) of the excess enzyme has occurred, and the usual low level of the enzyme in the circulation is restored.

Each enzyme has some tissue specificity. By knowing the tissue specificity of any enzyme, we can better ascertain where the damage occurred in the body. Enzymes of high specificity (amylase, acid phosphatase) are found predominantly in one type of tissue. Those enzymes which have moderate specificity are more widely distributed in the body. Some enzymes (alkaline phosphatase and lactate dehydrogenase) are ubiquitous - they are found everywhere. Even with this very wide distribution, these enzymes can provide valuable diagnostic information if we study the proper parameters. In case of lactate dehydrogenase the estimation of isoenzymes will increase the tissue specificity, as described above. Another enzyme, where the isoenzymes are a useful tool, is creatine kinase.

The following enzymes are routinely assayed in blood serum in clinical laboratories:

Enzyme	Normal value	Organ or disease of interest (In adults)
Aldolase	< 50 ncat/l	Liver, muscle
Acid phosphatase	< 108 ncat/l	
Acid phosphatase (prostatic)	< 24 ncat/l	Prostatic carcinoma
Alkaline phosphatase	< 2.3 µcat/l	Liver, bone disease
Alanine aminotransferase	< 0.67 µcat/l	Liver, heart disease
Aspartate aminotransferase	< 0.67 µcat/l	Liver, heart disease
γ-glutamyl transferase	< 1.7 µcat/l	Liver disease
Lactate dehydrogenase	< 8.0 µcat/l	Liver, heart, RBC
β-hydroxybutyrate dehydrogenase	< 6.0 µcat/l	Heart disease
Choline esterase	76-230 µcat/l	Liver disease
Creatine kinase	< 3.2 µcat/l	Heart, muscle
α-amylase	< 3.3 µcat/l	Pancreatic disease
Lipase	< 3.2 µcat/l	Pancreatic disease

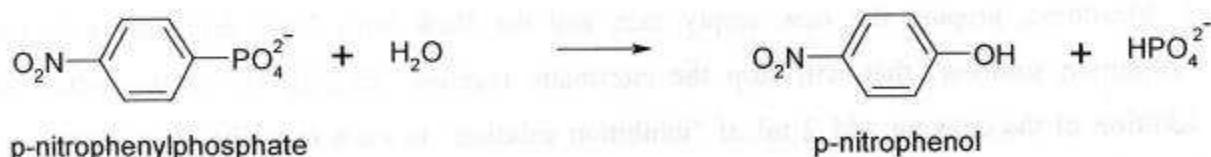
ENZYMOLOGY I

1. Kinetics of enzymatic reaction -  $K_M$  estimation of acid phosphatase
  2. Measurement of RBC catalase activity

## 1. Kinetics of enzymatic reaction - $K_M$ estimation of acid phosphatase

### ***Introduction:***

The acid phosphatase hydrolyses p-nitrophenylphosphate (an artificial substrate) to nitrophenol and inorganic phosphate. Nitrophenol exhibits a yellow colour in alkaline media and can be estimated photometrically.



Solutions

1. Citrate buffer pH 4.7 (0.05 mol/l)
  2. Substrate solution (2.5 mmol/l p-nitrophenylphosphate in citrate buffer ) - 1 tablet of substrate in 20 ml of citrate buffer
  3. Enzyme solution (partially purified plant acid phosphatase)
  4. Inhibition solution (NaOH 0.1 mol/l - EDTA 30 mmol/l)

Solutions of enzyme and substrate should be kept permanently in the refrigerator and are allowed to be taken out only for the necessary time of pipetting.

#### *Procedure:*

**Read the whole procedure before you start the experiment!**

1. Prepare a set of 10 small test-tubes. Pipette according to the table. Use the automatic micropipettes of following fixed volumes: 50, 100, and 200  $\mu$ l. Pipette the citrate buffer first and than the substrate solution. This way you can use the same disposable tip. Discard the tip after you have finished with the substrate solution.

<b>Tube No</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>
Citrate buffer ( $\mu$ l)	450	400	350	300	250	200	150	100	50	-
Substrate ( $\mu$ l)	50	100	150	200	250	300	350	400	450	500

2. Place the rack with all the test/tubes into the water bath ( $37^{\circ}\text{C}$ ) and leave it there for 5 minutes to warm up.
3. Take the enzyme solution (acid phosphatase in the Eppendorf tube) from the refrigerator, prepare the  $50 \mu\text{l}$  pipette with a clean tip and the stop-watch. Add  $50 \mu\text{l}$  of the enzyme to the test-tube No 1, press the stop-watch and let it running. Pipette the same volume of the enzyme to all the other test-tubes in precisely same periods of time (every 15 seconds is quite useful). Be sure that the enzyme drop came to the solution, not to the upper part of the tube wall, but don't touch the substrate containing solution with the tip. Shake each tube immediately after the enzyme is added and put it back to the water bath. Leave the stop-watch running and let the reaction proceed for exactly 10 minutes.
4. Meantime, prepare the new empty rack and the flask with 2 ml dispenser with the "inhibition solution" that will stop the enzymatic reaction. Exactly 10 minutes after the addition of the enzyme add 2 ml of "inhibition solution" to each test-tube in exactly same intervals as you have added the enzyme. So, in every test-tube the reaction is allowed to run for the same period of time. The individual tubes are not returned to the water bath but to the new rack.
5. Perform the photometric measurements on Specol 11 or on another photometer in 1 cm cuvettes at 405 nm against water. Don't rinse the cuvettes between the samples, only pour them always carefully and remove the drops at the edge with a filter paper.
6. The result (absorbance) is proportional to the rate of enzymatic reaction in each tube, because the rate is the amount of substrate changed per time unit. In this case the result represents the amount of product formed in 10 minutes. Calculate the concentration of substrate (p-nitrophenylphosphate) in each reaction mixture. The stock solution of substrate is 2.5 mmol/l. Construct the graph using the double reciprocal values and estimate graphically the  $-1/K_M$  and  $K_M$ , or transfer your results into the Excel table and produce the final results, i.e. estimate the  $K_M$ .

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1
0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1

## 2. Measurement of RBC catalase activity

### Introduction:

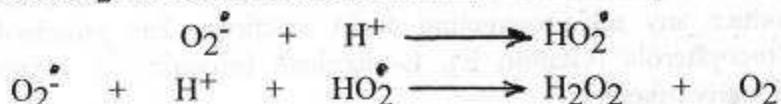
Molecular oxygen is a biradical that has two unpaired electrons. These two electrons have the same quantum spin number (parallel spin) and require incoming electrons to be also of parallel spin so as to fit into the vacant spaces in the  $p$  orbitals. In accordance with Pauli's exclusion principle, a pair of electrons from an atomic or molecular orbital would have antiparallel spins. These conditions impose restrictions involving spin inversions on oxidations by  $O_2$  that tend to make it accept its electrons one at a time. The advantage of this process for aerobic life is a considerable slowing down of reactions of oxygen with non-radicals. The disadvantage, however, is that one-electron reduction of  $O_2$  leads to the formation of reactive oxygen species.

One-electron reduction product of  $O_2$  is the **superoxide radical (anion)** ( $O_2^-$  or  $O_2^{\bullet-}$ ) and its protonated form is the **hydroperoxyl radical** ( $HO_2^{\bullet}$ ). The  $pK_A$  for its dissociation is approximately 4.8, there is likely to be little  $HO_2^{\bullet}$  present at physiological pH and the superoxide anion, therefore, is the main one-electron reduction product of dioxygen in aqueous biological media.



Generation of  $O_2^-$  in solution has been observed to kill or inactivate bacteria and other cells, stimulate lipid peroxidation, and damage DNA, carbohydrate, and protein. Chemical studies, however, cast considerable doubt on this, for in aqueous solution  $O_2^-$  is a weak oxidizing agent and moderately strong reducing agent. Most, if not all, damage associated with the generation of  $O_2^-$  must be due to other species whose formation depends on it. The hydroperoxyl radical has a greater oxidizing potential than  $O_2^-$  and may be important at sites with an acid pH or within the lipophilic membrane interior.

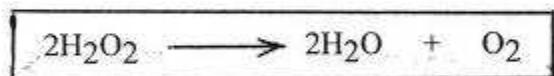
The major reaction of  $O_2^-$  is the dismutation reaction, which takes place in two stages:



Overall reaction:



Since the concentration of  $H^+$  is low at physiological pH the spontaneous dismutation reaction is slow, allowing  $O_2^-$  to diffuse from its site of formation. To prevent this, most of the aerobic organisms contain superoxide dismutase, an enzyme which is able to speed up the above reaction. It is usually combined with the action of catalase, which completes the oxygen radical detoxification process by disproportionating the  $H_2O_2$  to  $H_2O$  and  $O_2$ :



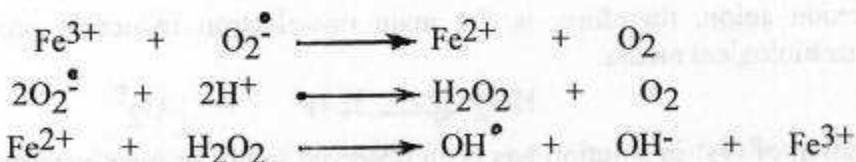
Hydrogen peroxide ( $H_2O_2$ ) is the two-electron reduction product of  $O_2$  and since it has no unpaired electrons it cannot be called a radical. It is the most stable of the intermediates formed in the reduction of  $O_2$  to water. It thus can diffuse from its site of formation and can

also cross cell membranes, unlike  $O_2^-$ , which does not cross membranes unless there is a specific channel for it.

Addition of an electron to hydrogen peroxide gives the **hydroxyl radical** ( $OH^\bullet$ ):



This is a highly active and most dangerous species reacting at a near diffusion controlled rate *with almost any biological molecule*. Therefore, it cannot move far from its site of generation. Hydroxyl radicals react by several mechanisms, the most important being the hydrogen abstraction, which is also the basis of lipid peroxidation (see Harper's Biochemistry p. 151). The chemists suggested that  $O_2^-$  and  $H_2O_2$  can directly react to give  $OH$  (Haber-Weiss reaction). However, it was soon clear that this reaction was not biologically feasible but could occur through metal ion catalysis. Participation of trace amounts of iron salts in the reaction is essential and, therefore, non-bound iron or other metal ions are considered as dangerous.



A highly susceptible target for the action of oxygen radicals is the polyunsaturated fatty acid side chains of cell and organelle membranes. The above mentioned lipid peroxidation can result in a serious damage of membrane structures and in the eventual death of the cell.

Under normal circumstances the reactive oxygen species formation is in a balance with the activity of **antioxidants**, i.e. the superoxide dismutase and other enzymes involved in radical destruction or repair of peroxidated structures, and also such called **free radical scavengers**. These are substances able of interaction with any free radicals produced, but do not in turn produce any self-propagating chain reactions. The principal ones present in humans are  $\alpha$ -tocopherols (vitamin E), L-ascorbate (vitamin C), retinoids (vitamin A), ubiquinone, and many others.

#### Solutions:

1. Substrate solution (hydrogen peroxide 10 mmol/l in phosphate buffer pH 7.4)
2. Standard solution of potassium permanganate 2 mmol/l in burette
3. Sulfuric acid 1 mol/l
4. Enzyme solution (diluted hemolyzed red blood cells)

#### Procedure:

**Read the whole procedure before you start the experiment!**

1. Prepare 10 titration flasks, mark them 0 - 9 and add 2.5 ml of sulfuric acid (1 mol/l) into each. (It will later stop the enzymatic reaction and also is necessary for the manganometric titration.)

2. Set up the reaction mixture. Into a 100 ml Erlenmayer flask transfer 60 ml of substrate solution, add the glass stirrer and place the flask on a magnetic stirrer.
3. Take a 5 ml sample from the reaction mixture (before the addition of enzyme) and transfer it into the first titration flask marked "0". Be careful not to get the sulfuric acid on your pipette tip.
4. Take 1 ml of enzyme solution and add it to the reaction flask on a stirrer and start the stopwatch.
5. Take additional 5 ml samples in 1 minute intervals and blow out the samples into prepared titration flask with sulfuric acids. Your pipetting must be done in such a way that the reacting solution comes in contact with the sulfuric acid exactly in the desired time, so the pipette must be filled before.
6. Titrate all the samples with permanganate. The amount of used standard is proportional to the amount of hydrogen peroxide in each sample.
7. Make a graph:  $x$  - time;  $y$  - moles of disproportionated  $H_2O_2$ . Construct also a graph that would express the velocity of the enzymatic reaction and its dependence on time. You may use the prepared Excel program.
8. Repeat the whole experiment with 10 times diluted enzyme solution (with distilled water).

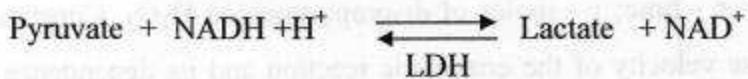
## ENZYMOLOGY II

1. Estimation of lactate dehydrogenase activity in serum – Warburg's optical test
2. Alanine aminotransferase and aspartate aminotransferase in liver
3. Estimation of alkaline phosphatase in serum

### 1. Estimation of lactate dehydrogenase activity in serum – Warburg's optical test

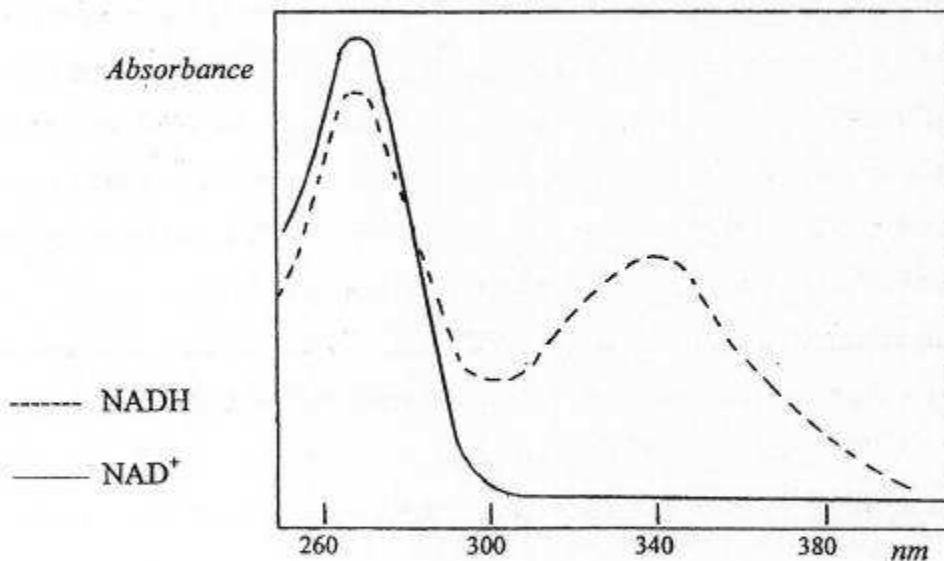
#### Principle:

Lactate dehydrogenase (LDH) catalyses the reduction of pyruvate to lactate accompanied by oxidation of  $\text{NADH} + \text{H}^+$  to  $\text{NAD}^+$ . This is extremely important in anaerobic glycolysis when reduced  $\text{NADH} + \text{H}^+$  cannot sent to the mitochondrial respiratory chain for reoxidation. Instead, the coenzyme reoxidation is achieved by reduction of pyruvate to lactate.



Absorption spectra of reduced and oxidised NAD differ in region around 340 nm where NADH has the second absorbing peak while  $\text{NAD}^+$  does not absorb in this region of spectrum. This measurement principal is known as Warburg's optical test and it can be used in estimation of any enzyme exploiting NAD.

#### Warburg's optical test



LDH is present in all the cells. High levels are present in liver, heart, kidney, skeletal muscles and RBC. Levels in serum or plasma are increased in patients with liver diseases, renal disorders, myocardial infarction, malignancies, muscular dystrophy and haemolysis. *Normal values for adults using this method are below 3.5 µcat/l at 25°C and below 8 µcat/l at 37°C.*

**Solutions:**

1. "Reagent solution" (ready to use!) was prepared from 4 ml of solution A (tris 100 mmol/l, pyruvate 2.75 mmol/l, NaCl 222 mmol/l, pH 7.2) and 1 ml of solution B (NADH 1.55 mmol/l, sodium azide 9.5 g/l – POISON !)
2. "Standard solution" of the enzyme
3. Serum sample

**Procedure:**

1. Switch on photometer ECOM E 6125 and the printer (put only one paper into its printer). Depress the key ABSORBANCE and with arrow keys set the wavelength to 340 nm. Rinse several times the cuvette in photometer with distilled water. The cuvette is emptied with the pump (ask for the demonstration how to use it).
2. Stop the pump and fill the cuvette with distilled water as blank. Check the display of the photometer. It should show ABSORBANCE 340 nm BLANK. If not, used the key BLANK to obtain this parameter. Depress the key ENTER – MEASURE, the absorbance on the display should be 0.000.
3. *Standard measurement:* Empty the cuvette and switch off the pump. Check the display. After the blank measurement the display should show ABSORBANCE 340 nm SAMPLE. If not, use the key SAMPLE. Get ready the stop-watch and then prepare the standard reaction mixture in a small test-tube from 20 µl of "Standard solution" of the enzyme and 1000 µl of the "Reagent solution" and start the stop-watch. Mix with the last (1000 µl) pipette and immediately transfer with the same pipette into the cuvette. Measure the absorbance of the sample exactly after 1, 2, 3 and 4 minutes. The measurement is achieved by depressing the key ENTER – MEASURE: The result of measurement is always printed out.
4. *Sample measurement:* Rinse the cuvette repeatedly with distilled water, make sure that the cuvette is empty and get ready the stop-watch. Mix in a small test-tube 20 µl of serum sample and 1000 µl of the "Reagent solution" and start the stop-watch. Mix with the last (1000 µl) pipette and immediately transfer with the same pipette into the cuvette. Measure the

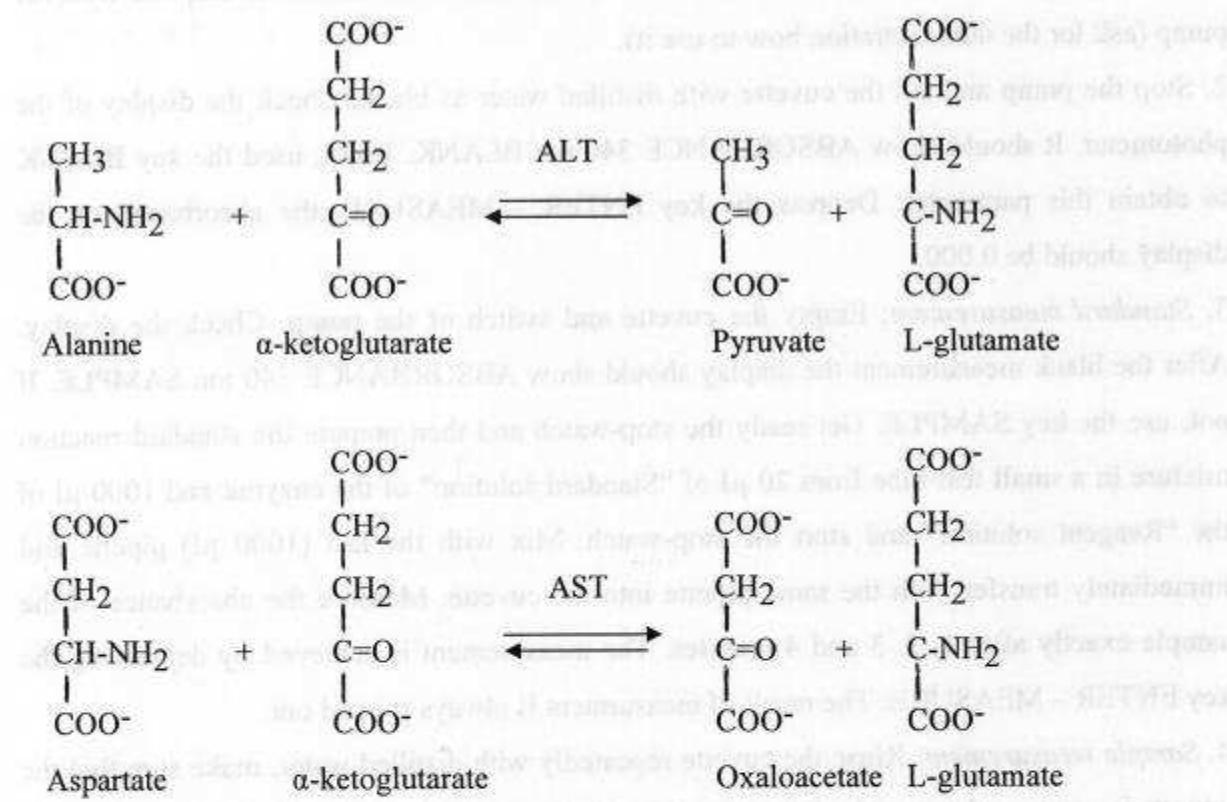
absorbance of the sample exactly after 1, 2, 3 and 4 minutes. The measurement is achieved by depressing the key ENTER – MEASURE: The result of measurement is always printed out.

5. Rinse several times the cuvette with distilled water and switch off the pump, photometer and printer. Transfer (type) the obtained values into the table of the computer program "Lactate dehydrogenase". Print out the results and make the evaluation.

## 2. Alanine aminotransferase and aspartate aminotransferase in liver

### Introduction:

Aminotransferases catalyse reactions called transamination in which the equilibrium between the  $\alpha$ -amino acids and  $\alpha$ -keto acids is re-established. The  $\alpha$ -amino group is usually transferred from an amino acid to  $\alpha$ -ketoglutarate which is converted to L-glutamate. The most frequent and clinically used are two aminotransferases: alanine aminotransferase (ALT) and aspartate aminotransferase (AST):



Both of these enzymes are intracellular and appear in the blood serum only in very low concentrations ( $< 0.67 \mu\text{kat/l}$ ). They both are present in high concentrations in liver and muscle tissues (both cardiac and skeletal muscles). AST is known in two isoenzymes,

cytoplasmic and mitochondrial. The mitochondrial isoenzyme is released only in serious damage of the cell and, therefore, it serves as an indicator of development of liver diseases. The aminotransferases are suitable for diagnosis of acute liver diseases (mostly ALT is elevated) and myocardial infarction (mostly AST is elevated with the maximum in 24-48 hours after the infarction).

The estimation of ALT activity is based on the reaction of products ( $\alpha$ -keto acids) with 2,4-dinitrophenylhydrazine and formation of red hydrazone. It is important that the hydrazone of pyruvate gives at 505 nm higher absorbance than hydrazone of  $\alpha$ -ketoglutarate. The concentration of the latter substrate is also kept low in order to get a good result. In estimation AST the same approach is used, because the oxaloacetate produced in this reaction decarboxylates spontaneously forming the pyruvate.

*Solutions:*

1. Substrate for ALT (DL-alanine 0.2 mol/l, d-ketoglutarate 0.002 mol/l, phosphate buffer pH 7.4 0.1 mol/l)
2. Substrate for AST (L-aspartate 0.1 mol/l, d-ketoglutarate 0.002 mol/l, phosphate buffer pH 7.4 0.1 mol/l)
3. 2,4-dinitrophenylhydrazine (0.1 g DNP-hydrazine in 500 ml of HCl 1 mol/l)
4. Sodium hydroxide 0.4 mol/l
5. Phosphate buffer pH 7.4 0.1 mol/l

*Procedure:*

1. Pipette into 4 marked test-tubes according to the table:

Tube	1	2	3	4
Substrate ALT (ml)	0.5	0.5	-	-
Substrate AST (ml)	-	-	0.5	0.5

Put the test-tubes to the 37°C water bath. Meanwhile, prepare the cell extract. Take about 1 cm<sup>3</sup> of fresh or frozen liver tissue and homogenize it with 5 ml of phosphate buffer in a mortar. Transfer the homogenate into the centrifugation tube and centrifuge at 2500 rpm for 5 minutes. Take the supernatant as the enzyme solution. Then add to the previous test-tubes:

Tube	1	2	3	4
Enzyme (ml)	0.1	-	0.1	-
Do not discard the rest of the enzyme yet. Leave the tubes on the water bath at 37°C 30 minutes. Then add:				
DNP-hydrazine (ml)	0.5	0.5	0.5	0.5
Mix and leave to stay for 15 minutes. Then add:				
NaOH (ml)	5.0	5.0	5.0	5.0
Enzyme (ml)	-	0.1	-	0.1

2. Mix and wait for 15 minutes to develop the colour. Measure on spectrophotometer at 506 nm in 1 cm cuvettes always tube No.1 (ALT) against No.2 (blank) and tube No.3 (AST) against No.4 (blank). The enzyme activity can estimated from graph.

### 3. Estimation of alkaline phosphatase in serum

#### *Introduction:*

Phosphatases hydrolyse phosphate esters. According to the pH optimum they can be divided into alkaline (optimum pH 7 - 10) and acid (optimum pH 4 - 6).

Alkaline phosphatase (ALP) is present mainly in the intestinal cells (necessary for absorption), in the bone (takes part in reconstruction of the bone mineral), and in the liver (takes part in carbohydrate metabolism and others). The enzyme is usually bound to the cytoplasmic membrane. It is activated with  $Mg^{2+}$ . There are known several isoenzymes, two of them, hepatic and bone, are of clinical importance. The hepatic alkaline phosphatase is increased in blood serum in liver diseases connected with cholestasis. Increased bone alkaline phosphatase is characteristic for bone diseases such as rickets, osteomyelitis, bone tumours, both, primary and metastatic. Higher levels of this enzyme are physiologically found in children ( $<7 \mu\text{cat/l}$ ) while in adults the normal level is  $<2.3 \mu\text{cat/l}$ .

Acid phosphatase (ACP) is present predominantly in the prostatic gland. Other tissues contain this enzyme in concentrations of several orders of magnitude lower. The enzyme is localized in lysosomes, so its release to the extracellular fluids is connected with severe changes in cellular structure. Higher ACP is characteristic for prostatic cancer and for its bone metastases. In these cases the ACP prostatic isoenzyme is *L-tartarate sensitive*, which is quite

characteristic for the condition. ACP is present also in the spermatic fluid and this is often used in forensic examinations.

The phosphatase reaction is measured using an artificial substrate p-nitrophenylphosphate which is hydrolysed to 4-nitrophenol and inorganic phosphate. Nitrophenol exhibits a yellow colour in alkaline media and can be estimated photometrically. For details see page 95 of these manuals.

*Solutions:*

1. Buffer (methylglucosamine + magnesium sulfate)
2. Substrate (p-nitrophenylphosphate + glucose)
3. Inhibition solution (EDTA + NaOH)
4. Standard solution of 4-nitrophenol

*Procedure:*

Pipette according to the table:

Tube	1 Sample	2 Blank
Buffer (ml)	1.0	1.0
Serum (ml)	0.02	-

Put to the water bath of 37°C and leave for 5 minutes. Then add:

Substrate (ml)	0.2	0.2
Mix and let to react exactly 10 minutes at 37°C. Then add:		
Inhibition solution (ml)	0.5	0.5
Serum	-	0.02

Measure the sample (1) against blank (2) at 420 nm in 1 cm cuvettes, or measure each sample against water and then calculate  $A_1 - A_2$ . Calculate the activity according to the formula:

$$(ALP) \mu\text{cat/l} = (A_1 - A_2) \times 10.263$$

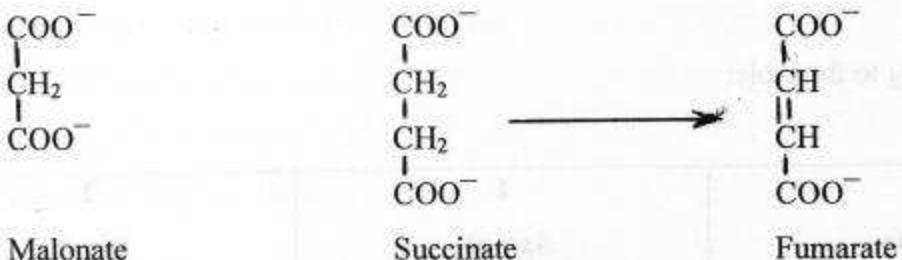
### ENZYMOLOGY III

1. The inhibition of succinate dehydrogenase with malonate
2. Estimation of aldolase activity
3. Demonstration of xanthine oxidase activity in the milk

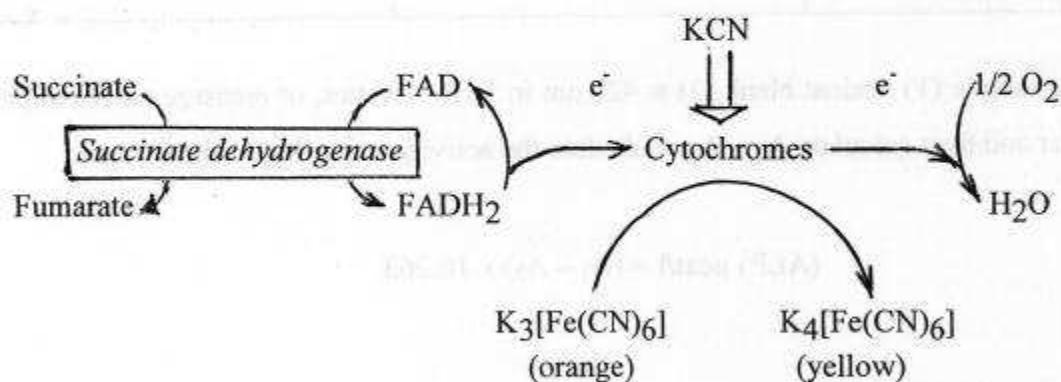
#### 1. The inhibition of succinate dehydrogenase with malonate

##### Introduction:

Succinate dehydrogenase is an enzyme of citrate cycle. It belongs to the riboflavin-linked dehydrogenases and present in the inner mitochondrial membrane. It converts succinate to fumarate under normal conditions. Malonate is known as competitive inhibitor of this reaction (notice the similarity of the inhibitor to the substrate).



In natural conditions this enzyme transfers electrons through the respiratory chain to oxygen. In order to measure this reaction in our experiment we shall block the function of cytochromes with KCN and we supply an artificial electron acceptor - potassium hexacyanoferrate(III),  $\text{K}_3[\text{Fe}(\text{CN})_6]$ , which is reduced to potassium hexacyanoferrate(II),  $\text{K}_4[\text{Fe}(\text{CN})_6]$ , and the colour change is registered photometrically.



*Solutions:*

1. Sodium succinate 0.2 mol/l
2. Potassium cyanide 0.1 mol/l POISON!
3. Potassium hexacyanoferrate(III) 0.01 mol/l
4. Sodium malonate 0.01 mol/l
5. Phosphate buffer pH 7.2 0.1 mol/l

*Procedure:*

**A) Preparation of enzyme**

1. Put a mortar with a pestle to pre-cool into the refrigerator. Prepare the ice bath: pieces of ice and small amount of water to the beaker. Leave all the solutions in the refrigerator and take only the necessary ones just when you need it.
2. Take 2 ml of phosphate buffer to the test-tube and add 8 ml of distilled water, mix and put into the ice bath. Put also another test-tube with distilled water to cool into the ice bath.
3. Take a small piece of beef heart, rinse it in order to remove all the blood and cut it with scissors to small pieces into the mortar. Wash the pieces with diluted buffer to remove again the blood and decant the buffer. Add about 5 ml of fresh diluted phosphate buffer and homogenize the tissue for a while.
4. Transfer the crude homogenate to the centrifugation tube and spin it down for 3 minutes at 1500 rpm. Meanwhile, clean the mortar and pestle.
5. When the centrifugation is over pour of the supernatant and resuspend the sediment in another 3 ml of diluted phosphate buffer. It should not contain any blood, its colour must not be even pink. Any colour of this type would interfere with the later photometric measurements. If necessary, repeat the washing and centrifugation. Add pieces of glass. Repeat the homogenisation in mortar now to the completion.
6. Repeat the centrifugation at 1500 rpm for 5 minutes but now collect the supernatant to the clean test-tube and place it to the ice bath. This is your enzyme solution.

**B) Enzymatic reaction**

1. Set up the spectrophotometer to the wavelength t 415 nm.
2. Take the other solutions from the fridge and prepare the "basic solution":

Phosphate buffer 0.1 mol/l 10 ml

KCN 0.1 mol/l 3 ml POISON!

$K_3[Fe(CN)_6]$  3 ml

Mix well and leave on the ice bath.

3. Prepare the reaction mixtures in small test-tubes according to the table. Keep them in the ice bath.

Tube	Blank	1	2	3
Basic solution (ml)	-	2.0	2.0	2.0
Sodium succinate (ml)	-	1.0	1.0	-
Sodium malonate (ml)	-	-	0.5	0.5
Cold distilled water	3.5	0.5	-	1.0

4. Take 0.5 ml of enzyme and add it to the blank. Pour it to the photometric cuvette and set up the blank on photometer.

5. Prepare the stop-watch. Take the mix 1, add 0.5 ml of enzyme, quickly mix, immediately pour into the cuvette, and measure the absorbance against the blank. Start the stop-watch in the moment of reading. Leave the cuvette in the spectrophotometer and repeat the reading every minute. Always check the blank between the measurements. Continue the measurement for 20 minutes.

Alternatively, you can start the reaction in two test-tubes (mix 1 and mix 2) and measure No.2 always 30 seconds after the No.1, each tube in 1 minute intervals.

6. Repeat the same procedure with mixes 2 and 3. Create the graph or use the Excel program.

## 2. Estimation of aldolase activity

### Introduction:

Aldolase is an glycolytic enzyme catalysing the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde phosphate and dihydroxyacetone phosphate. Aldolase is an intracellular enzyme present mainly in the cytosol of muscle and in some lesser extend in hepatic cells. In cases of damage of these tissues the enzyme may appear in blood serum. In this examination we must avoid any hemolytic sera because the aldolase activities are 10 times higher in RBC compared to those in plasma.

In order to estimate the activity we let the products of aldolase splitting to react with dinitrophenylhydrazine in alkaline media. In order to stop the possible oxidative degradation of the mentioned products with serum dehydrogenases we block their thiol groups with monoiodoacetate. Physiological values should not exceed 50 ncat/l.

*Solutions:*

1. Substrate solution (fructose-1,6-bisphosphate - 0.02 mol/l)
2. Monoiodoacetate (0.002 mol/l)
3. Hydrazine sulfate (7.3 g/100 ml)
4. Collidine buffer pH 7.4
5. Dinitrophenylhydrazine 0.1% in HCl 2 mol/l
6. Trichloroacetic acid 10%
7. Sodium hydroxide 3%

*Procedure:*

Pipette the following reagents into 4 centrifugation (I) test-tubes:

Tube	1	2	3	4
Collidine buffer (ml)	0.2	0.2	0.2	0.2
Serum (ml)	0.2	0.2	-	-
Hemolytic serum (ml)	-	-	0.2	0.2
Hydrazine sulfate (ml)	0.05	0.05	0.05	0.05
Monoiodoacetate (ml)	0.05	0.05	0.05	0.05
Trichloroacetic acid (ml)	-	0.4	-	0.4
Mix the content of all tubes and let to stay on the water bath (37°) for 5 minutes. Then add:				
Substrate (ml)	0.1	0.1	0.1	0.1
Mix again and incubate in the water bath (37°) for 30 minutes. ( <i>In tubes 2 and 4 due to the trichloroacetic acid the reaction did not proceed.</i> ) After the incubation add the trichloroacetic acid to the remaining tubes and mix:				
Trichloroacetic acid (ml)	0.4	-	0.4	-
Centrifuge all 4 tubes at 1,500 rpm for 10 minutes. Transfer 0.5 ml of clear supernatant to the new clean tubes. Add:				
Sodium hydroxide (ml)	0.5	0.5	0.5	0.5
Leave 10 minutes at room temperature, then add:				
Dinitrophenylhydrazine (ml)	0.5	0.5	0.5	0.5
Mix well and transfer again to the water bath (37°) for 10 minutes. Take off the bath and add:				
Sodium hydroxide (ml)	3.0	3.0	3.0	3.0
Let to stay for another 5 minutes and measure the samples against blanks, i.e. 1 against 2, and 3 against 4 at 540 nm in 1 cm cuvettes. Calculate the enzymatic activity:				
$A_{540} \times 0.98 = (\text{ALD}) \text{ ncat/ml}$				

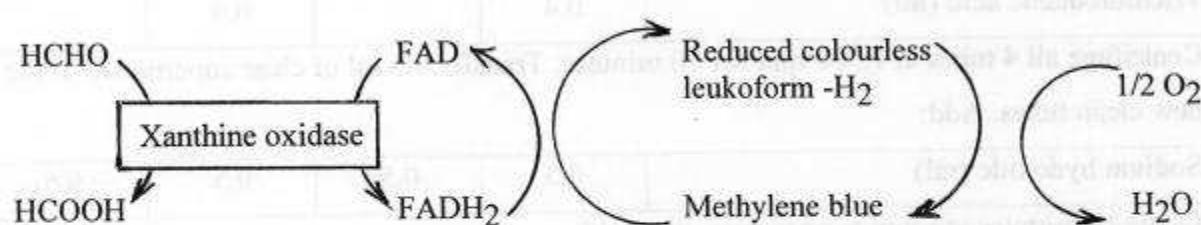
### 3. Demonstration of xanthine oxidase activity in the milk

#### Introduction:

Xanthine oxidoreductase (XOR) is a complex molybden-containing flavoprotein. The complete enzyme is a dimer where each subunit contains one molybden atom, one FAD, and two redox centres with iron bound to protein sulphur. XOR is able to react with a broad spectrum of substrates and catalyze both, their oxidation and reduction. Therefore, it is an exceptional enzyme lacking the strict substrate specificity. Traditionally its main function is considered to be the oxidation of hypoxanthine to xanthine and xanthine to uric acid with the simultaneous reduction of molecular oxygen to hydrogen peroxide or to superoxide ion. This formation of the mentioned *reactive forms of oxygen* is not fully understood. In medicine is studied mainly its possible negative role in tissue hypoxia and reperfusion, but the produced uric acid is antioxidant able to react with oxygen and other free radicals. It was also shown that XOR can reduce nitrates or nitrites to NO (nitric oxide) which is an important signalling molecule, but also another source of free radicals.

XOR exists in two convertible forms – *xanthine dehydrogenase* and *xanthine oxidase*. Both forms can exploit molecular oxygen but only *xanthine dehydrogenase* can use also NAD and seems to be the preferred isoform *in vivo*. XOR is present in milk of all mammals in large amounts bound to the membrane of fat droplets. The explanation seems to be the ability to form the reactive oxygen species with antibacterial activity in the digestive tract of newborns.

In our demonstration we shall use the formaldehyde as substrate for *xanthine oxidase* and methylene blue as the first electron acceptor. The final electron acceptor is the atmospheric oxygen.



#### Solutions:

1. Methylene blue 0.01% solution
2. Formaldehyde 2% solution
3. Fresh milk (non-pasteurised)

**Procedure:**

1. Prepare a water bath ( $40^{\circ}\text{C}$ ) to accommodate 4 test-tubes. Mark 4 test-tubes and pipette according to the table:

Tube	1	2	3	4
Fresh milk (ml)	5	5	5	5
Boil (over flame)	No	No	Yes	Yes
Methylene blue (drops)	5	5	5	5
Formaldehyde (drops)	5	-	5	-

2. Shake well the tubes and place them on the water bath. Follow the reaction and pay special attention to those where the colour disappears. Explain the observation.
3. Shake vigorously the tube where the colour of methylene blue disappeared in order to get enough air into the solution. Keep shaking until the blue colour is restored. Then continue the reaction on the water bath.
4. You can repeat this cycle several times until all the formaldehyde is exhausted. Then the methylene blue will not loose the colour any more. In such a case few drops of additional formaldehyde will restore this ability again.

## ENZYMOLOGY IV

1. Electrophoretic fractionation of haptoglobins (phenotyping)
2. Estimation of the haptoglobin gene type (genotyping)

### 1. Electrophoretic fractionation of haptoglobines - estimation of haptoglobin phenotype

**Introduction:**

Haptoglobin belongs to the  $\alpha_2$ -globulin fraction of plasma proteins. Its function is not totally clear but its most striking property is the ability to form a stable complex with hemoglobin. Thus, it prevents the decomposition of free hemoglobin in blood plasma and its losses by the urine. The haptoglobin-hemoglobin complex is recognised by a specific receptor on the liver parenchymal cells, is taken up and metabolised. In addition to that haptoglobin is considered to be a member of the acute-phase reactants and seems to modulate the immune



response. For example, haptoglobin is expressed at high levels in specific cells, including alveolar macrophages and eosinophils in diseased or inflamed human lung tissue, but not in the normal lung.

Haptoglobin itself is a tetrameric protein containing two  $\alpha$  and two  $\beta$  chains. Phenotypically, there were identified 2 main types of haptoglobin – haptoglobin 1 (Hp1) and haptoglobin 2 (Hp2) which differ by the length of their  $\alpha$  chains. In both types there were found several variants that differ by their amino acid composition. Thus, in Hp1 type exist as variants Hp1S (slow) and Hp1F (fast) named according to the electrophoretic mobility. Variant Hp1S contains in position 70 asparagine and in position 71 glutamic acid, while variant Hp1F contains in same position aspartic acid and lysine. In Hp2 more variants were described.

The amino acid sequences are shown in one-letter code (see Harper – chapter 4-Amino Acids – Table 4-3, p.27). Hp1 and Hp2 differ in size by 59 amino acids. This is the result of partial gene duplication in case of Hp2 and it is apparent as a sequence repeat (shown in Italics). The Hp1 subtypes – Hp1F and Hp1S differ in positions 70 and 71 as shown in bold. In case of Hp2 more subtypes exist due to the doubled region with four positions of possible variation.

#### Haptoglobin 1 F precursor:

**Amino acid sequence:** 347 amino acids

1 MSALGAVIAL LLWGQLFAVD SGNDVTDIAD DGCPKPPEIA HGYVEHSVRY  
51 QCKNYYKLRT EGDGVYTLND **KKQWINKAVG** DKLPECEAVC GPKKNPANPV  
101 QRILGGHLDA KGSFPWQAKM VSHHNLTTGA TLINEQWLLT TAKNLFLNHS  
151 ENATAKDIAP TLTLYVGKKQ LVEIEKVVLH PNYSQVDIGL IKLKQKVSVN  
201 ERVMPICLPS KDYAEVGRVG YVSGWGRNAN FKFTDHLKYV MLPVADQDQC  
251 IRHYEGSTVP EKKTPKSPVG VQPILNEHTF CAGMSKYQED TCYGDAGSAF  
301 AVHDLEEDTW YATGILSFDK SCAVAEYGVY VKVTSIQDWV QKTIAEN

#### Haptoglobin 1 S precursor:

**Amino acid sequence:** 347 amino acids

1 MSALGAVIAL LLWGQLFAVD SGNDVTDIAD DGCPKPPEIA HGYVEHSVRY  
51 QCKNYYKLRT EGDGVYTLNN **EKQWINKAVG** DKLPECEAVC GPKKNPANPV  
101 QRILGGHLDA KGSFPWQAKM VSHHNLTTGA TLINEQWLLT TAKNLFLNHS  
151 ENATAKDIAP TLTLYVGKKQ LVEIEKVVLH PNYSQVDIGL IKLKQKVSVN  
201 ERVMPICLPS KDYAEVGRVG YVSGWGRNAN FKFTDHLKYV MLPVADQDQC

251 IRHYEGSTVP EKKTPKSPVG VQPILNEHTF CAGMSKYQED TCYGDAGSAF  
301 AVHDLEEDTW YATGILSF DK SCAVAEYGVY VKVTSIQDWV QKTIAEN

**Haptoglobin 2 precursor:**

**Amino acid sequence: 406 amino acids**

1 MSALGAVIAL LLWGQLFAVD SGNDVTADIAD DGCPKPPEIA HGYVEHSVRY  
51 QCKNYYKLRT EGDGVYTLND **KK**QWINKAVG DKLPECEADD GCPKPPEIAH  
101 GYVEHSVRYQ CKNYYKLRT EGDGVYTLN**ME** KQWINKAVGD KLPECEAVCG  
151 KPKNPANPVQ RILGGHLDAK GSFPWQAKMV SHHNLTTGAT LINEQWLLTT  
201 AKNLFLNHSE NATAKDIAPT LTLYVGKKQL VEIEKVVLHP NYSQVDIGLI  
251 KLKQKVSVNE RVMPICLPSK DYAEVGRVGY VSGWGRNANF KFTDHLKYVM  
301 LPVADQDQCI RHYEGSTVPE KKTPKSPVGQ QPILNEHTFC AGMSKYQEDT  
351 CYGDAGSAFA VHDLEEDTWY ATGILSF DKS CAVAEGVYV KVTSIQDWVQ  
401 KTIAEN

All variants of Hp are synthesised as a single-chain precursor and later are cleaved. First 18 amino acids represent the signal peptide in all forms. In Hp1 the amino acids 19-101 are cleaved as  $\alpha$ -chain and amino acids 103-347 represent the  $\beta$ -chain.  $\alpha$  and  $\beta$  chains are connected with a disulfide bond formed by cysteines 90 and 207, while two  $\alpha$  chains are connected between cysteines in position 33. Additional disulfide bonds are present within the chain  $\beta$  in two locations (cysteines 250, 281 and 292,322). Four glycosylation sites are the asparagine residues (125, 148, 152, 182). In Hp2  $\alpha$ -chain is larger and is represented by amino acids 19-160, the  $\beta$ -chain being formed by amino acids 162-406. The disulfide bonds and glycosylation sides are analogical to Hp1.

All the described variations contribute to the changes in electrophoretic mobility of haptoglobins as proteins. Remember that electrophoretic behaviour depends on size of the separated molecule and on its charge. Therefore, Hp1 is generally faster than Hp2, but the subtypes show additional differences. Since every person is equipped with two Hp genes we can see in the phenotype one of the following three possibilities: Hp1-1, Hp2-1 or Hp2-2.

Electrophoresis, the migration of ions in an electric field, is widely used for the analytical separation of biological molecules. The use of electrophoresis to separate proteins was first reported in 1937 by the Swedish biochemist Arne Tiselius. In the technique he introduced, *moving boundary electrophoresis*, a protein solution is placed in a U-shaped tube and a protein-free buffer solution is carefully layered over both ends of the protein solution. An electric field between electrodes immersed in the buffer on either side of the protein

solution causes the charged protein molecules to migrate towards the electrodes of opposite polarity. Different proteins move at different rates as a result of different charges and fractional coefficients so that the leading and trailing edges of the migrating protein columns of each species form separate moving boundaries (fronts) in the buffer solution.

This technique has been later supplanted by *zone electrophoresis*, in which the sample is constrained to move in a solid support such as filter paper, cellulose, or gel. The small quantity of material that is used in zone electrophoresis permits the various sample components to migrate as discrete bands (zones).

In *paper electrophoresis*, the sample is applied to a point on a strip of filter paper or cellulose acetate moistened with buffer solution. The ends of the strip are immersed in separate reservoirs of buffer in which the electrodes are placed. An ion's migration rate is influenced, to some extent, by its interaction with this support matrix, but is largely a function of its charge. Upon completion of the electrophoretogram the strip is dried and the sample components are located using suitable detection techniques. An electric field of  $20 \text{ V.cm}^{-1}$  is adequate for many separations. The high diffusion rates of small molecules such as amino acids and small peptides, however, limits their resolution in complex mixtures. This difficulty can be reduced and the separation greatly speeded up by using electric fields of  $200 \text{ V.cm}^{-1}$ . In this *high voltage electrophoresis*, the paper is clamped between two cooled plates to carry off the heat generated by the high voltage current flow.

Paper electrophoresis and paper chromatography are superficially similar. However, paper electrophoresis separates ions largely on the basis of their charge, whereas paper chromatography separate molecules on the basis of their polarity. The two methods are often combined in a two-dimensional technique known as *fingerprinting* in which a sample is first treated as in two-dimensional paper chromatography but is subjected to electrophoresis in place of the second chromatographic step.

*Gel electrophoresis* is among the most powerful and yet conventionally used methods of macromolecular separation. The gels in common use, *polyacrylamide* and *agarose*, have pores of molecular dimensions whose sizes can be specified. *The molecular separations are therefore based on gel filtration as well as the charges of the molecules being separated.* Gel electrophoresis is used in both, protein separation in estimation of haptoglobin phenotype (starch gel), and DNA separation in estimation of haptoglobin genotype (agarose gel).

The selection of a proper buffer is of particular importance. Depending on the relationship of the buffer pH to the pI (isoelectric point of the molecule to be separated), the molecule will either move toward the cathode or the anode, or remain stationary ( $\text{pH} = \text{pI}$ ).

In our laboratory practical the buffer pH is above pI, and therefore both, proteins and DNA fragments will migrate towards anode (+) and will be separated in principle according to their size.

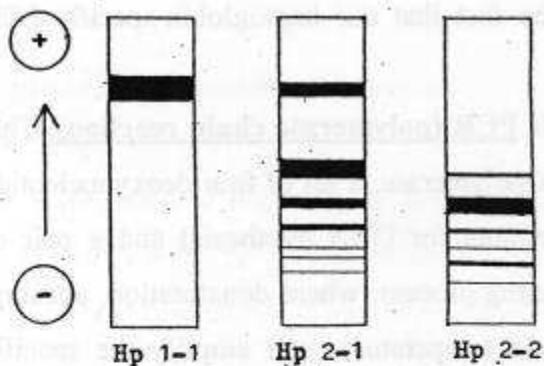
#### *Solutions:*

1. Hemoglobin solution 0.3%
2. Sera containing individual types of haptoglobin
3. Physiologic solution (0.9% NaCl)

5. Staining and destaining bath for proteins
6. Tolidine, hydrogen peroxide, acetic acid

**Procedure:**

1. You receive the preincubated serum samples containing different haptoglobin types. The samples were prepared as follows: 5 drops of serum was measured into each tube and 2 drops of hemoglobin solution. The mixture was incubated for 30 minutes at 37°C.
2. You receive also starch gel frame. Cut the gel with a lancet in the starting line. Take small square piece of filter paper into a forceps wet it in the first serum (haptoglobin) sample and place it into the starch gel. Repeat this procedure with all the samples. Apply all the samples in duplicate.
3. Cover both gels with a plastic foil and ask the personnel to start the electrophoresis. Leave it running at 220 V, 35-40 mA for 90 minutes. (*Meanwhile proceed to the genotype analysis.*)
4. When the electrophoresis is over, ask the technician to stop it and get your starch gels. Cut the gels in the frames with the help of a nylon thread into two slices each and start the staining. Prepare the tolidine reagent (always fresh just before use): 3 ml of water, 3 ml of acetic acid, 0.5 ml of hydrogen peroxide, tolidine on the tip of the knife. Apply the reagent on the inner surfaces of gel containing Hp-Hb with a cotton on a toothpick or cotton in the forceps. Wait for the colour to develop. Compare the unknown sample with the standard. Make a drawing into your protocol.

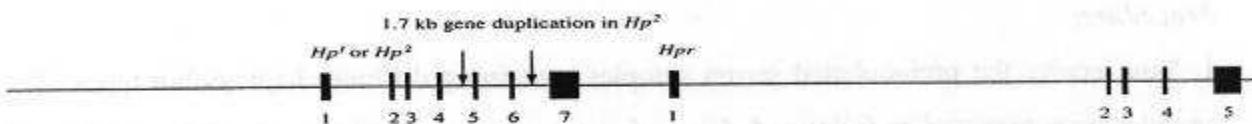


**2. Estimation of the haptoglobin gene type (genotyping)**

Please, study the necessary background in Harper's Biochemistry – chapter 42 - „Recombinant DNA technology“, especially clarify terms: *primer, template, polymerase chain reaction (PCR), intron, exon, restriction endonuclease, reverse transcription.*

### **Introduction:**

The *haptoglobin* gene is localised on chromosome 16 (locus 16q22.1) and contains 5



(in *Hp<sup>1</sup>*) respectively 7 exons (in *Hp<sup>2</sup>*). There is partial gene duplication (1.7 kb) including 2 exons in *Hp<sup>2</sup>* gene. Protein is synthesised as a single precursor and later it is cleaved to release a signal peptide and both,  $\alpha$  and  $\beta$  chains. Due to the gene duplication in *Hp<sup>2</sup>* we find the repeated stretch of 59 amino acids within the structure of *Hp<sup>2</sup>* protein while in *Hp<sup>1</sup>* this region is present only once. Thus *Hp<sup>2</sup>*  $\alpha$  chains are longer compared to their equivalents in *Hp<sup>1</sup>*.

Genes are rarely solitary or unique, they frequently have several variants of more or less identical structure (gene families). This is also the case of haptoglobin. Apart of the true haptoglobin gene, there is a similar gene for *haptoglobin related protein (HPRP)*. Because its structure is very similar, we must count with the fact that our haptoglobin-specific PCR amplification will also amplify this gene.

A standard technique for such estimation is **PCR (polymerase chain reaction)**. This amplification technique uses thermostable DNA polymerase, a set of four deoxynucleotide triphosphates (dATP, dGTP, dCTP, dTTP - precursors for DNA synthesis) and a pair of specific primers (oligodeoxynucleotides). The cycling process, where denaturation, annealing and synthesis stages are introduced by changes in temperature, will amplify the specific region of DNA given by the position of the two primers and the amplified product can be later visualised on electrophoretic gel.

You will use two sets of primers in PCR reaction:

1. Set A (A-forward and A-reverse primers) where the primers are interexonic, i.e. they bind to DNA sequences in different exons and therefore, the PCR product in amplification of genomic DNA will be considerably longer than that in amplification of cDNA (reverse transcribed mRNA where introns were removed).
2. Set B (B-forward and B-reverse primers) where the primers are intraexonic, i.e. they bind to DNA sequences in the same exon and therefore, the PCR product in amplification of genomic DNA will have the same length as that in amplification of cDNA.

At the end of this manual you find the nucleotide sequences of Hp1F, Hp1S, Hp2 and HpRP genes and the sequences of appropriate cDNAs with marked positions of primers A and B.

### Calculate the lengths of expected PCR products in all the genes!

A complementary technique, which can specify additional details in genotyping, is the cleavage of PCR products with restriction endonuclease, and analysis of obtained restriction fragments. Restriction endonucleases are specific enzymes of bacterial origin, able to cleave the DNA in specific points, given by the sequence of 4 – 6 base pairs. There are hundreds of such enzymes available, specific towards many different target sequences. In case of analysis of Hp gene, the restriction enzyme **Rsa I** (isolated from *Rhodopseudomonas sphaeroides*) was chosen with the specificity towards the sequence 5'-GTAC-3', cleaving it in the middle. This target sequence is present in Hp1S gene in the position that codes for valine (65) and tyrosine (66) (codons GTG TAC), but it is absent in Hp1F gene where the same amino acids are encoded by GTA TAC (valine is encoded by four codons – GTT, GTC, GTA and GTG). Therefore, the number and length of Rsa I restriction fragments can be used in determination of Hp1 subtype in both, genomic and cDNA.

In this laboratory you will be dealing with the following techniques:

- polymerase chain reaction (PCR)
- reverse transcription followed by polymerase chain reaction (RT-PCR)
- restriction enzyme digestion of amplified DNA fragment
- agarose gel electrophoresis to identify DNA fragments
- starch gel electrophoresis to identify haptoglobin types

#### **Procedure:**

In standard (time saving) procedure you receive the samples containing loading buffer (blue colour and higher density) ready to be applied to the agarose gel for electrophoretic separation. Load the following amounts of individual samples into the wells of agarose gel:

1. 5 µl gHpA – PCR product of genomic DNA amplified with primers A
2. 5 µl cHpA – PCR product of cDNA amplified with primers A
3. 5 µl gHpB – PCR product of genomic DNA amplified with primers B
4. 5 µl cHpB – PCR product of cDNA amplified with primers B
5. 8 µl lambda/Pst I (marker)
6. 10 µl gHp1A/Rsa I - PCR product of genomic DNA amplified with primers A cleaved with Rsa I

Ask personnel to start the electrophoresis (70 V for 45 minutes).

Full procedure to obtain all the product mentioned above:

1. Take the necessary solutions in Eppendorf tubes from the freezer and put them on ice.
2. You receive the instruction sheet with recommended amounts of DNA to be taken for the PCR reaction. (The individual DNA preparations are not identical and the amount necessary for PCR must be adjusted.) Pipette into 4 PCR micro-tubes:

<b>gHp-A</b>	6,5 µl PCR Master Mix Hp-A	..... µl d. water	..... µl genomic DNA
<b>gHp-B</b>	6,5 µl PCR Master Mix Hp-B	..... µl d. water	..... µl genomic DNA
<b>cHp-A</b>	16 µl RT-PCR Master Mix Hp-A	---	4 µl 1 <sup>st</sup> strand cDNA
<b>cHp-B</b>	16 µl RT-PCR Master Mix Hp-B	---	4 µl 1 <sup>st</sup> strand cDNA

(Master Mix contains buffer, two specific primers, Taq DNA polymerase and dNTPs).

3. Spin down shortly on the centrifuge and place the tubes into the cycler and start programme "Hapto-PCR". Let the cycler running till the end of the programme (about 100 minutes).

*Used Master Mixes:*

*PCR Master Mix Hp-A:*

Reagent	Final concentration	1 reaction (20 µl)	Master Mix (2 x 20 µl)
PCR buffer (10x)	1 x	2 µl	4 µl
dNTP (10 mM each)	0,2 mM each	0,4 µl	0,8 µl
Primer HP A-F (10 pmol/µl)	1 pmol/µl	2 µl	4 µl
Primer HP A-R (10 pmol/µl)	1 pmol/µl	2 µl	4 µl
Taq polymerase (5 U/µl)	0,5 U/20 µl	0,1 µl	0,2 µl
Total		6,5 µl	2 x 6,5 µl

*PCR Master Mix Hp-B:*

Reagent	Final concentration	1 reaction(20 µl)	Master Mix (2 x 20 µl)
PCR buffer (10x)	1 x	2 µl	4 µl
dNTP (10 mM each)	0,2 mM each	0,4 µl	0,8 µl
Primer HP B-F (10 pmol/µl)	1 pmol/µl	2 µl	4 µl
Primer HP B-R (10 pmol/µl)	1 pmol/µl	2 µl	4 µl
Taq polymerase (5 U/µl)	0,5 U/20 µl	0,1 µl	0,2 µl
Total		6,5 µl	2 x 6,5 µl

*RT-PCR Master Mixes:*

	<i>RT - PCR Master Mix HP-A (for 100 µl)</i>	<i>RT - PCR Master Mix HP-B (for 100 µl)</i>
water	65,5 µl	65,5 µl
reaction buffer (10x conc.)	8 µl	8 µl
MgCl <sub>2</sub> (25mM)	2 µl	2 µl
HP A-F (10 pmol/µl)	2 µl	---
HP A-R (10 pmol/µl)	2 µl	---
HP B-F (10 pmol/µl)	---	2 µl
HP B-R (10 pmol/µl)	---	2 µl
Taq polymerase (5 U/µl)	0,5 µl	0,5 µl

4. Set up the reaction with restriction endonuclease Rsa I: Take a tube containing 12 µl of RsaI Master Mix and pipette 8 µl of purified PCR product of gHpl-A. Mix by pipetting up and down and spin briefly. Place into the 37°C water bath for 60 minutes.

*Restriction Master Mix Rsa I contains (for 10 reactions 20 µl each):*

water	93 µl
RE buffer (10x conc.)	20 µl
BSA (10 mg/ml)	2 µl
Rsa I (10 U/µl)	5 µl

5. Calculate the expected lengths of all of your samples in both, PCR and restriction analysis. Prepare yourself for the evaluation.

6. 30 minutes before the cycling has finished, prepare the agarose gel for DNA electrophoresis. Take an Erlenmayer flask with 0.25 g of agarose, add 25 ml of 1xTAE (tris-acetate-EDTE) buffer and boil on a water bath until all the agarose dissolves. Let it to cool to 50 °C, add 100 µl of ethidium bromide (0.125 mg/ml) and pour it into the casting device with prepared comb. Let it to stay for at least 10 minutes. Then remove carefully the comb and rubber pieces and put the gel into the apparatus (the starting wells are on the minus site). Fill the apparatus with TAE buffer 1 mm above the gel.

**Warning! Ethidium bromide is a potential carcinogen. During the manipulation use the gloves and work carefully.**

7. When cycling is over, take the samples out, add 3 µl of loading buffer (glycerol and bromphenol blue) to each and briefly spin. Add 3 µl of loading buffer also to the sample incubated with the restriction enzyme. Apply carefully samples into wells in agarose gel – see the beginning of this procedure – page 117.

Back to the standard procedure:

8. Calculate the expected lengths of all of your samples in both, PCR and restriction analysis. Prepare yourself for the evaluation.

9. When the DNA electrophoresis is over, ask to stop it and transfer the gel onto the UV transluminator. Cover it with the safety shield and switch on. Evaluate the lengths of your fragments comparing them with marker and with calculated values.

**Use gloves and use the UV light only when covered with the special shield!**

10. Evaluate your results with respect to phenotype and genotype.

## APPENDIX – SEQUENCES OF GENES AND mRNAs

### Haptoglobin precursor 1S: mRNA (cDNA) and translation

15 atgagtgcctggagctgtcattgcctcctgtctggggacag  
M S A L G A V I A L L L W G Q  
60 cttttgcagtgg **ACTCAGGCAATGACGTCA**Gatatcgcat **primer HP A-F**  
L F A V D S G N D V T D I A D  
105 gacggctgcccgaagccccccgagattgcacatggctatgtggag  
D G C P K P P E I A H G Y V E  
150 cactcggtcgctaccagtgtaaagaactactacaaactgcgcaca  
H S V R Y Q C K N Y Y K L R T  
195 gaaggagatggagt **Gtac**acccttaaa**CA**at**G**agaaggcagtggata ← **gtac - Rsa I**  
E G D G V Y T L N **N E** K Q W I  
240 aataaggctgttggagataaaacttcctgaatgtgaaggcagtatgt  
N K A V G D K L P E C E A V C  
285 gggaaagcccaagaatccggcaaaccaggcagtgccggatcctgggt  
G K P K N P A N P V Q R I L G  
330 ggacacctggatgccaaaggcagcttccctggcag **GCTAAGATG** **primer HP B-F**  
G H L D A K G S F P W Q A K M  
375 **GTTC**CCCACCC**AATA**CTCAC**CCACAGG**gtccacgctgtatcaatgaa **primer HP A-R**  
V S H H N L T T G A T L I N E  
420 caatggctgctgaccacggctaaaaatctcttcctgaaccattca  
Q W L L T T A K N L F L N H S  
465 gaaaatgcaacagcgaaagacattggccctactttaacactctat  
E N A T A K D I A P T L T L Y  
510 gtggggaaaaagcagctttagagattgagaaggttttcacac  
V G K K Q L V E I E K V V L H  
555 cctaactactcccaggttagatattggcgtcatcaaactcaaacag  
P N Y S Q V D I G L I K L K Q  
600 aagggtgtctgttaatgagagagtgatgccatctgcctacattca  
K V S V N E R V M P I C L P S  
645 aaggattatgcagaagttagggcgtgtggttatgtttctggctgg  
K D Y A E V G R V G Y V S G W  
690 gggcgaaatgccaattttaaatttactgaccatctgaagtatgtc  
G R N A N F K F T D H L K Y V  
735 atgctgcctgtggctgaccaagaccaatgcataaggcattatgaa  
M L P V A D Q D Q C I R H Y E  
780 ggcagcacagccccgaaaagaagacaccgaagagccctgttaggg  
G S T V P E K K T P K S P V G  
825 gtgcagccatactgaatgaacacacccctctgtgctggcatgtct  
V Q P I L N E H T F C A G M S  
870 aagtaccaagaagacacccctgtatggcgatgcggcagtgcctt ← **gtac - Rsa I**  
K Y Q E D T C Y G D A G S A F  
915 gccgttacgacacctggaggaggacacctggatgcactggatc  
A V H D L E E D T W Y A T G I  
960 ttaagcttgataagagctgtgtggctgagtatgggtgttat  
L S F D K S C A V A E Y G V Y  
1005 gtg **AAGGTGACTTCCATCCAGG**actgggttcagaagaccatagct **primer HP B-R**  
V K V T S I Q D W V Q K T I A  
1050 gagaactaa 1058  
E N \*

### Haptoglobin precursor 1F: mRNA and translation

15 atgagtgccctggagctgtcattgccctcctgctgtggggacag  
M S A L G A V I A L L L W G Q  
60 cttttgcagtggACTCAGGCAATGATGTCACGgatatcgccagat **primer HP A-F**  
L F A V D S G N D V T D I A D  
105 gacggctgcccgaagccccccgagattgcacatggctatgtggag  
D G C P K P P E I A H G Y V E  
150 cactcggttcgttaccagtgttaagaactactacaaactgcgcaca  
H S V R Y Q C K N Y Y K L R T  
195 gaaggagatggagtAtacacacctaaaTGatAagaaggcagtggata  
E G D G V Y T L N D K K Q W I  
240 aataaggctgttggagataaaacttcctgaatgtgaaggcagtatgt  
N K A V G D K L P E C E A V C  
285 gggaaagccaagaatccggcaaaccaggatgcagcggatcctgggt  
G K P K N P A N P V Q R I L G  
330 ggacacccatgtgccaaaggcagcttccctggcagGCTAAGATG **primer HP B-F**  
G H L D A K G S F P W Q A K M  
375 GTTTCCCACCATAATCTCACCACAGGtgccacgctgtcaatgaa **primer HP A-R**  
V S H H N L T T G A T L I N E  
420 caatggctgctgaccacggctaaaaatcttcctgaaccattca  
Q W L L T T A K N L F L N H S  
465 gaaaatgcaacagcgaaagacattgcccactttaacactctat  
E N A T A K D I A P T L T L Y  
510 gtggggaaaaagcagcttgttagagattgagaagggttctacac  
V G K K Q L V E I E K V V L H  
555 cctaactactcccaggtagatattggctcatcaaactcaaacag  
P N Y S Q V D I G L I K L K Q  
600 aagggtgtctgttaatgagagagtgtatgcccattctgcctaccctca  
K V S V N E R V M P I C L P S  
645 aaggattatgcagaagttagggcgtgtggttatgtttctgggtgg  
K D Y A E V G R V G Y V S G W  
690 gggcggaaatgccaattttaaatttactgaccatctgaagtatgtc  
G R N A N F K F T D H L K Y V  
735 atgctgcgtgtggctgaccaagaccaatgcataaggcattatgaa  
M L P V A D Q D Q C I R H Y E  
780 ggcagcacagccccgaaaaagaagacaccgaagagccctgttaggg  
G S T V P E K K T P K S P V G  
825 gtgcagcccatactgaatgaacacacccctctgtgtggcatgtct  
V Q P I L N E H T F C A G M S  
870 aagtaccaagaagacacccgttatggcgatgcggcagtgcctt ← gtac - **Rsal**  
K Y Q E D T C Y G D A G S A F  
915 gccgttacgacccatggaggaggacacccgttatgcgactggatc  
A V H D L E E D T W Y A T G I  
960 ttaagcttgataagagctgtgtggctgatgtgttat  
L S F D K S C A V A E Y G V Y  
1005 gtgAAGGTGACTTCCATCCAGGActgggttcagaagaccataact **primer HP B-R**  
V K V T S I Q D W V Q K T I A  
1050 gagaactaa 1058  
E N \*

### Haptoglobin 2 precursor: mRNA (cDNA) and translation

3 atgccccacagcaactgctttccagaggcaagaccaccatgc  
M P H S T A L P E A R P T K M  
48 agtgcctggagctgtcattgccttcgtctggggacagctt  
S A L G A V I A L L W G Q L  
93 tttgcagtggactcaggcaatgtcacggatatcgccatgc  
F A V D S G N D V T D I A D D  
138 ggctgcccgaagccccccgagattgcacatggctatgtggagcac  
G C P K P P E I A H G Y V E H  
183 tcggttcgttaccagtgttaagaactactacaactgcgcacagaa  
S V R Y Q C K N Y Y K L R T E  
228 ggagatggagtatacaccttaatgataagaaggcgtggataaat  
G D G V Y T L N D K K Q W I N  
273 aaggctgtggagataaaacttcctgaatgtgaaggcagatgcggc  
K A V G D K L P E C E A D D G  
318 tgcccgaagccccccgagattgcacatggctatgtggagcactcg  
C P K P P E I A H G Y V E H S  
363 gttcgcttaccagtgttaagaactactacaactgcgcacagaaagg  
V R Y Q C K N Y Y K L R T E G  
408 gatggagtgtacaccttaaacaatgagaaggcgtggataataag  
D G V Y T L N N E K Q W I N K  
453 gctgttggagataaaacttcctgaatgtgaaggcagatgtggaaag  
A V G D K L P E C E A V C G K  
498 cccaagaatccggcaaaccaggcgtgcagcggatcctgggtggacac  
P K N P A N P V Q R I L G G H  
543 ctggatgccaaggcagcttcctggcaggctaagatggttcc  
L D A K G S F P W Q A K M V S  
588 caccataatctcaccacagggtgccacgctgtcatgaacaatgg  
H H N L T T G A T L I N E Q W  
633 ctgctgaccacggctaaaaatcttcctgaaccattcagaaaat  
L L T T A K N L F L N H S E N  
678 gcaacagcgaaagacattgcctacttaacactctatgtgggg  
A T A K D I A P T L T L Y V G  
723 aaaaagcagtttagagattgagaagggtttctacaccctaac  
K K Q L V E I E K V V L H P N  
768 tactcccaggttagatattggcgtcatcaaactcaaacagaagg  
Y S Q V D I G L I K L K Q K V  
813 tctgttaatgagagagtgtatgcctacccatctgccttcaaaggat  
S V N E R V M P I C L P S K D  
858 tatgcagaaggtagggcgtgtgggttatgtttctggctggggcga  
Y A E V G R V G Y V S G W G R  
903 aatgccaattttaaatttactgaccatctgtcatgtatgtcatgt  
N A N F K F T D H L K Y V M L  
948 cctgtggctgaccaagaccaatgcataaggcattatgaaggcag  
P V A D Q D Q C I R H Y E G S  
993 acagtccccgaaaagaagacaccgaagagccctgttaggggtgcag  
T V P E K K T P K S P V G V Q  
1038 cccataactgtaatgaaacacacccctgtgtcatgtctaaatgc  
P I L N E H T F C A G M S K Y  
1083 caagaagacacccgtatggcgatgcggcactgccttgcgtt  
Q E D T C Y G D A G S A F A V  
1128 cacgacctggaggaggacacccgtatgcgtactggatcttaagc

```

H D L E E D T W Y A T G I L S
1173 tttgataagagctgtgctggctgagtatggtgttatgtgaag
F D K S C A V A E Y G V Y V K
1218 gtgacttccatccaggactgggttcagaagaccatagctgagaac
V T S I Q D W V Q K T I A E N
1263 taa 1265

```

**Haptoglobin 1S gene partial sequence (exons are in bold):**

121 CGACTTTCTT TTCTGGCTGC TAAGTGGGAG GAGTGTGTGT GTATGCATGT GTGTGTGTGT  
 181 GTGT **GTACAT** GCATGTGTGT GTGGATGCAT GCATGTGCTG TGAAGCAGGG AGACTAGCTT - Rsa I  
 241 TCCACTCCTC CTTGTCCTCT **CTCTGCAGTG CCCTGGGAGC TGTCATTGCC CTCCGCTCT**  
 301 **GGGGACAGCT TTTGCAGTG GACTCAGGCA ATGATGTCAC GGATATCGCA GGTCAGTCTT** HP A-F  
 361 TGGTTGGGTA GGAGTGTGCA TCCCACACTG ACCCTCTCGG GTCTGCACTC TCTCTGAGAA  
 421 CACCCAATTTC CCCCTTCTTA TCTCGACCTC TGGGCTTCA GGACCATAAA GAACATTGGG  
 481 GTTCTGCCA GAAATGAGGG GAGCTTGCT TTCCATTGGC TTCTATTCCG GGTGGAAGGA  
 541 GATTGATGTG CAGAGCAGCT CCCGCTCATC TGACTTTCA CGGTTCACTG GGAACAATT  
 601 CCAAATAGCA AACTCTCTGG CTTCTCTCTC **TTTGCAGATG ACGGCTGCC GAAGCCCCC**  
 661 **GAGATTGCAC ATGGCTATGT GGAGCACTCG GTTCGCTACC AGTGTAAAGAA CTACTACAAA**  
 721 **CTGCGCACAG AAGGAGATGG** TAAGATGTGG ACAACTGTCT CCATGCCCTA CATACAAACCC  
 781 CCTTCTCTGA CATTCCATG ATGGGTGGTG CTGAGGTGAT TCGCCAGAAA GTTCGTTGCT  
 841 CTCCTGGAG CCAGGAGATT TAGATTCTAA TAAGCGTTT GTGCCAGTA GCCATGGCCC  
 901 TTTGGGCAGA CTAACCTTTG TCAGCCTCAA GTTTCTGTT TTGTTAAGGG GAGGCGATGC  
 961 CATGCAGCCT ACCTCATGTA AATCTCAGAG TCACATTAC ATCTCCAGCA GATGTGGGAA  
 1021 AAGAAGGAAT GCTGATGTG ATGTCACCT CACCTAGTGA GTCTGCTGT CCTGGCACTG  
 1081 CTCTAAGGGC TTTATACTTA TTTGCTCACT TAGCCTCAC AGTATCCCTC TGAACAGAGT  
 1141 TTATTGTTT CACTTGCTG ATAAGGAAAC TGAGGCACAG ACAGGTTGAG TATCTTGC  
 1201 AAATTCAAGGC AGCCTGTAAG AGGCAGAGTC AGGATTGAA CCCTGAGCCC TCCCT **GTACT**  
 1261 GCTTGGCTGT GACGCCATG ACCACAGTGT GTTCTGCTGG GCTTAACTGG CATCCAGGCA  
 1321 CTTGGCTTCC AGCACAGCAC TCTTCCCTT CCTCCTTCTC ATATACTCTC TCCCTTCCC  
 1381 CTTCTTTT GTCCCCTTT CCTCTTCTT TTAGTTCTC TCTTTAAATG CCTCTCACT  
 1441 CTGCACGGGG TCTAGACTTG ACTTCTCCTT TGGCTCACTT CTTGCCCTT GTTCAGGAG  
 1501 **TGTACACCTT AAACAATGAG AAGCAGTGGAA TAAATAAGC TGTTGGAGAT AAACTCCCTG** - Rsa I  
 1561 **AATGTGAAGC AGGTGGGTGC TGAGCACTTA AGAGAGCAGG CAGGCGTCCA GCGGGGAACG**  
 1621 TCCTAGAGGC ACAGCCTTCC AGTGCCTGTT CCTCTGAGCA CACAAGAGCC AGGAGGAGGG  
 1681 ATGTGGGAGA ACCGCAGCTG GCCAGGGAGA GACTTAAGCA GTTAGGTGAT GACTCCCTAA  
 1741 GGGTCACCAA GGGTCTGTT CATTAGGGCC TGAAGGGCAC TGGCTGAATC CATTGTC  
 1801 ATCGCCCACA GATTAGGAGA GCCTGTGCAT ACAGAGAGCC TGCTAGAGAG CCCTGGGTCT  
 1861 AAGGAGAACG AAGCTCCAGG GAGAACAAAGT CAAGGAATGA CATAAAATCT TAATCCATGG  
 1921 AAGCCTAGCA GGAGGCTGGA CATGGGTGG AACTCCTGCT TCTCGTTATT AGGAGGAGCT  
 1981 GTTGTCTCT CCTTCATTC TCAGAACAAAG AGGCAAAGGC CCAGCCTCTT CTGCTCTTAC  
 2041 TGGTGTGGAA ATGCCAACCT GCCTCGTATT AACTGCACCA TCTACAAAAT CTGAGCTCCA  
 2101 GCCAGTGCTG CTCTAGATTC ATCTTCTT AGAGAGAATG AATTATTGTA GCCCCTAGCC  
 2161 CTTCAATGA ATTTCAGGGA ATTGTGGAA TTCCTTATT GGGATAATTG TTTAAATATA  
 2221 ATACAGTTCA CCAGCCAGGG CTCAAAATC TCAGTATTTC CCACCTCCTT TGTTAGAAAA  
 2281 GTGGGAAATA GAGCTTTTG TAATGTAAAC AATTAAAAAA ACAGAATTAT TTTAAAATCTG  
 2341 CAACTATTGG AAATGAGATC AGCAGGTGGT AAGGGCAAAG CATTAAATC TTTCTACTTT  
 2401 ACGCAGCAGT GACAGCCGCC CATGCTTCA CCCCTTCTC AGATGAAAG GCTCTTGAC  
 2461 ATTTCCACTC ACGAGTGTCT TGCTCTCCTT GACAGTATGT **GGGAAGCCCA AGAATCCGGC**  
 2521 **AAACCCAGTG CAGCGGATCC TGGGTGGACA CCTGGATGCC AAAGGCAGCT TTCCCTGGCA**  
 2581 **GGCTAAGATG GTTCTCCACC ATAATCTCAC CACAGGTGCC ACGCTGATCA ATGAAACAATG** HP B-F

2641 GCTGCTGACC ACGGCTAAAA ATCTCTTCC CAACCATTC GAAAATGCAA CAGCGAAAGA HP A-R  
 2701 CATTGCCCT ACTTTAACAC TCTATGTGGG GAAAAGCAG CTTGTAGAGA TTGAGAAGGT  
 2761 TGTTCTACAC CCTAACTACT CCCAGGTAGA TATTGGGCTC ATCAAACTCA AACAGAAGGT  
 2821 GTCTGTTAAT GAGAGAGTGA TGCCCATCTG CCTACCTTC AAGGATTATG CAGAAGTAGG  
 2881 GCGTGTGGGT TATGTTCTG GCTGGGGCG AAATGCCAAT TTTAAATTAA CTGACCATCT  
 2941 GAAGTATGTC ATGCTGCCTG TGGCTGACCA AGACCAATGC ATAAGGCATT ATGAAGGCAG  
 3001 CACAGTCCCC GAAAAGAAGA CACCGAAGAG CCCTGTAGGG GTGCAGCCC TACTGAATGA  
 3061 ACACACCTTC TGTGCTGGCA TGTCTAAGTA CCAAGAAGAC ACCTGCTATG GCGATGCAGG - Rsa I  
 3121 CAGTGCCTT GCCGTTCACG ACCTGGAGGA GGACACCTGG TATGCGACTG GGATCTTAAG  
 3181 CTTGATAAG AGCTGTGCTG TGGCTGAGTA TGGTGTGTAT **GTGAAGGTGA** CTTCCATCCA HP B-R  
 3241 GGACTGGGTT CAGAAGACCA TAGCTGAGAA CTAATGCAAG GCTGGCCGG AGCCCTTGCC  
 3301 TGAAAGCAAG ATTCAGCCT GGAAGAGGGC AAAGTGGACG GGAGTGGACA GGAGTGGATG  
 3361 CGATAAGATG TGGTTGAAG CTGATGGGTG CCAGCCTGC ATTGCTGAGT CAATCAATAA  
 3421 AGAGCTTTCTT TTGACCCAT TTCTGTGTTG TGTCAGTCT TGAGTCTTT TTATTTGCTC  
 3481 CTTTATGGTC CAGG

### Haptoglobin 2 gene full sequence (exons are in bold and in capital letters):

1 **AGATGCCCA** CAGCACTGCT CTTCCAGAGG CAAGACCAAC **CAAGATGAGg** tgggtccaca EX 1  
 61 gcttccctc ctgcctttcc tctggttctt tatttcagtc ttttttgcat acatcgtag  
 121 agatgcagaa atagaacaaa gaaacggca aatggctaa attatagtga accaaaggc  
 181 ttagtgtttt aaatcttctc ctttctgca tccatagaag acagtgtgc tgtctttccc  
 241 aggagataag atttactctc aggagtgtct ttttcctca gtttacattt ttgactttat  
 301 aggttatgtc atcagctccc gtggtaggtc tccctggcattt ctgagttat ttatttagcag  
 361 atattttctt ctttaaaaat **gtacaataag** gaagactaat agtaacacat ttgaatgaca -Rsa I  
 421 caattaatttq actagtac gggatacaca ctaataccctg gatacatct aattaaggca -Rsa I  
 481 ctttagatctt ataaaaataa acactttttt aatgttcaa ataataagac tagaaacttt  
 541 tttttttttt gagatggagt ctcgtttgtt caccaggctg cagtgcagtgc gcatgatctc  
 601 ggctcaactgc aacctccacc tcccagggttc aagtgttctt cctgcctcag cctcccaaggt  
 661 agctggact acaggcgtgc gccactatgc ccacctaattttt tttgttatttt tagtagagac  
 721 gggtttcaac catgttggcc aggtgtaccc cgtctcttgc acctcgtgtt ctgcctgcct  
 781 tggctccca aagtgttggg attacaggca tgagccaccg tgtctggcct agaaactatt  
 841 ttaatagaag caagttagtgc cccaaatgtt ggcattttt aatgttggatgg tgaactggca  
 901 gacggcacct gtgggtcaat gccatggcc accgtctgc ttttggacac cagttctt  
 961 ccaggttaacc ttctggcatt ttgggtttt agataccatt tcctaaaggtaa gaattattat  
 1021 aaaatacttag aaataccac gtgtttaaaa ttatataattt aaggaacattt ttattacgg  
 1081 aaatatcaag aagttaggaa atagcacat aagccaaag ttcccatctc tgagttatct  
 1141 acattttattt accactatct ttgcgtgtc tgaggaggt ttcttttcc tggagggtctc  
 1201 ctgtattttt **gccaatgtac** ttccctgaat gcagccagaa actgagccca cccctccacc -Rsa I  
 1261 tatgtgcctt tctatccctc tctgaagttt ctgcagaattt cccagcagga cagggtttgc  
 1321 tggaaagcttgc gtatgtctcg aagctgtcaa agtgtgtatg ggcagggtgtt gggcaattt  
 1381 ctgggtccat gcaactccat atatcgactt tctttctgg ctgcttaatgtt ggaggaggt  
 1441 gtgtgtatgc atgtgtgtgtt gtgtgtgtgtt acatgcattt gtgtgtgtt gcatgcattt  
 1501 gctgtgaagc agggagacta gctttccact ctccttgc ttctctctgc ag**TGCCCTGG**  
 1561 **GAGCTGTCA** TGCCCTCCTG CTCTGGGGAC **AGCTTTTGC** **AGTGGACTCA** GGCAATGATG EX 2  
 1621 **TCACGGATAT** CGCAGgtcag tctttggttt ggttaggatgt tgcatccac tctgaccctc A-F  
 1681 tcgggtctgc actctctctg agaacaccca attccccctt ttatctcgat cctctggct  
 1741 ttcaaggacca taaagaacat tgggttccctt gccagaaatg aggggaggtt gccttccat  
 1801 tggcttctat tcgggttggaa aggagatgtt tgcgtgtgtt gcatgcattt  
 1861 ttacacgggtt actggaaaca atttccaaat agcaaaactt ctggcttctc ttcttttgc  
 1921 g**ATGACGGCT** GCCCGAAGCC CCCCGAGATT GCACATGGCT ATGTGGAGCA CTCCGGTTCGC EX 3  
 1981 **TACCAAGTGT** AAGAACTACTA CAAACTGCGC ACAGAAAGGAG ATGgtaaat gtggacaact  
 2041 gtctccatgc cttacatatac acccccttctt ctgacatttc catgtgttgggtt ggtgtgtt  
 2101 tgattcgcca gaaagttcgt tgctctcattt ggagccagga gatttagatt ctaataagcg

2161 ttttgcgcc agtagccatg gccctttggg cagactaact tttgtcagec tcaagtttc  
 2221 tgtttgtta agggggaggcg atgcccattca gcctacccata tgtaaatctc agagtcacat  
 2281 ttacatctcc agcagatgtg gaaaaagaag gaatgtgtat gatgtatgtca ccctcaccta  
 2341 gtgagtcttg ctgtccctggc actgctctaa gggcttata cttatgttgc cacttagtcc  
 2401 tcacagtatc cctctgaaca gagtttattt ttttcaactt gctgataagg aaactgaggc  
 2461 acagacaggt tgagtatctt gcccaaattt aggccggctg taagaggcag agtcaggatt  
 2521 tgaaccctga gccctccctg tactgcttgg ctgtgaccgc catgaccaca gtgtgttctg  
 2581 ctgggcttaa ctgggttcca ggcacttggc ttcacgaca gcactcttc ccttcctc  
 2641 tctcatattc tctctcctt ctccttcctt gtctgcctcc tttcttcctt ttctttttaa  
 2701 ttcttctctt taaatgcctt ctcactctgc tctgggttgc gacttgactt ttcttttggc  
 2761 tcatttcttgc cttttgttt cag**GAGTATA CACCTAAAT GATAAGAACG AGTGGATAAA** EX 4  
 2821 **TAAGGCTGTT GGAGATAAAAC TTCCCTGAATG TGAAGCAGgt** ggggtgttag cactgagcac  
 2881 ttaagagagc aggccggcgt ccagccccggg acgtccctaga ggcacagccct tccagtgcgg  
 2941 cttccctctga gcacacaaga gccaggaggaa gggatgtggg agaaccgcag ctggccagg  
 3001 agagacttaa gcagtttagt gatgactccc taagggtcac caagggttctt gttcatttggg  
 3061 gcctgaaggg cactggctga atccactgtc ggcactgccc acagatcagg agagccgtgt  
 3121 catacagaga gcctgtctaga aagccctggg tctaaggaga agcaagctcc agggagaaca  
 3181 agtcaaggaa tgacataaaaa tcttaatcca tggaaagccta gcaggaggct ggacatggc  
 3241 tggaaactctt gcttctcggtt attaggagga gctgtgtctt ttcctttca ttctcagaac  
 3301 cagaggcaaa gacccaggctt cttctgtctt tactgggtgtg gaaatgcctt cctgcctcgt  
 3361 attaactgca ccatctacaa aatctgagctt ccagccaggctg ctgtctctaga ttcatcttc  
 3421 tttagagaga atgaatttattt gtagccctta gccccttcaa tgaattttag ggaatttggg  
 3481 aaattccctt attgggataa ttgtttaaaaa ataatacagt tcgcgagctt ctattcgggg  
 3541 tggaaaggaga ttgtatgtca gaggcagctcc cgctcatctg acttttcacg gttcacttggg  
 3601 aacaatttcc aaatagcaaa ctctctggct tctctctt **TGCAGATGAC GGCTGCCCGA**  
 3661 **AGCCCCCGA GATTGCACAT GGCTATGTGG ACCACTCGGT TCGCTACCCAG TGTAAGAACT** EX 5  
 3721 **ACTACAAACT GCGCACAGAA GGAGATGgtt** agatgtggac aactgtctcc atgccttaca  
 3781 tacaaccccc ttctctgaca tttccatgtt ggggtgtgtt gagggtattt gccagaaaagt  
 3841 tcgttgcctt ctttggagcc aggagatttta gattctaata agcgttttgc cggccaggtagc  
 3901 catggccctt tggccagact aacttttgc tggccatgtt tttctgtttt gtttgggg  
 3961 ggcgtatgcca tgcagccatcc ctcatgtaaa ttcagatgtc acatttacat ctccagcaga  
 4021 tggggaaaaa gaaggaatgc tggatgtat gtcaccctca cctagtgtt gtttgcgttcc  
 4081 tggcactgtt ctaagggttt tataacttattt tggccatgtt gtcctcacag tatccctctg  
 4141 aacagagttt attgtttca ctttgcgtat aaggaaaactg aggccacagac aggtttagt  
 4201 tcttgcccaa attcagggcag cctgttaagag gcaaggatcag gatttgaacc ctgagccctc  
 4261 cctgtactgc ttggctgtt gggccatgtt cacagtgtt ttcgttggc ttaactggca -Rsal  
 4321 tccaggcact tggcttccat cacagcactt ttcccttcc tcccttcat atactcttc  
 4381 cttttccctt tcccttttgc ccccttttcc tccctttttt agtttcttc tttaaatgcc  
 4441 ttctcaacttgc acacggggc tagacttgac ttcccttttgc tccacttttgc tcccttttgc  
 4501 ttcaag**GAGTG TACACCTTAA ACAATGAGAAA GCAGTGGATA AATAAGGCTG TTGGAGATAA** -Rsal  
 4561 **ACTTCCTGAA TGTGAAGCAG** gtgggtgtt agcacttaag agaggaggca ggcgtccagg EX 6  
 4621 ggggaacgtc ctagaggccac agccttccag tggccgttcc tctgagcaca caagagccag  
 4681 gagggggat gttggagaac cgcagctggc cagggagaga cttaaaggctt taggtgtat  
 4741 ctccttaagg gtcaccaagg gtcgttgc ttagggctt aaggccactt gtcgttgc  
 4801 ttgtctacat cggccacaga ttagggatgc ctgtgcatac agagccctt ctagagagcc  
 4861 ctgggtctaa ggagaagcaa gtcggggaa gaacaagtca aggaatgaca taaaatctt  
 4921 atccatggaa gccttagcagg aggtggaca tgggtggaa ctccctgttcc tccgttattt  
 4981 gagggactgt tgcgttctcc ttccatttctt agaacaagag gcaaggccc agcccttttct  
 5041 gctcttactg gtgtggaaat gccaacctgc ctgttatttca ctgcaccatc tacaaaatct  
 5101 gagctccagc cagtgtgtt ctagattcat ctttctttag agagaatgaa ttattgtat  
 5161 cccttagccct ttcaatgtt ttcaggaaat tggggaaatt ctttatttgg gataattgtt  
 5221 taaatataat acagttcacc agccagggtt caaaaatctc agtatttccc acttccttgc  
 5281 ttagaaaaat gggaaataga gtttttgc tttttttttt tttttttttt agaatttattt  
 5341 taaaactgca actattggaa atgagatcag caggtggtaa gggccaaagca tttttttttt  
 5401 tctacttttgc gcaaggatgtt cagccggccca tgcttgcacc ctttcttc tttttttttt  
 5461 tcttgccat tccacttgc gatgtgtttt ctctcttgc cag**TATGTGG GAAGCCCAAG**  
 5521 **AATCCGGCAA ACCCAGTGCA GCGGATCCTG GGTGGACACC TGGATGCCAA AGGCAGCTTT** EX 7  
 5581 **CCCTGGCAGG CTAAGATGGT TTCCACCAT AATCTCACCA CAGGTGCCAC GCTGATCAAT** B-F A-R  
 5641 **GAACAATGGC TGCTGACCCAC GGCTAAAAAT CTCTTCCCTGA ACCATTCAAGA AAATGCAACA**  
 5701 **GCAGAAAGACA TTGCCCCCTAC TTTAACACTC TATGTGGGGAA AAAAGCAGCT TGTAGAGATT**  
 5761 **GAGAAGGGTTG TTCTACACCC TAACTACTCC CAGGTAGATA TTGGGCTCAT CAAACTCAAA**  
 5821 **CAGAAGGGTGT CTGTTAATGAGAGTGATG CCCATCTGCC TACCTTCAAA GGATTATGCA**  
 5881 **GAAGTAGGGC GTGTGGGTTA TGTTCTGGC TGGGGCGAA ATGCCAATT TAAATTTACT**

5941 GACCATCTGAGTATGTCATGCTGCCTGTGGCTGACCAAGACCAATGCAT AAGGCATTAT  
 6001 GAAGGCAGCACAGTCCCCGA AAAGAAGACA CCGAAAGAGCC CTGTAGGGGT GCAGCCCATA  
 6061 CTGAATGAACACACCTTCTGTGCTGGCATGTCTAAGTACCAAGAACACACCTGATGGC -RsaI  
 6121 GATGCGGGCA GTGCCCTTGC CGTTCACGAC CTGGAGGGAGG ACACCTGGTA TGGCACTGGG  
 6181 ATCTTAAGCT TTGATAAGAG CTGTGCTGTG GCTGAGTATG GTGTGTATGT GAAGGTGACT B-R  
 6241 TCCATCCAGG ACTGGTTCA GAAGACCATA GCTGAGAACT AATGCAAGGC TGGCCGGAAAG  
 6301 CCCTTGCCCTG AAAGCAAGAT TTCAGCCTGG AAGAGGGCAA AGTGGACCGG AGTGGACAGG  
 6361 ACTGGATGCG ATAAGATGTG GTTTGAAGCT GATGGGTGCC AGCCCTGCAT TGCTGAGTCA  
 6421 ATCAATAAAAG AGCTTTCTTTTGACCCATTTC

#### **Haptoglobin related protein (HRP) gene sequence:**

1 ACTGCTCTTC CAGAGGCAAG ACCAACCAAG ATGAGgtggg tccacagctt tccctcctgc  
61 ctteectctg gttctttatt tcagtcttt ttgcatacat tggtacatat gcagaatatag **RsaI**  
121 aacaaagaaa cagggcaaat gggctaaatt atagtgaacc aaaggctta gtgtttaaa  
181 tcttetcctt ttctgcatcc ataqaaqaca gtqctgtqt tttccccagg agataagatt

### *intron 1*

10441 aagcagctaa agcgtgtatg tggggccggag ggtggggca acttcttggt cctagcacct  
 10501 ccatatatgg atttctttt ctggctgtt agtgggagga gtgtgtgt atgcgtgt  
 10561 gtgtgtgcgt gtgtgtgtgt gtgtacatgc ctgtgtgtgt ggatgcacatc atgtgtgtgt **Rsal**  
 10621 aagcaggag accagtttc cgctccttct tggggctct ctgcag**TGAC CTGGGAGCTG A-F**  
 10681 **TCATTTCCCT CCTGCTCTGG GGACGACAGC TTTTGCACT GTACTCAGGC AATGATGTCA RsaI**  
 10741 **CGGATATTC AGgtcagtct ttgagttggg taggagcatg catccctggc actgccacat**  
 10801 cccactctga ctctctcggt tctgcattct ttctttgaga acacacagtt cccatttt  
 10861 atcctgacct ctgggcttcc aggactgcca agaacattgg ggatcctgcc agaaatggg  
 10921 ggagctttag cttcgttgg ctcttatttgg ggtttagaagg agattgtatgt gcagagcagc  
 10981 ttccactcat ctgactttc atgggttctt ggaacaatt tccaaatggt aaactctctg  
 11041 gtttctcttctt cttgcag~~AT~~ GACCGCTTCC CGAACCCCCC TGAGATTGCA AATGGCTATG  
 11101 **TGGAGCACTT GTTCGCTAC CAGTGTAAAGA ACTACTACAG ACTGCCACA GAAGGAGATG**  
 11161 gtaagacctg gacaactatac tctgtgtctt acttacaacc cctgtctga cattttcatg  
 11221 atgggtgttctt ctgagggttatg ttgcaggaaa gtcgttgct ctcttggag ccaggagatt  
 11281 tagattctga taagcggttt gtcgcagta gcatggccc ttggcaga ctaactttt  
 11341 tcagcctcag gtttctgtt ttgttaaggg gaggtgtatgc catgcagcctt acctcatgt  
 11401 aatctcagag tcagatttac atetccagca gatgtggaa aagaaggaat gctgtatgt  
 11461 atgtcacccct cacctagtga gtcttgcgtt cctggcaactg ctctaaggc ttatattt  
 11521 ttgtctact tagtccttac agttccac tgaatagagg ttattattct cacttgcgt  
 11581 ataaggaaac agaggcaccg acagggttag tatcttgcoc aaattcagg ggcctgt  
 11641 aggcagagtc aggattttaga ccctgagccc tccctgtact gcctggctgt gaccgcacat **RsaI**  
 11701 accacagtgt gtctgtctgg gcttaactgg tgccaggca ctgggttcc agcacagcac  
 11761 tctttccctt ctccttcctc gtattcttc tctttctcc ctcttgcgtt gcctctttc  
 11821 ttcttcttctt tttaatttct tctctttaaa tgcccttcctca ctctgcgtt ggtcagact  
 11881 tgacttttcc ttgggtctat ttcttgcctt ttgtttcagg **AGTATAACACC TTAAATGATA**  
 11941 **AGAACGAGTG GATAATAAG GCTGTTGGAG ATAAACTTCC TGAAATGTGAA GCAGgtgggt**  
 12001 gctgagcaactt gggacttgg gagggcggc aggccgtccag cggggaaacgt cctagaggca  
 12061 cagccttcca gtgcggctt ctctgagcac acaagagcca ggaggaggga tgtgggagaa  
 12121 ccgcagctgg ccaggggagag acttaagcag ttaggtgtatg actccctaag gatcaccagg  
 12181 gtcttgcgttca ttggggcctt aagggcaactg gctgaatcca ctgtccgtat tgccacacaga  
 12241 tcaggagagc ctgtgcatac agagagcctg cttagagagcc ctgggtctaa ggagaagcaa  
 12301 gctccaggga gaacaagtca agggataaca taaaatctt atccatggaa gcctagcagg  
 12361 aggctggaca tgggtggaa ctcttgcctt tcgttattttag gaggaactgt tgctcttcc  
 12421 ttccctgttc agaaccagag gcaaaaggccc agcctttctt gctgtgatgt gtgtggact  
 12481 accaacctgc ctgttattaa ctgcaacatc taaaatgtat gagctccagc caatgtgtct  
 12541 cttagatttctt ctgttgcgtt agatgtatgaa ttattgtatc tccttgcctt ttctttttc  
 12601 ttctttttt tttttttttt tttgagacag agttttgtct tcgttgcctt ggctggagtg  
 12661 cagttgtgtt aatttgcgtt ctcagggtat ccaactgcct cagcccttcca aagtgtctgg  
 12721 attacaggcg ttagccaccg catctggccc ctggccctt caatgtatc cagggaaattt  
 12781 tgaaaaatttcc ttgttgcgtt taattgtatc aatataatat agtttgcctt ccaggccctca  
 12841 aaaaactcaq tatttgcgtt ttccttattt agaaatagag cttttgtaa tgtaaaacaat

12901 taaaaaaatt attttaaac tgcaactatt ggaaatgaga tcagcaggtg gtaaggacaa  
 12961 agcatttaaa tctttccagt ttatgcagca gtgacagccg ccaatgcctt caccctttc  
 13021 tcagatggaa aggcttgc acatttccac tcacgagtgt ctgtctcc ttgacag**TAT**  
 13081 **GTGGGAAGCC** **CAAGAATCCG** **GCAAACCCAG** **TGCAGCGGAT** **CCTGGGTGGA** **CACCTGGATG**  
 13141 **CCAAAGGCAG** **CTTCCCTGG** **CAGGCTAAGA** **TGGTTTCCA** **CCATAATCTC** **ACACACAGGGG** **B-F**  
 13201 **CCACGCTGAT** **CAATGAACAA** **TGGCTGCTGA** **CCACGGCTAA** **AAATCTCTC** **CTGAACCATT** **A-R**  
 13261 **CAGAAAATGC** **AACAGCGAAA** **GACATTGCC** **CTACTTTAAC** **ACTCTATGTG** **GGGAAAAAGC**  
 13321 **AGCTTGTAGA** **GATTGAGAAG** **GTGGTTCTAC** **ACCCCTAACTA** **CCACCAGGTA** **GATATTGGGC**  
 13381 **TCATCAAAC** **CAAACAGAAG** **GTGCTTGTTA** **ATGAGAGAGT** **GATGCCCATC** **TGCCTACCTT**  
 13441 **CAAAGAATT** **TGCAGAAGTA** **GGGCGTGTGG** **GTTACGTGTC** **TGGCTGGGGA** **CAAAGTGACA**  
 13501 **ACTTTAAACT** **TACTGACCAT** **CTGAAGTATG** **TCATGCTGCC** **TGTGGCTGAC** **CAATACGATT**  
 13561 **GCATAACGCA** **TTATGAAGGC** **AGCACATGCC** **CCAAATGGAA** **GGCACCGAAG** **AGCCCTGTAG**  
 13621 **GGGTGCAGCC** **CATACTGAAC** **GAACACACCT** **TCTGTGTCGG** **CATGTCTAAG** **TACCAAGGAAG** **RsaI**  
 13681 **ACACCTGCTA** **TGGCGATGCG** **GGCAGTGCCT** **TTGCCGTTCA** **CGACCTGGAG** **GAGGACACCT**  
 13741 **GGTACCGCGC** **TGGGATCCTA** **AGCTTTGATA** **AGAGCTGTGC** **TGTGGCTGAG** **TATGGTGTGT** **RsaI**  
 13801 **ATGTGAAGGT** **GACTTCCATC** **CAGCACTGGG** **TTCAGAAAGAC** **CATAGCTGAG** **AACTAATGCA**  
 13861 **AGGCTGGCG** **GAAGCCCTTG** **CCTGAAAGCA** **AGATTCAGC** **CTGGAAGAGG** **GCAAAGTGG**  
 13921 **CGGGAGTGG** **CAGGAGTGG** **TGCGATAAGA** **TGTGGTTGA** **AGCTGATGGG** **TGCCAGCCCT**  
 13981 **GCATTGCTGA** **GTCAATCAAT** **AAAGAGCTTT** **CTTTGACCC** **A**

**RNA codon inverse table:**

<b>Ala/A</b>	GUU, GCU, GCC, GCA, GCG	<b>Leu/L</b>	UUA, UUG, CUU, CUC, CUA, CUG
<b>Arg/R</b>	CGU, CGC, CGA, CGG, AGA, AGG	<b>Lys/K</b>	AAA, AAG
<b>Asn/N</b>	AAU, AAC	<b>Met/M</b>	AUG
<b>Asp/D</b>	GAU, GAC	<b>Phe/F</b>	UUU, UUC
<b>Cys/C</b>	UGU, UGC	<b>Pro/P</b>	CCU, CCC, CCA, CCG
<b>Gln/Q</b>	CAA, CAG	<b>Ser/S</b>	UCU, UCC, UCA, UCG, AGU, AGC
<b>Glu/E</b>	GAA, GAG	<b>Thr/T</b>	ACU, ACC, ACA, ACG
<b>Gly/G</b>	GGU, GGC, GGA, GGG	<b>Trp/W</b>	UGG
<b>His/H</b>	CAU, CAC	<b>Tyr/Y</b>	UAU, UAC
<b>Ile/I</b>	AUU, AUC, AUA	<b>Val/V</b>	GUU, GUC, GUA, GUG
<b>START</b>	AUG	<b>STOP</b>	UAG, UGA, UAA

## RNA codon table

The table shows the 64 codons and the amino acid for each. The direction of the mRNA is 5' to 3'.

		2nd base			
		U	C	A	G
U	UUU (Phe/F)Phenylalanine	UCU (Ser/S)Serine	UAU (Tyr/Y)Tyrosine	UGU (Cys/C)Cysteine	
	UUC (Phe/F)Phenylalanine	UCC (Ser/S)Serine	UAC (Tyr/Y)Tyrosine	UGC (Cys/C)Cysteine	
	UUA (Leu/L)Leucine	UCA (Ser/S)Serine	UAA Ochre ( <i>Stop</i> )	UGA Opal ( <i>Stop</i> )	
	UUG (Leu/L)Leucine	UCG (Ser/S)Serine	UAG Amber ( <i>Stop</i> )	UGG (Trp/W)Tryptophan	
C	CUU (Leu/L)Leucine	CCU (Pro/P)Proline	CAU (His/H)Histidine	CGU (Arg/R)Arginine	
	CUC (Leu/L)Leucine	CCC (Pro/P)Proline	CAC (His/H)Histidine	CGC (Arg/R)Arginine	
	CUA (Leu/L)Leucine	CCA (Pro/P)Proline	CAA (Gln/Q)Glutamine	CGA (Arg/R)Arginine	
	CUG (Leu/L)Leucine	CCG (Pro/P)Proline	CAG (Gln/Q)Glutamine	CGG (Arg/R)Arginine	
1st base	AUU (Ile/I)Isoleucine	ACU (Thr/T)Threonine	AAU (Asn/N)Asparagine	AGU (Ser/S)Serine	
	AUC (Ile/I)Isoleucine	ACC (Thr/T)Threonine	AAC (Asn/N)Asparagine	AGC (Ser/S)Serine	
	AUA (Ile/I)Isoleucine	ACA (Thr/T)Threonine	AAA (Lys/K)Lysine	AGA (Arg/R)Arginine	
	AUG (Met/M)Methionine, Start <sup>U</sup>	ACG (Thr/T)Threonine	AAG (Lys/K)Lysine	AGG (Arg/R)Arginine	
A	GUU (Val/V)Valine	GCU (Ala/A)Alanine	GAU (Asp/D)Aspartic acid	GGU (Gly/G)Glycine	
	GUC (Val/V)Valine	GCC (Ala/A)Alanine	GAC (Asp/D)Aspartic acid	GGC (Gly/G)Glycine	
	GUA (Val/V)Valine	GCA (Ala/A)Alanine	GAA (Glu/E)Glutamic acid	GGA (Gly/G)Glycine	
	GUG (Val/V)Valine	GCG (Ala/A)Alanine	GAG (Glu/E)Glutamic acid	GGG (Gly/G)Glycine	

## ENZYMOLOGY V

1. The dependence of activity of  $\alpha$ -amylase on pH
2. Enzyme specificity
3. Polarimetric estimation of sucrase activity

### 1. The dependence of activity of $\alpha$ -amylase on pH

#### Introduction:

Most of the enzymes are strongly pH-dependent in their activity. This will be demonstrated on  *$\alpha$ -amylase* ( $\alpha$ -1,4-glucan-glucan hydrolase, E.C.3.2.1.1.) which is present in the saliva of oral cavity and in the pancreatic juice. It hydrolyses starch and glycogen to maltose, maltotriose, and a mixture of branched oligosaccharides, non-branched oligosaccharides, and some glucose. At pH 4 the enzyme is completely inactivated.

#### Solutions:

1. Starch - 1% solution in water (non-buffered)
2. Solution of iodine (Lugol)
3.  $\text{Na}_2\text{HPO}_4$  0.2 mol/l
4. Citric acid 0.1 mol/l

#### Procedure:

1. Prepare a set of 7 test-tubes. Pipette the buffers according to the table:

Test-tube	$\text{Na}_2\text{HPO}_4$ (ml)	Citric acid (ml)	pH
1	0.58	0.42	5.6
2	0.63	0.37	6.0
3	0.69	0.31	6.4
4	0.77	0.23	6.8
5	0.87	0.13	7.2
6	0.94	0.06	7.6
7	0.97	0.03	7.8

2. Add 0.5 ml of 1% starch solution (not containing buffer!) into each tube as substrate.
3. About 0.5 ml of saliva dilute with 20 ml of water (not buffer). Add 0.5 ml of the enzyme solution to each tube and leave in water bath ( $37^\circ\text{C}$ ) for 15 minutes.
4. After 15 minutes of incubation pour approximately half of the reaction mixture into another set of tubes and the remaining half leave on the water bath for another 15 minutes.

5. To each of the samples taken of the incubation add 5 ml of water and 1 drop of iodine solution. Register the colour that develops.
6. After the second incubation is completed take the samples of the bath, add again 5 ml of water to each tube and again 1 drop of iodine. Register the colour again.
7. Evaluate the results:
  - (a) What pH is the most suitable for  $\alpha$ -amylase?
  - (b) What colours develop in reaction of iodine with hydrolytic products of starch?

## 2. Enzyme specificity

### *Introduction:*

Most of the enzymes are highly specific for the reaction to be catalysed and for the substrate to be selected (see also Harper's Biochemistry pp.62-63). This property will be demonstrated in reaction with adequate and inadequate substrate. We shall demonstrate also the thermostability of enzymes. We shall use  $\alpha$ -amylase ( $\alpha$ -1,4-glucan-glucan hydrolase, E.C.3.2.1.1.) from saliva and sucrase ( $\beta$ -D-fructofuranoside-fructohydrolase, E.C.3.2.1.26.) of baker's yeast (*Saccharomyces cerevisiae*). The latter hydrolyses the  $\beta$ -glycosidic bond of fructofuranose. This enzyme is different from sucrase-isomaltase complex (E.C.3.2.1.48) in the intestinal juice of mammals which splits the  $\beta$ -glycosidic bond of glucose. However, in both cases sucrose is digested to glucose and fructose.

### *Solutions:*

1. Starch 1% solution
2. Sucrose 2% solution
3. Fehling I, II
4. Solution of iodine (Lugol)

### *Procedure:*

1. Take approximately 0.5 g of yeast and suspend in 2 ml of distilled water. This is the sucrase solution.
2. About 0.5 ml of saliva dilute with 20 ml of water. This is the amylase solution.
3. Prepare a set of 6 test-tubes and pipette according to the table. Perform the described experiment:

Test-tube number	1	2	3	4	5	6
Amylase (ml)	0.5	0.5				0.5
Sucrase (ml)			0.5	0.5	0.5	
Boil		Yes			Yes	
Starch (ml)	2.0	2.0	2.0			
Sucrose (ml)				2.0	2.0	2.0
Place on the water bath (37°C) for 30 minutes. Then divide the content of each tube into two parts. The first portion will be examined with Fehling reagent and the second with iodine (1 drop of iodine solution + 5 ml of water). Record the result:						
Fehling (positivity)						
Iodine (positivity)						

4. Evaluate the result in each test-tube and make conclusions in your protocol.

### 3. Polarimetric estimation of sucrase activity

#### *Introduction:*

The properties of *yeast sucrase* are described in previous experiment. Substrate (sucrose) and products (glucose and fructose) have different ability to rotate the plane of polarized light. (Sucrose: + 66.6°; glucose: + 52°; fructose: -100.3°; equimolar mixture of glucose and fructose: -48.3°). This can be used for measurement of enzyme activity.

#### *Solutions:*

1. Sucrose 5% solution (buffered to pH 6.0)
2. Fresh baker's yeast (*Saccharomyces cerevisiae*)
3. KOH 2 mol/l
4. "Albil" (denaturing and precipitation agent)

#### *Procedure:*

1. Measure 150 ml of sucrose solution and transfer it to a reaction flask and place on the water bath (25°C).
2. Prepare a set of 8 large test-tubes that should accommodate 15 ml of sample and mark them 0 - 7. Pipette 0.5 ml of KOH into each. This will later stop the reaction in each sample. Take

first 15 ml sample out of the reaction mixture before you have added the yeast and transfer it to the test-tube "0".

3. Suspend approximately 0.5 g of baker's yeast in 2 ml of water and add it to the reaction jar. Register time. The next sample will be taken after 15 minutes. Mix occasionally the content of the reaction flask and keep the temperature at 25°C.

4. Take 15 ml sample every 15 minutes and transfer it to the prepared test-tube. Mix it with KOH well to stop the reaction. Let it stay in the rack.

5. When all the samples are taken, divide the content of each tube into two centrifugation tubes (mark them well) and spin down the content in the centrifuge (only a technician is allowed to start the centrifuge). When finished, carefully pour out the supernatants to the clean test-tubes, combine again the original fractions, and measure the supernatants in polarimeter. The samples must not be turbid. If necessary add "Albil" into the samples and repeat the centrifugation.

6. Construct a graph: x - time; y - rotation angle.