CONTENTS

1. INTRODUCTION TO THE STUDY OF PHYSIOLOGY

The experimental method is a systematic and scientific approach to research in which the researcher manipulates one or more variables, and controls and measures any change in other variables.

Classification criteria of the experiments:

1) after the time of the result:

a) *Acute experiment:* evaluate the result immediately (seconds, minutes, hours). Example: demonstration of spinal reflexes laws on the spinal frog.

b) *Chronic experiment:* the result is evaluated after a long time (days, weeks, years) from the start of the experiment. Examples: conditioned reflexes, the action of stress on the organs by the appearance of lesions in gastric mucosa.

2) after the environment where the experiment is performed:

a) *in vivo:* studied biosystem is a living organ or tissue or body, the changes induced by the researcher being reversible. Examples: determination of respiratory volumes, clinical trials.

b) *in situ:* biological subsystem studied is not removed from the body, but his integrity is much diminished, and the induced changes are irreversible. Conditions allow nutrition and other functions of the body or tissue examined. Examples: frog heart *preparation in situ*, ligatures of Stannius, the law of the heart.

c) *in vitro* biological subsystem is studied separately from the body in a controlled environment where there are the same conditions like in source organism. Example: *in vitro* fertilization, organ bath. Many experiments in cellular biology are conducted outside of organisms or cells. Since the test conditions do not coincide with those of the body, the results are not always conclusive. Therefore, these experimental results are often annotated with in vitro.

d) *in silico:* the experiment is simulated on the computer.

3) after the time of the investigated parameter:

a) *direct experiment*: the studied parameter is measured directly. Example: determining the vital capacity by spirometry.

b) *indirect experiment:* the parameter investigated is calculated using other parameters more easily measured. Example: determining total lung capacity (CPT = CV VER VIR).

4) by means of contact with the body's internal environment:

a) bleeding experiments: investigation transducers and equipment are in direct contact with the body's internal environment. Example: determining blood pressure in rats.

b) non-bleeding experiments: transducers and equipment are not in contact with the body's internal environment research. Example: determining blood pressure by the indirect method (palpatory or ascultatory method).

The experiment should be characterized by good reproducibility, sensitivity and precision.

Most commonly used laboratory animals are: frog, rat, guinea pig.

To remove the resistance produced by animal, the anesthesia is performed. Methods of anaesthesia are frog-pitting (destruction of nervous centers from spinal cord) and on other animals, administration of various analgesic substances (ether, chloroform, ketamine, etc.). By anaesthetic muscle relaxation, we could prevent the surgical shock and the harmful neuro-

somatic-vegetative reflexes. In chronic experiments, anaesthesia is necessary for the animal to wake up vigil after the experiment.

Investigation of functional parameters (data acquisition, storage, data analysis) requires a measurement chain (or bioelectrometric chain). It consists of detection, amplification, filtration and recording / displaying assembly for biological signals.

- **Detection components** involves
  - sensors – physical devices which can detect energy variations of some paramenters and can modify their internal state depending on sensed parameters: temperature, resistance, transparency, etc
  - transduction devices which transform variations of physical parameters of the sensors in electrical signal who can be modulated.
  - usually the sensors and the trasduction devices form functional units.
- **Amplifiers** magnify the extreme low energy variation of the biological signals (e.g. micro/mili Volts)
- **Filtration** is necessary to remove undesired signals that affect the studied biological signal.
- **Recording / displaying assembly** includes kymograph (e.g. experiments with nerve muscle preparation), oscilloscope display / computer display, audio station, etc. It helps to visualise biological signal after amplifying and filtration, in order to be analysed and interpreted.
- **Data analysis** uses special computer software's for analysing the collected data and includes *statistical analysis* that validate the results obtained.

An example of biological measurement chain is the recording of electrical activity within a muscle named electromyogram: bioelectrical signals generated by the muscles are detected and collected with surface electrodes, than summed muscle action potentials are amplified and filtrated by the corresponding system and finally they are recorded, displayed and analysed by a computer digital-acquisition system, called A/D converter. Basically, an A/D converter maps the analogue signal into equivalent digital steps. These steps then allows us to perform a number of analyses on the data stream (figure 1.1).

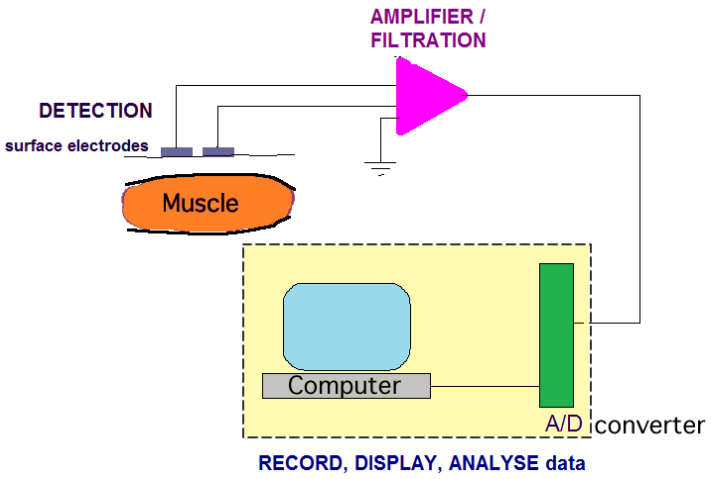


Figure 1.1. Diagram of measurement chain used in digital acquisition of electromyography data (see section 3.7)

2. GENERAL PHYSIOLOGY

2.1. Membrane transport

Cell membrane separates the interior of all cells from the outside environment. The membrane is selectively permeable and able to regulate the movement of substances in and out of cells, thus facilitating the transport of materials needed for survival.

Classification of membrane transport *(for details see the lecture)*:

I. în terms of *energy consumption:*
   a. active transport
   b. passive transport

II. in terms of *particle size transported*:
   a. microtransfer
   b. macrotransfer

III. in terms of number of species transported
   a. unitransport
   b. cotransport – simport and antiport

Passive transport:

1. simple diffusion (osmosis)
2. facilitated diffusion through channel proteins, transporters, ionophores

Active transport:

1. ionic pumps (ATPases)
2. macrotransfer systems - endocytosis - phagocytosis
   - pinocytosis
   - exocytosis
   - transcytosis

2.2. Osmotic fragility of red blood cells

Definition: Osmosis, osmolarity, tonicity – *see the lecture*

Osmotic fragility of RBCs represents the susceptibility of erythrocyte membrane to get lysed due to changes in the osmotic pressure of the solution in which they are suspended.

Principle:

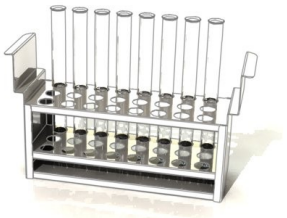
Membrane transport – *see the lecture*

Decreasing osmolarity of external environment will increase the cell volume (RBCs will gain water, swell and burst = hemolysis).

Increasing osmolarity of external environment will decrease the cell volume (RBCs will lose water and shrink, crenate = ratatination).

Materials required:

– 22 test tubes labelled from 1 to 22;
– Saline solution, NaCl 7‰;
– freshly drawn anticoagulated rat blood sample;
– 2 Pasteur pipettes (droppers).



Technique: RBCs are suspended in a series of 22 tubes containing serial dillutions of buffered 7‰ NaCl ranging from 0.68% to 0.24% concentration in increments of 0.02%.

Drop dilution method is used (Table 2.1):

1st tube contain 34 drops of 7‰ NaCl + 1drop of distilled water - hypertonic solution

2nd tube contain 33 drops of 7‰ NaCl + 2 drops of distilled water

..... ……..

22 tube contain 13 drops of 7‰ NaCl + 22 drops of distilled water - hypotonic solution

With a new Pasteur pipette add 1 drop of blood in every test tube and gently mix;

Allow to stand for 3 hours at room temperature or 24 hours at 4ºC or centrifuge the samples in order to speed up the sedimentation process;

Test tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Water drops No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
NaCl 7‰ drops No.	34	33	32	31	30	29	28	27	26	25	24	23	22	21	20	19	18	17	16	15	14	13
Saline solution **concentration (%)**	0,68	0,66	0,64	0,62	0,60	0,58	0,56	0,54	0,52	0,50	0,48	0,46	0,44	0,42	0,38	0,36	0,34	0,32	0,30	0,28	0,26	0,24

Table 2.1 Drop dilution method

Results: Examine the tubes from higher to lower concentrations (figure 2.1 and 2.2):

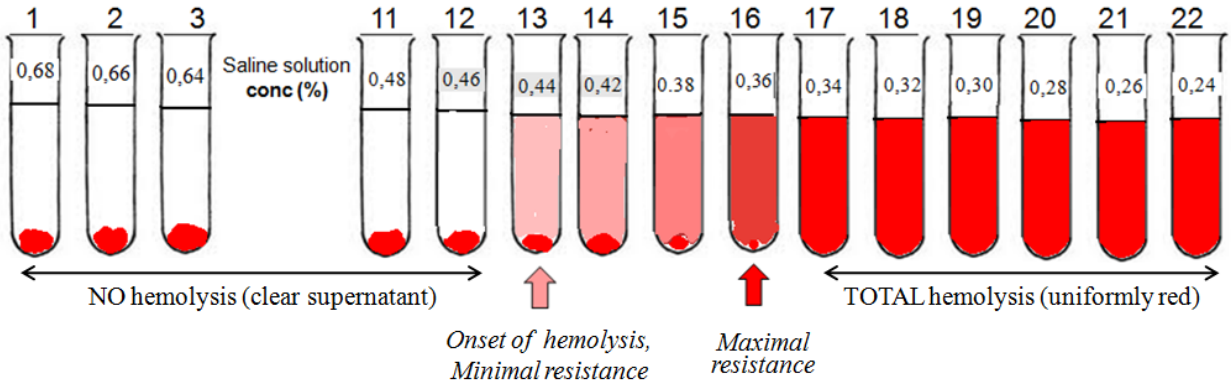
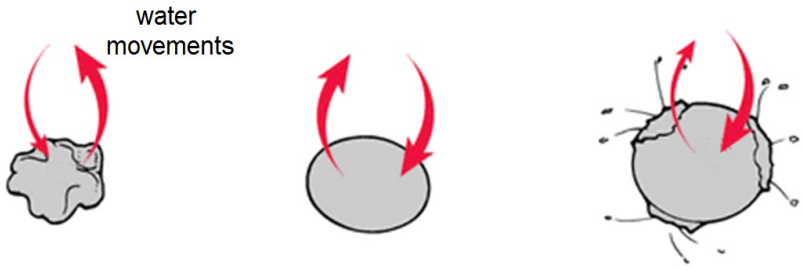


Figure 2.1 RBCs osmotic fragility estimation by drop dilution method

- tubes with solutions of higher concentration have sedimented RBC on the bottom of the tube and clear, colourless supernatant – NO HEMOLYSIS *(Why?);*
- as the concentration decreases gradually the tubes have reduced sediment and light red supernatant – PARTIAL HEMOLYSIS *(Why?)*. Concentration of saline solution in the first tube with light red supernatant determines the **minimal resistance (0.46-0.42)** corresponding to the 12 – 14th tube where the concentration was 0,46% - 0.42%;
- last tubes have no sediment, but red supernatant – TOTAL HEMOLYSIS (haemolysis is complete and all RBCs are lysed - *Why?*). Concentration in the last tube with sediment (last tube with partial hemolysis) determines the **maximal globular resistance (0.38-0.34)**, corresponding to the 15 – 17th tube where the concentration was 0,38% - 0.34%.

– osmotic fragility is expressed as the range of saline concentrations in which, beginning and completion of haemolysis occurs.



a. b. c.

Figure 2.2. Osmosis in RBCs placed in a hypertonic (a), isotonic (b) and hypotonic (c) environement.

Discussions:

As cells are exposed to progressively more dilute solutions (of decreasing osmolarity) water is osmotically drawn into them, causing RBCs to swell and become spherical until the **critical volume** is reached (the RBC volume before it bursts, is about 165% of normal). Afterward the membrane at first leaks and then bursts releasing haemoglobin (haemolysis). The membrane has a limited ability to stretch, so surface area is a limiting factor in determining the volume of water that can be accommodated. The susceptibility to osmotic lysis is primarily determined by the **surface area to volume ratio (SAVR)** of RBCs.

Explain the SAVR in all three states.

The ability of the normal RBC to resist hypotonicity results from its biconcave shape, which allows the cell to increase its volume by ~70% before the surface membrane is stretched. Once this limit is reached, lysis occurs.

A spherocyte will burst at higher saline concentrations ⇨ **SAVR is less** because spherocytes cannot expand as much as normal discoid erythrocytes and result osmotically more fragile. The RBC's ability to take in water before stretching the surface membrane is thus more limited than normal, and it is, therefore, particularly susceptible to osmotic lysis. Thus, in this case, the osmotic fragility curve will shift to the right. That is, the lysis will start at an even lower hypotonic challenge.

Spherocytes (figure 2.3) have increased thickness (about 3 µm), normal volume, and biconvex shape. Are associated with autoimmune hemolytic anemia, spherocytosis, etc

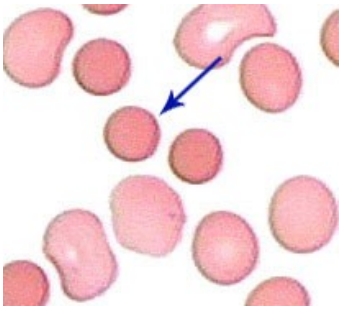


Figure 2.3. Spherocytes

A leptocyte has **a higher SAVR** to begin with. Thus it will accommodate the osmotic challenge of lower saline concentrations and will burst after taking more water. As a result, there will be a shift to the left in the curve. That is, the lysis starts at a higher hypotonic challenge.

Leptocytes (figure 2.4) are erythrocytes of normal volume but much reduced thickness (target cell). Are associated with thalasemia, iron deficiency anemia, as a result of disturbances in the hemoglobin synthesis. etc

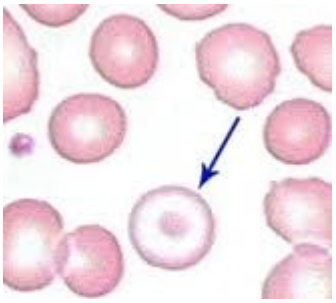


Figure 2.4. Leptocytes

Why do we perform osmotic fragility test?

- it is used to evaluate and manage suspected disorders of the red blood cell membrane (e.g. diagnosis of hereditary spherocytosis; screening test for beta-thalassemia).

Data interpretation/ Clinical significance:

Normal values:

- minimum osmotic resistance: 0.44% (4.4 g/l),
- maximum osmotic resistance: 0.3-0.32% (3-3.2 g/l)

Higher values: increased osmotic fragility / decreased osmotic resistance:

- spherocytosis when RBCs become spherical
- altered red blood cell membrane (aging red blood cells due to decreased turnover, autoimmune hemolytic anemia)
- deficiency of glucose-6-phosphat dehydrogenase (sex-linked inherited disease) increase the tendency of RBCs to get hemolysed by oxidizing agents like antimalarial drugs
- venum of cobra and other insects contains lecithinase which dissolves lechitin from RBC membranes making them more fragile

Lower values: decreased osmotic fragility / increased osmotic resistance:

- iron deficiency anaemia when RBCs become very thin and contain less haemoglobin, therefore, they can swell to a greater extent before they rupture
- sickle cell anemia, after splenectomy

3. NERVE MUSCLE PHYSIOLOGY

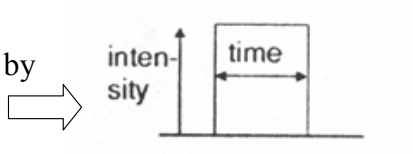
3.1. Excitability on frog neuromuscular preparation

Excitability is the property of the living matter consisting of a specific response (muscle contraction, nerve impulse, secretion, etc.) to an adequate (type, intensity, duration) endo- or exogenous stimulus.

An **excitable cell** is a cell which readily and rapidly responds to suitable stimuli, and in which the response includes a fairly rapid electrical change at the cell membrane. There are two types of excitable cells: nerve cells, which transmit and modify impulses within the nervous system, and muscle cells, which contract either in response to nerve stimuli or autonomously.

The reactivity of the nerve and muscle to different stimuli depends on the type (physical, chemical, mechanical, electrical), the strength of the stimulus, and the duration of the stimulation. In the physiology lab, *the most used stimulus is galvanic current* because of some advantages:

- it is easy to deliver (the operator can handle it properly)
- it can be well controlled and easy to regulate (it can be applied with the desired strength, frequency and duration)
- it can be accurately localized on the tissue
- it is slightest injurious to the tissue
- it is used *rectangular electrical current* characterized by intensity (amplitude, strength) and duration (time)



The most representative parameter defining the excitability of a living structure is **stimulus intensity**. According to this criterion, stimuli may be:

- ***subliminal (below threshold or subthreshold)*** - the intensity of the stimulus can trigger only locally changes (at the place of stimulus action), without any specific response from the investigated structure.
- depolarization that reaches the critical threshold and triggers the action potential
- ***supraliminal (above threshold or suprathreshold)*** up to submaximal - the intensity of the stimulus that it causes a response proportional to the its value applied;
- ***maximal*** - produces the maximum possible response from a living structure;
- ***supramaximal*** – the intensity of the stimulus can cause injuries on the cell, tissue or organ, it is inadequate as it even produces the latter's necrosis.

The response of the nerve and muscle to various intensity stimuli is studied *in situ*, on frog neuromuscular preparation (gastrocnemius muscle and sciatic nerve).

Determination of the excitation threshold and graded response of muscle

Definition: Excitation threshold = the minimal current intensity that triggers a muscle contraction

Materials required (figure 3.1.):

1. for the electrical part:

- *source of stimulation* gives galvanic current with adjustable voltage.

- stimulating device – *induction coil* convert galvanic current (low voltage) into induction faradic current (high voltage). It consists of: (i) primary coil is a thin copper wire wrapped on an

iron core and (ii) secondary coil is wrapped on a hollow wooden frame mounted on slide rails that can be moved in relation to the primary coil. The primary coil is connected through wires to the stimulation source; the secondary coil is connected to the stimulating electrodes. The magnitude of the induced current is selected by changing the distance between the coils (the maximum intensity of the induced current is obtained when there is 0 distance and overlapping of the coils and minimum when the distance is maximum between primary and secondary coils). The advantages of the induced current are the high intensity (can be conveniently controlled by simply changing the distance between the coils) and short duration (helpful for our experimental purpose of stimulating nerve and muscles).

- recording device - the *kymograph apparatus* consists of a base with motor and a straight shaft over which the drum (rotating cylinder) is fixed. The drum can be moved up and down as desired by moving a screw on the top of the shaft. Below the base of the kymograph are present levelling screws. The drum is wrapped over by a glazed paper which is smoked uniformly and thin in order to obtain good tracings. The kymograph has provision for off and on, locking system, speed selector for rotating drum.

- nerve-muscle preparation

- *stimulating electrodes* are 2 thick wires with a non conducting material between them and is used to deliver the current to the nerve / muscle

- *switch* between current source and induction coil used to on/off the current in the circuit

- *electromagnetric pen signal* connected in parallel to the source, used to mark the exact moment of stimulus administration.

2. for the non-electrical part

- *muscle lever (myograph lever)* – consists of a horizontal long arm to which is fitted a writing pen at one end and at the other is attached directly (or via a short vertical arm) by the *myograph board* to thread which is tied by a hook to the muscle tendon and is connected with a weight (which is hang over the long arm). It can be adjusted in such way that the muscle and the treated are not freely stretched by the load during the rest. The lever magnifies the contraction and records it on the rotating drum.

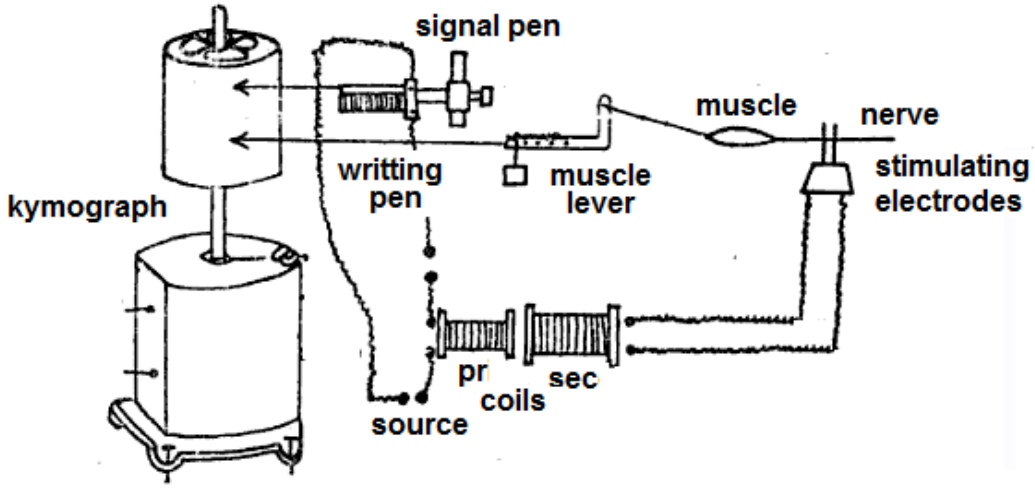


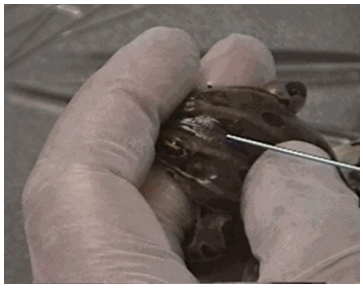
Figure 3.1. The set up for recording muscle contraction

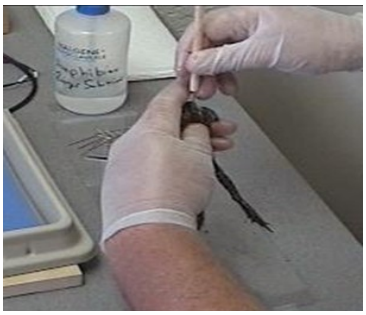
- fresh prepared frog neuromuscular preparation: living frog, dissecting set (dissection board, scissors with blunt and sharp end, pins to fix the frog on the board), pithing needle, gloves, Ringer's solution (13g of NaCl, 0.28g of KCl, 0.3g of $CaCl_2 2H_2O$, 0.4g of $NaHCO_3$ for 2L of distilled water), Pasteur pippet, glass probe, wipes.

Technique:

1. *pithing of the frog (destruction of spinal cord of the frog with a needle)*

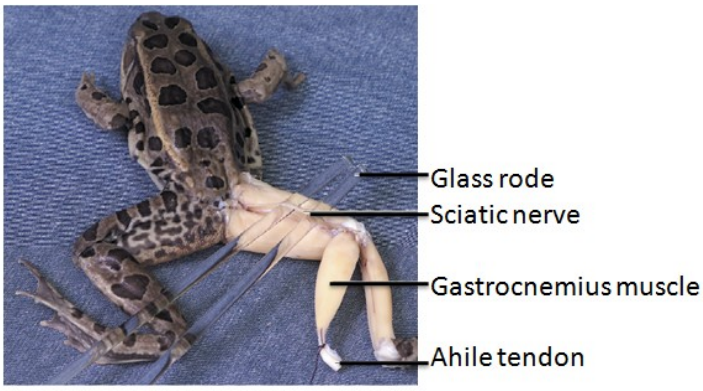
- catch the frog gently, but firmly with a dry cloth or cotton
- hold the frog with left hand tightly and fix the head of the frog by keeping the index finger on the head, thumb on the back and other three fingers on the belly of the frog
- press between the head and trunk with index finger and feel the depression at the atlanto-occipital joint
- pass the needle through the depression and move it inside vertebral canal towards the lower side; rotate the needle in all directions to destroy the spinal cord properly (the needle is correctly positioned because immediately after the needle is directed into the spinal cord, the muscles of the trunk and lower limb become spastic; they become flaccid after destruction of spinal cord)
- after pithing the frog loses its voluntary and reflex movements, but is still alive and can be used for experiments.





2. dissection:

- place the frog on the dissecting board on its abdomen with the pins in their extremities.
- remove the skin from one leg by first cutting the skin as high as possible around the thigh. Roll the loosened skin back a short distance. Peel the skin completely off the leg by pulling it quickly
- carefully insert a glass probe to separate the thigh muscles and to expose the sciatic nerve Do not pull the nerve; do not touch with any metal object; use only glass rod for handling the nerve.
- use the probe again to separate and free the gastrocnemius muscle from the other muscle of the frog's leg. Locate the Achilles tendon and cut the tendon closer to the animal's heel bone. put the distal end of the tendon in the hook attached to a recording pen;
- keep the frog's tissues moist with Ringer's solution during experiment to avoid drying



3. *determination of the excitation threshold and graded response of muscle:*

- writing pen of the lever (for recording muscle contraction) and the signal pen of the parallel circuit (for marking the moment when stimulus is applied) are brought in contact with the smoked surface of the drum. The two pens must be at the same level on the kymograph cylinder. Rotate the drum with low speed.
- the sciatic nerve is stimulated at progressively increasing intensities (sliding the secondary coil against primary coil) by delivering one single stimulus to the muscle by pressing the switch (which turn on/off the electric circuit supplying the induction coil) and the responses of the gastrocnemius muscle are recorded on the drum.
- administer single stimuli to the muscle at approximately 2-5 seconds intervals, increasing the intensity of the stimulation slightly until a contraction is obtained (shown by a spike on the paper). The smallest intensity stimulus at which a muscle contraction occurs is known as the ***threshold (minimal) stimulus.***
- all stimuli applied prior to this point are termed ***subthreshold (subminimal) stimuli*** because at those intensities no contraction was elicited.
- continue to increase the stimulus strength until you reach the point where further increases in stimulus intensity will not produce a larger muscle contraction ***(maximal stimulus)***. Stimulus intensities from threshold point to the maximal point are known as ***submaximal stimuli.*** Any stimulus greater than maximal is said to be ***supramaximal*** (figure 3.2)

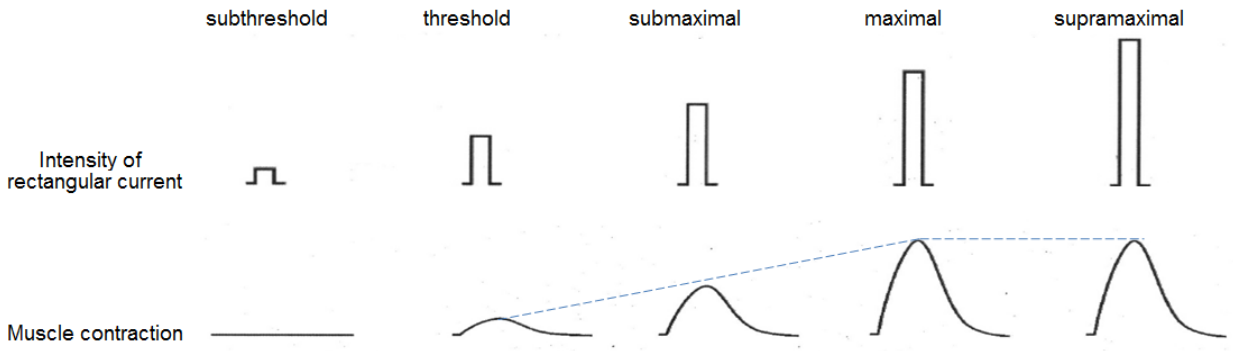


Figure 3.2. Stimulus intensity and the response of the muscle

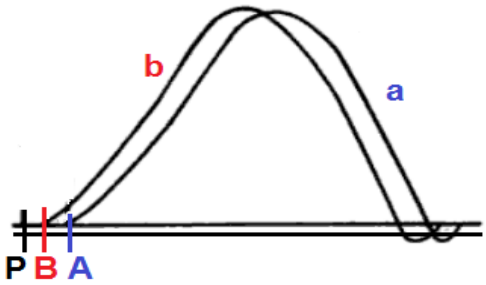
- excitation is achieved using electric current (rectangular current impulses of different durations and intensities), either **indirectly** (by placing the electrodes under the sciatic nerve), or **directly** (by placing a pair of electrodes on the muscle surface). *Compare excitability threshold in direct muscular stimulation and nerve stimulation (indirect stimulation of muscle).*

3.2. Conductivity on frog neuromuscular preparation

Principle: velocity of the nerve impulse can be determined in frog nerve muscle preparation.
Technique:

- it is used the same technique described above to obtain a nerve muscle preparation and a stimulation circuit
- place the stimulation electrodes on the sciatic nerve in 2 points: *point A* close to the vertebral end of the nerve and *point B* close to the muscle end of the nerve
- stimulate the nerve with a maximal stimulus in point A, respectively B and record on the rotating drum the corresponding twitches (figure 3.3).

Figure 3.3. Determination of nerve conduction velocity in frog neuromuscular preparation, where
P = point of stimulus application
a,b = corresponding twitches
PB = latent period corresponding to twitch following stimulation of the nerve close to the muscle
PA= latent period corresponding to twitch following stimulation of the nerve close to the vertebra



Calculation:

- distance between the points of stimulation A, B is measured with a ruler and is noted with D
- difference between latent periods: PA – PB = T (time)
- apply the formula: $velocity = \frac{distance\ travelled}{time\ consumed} = \frac{D}{T}\ metres/seconds$

Normal values: 24-30 m/s

Major factors that affect nerve conduction velocity: diameter of the axon (varies direct proportional), myelin sheath (myelinated fibers > unmyelinated fibers), temperature (varies direct proportional).

Neuronal conductivity is the ability of the nerve structure to propagate the action potential (it is a membrane phenomenon).

Conductivity is realized differently, depending on the type of nerve fiber:

- *in unmyelinated fibers* – use Hermann electric currents, it is slowly, step by step (figure 3.4).
  - propagation of the action potential from the site of initiation to other locations along the nerve cell is caused by the positive charges in the cell leaking to an adjacent (un-stimulated) region and depolarizing that region enough to create an action potential there;
  - a membrane potential reversal occurs (the load-carrying cations migrate towards the polarized area (at rest) and gather there triggering depolarization) on a particular membrane length (nerve impulse "wave length": 16—60 mm), when the action potential arises.
  - in this way, the signal moves from one region of the axon to the adjacent one, and ultimately to the end of the axon.

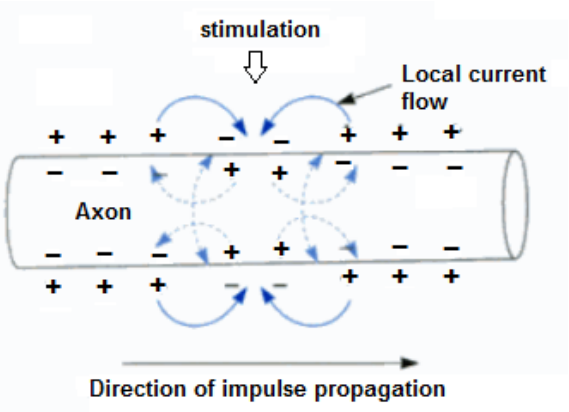


Figure 3.4. Conduction of action potential in unmyelinated fibers

- *in myelinated fibers* - saltatory conduction, very rapid (figure 3.5)
  - the axon is covered with a series of Schwann cells, a type of glial cell which electrically insulates the axon. The spaces between adjacent Schwann cells are called the nodes of Ranvier, and they are the only regions along the axon where the membrane is exposed to the extracellular fluid. The myelin insulation prevents the current associated with the action potentials from leaking out of the membrane until they reach a node. So, action potentials are only regenerated at the nodes in myelinated cells.
  - the fact that the internode is natrium tight and its reduced capacity limit the amplitude losses suffered by the potential during electrotonic diffusion, cause depolarization, which "jumps" to the next node, yet it remains sufficient to trigger the nodal amplifier that generates a new action potential.

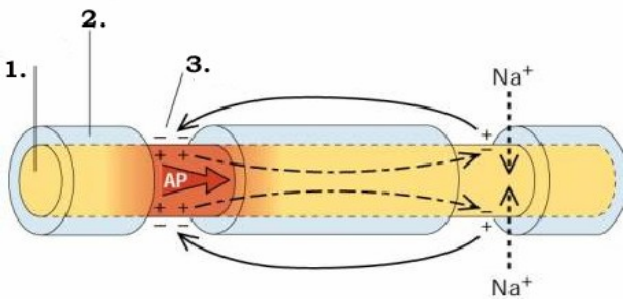


Figure 3.5. Saltatory conduction in myelinated fibers (1. axon, 2.myelin, 3. Ranvier node where action potential, AP, is regenerated)

3.3. Contractility on frog neuromuscular preparation

Principle: to demonstrate a simple muscle curve, incomplete / complete tetanus, on a frog gastrocnemius-sciatic preparation.

Technique:

- it is used the same technique described above (section 3.1.) to obtain a nerve muscle preparation and a stimulation circuit
- indirectly it is stimulated the muscle (the stimulation electrodes are applied on the sciatic nerve)
- when the gastrocnemius muscle is stimulated with a single electrical stimulus the response will be contraction and then relaxation. Simple contraction of the muscle is called **muscle twitch** and graphical recording of this is called **simple muscle curve.** The contraction is recorded as upward deflection and the relaxation is recorded as a downward deflection back to the baseline (figure 3.6).

Interpretation:

Four points could be marked in this curve:

- **SP** = stimulation point (the exact moment when stimulus is applied);
- **CP** = contraction point (indicate the time when the muscle begin contraction);
- **MCP** = maximum contraction point (mark the end of the contraction and the beginning of the relaxation);
- **MRP** = maximum relaxation point (show the complete relaxation of the muscle).

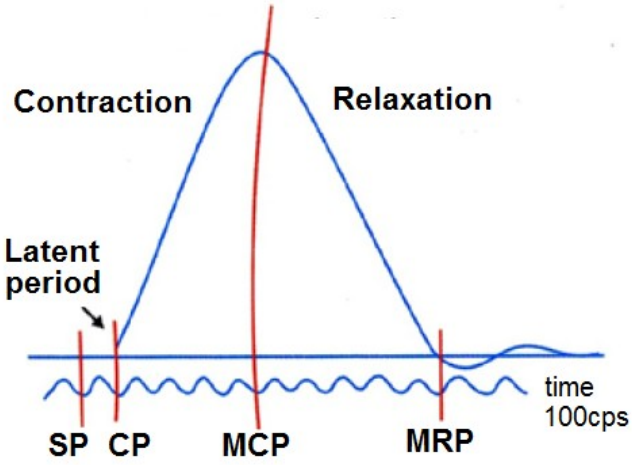


Figure 3.6. Simple muscle curve

These points split *simple muscle curve* into 3 phases:

1. *Latent period* is the time interval between stimulus application and beginning of the contraction (from point SP to point CP). No mechanical activity is recorded in the muscle during this period. Duration: 0.01 seconds.
2. *Contraction period* is interval between beginnings of the contraction to maximum contraction (CP - MCP). The muscle contracts during this phase. Duration: 0.04 seconds
3. *Relaxation period* is interval from the peak of the contraction and the end of the relaxation (MCP - MRP). The muscle relaxes during this phase. Duration: 0.05 seconds.

Total twitch duration = 0.1 seconds.

The latent period is due to:

- time needed for stimulus to travel along the nerve to the neuromuscular junction (in indirect stimulation of the muscle)

- time needed for impulse to cross the neuromuscular junction, to stimulate the muscle
- time needed for excitation – contraction coupling to occur
- time needed for chemical events to occur (sliding phenomenon)
- time needed for development of tension in the muscle
- time taken by inertia of recording device

Contraction phase is always shorter than relaxation phase because contraction is an active process and relaxation, a passive process.

Total witch duration (contraction time) depends on the types of the fibers:

- fast (white) muscle fibers have shorter contraction time (0.025 s), e.g ocular muscles, hand muscles (for fine movements);
- slow (red) fibers have longer contraction time, 0.1s , e.g. back muscles

Total witch duration varies with species: human skeletal muscle (0.03 – 0.05 s) < frog muscle (0.1 s)

Discussions: In the normal situation, muscle is stimulated by flux of the nerve impulses rather than single impulse. Therefore, the simple twitches do not occur naturally.

Factors affecting contractile response (force of contraction):

1. strength of stimulus
2. frequency of stimulus
3. load on the muscle
4. initial length of the muscle
5. temperature

*1. Strength of stimulus (**spatial summation**).*

- *A single muscle fiber* obeys the all or none law, i.e. a subthreshold stimulus determines no response, and with threshold, maximal, supramaximal stimuli the contractile response remain the same.
- *The whole muscle* when stimulated with different intensity stimuli respond gradually (see section 3.1.)
- In the body, the fibers of skeletal muscles are stimulated by the alpha motor axons from anterior horn of spinal cord, i.e., by *recruitment of motor units*. Small motor units are recruited first; they demonstrate greater fatigue resistance and remain active as long as the muscle contracts. With increasing activity, large motor units are recruited into activity in order to increase contractile force of the muscle; this process is called *multiple motor unit summation*

*2. Frequency of stimulus **(temporal summation)***

The effect of repeated stimuli on the contractile response of the skeletal muscle depends on the number of stimuli (frequency). The muscle response depends upon where the next stimulus falls. First, it will be presented the effect of two successive stimuli, then the effect of repetitive multiples stimuli.

The effect of two successive stimuli

a. if the second stimulus is applied following the relaxation period of the first twitch (just after the first twitch touches the baseline), it will be recorded 2 complete individual twitches (figure 3.7). The second one will have higher amplitude than the first, due *to beneficial effect* of first contraction.

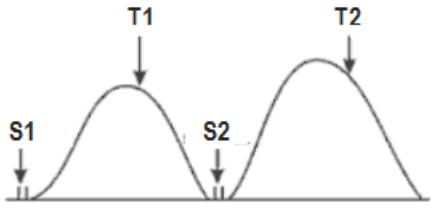


Figure 3.7. 2nd stimulus falls following the relaxation period of the 1st twitch

b. The causes of this effect are:
   - the Ca^{2+} ions left in the sarcoplasm after the first contraction. Normally, the Ca^{2+} released from the terminal cisterns during muscle contraction are

pumped back into reticulum during relaxation. When the second stimulus falls in the relaxation or following the relaxation period of the first one, some amount of Ca^{2+} is left in sarcoplasm. Therefore increases the $[Ca^{2+}]$ for the second stimulus and explains why the height of the contraction increases with the second stimulus;
- the increase in temperature,
- the decrease in the viscosity of the muscle by the first contraction

c. if the second stimulus is applied during the relaxation period of first twitch, the relaxation is arrested and the second contraction occurs (figure 3.8). It will result a *curve summation* with the second curve higher than the first one, due to beneficial effect explained above. The increased magnitude of contraction is due to the summation of the responses (contractions) rather than the summation of stimuli.

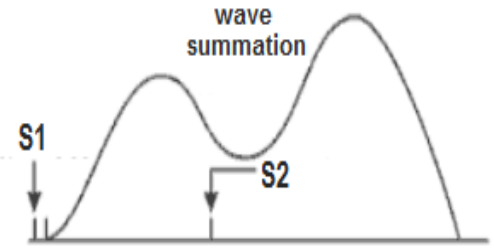


Figure 3.8. 2nd stimulus falls within relaxation period of the 1st twitch

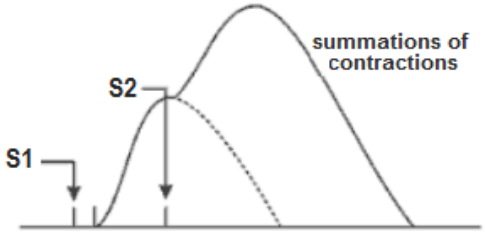


Figure 3.9. 2nd stimulus falls during the contraction period of the 1st twitch

d. if the second stimulus is applied during the contraction period of first twitch it will result two curves fused into one whose height is greater than single stimulation of the same strength (figure 3.9). This is named *summation of contractions.*

e. if the second stimulus is applied during second half of latent period (LP) of first twitch, then the effect of two stimulations are added together giving a single twitch with higher amplitude (figure 3.10). This is named *summation of stimuli.*

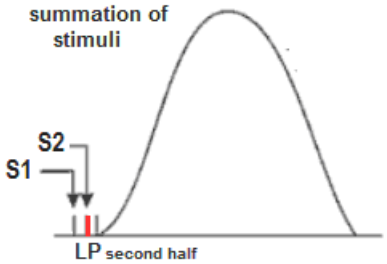


Figure 3.10. 2nd stimulus falls during second half of latent period (LP) of the 1st twitch

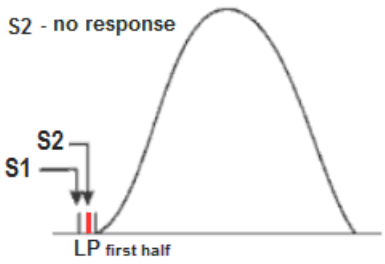


Figure 3.11. 2nd stimulus falls during first half of latent period (LP) of the 1st twitch

f. if the second stimulus is applied during first half of latent period (LP) of first twitch, it will not evoke any response because the second stimulus falls within the absolute refractory period of the first contraction (figure 3.11). During this period the second stimulus of whatever strength is ineffective.

The effect of repetitive multiple stimuli

Depending on frequency of stimulation following phenomenon are observed:

a. *Staircase or treppe* (from german staircase) (figure 3.12) – if a fresh nerve muscle preparation is stimulated with certain strength of stimuli, then simple muscles curves of certain amplitude are recorded. If 2nd stimulus followed by 3rd, 4th, 5th are applied at short interval of about 1 second, for 5-6 stimulations, gradually increased amplitude of twitches is observed. This is due to the beneficial effect explained above.

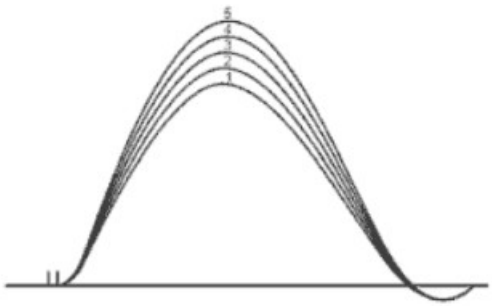


Figure 3.12. Staircase or treppe phenomenon

b. *Incomplete tetanus* or experimental clonus - when the frequency of multiple stimuli is such that the next successive stimulus falls on the relaxation phase of the previous twitch, then the succeeding contractions obtained, will be superposed over the previous twitch due to incomplete summation of waves. In incomplete tetanus the relaxation is incomplete.

c. *Complete tetanus* is a state of sustained contraction. When the frequency of multiple stimuli is such that the next successive stimulus falls during the contraction phase of the previous twitch (i.e. before relaxation begins), then due to the complete summation effect, the muscle will remain in a state of tonic, forceful contraction without relaxation.

Significance: The muscles of our body that are involved in maintenance of posture exhibit titanic contractions. Also, titanic contractions of skeletal muscles are seen during an isometric exercise. In clinic, the persons infected with bacteria called *Clostridium tetani* show tetanic contractions of facial muscles (commonly affected) or of all skeletal body muscles in severe cases. Tetanus results because the bacteria release titanic toxin which stimulates motor neurons repeatedly at higher frequency. The disease can be prevented by prior immunization with tetanus vaccine or receiving vaccine within 12 hours of injury (cut, wound) through which bacteria enter the body.

Recording the incomplete / complete tetanus on the moving drum of the kymograph

The materials required and procedure is the same as in case of muscle twitch (figure 3.13).

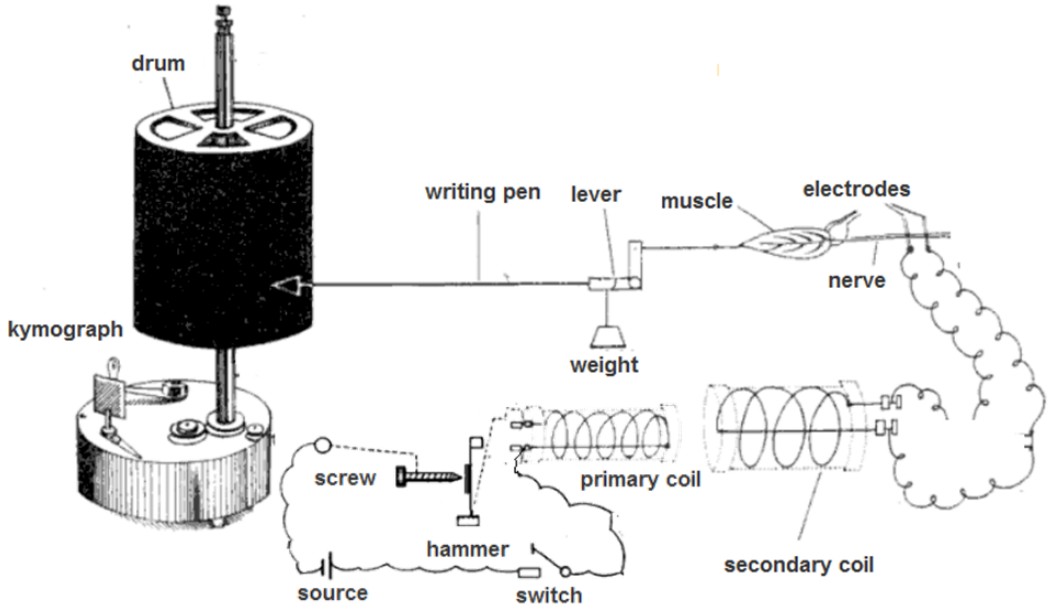


Figure 3.13. Stimulation circuit to record tetanus.

The primary coil has metallic plate with a hammer (variable interrupter) and a movable screw to regulate the frequency of applied stimuli (by rotating the screw it is adjust the rate of movement of the hammer of the induction coil; when the screw is taken away the frequency of the stimuli decreases and when is pushed, the frequency increases). When circuit is closed from the switch, the movement rate of the hammer cause the same rate of interruptions of the current in the circuit.

Rapid and multiple stimulation (25-30 stimuli/s) to muscle fibers leave no time to relax them, so successive contractions fused together as a continuous line is recorded on the moving drum – *complete tetanus (figure 3.5).*

In case of *incomplete tetanus*, less rapid stimulation are given, in this case, contraction is not smooth and individual waves may be discerned (incomplete relaxations) (figure 3.14 and figure 3.15.)

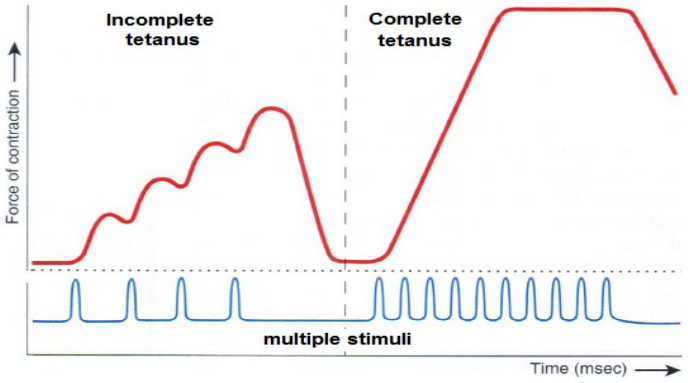


Figure 3.14. Tetanus

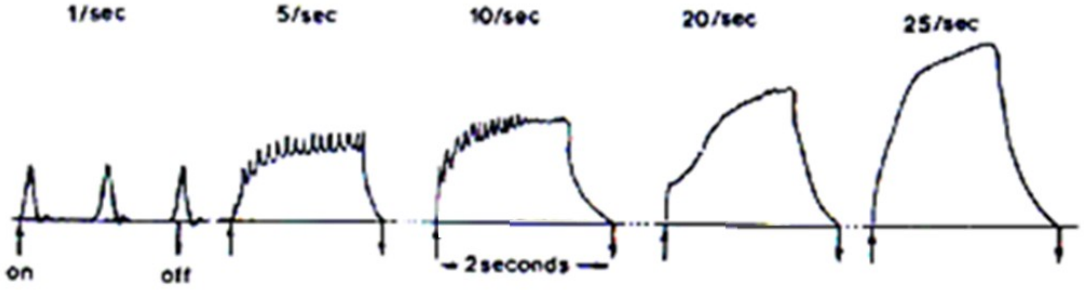


Figure 3.15. Effect of increasing the frequency of stimuli

Most sustained voluntary skeletal muscle contractions are unfused tetanic contractions with different motor units stimulated at different times (asynchronous contractions). The asynchronous contractions delay muscle fatigue, which is an inability to contract caused by long periods of muscle contraction.

d. fatigue: when repeatedly stimulated, muscle looses its excitability, become gradually less excitable and ultimately stops to respond (figure 3.16).

Fatigue can be demonstrated on nerve muscle preparation by applying repeated stimuli over a prolonged period. Initially, for the first few contractions, staircase phenomenon (treppe) is observed (curves 1, 2, 3 due to the beneficial effect). Following contractions gradually contracture is observed: the muscle contracts but fail to relax; the contraction period become smaller and the relaxation period are progressively increased (early sign of fatigue).

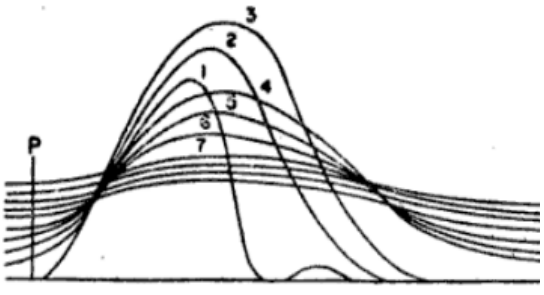


Figure 3.16. Graph showing the effect of the fatigue on frog isotonic muscle twitch

Finally, the tracing fail to return to the baseline during relaxation – this is called contraction reminder (traces 4-7). It occurs due to a decrease in ATP content and accumulation of the metabolites in the muscle.

Causes of fatigue: lack of energy in muscle; accumulation of lactic acid in the muscle; depletion of acetylcholine (neurotransmitter of neuromuscular junction); raised intramuscular tension hampers passage of blood through muscle.

- *The first site of the fatigue in this experiment is neuromuscular junction*.
- *The muscle is not the first site of the fatigue* because the direct stimulation of the muscle gives a response even after the fatigue is obtained when the muscles is stimulated through the nerve.
- *The nerve is not the first site of the fatigue* because of the next experiment: Two nerve muscle preparations named N1 and N2 are fixed in such manner that both can be stimulated simultaneously. A piece of ice is kept on nerve just before the neuromuscular junction on the N2 preparation: it blocks the conduction of stimulation on the nerve itself. Both, N1 and N2 are stimulated till the N1 muscle does not contract i.e. the muscle is fatigue. The ice is removed from N2 and again both preparations are stimulated. N1 will not respond, but N2 respond.

In intense physical effort muscular fatigue is due to the events at the synapses in the central nervous system. This is considered to be a protective phenomenon. The central fatigue is manifested before there is any block at neuro-muscular junction and long before the muscle completes fatigued.

3. Effect of load on the muscle – load is the force exerted by an object on the muscle. It opposes to the muscle tension (the force exerted by the contracting muscle on an object). The load acting on the muscles is two types: free-load (pre-load) and after-load.

Free-load starts acting on the muscle before the muscle it starts to contract (e.g. filling water from a tap by holding a bucket in the hand). Free-load stretches the muscles passively and develops a passive tension across the muscle. This passive tension increases the force of contraction in two ways: by increasing initial length of the muscle to its resting length at which maximum force is generated, and, by adding an elastic recoil force to the muscle during its contraction.

After-load acts on the muscle after the beginning of muscular contraction. It opposes the force produced by muscle contraction. The work done in an afterloaded muscle is less than that of a free-loaded muscle. An example of after-load in intact body is lifting an object fron the ground.

On the basis of the pattern of tension production, the muscles contraction can be divided in

a. isometric contraction - the muscle is developing force but not shortening and no visible movement is seen. In this case, the force developed by the muscle equals the force (load) it is contracting against. *Examples of isometric contractions*:

- holding a heavy weight above the ground
- pushing against a locked door
- trying to pick up a car
- postural muscle that keep your body upright when you stand or sit involve the isometric contractions of muscles that oppose the force of gravity; the entire amount of energy is turned into heat.

b. isotonic contractions - the muscle is generating a constant force to move a load (it performs mechanical work.). Examples: lifting an object off a desk, walking, and running involve isotonic contractions. There are two types of isotonic contraction:

- *concentric isotonic contractions - the muscle is shortening while it is contracting* (the muscle tension exceeds the resistance and the muscle shortens). An example of this is using the biceps brachii (large anterior muscle of arm) to lift a book off the table.
- *eccentric isotonic contractions - the muscle is lengthening while it is contracting* (the peak tension developed is less than the resistance, and the muscle elongates owing to the contraction of another muscle or the pull of gravity). This occurs when you slowly lower your

arm to return the book to the table. The biceps brachii is still contracting, but it is lengthening while it is contracting. This enables you to lower the book in a controlled manner. By varying the tension in an eccentric contraction, you can control the rate of elongation, just as you can vary the tension in a concentric contraction. For example, precisely controlled eccentric contractions occur each time you walk down stairs or settle into a chair.

Normal daily activities therefore entail a mixture of isotonic and isometric muscular contractions. As you sit and read this text, isometric contractions of postural muscles stabilize your vertebrae and maintain your upright position. When you turn a page, the movements of your arm, forearm, hand, and fingers are produced by a combination of concentric and eccentric isotonic contractions.

Isotonic versus isometric twitch on frog neuromuscular preparation (figure 3.17)

- in isotonic recording the muscle is made to contract against various loads and the shortening is recorded using a system of levers; all isotic contractions begins with a brief phase of isometric contraction, which accounts for a part of the latent period of isotonic contractions;
- in isometric recording the muscle is made to produce a very small displacement of a very heavy load and the muscle tension is recorded using a tension transducer

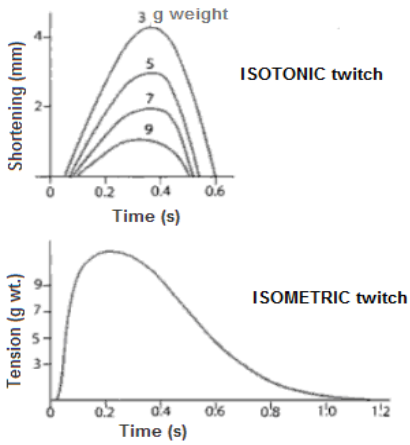


Figure 3.17. Traces of isotonic / isometric twitch of frog gastrocnemian muscle *(after Sabyasachi Sircar, 2007)*

4. Initial length – length tension relationship graph (see the lecture)

5. Effect of temperature on the contractile response (figure 3.18.)

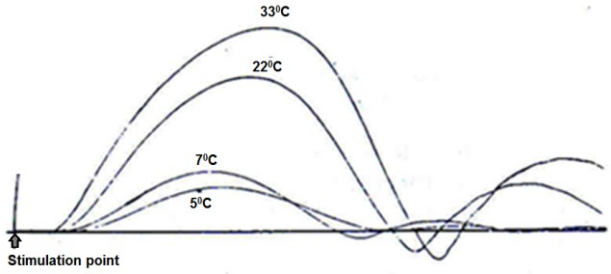


Figure 3.18. Traces showing the effect of temperature on frog isotonic gastrocnemian muscle contraction

- at moderate high temperature (40^{0}C) – increase amplitude of muscle curve due to increase in isotonic shorting of the muscle (decrease the internal viscoelastic resistance)
- at low temperature (5-10^{0}C) the reverse changes occurs; reversible phenomenon; if the muscle is gradually re-warmed, excitability is regained.
- at high temperature (50 -60^{0}C) – coagulation of muscle proteins – heat rigor – irreversible phenomenon.

3.4. Contractility of smooth muscle

Functional basis of smooth muscle contraction, skeletal *versus* smooth muscle contration – see lecture

Experimental, smooth muscle contraction can be demonstrated *in vitro* using organ bath.

Principle: experimental analysis of smooth muscle contraction in organ bath

Materials required: small intestine of rat (smooth muscle), organ bath assembly, lever, and kymograph

Technique: anesthetize the rat, open abdominal cavity, take a ring of small intestine (2 mm), rinse with Krebs-Henseleit solution and tie both the ends of the intestine with threads and leave it for

10 minute in buffer solution. Than tie the free end of one thread in the "L" shaped bent end a glass tube (to be place in the organ bath chamber) and the free end of the other thread to long arm of the leaver (or an isometric force transducer connected to a computer). Fill the chamber with Krebs-Henseleit solution (isotonic with internal environment of the rat) at 37^0C and bubbles air (O2) through glass tube L shaped. Attach the writing pen to a smoked drum or other recording device and observe after 5-10 minutes spontaneus contractions of small intestine. Note the rate and the amplitude of the contractions.

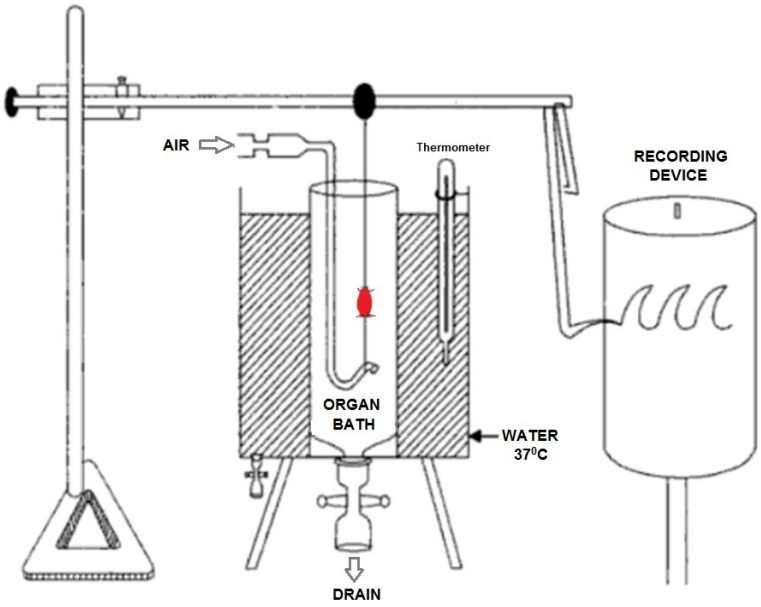


Figure 3.19. Experimental set up for recording of smooth muscle contraction *(after Rastogi Sc, 2005)*

In order to study the drug action, add them to the organ bath and after recording the effect of each drug, the Krebs Henseleit solution should be drained and replaced. The effect of the next drug should be recorded after the intestine is allowed too equilibrate, means it shows normal spontaneus movements.

Interpretation:

Two drugs are choosing for study: acetylcholine and epinephrine, because smooth muscle has dual innervation: postganglionic sympathetic fibers discharge epinephrine and parasympathetic fibers release acetylcholine. Acetylcholine accelerates contraction and adrenaline inhibits smooth muscle and the resting potential is made more negative by making the muscle less active.

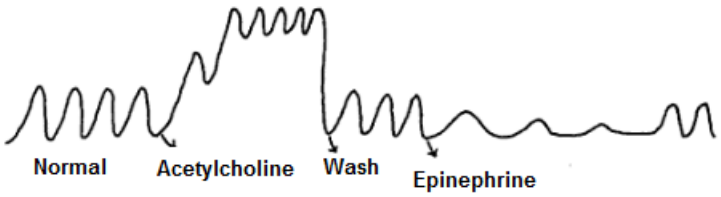


Figure 3.20. Effects of drugs on intestine smooth muscle.

3.5. Determination of compound action potential (electronervogram)

Definition: Compound action potential (CAP) is the algebraic sum of individual "all or none" action potentials produced by all the fibers (motor and sensory) from a mixed (large, compound) nerve that were fired up by an electric stimulus.

What is the difference between nerve and neuron? (see lecture)

The axons within a mixed nerve include afferent (sensory) fibers and efferent (motor) fibers. Individual axons vary in diameter, myelination, excitability, threshold and conduction speed (Table 3.1.)

The CAP is *not* a naturally occurring phenomenon. It is elicited experimentally or clinically, by stimulating the whole nerve (e.g. the sciatic), with *extracellular stimulating electrodes* and is recorded, using *extracellular recording electrodes*, which measure the summed electrical response of all the excited axons or dendrites in the nerve.

Principle: graphical recording with extracellular electrodes of compound action potential from a mixed nerve (frog sciatic nerve)

Materials required: isolated sciatic nerve preparation, a stimulation set up (stimulator, amplifier, software for data acquisition and processing, lab computer) and a nerve chamber with electrodes, Ringer's solution.

Technique:

- obtain the isolated sciatic nerve using the same procedure from the sciatic nerve preparation (section 3.1.), cut the nerve and mount it into the nerve chamber; prepare the stimulation set-up (figure 3.21)

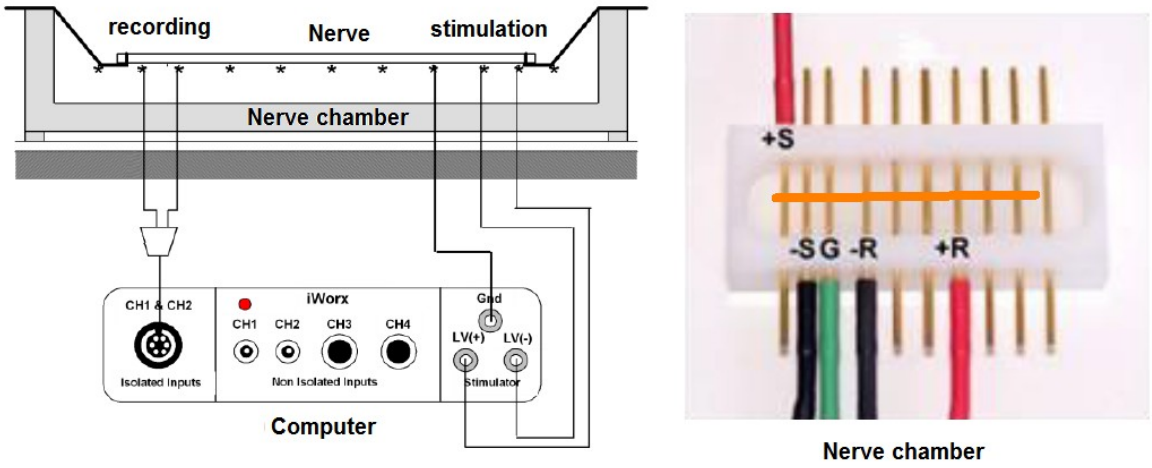


Figure 3.21. Experimental set-up for recording compound action potential on frog sciatic nerve.

- stimulating electrodes are placed to one end of the nerve; recording electrodes are located along the nerve. If the nerve is stimulated with a current pulse of sufficient amplitude, the action potentials produced in the fibers propagate toward the recording electrodes. The cumulative effect of the many action potentials is an extracellular wave of negative potential, moving along the surface of the nerve;
- if for the recording of CAP *two electrodes* are used it will be obtained a positive and negative deflection called *dysphasic CAP (figure 3.11)*:
  - when the nerve is inactive there is no potential difference between the electrodes and the trace is at baseline;
  - when the CAP arrives at the proximal electrode it becomes transiently negative (relative to the distal electrode) and the trace is an upward deflection
  - when the CAP is in between the proximal/distal electrodes there is no potential difference and the trace returns to baseline;
  - when the CAP arrives at the distal electrode it becomes negative (relative to the proximal electrode) and the trace is a negative deflection;
- if for the recording of CAP *one electrode* remain active it will be obtained a positive deflection called *monophasic CAP*. That means the CAP reaches the first recording electrode, but does not reach the second recording electrode because the propagation of CAP was blocked between the two recording electrodes by mechanical methods (pressure applied to the nerve or crushing the nerve with forceps) or chemical methods (applying potassium chloride, local anaesthetics, cocaine, or tetrodotoxin to the nerve) (figure 3.22).

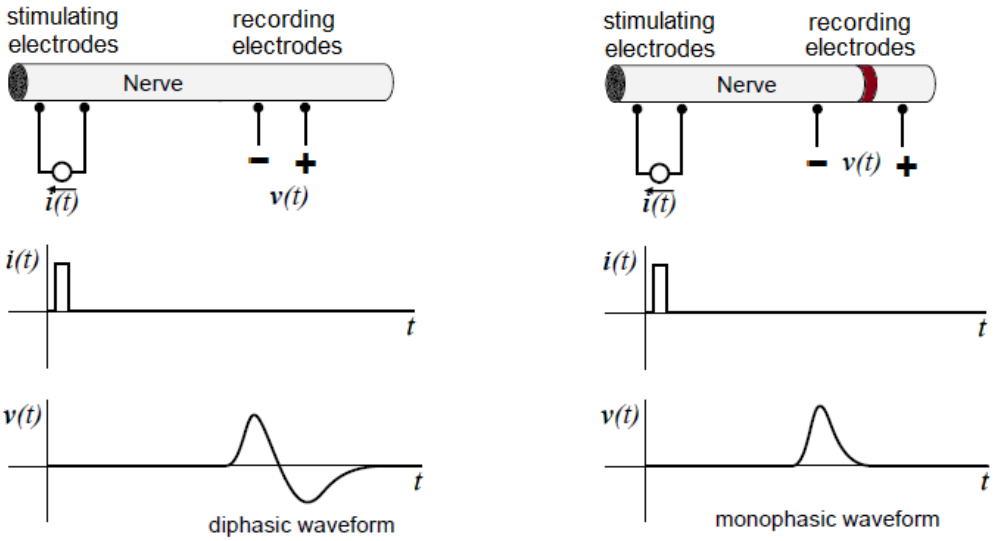


Figure 3.22. Extracellular recording of compound action potential as dysphasic or monophasic wave.

- if a maximal stimulus is applied to the nerve, all the fibers will be depolarized and the CAP will display a number of peaks, each corresponding to the group of axons with different velocities of conduction (figure 3.12).

Interpretation:

The CAP differs from individual AP of a single axon in several ways:

- the individual AP demonstrated by intracellular recording from a single axon obeys "all or none" law;
- the CAP demonstrated by extracellular recording from many axons, is a graded response whose magnitude increase with the intensity of stimulation, because different axons have *different thresholds of excitation.* The largest axons have the lowest threshold, i.e., they are the most excitable ($A\alpha > A\beta > A\gamma > A\delta > B > C$). At low stimulus intensities, only the larges axons are activated, but as the stimulus intensity is raised in steps, many small axons are progressively recruited and many peaks will appear on CAP trace. The conduction velocity of individual action potentials increases with axon diameter, thus, AP of largest axons will reach the recording electrodes first. (figure 3. 23, table 3.1).

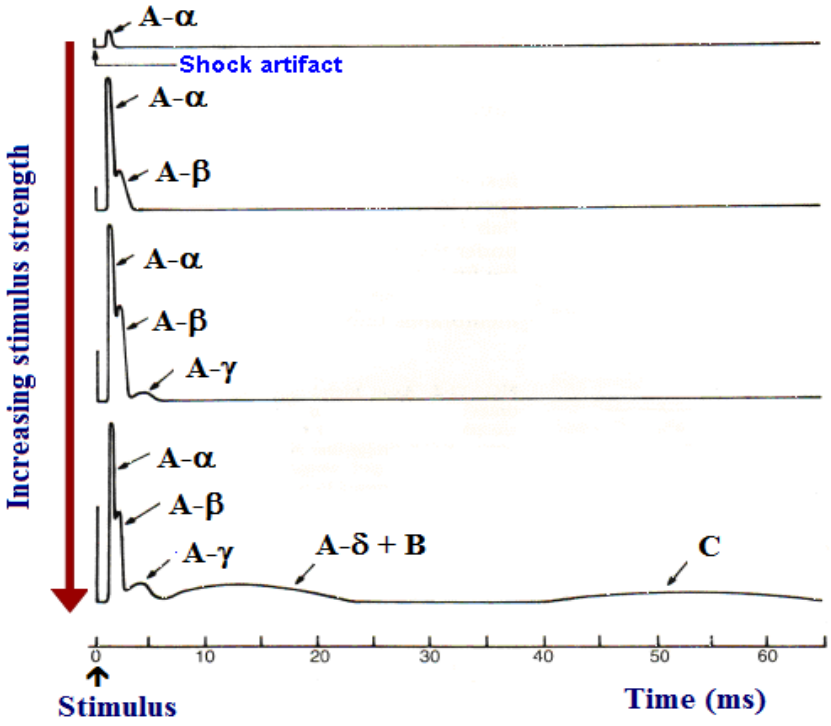


Figure 3. 23. Compound action potential (CAP) traces of the sciatic nerve determined electrical stimuli of increasing intensity applied to the nerve. The various components of the CAP are labelled where they appear.

Group / Type		Myelination	Diameter µm	Conduction velocity m/s	Function	Agents to which conduction is most susceptible
Erlanger & Gasser	Lyod & Hunt					
Aα	Ia, Ib	yes	13-20	70-120	Motor supply to skeletal muscles, Proprioception (Ia - sensory fibers, afferents of muscle spindles; Ib – afferents of Golgi)	pressure
A β	II	yes	4-13	25-70	Touch, pressure, kinesthetic sense, (secondary afferents of muscle spindles-innervate nuclear chain only)	pressure
A γ	-	yes	3-6	15-30	Motor supply to intrafusal fibers	pressure
A δ	III	yes	1-5	5-30	Pain, temperature, pressure, touch	pressure
B	-	yes	1-3	3-14	Preganglionic autonomic fibers	hypoxia
C	IV	no	0.2 - 1	0.2 - 2	Pain, temperature, pressure Postganglionic autonomic fibers	Local anaesthetics

Table 3.1. Classification of nerve fibers according to Erlanger & Gasser and the corresponding group in the classification of Lyod & Hunt (Lyod & Hunt consider only sensory fibers).

Practical importance of recording CAP:

- basic science: to find out how our nerves operate
- science applied to clinical problems: in nerve conduction studies, to detect/analyze nerve malfunction in patients suffering from neurological disorders.

3.6. Determination of rheobase and chronaxie

from strength-duration (intensity-time) curve

Rheobase and chronaxie are parameters of neuro-muscular excitability. These two are to compare excitability of different nerves or excitable tissues using electric stimuli (galvanic currents). Not any stimulus at any strength can get a response. For response to take place two factors are necessary: (i) minimum strength, and (ii) adequate duration of the stimulus (these two factors are inversely related to each other). The relationship between strength and duration of a stimulus has been studied by varying the duration of a stimulus and finding out the threshold strength for each duration. The record of results plotted gives the *strength-duration curve (figure 3.24)*:

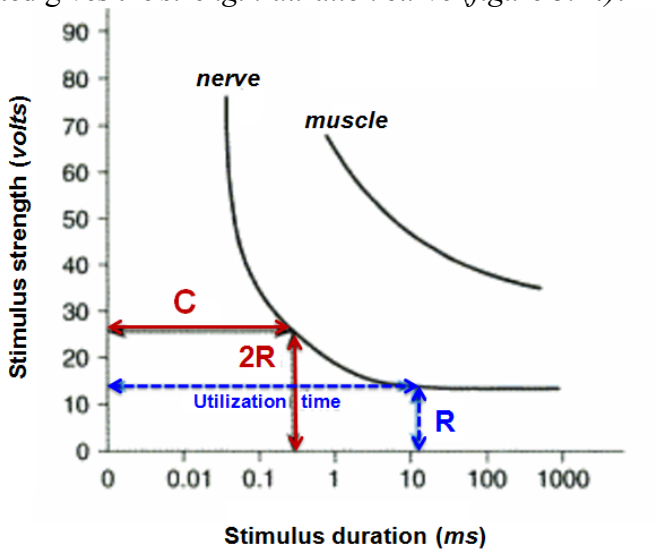


Figure 3.24. The strength-duration curve for the nerve and for the muscle (R = rheobase, C = chronaxie)

- **rheobase** refers to the
    - minimum intensity (strength, amplitude) of the electrical stimulus which if applied for adequate time (utilisation time) produces a response from the tissue, e.g., muscle or nerve. In practice, this minimum intensity it is called threshold, minimal or liminal stimulus.
    - minimum voltage of galvanic current which when allowed to indefinitely, will excite the tissue (by Lapique).
- **utilisation time** - time taken for excite the tissue when rheobase current is applied.
- **chronaxie** *(Greek: chronos, time, + axia, value)* refers to the
    - minimum time taken for response when twice rheobase current is applied
    - minimum needed by a stimulus with an intensity twice as high as the rheobase to elicit a response from an excitable tissue
    - shortest duration of a galvanic current which is twice as great as rheobase to excite the tissue when applied.
    - minimum duration of electric current required to induce muscular contraction at a voltage of two times the threshold voltage
- when a *stimulus of weaker intensity* than rheobase is applied, it will not produce a response whatever may be the duration of the stimulus; stimulus *with extremely short duration* will not elicit any response, no matter how intense it may be; in between, values of strength and duration are inversely related; this means that low intensity stimuli take longer time to excite an excitable tissue whereas stronger stimuli need shorter duration for the same.

Chronaxie is a measure of excitability; **shorter the chronaxie, more excitable is the tissue.**
Interpretation:
- Chronaxies differ in various species; in different tissues of the same species; in same group of tissues in the same body.
- Comparison of chronaxies of various excitable tissues:
    - in general, muscles have shorter chronaxies than nerves
    - myelinated nerves < unmyelinated nerve < skeletal muscle < smooth muscle < cardiac muscle
    - conducting system of the heart < cardiac muscle fibers;
    - nerve fibers of larger diameter have short chronaxie than short fiber of a small diameter. Somatic nerves are thick hence have shorter chronaxies. Autonomic nerves are thin hence have longer chronaxies;
    - the chronaxie of corresponding motor and sensory nerves are almost the same;
    - adults have shorter chronaxies than infants ;
    - pale fibers (fast-twitch, glycolitic) have shorter chronaxies than red fibers (slow twitch/ oxidative);
    - in the same limb the muscles with the same function have the same chronaxies;
    - chronaxie of the flexors = ½ chronaxie of the extensors;
    - in the same limb chronaxies of the distal muscles are longer as compare as chronaxies of the proximal muscles (forearm > arm; leg > thigh).
- temperature - cold increases and heat diminishes chronaxie within physiological limits
- tissue injury or degeneration diminishes chronaxie

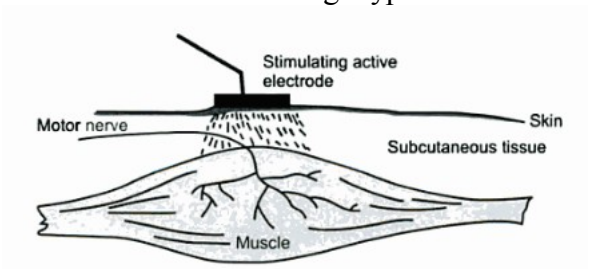
Practical significance:
1. In ***physiotherapy***, the strength / duration (S/d) curves are made for:
    a. *diagnostic* in order to determine the degree of a peripheral nerve lesions and the degree of denervation of the muscle tissue;

b. *therapy* because the the S/d curve gives information about the parameters for muscle stimulation (pulse duration, frequency, intensity of the current);
c. monitoring the progress of the lesion and denotes if the lesion is recovering / regressing.

2. In ***sport medicine***, chronaxie determination provides valuable information on their physical fitness, training and state of fatigue.
3. In ***cardiac pacing*** the strength / duration (S/d) curves is used for the proper programming of stimulus amplitude and pulse duration.

Technique:

- With a *neuromuscular stimulator* it is applied on motor points of the muscles rectangular impulses of various duration such as 0.1, 0.3, 1, 3, 10, 30, 100 ms, which may be constant current or constant voltage type.



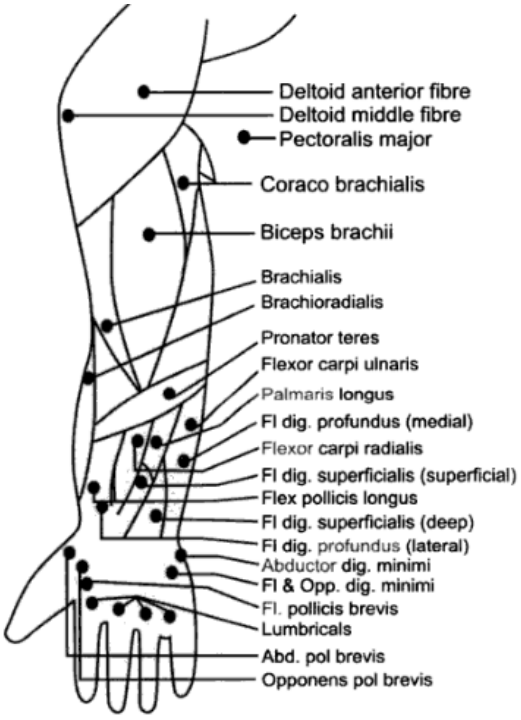


Figure 3.25. The motor point and the chart of motor points in the front of upper limb *(after de Nanda B, 2008)*

- *Motor point* is defined as point over the surface of the muscle where the main motor nerve enters it (with great density of terminal endplates). This is an area of low threshold excitability because of low skin resistance and good electrical conductivity. Through these points the muscle can be stimulated optimally at a relatively low intensity. There are charts with the exact location of the motor points in the muscles (figure 3.25).

- The negative electrode is placed on the motor point and positive electrode is placed proximally from the negative electrode.
- First is applied the stimulus with the longest duration and is observed visually the minimum perceptible contraction. It is noted the magnitude of the current / voltage required for the contraction (rheobase).
- (i) Gradually shorten the impulse duration and it is noted the corresponding current (voltage) required to get the same type of muscle contraction (chronaxie).
- *or* (ii) double intensity of the rheobase and set the time scale at the shortest duration (0.1 milliseconds). Gradually lengthen the impulse duration until the same type of muscle contraction is observed and it is noticed the time (chronaxie).
- The active electrode is kept over the same point over the muscle during the test. It is plotted the strength-duration cure from the results of this test by putting the durations (ms) along the abscissa and the corresponding strength (mA or V) along the ordinates.

Normal values:

- *Rheobase:* 10-20 volts for a constant voltage or 2-10mA for a constant current stimulator
- *Chronaxie*: < 1ms (0.08 – 0.32 ms) for a constant current and < 0.1ms for a constant voltage stimulator

3.7. Electromyography

Definition: refers to the technique of recording the electrical activity of the muscle by placing electrodes on the skin or inserting electrodes into the muscle. The recorded graph is called electromyogram (EMG).

Materials required: the electromyography recording set up includes: data collection electrodes, amplifier system and recording display system.

A. The data collection electrodes - 2 different types:

a. surface or skin electrodes are placed at 4 cm between them (one on the muscle surface and the other on the tendon) and they collect data about the potentials of all muscle fibers, thus they record a *global EMG*. These electrodes are flat, they have a button- or plate-like appearance and they are applied directly on the skin.

b. needle electrodes (intramuscular) collect data on the activity of a single motor unit that is almost isolated from the potentials of the neighbouring fibers (*motor unit EMG*).

B. Amplifier system produces an increase in the order of tens of microvolts and is capable of selective amplification, i.e. it only amplifies the electrode current and removes the currents coming from remote elements.

C. Display system: oscilloscope, chart recorder, audio for collected muscle potentials; provides data about shape, amplitude, duration and frequency of biocurrents (figure 3.26).

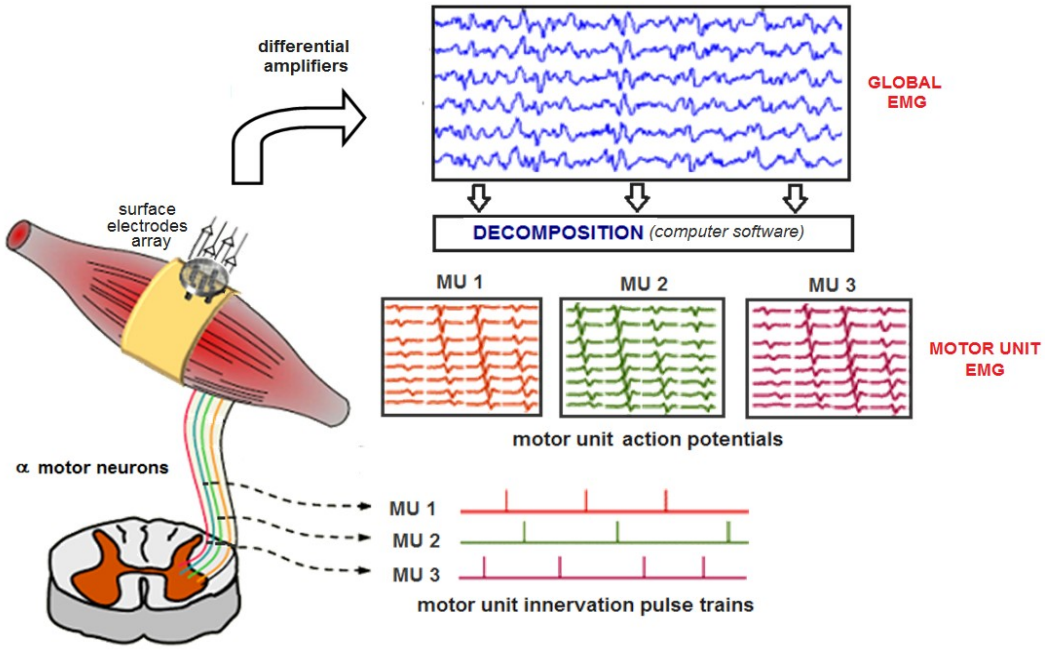


Figure 3.26.
The diagram of generation, detection (left), acquisition (top) and decomposition (bottom right) of multichannel surface electromyography signals.

Technique:

- patient in restful position (spontaneous movements can disturb the measurement) at a comfortable room temperature (23^0, to avoid shivering or sweating), in a faraday room
- the electrodes are placed on the skin / inserted into muscle and then are connected by cables to the myograph
- the muscle electrical activity is recorded in different conditions: during rest or graded voluntary effort in agonist / antagonist muscles (contraresistence)

- the trace obtained depends on the number /size of activated motor units of investigated muscle.

Normal EMG (figure 3.27):

A. at rest *no bioelectric activity* is detected ("electric silentium" state, A), except for:

- the insertion activity when a needle electrode is inserted into a muscle and it is recorded a discharge of action potentials of short duration / small amplitude. The insertion activity is prolonged in denervated muscles and absent when muscle is not viable.
- end plate noise is recorded as a negative monophasic potential after the cessation of insertion activity.

B. during minimal voluntary contraction, a few motor units discharge: some action potentials are recorded – *simple recording* (B)

C. with progressive increase of voluntary effort, the firing rate of small units increases until it reaches a certain frequency, when larger units are recruited giving a moderate force of contraction - *intermediary recording* (C, many peaks, butt distinguishable from one another)

D. during maximal contraction, most of the motor units discharge and a very high number of potentials are recorded; the graphic components belonging to one particular motor unit cannot be distinguish - *interference recording*(D)

E. during maximal contraction against resistance, a sinusoidal interference pattern appears – synchronous activity of spinal motor neurons – Piper rhytm (E)

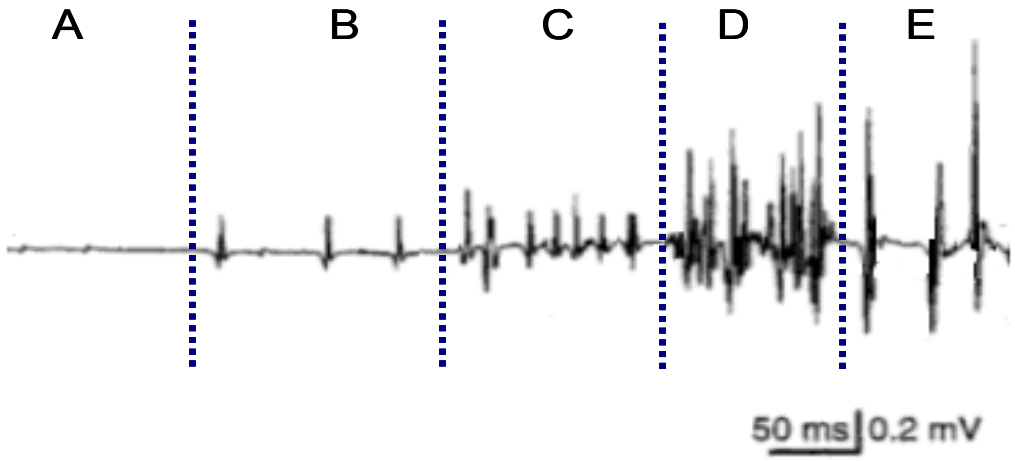


Figure 3.27. Types of electromyographic recording (A - isoelectrical activity, B – simple recording, C – intermediary recording, D – interference recording, E - Piper rhytm)

Clinical significance of EMG:

Electromyography is a useful tool for nerve and muscle function in order to:

- determine extent of disease and monitor progress
- measure dysfunction and propose solutions
- study normal anatomy and physiology

Usually electromyography is used to differentiate myopathies and neuropathies.

Clinical applications of electromyography:

- diagnosis of neuromuscular diseases (neuropathy, myopathy);
- evaluation, diagnosis in work medicine and ergometry studies (to measure muscle force exerted during physical activity);
- in sports medicine to measure muscle strength and onset of fatigue during exercise;
- in rehabilitation and physiotherapy to diagnose and monitor neuro-muscular disease

The parameters of muscles potential could be modified by a primary injury in the muscle fiber, by a motor neuron or motor nerve injury, or by local metabolism alterations. The pathological aspects of the EMG may be classified in myogenic or neurogenic recordings, and those generated by endocrine-metabolic disorders (figure 3.28):

- **neurogenic trace** occurs in case of spinal motor neuron (central neurogenic path) or motor nerve (peripheral neurogenic path) diseases such as radiculitis, neuritis or trauma. It is characterized by: the occurrence of action potential while at rest; the occurrence of greater amplitude and duration potentials both at rest and during contraction.
- **myogenic trace** occurs in primary muscle fiber diseases such as myasthenia, myodystrophy, etc. It is characterized by: the absence of electric activity while at rest or interference trace with polyphasic potentials of lower amplitude and duration, in addition to specific electric manifestations like *myotonic discharges* and *myasthenia*
- EMG trace in **endocrine-metabolic disorders** like spasmophilia. It is characterized by: repetitive double or triple electric potentials at rest, or potentials occurring spontaneously or only after path activation by compressing the limb in question using the sphygmomanometer band at mean blood pressure, for 10 min, in addition to other 5 minutes of hyperpnea.

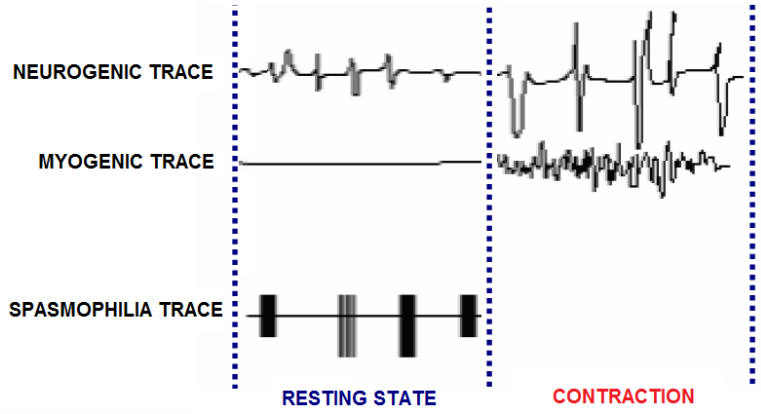


Figure 3.28. Pathological aspects of elecetromyographic recordings

4. BLOOD PHYSIOLOGY

4.1. Body fluids and compartments

Water is a major constituent of the body and comprises approximately 60% of an adult's body weight and as much as 70-80% of a child's weight. The remaining body weight is taken up with proteins, fats, sugars and minerals which, when distributed within the water, form the body fluids. The body fluids are contained in two compartments (figure 4.1):

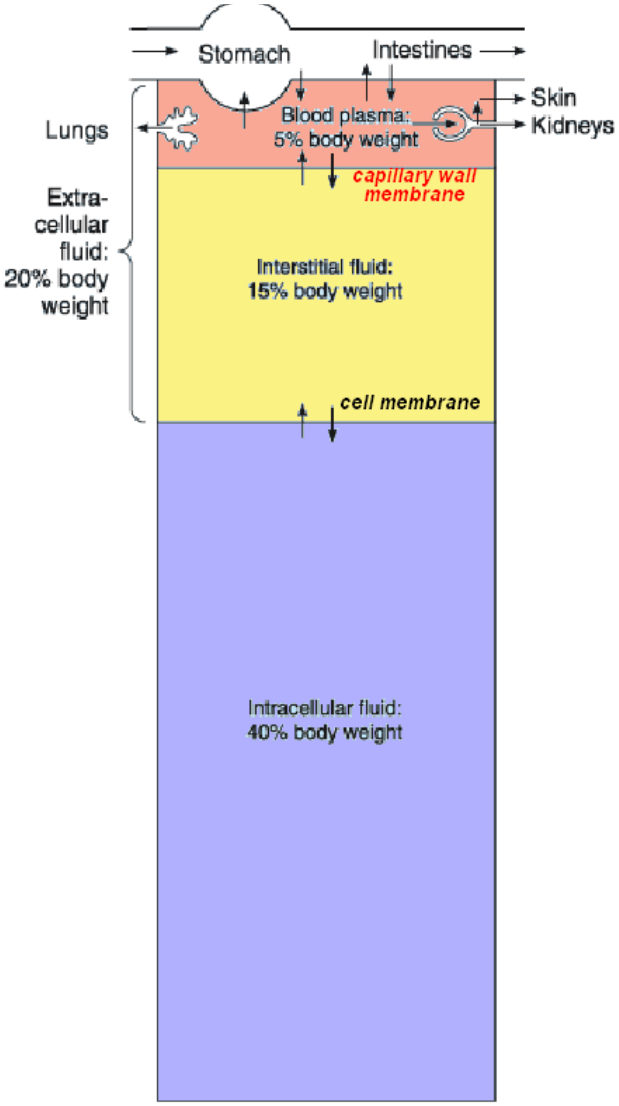


Figure 4.1 Body fluids compartments

1. **Intracellular Fluid (ICF)** - 2/3 of the body's water. If your body has 60% water, ICF is about 40% of your weight. The ICF a solution of potassium and organic anions, proteins etc. under control of cellular metabolism and cell membranes.
2. **Extracellular Fluid (ECF)**
   i. 1/3 of your body's water; ECF is about 20% of your weight
   ii. is primarily a NaCl and NaHCO3 solution
   iii. is subdivided into 3 subcompartments:
      i. **Interstitial Fluid (ISF)** surrounds the cells - 3/4 of the ECF
      ii. **Blood circulating plasma** - 1/4 of the ECF
      iii. Transcellular fluid represents 1-2 liters located in the peritoneal, pleural, pericardial cavities, as well as cerebrospinal fluid, ocular fluid, joints fluid, digestive secretions, etc. The main characteristic of this compartment is that significant gains or losses do not occur on a daily basis.

4.2. Functions and composition of blood

Blood is a specialized fluid connective tissue that contains cells suspended in a fluid matrix.
Functions of the blood include:

1. Distribution functions
   a. Carries O2 (from lungs) and nutrients (from GI tract and body stores) to all cells

b. Carries wastes from all cells to elimination sites (lungs for CO2; kidneys for nitrogenous wastes
c. Carries hormones (chemical signals) from endocrine organs to target tissues.

2. Regulatory functions
   a. Regulates body temperature by absorbing and distributing heat (e.g. heat loss via skin if hot; heat retention to brain and other vital organs via shunting)
   b. Maintains body fluid pH by its many buffers
   c. Maintains adequate body fluid volumes.
3. Protective functions
   a. Prevents blood loss by initiating clotting mechanisms in response to blood vessel damage
   b. Prevents infection via WBCs and plasma immune proteins.

Composition of blood:

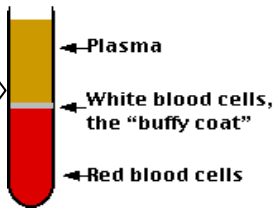
1. plasma: liquid matrix consisting of 91-92% **water** and 7-9% **dry residue** (7% **plasma proteins** – albumins, globulins, fibrinogen – 0.9% **anorganic compounds** like electrolytes – Na, K, etc – **organic compounds** like non-protein compounds of nitrogen (urea, creatinine, uric acid, ammonia and amino acids) and **hormones, antibodies, enzymes** (amylase, proteases, lipases, esterase)

2. formed elements:

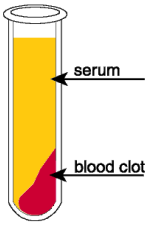
- **red blood cells (RBCs, erythrocytes)**; most abundant blood cells, essential for transport of respiratory gases in blood
- **white blood cells (WBCs, leukocytes);** less numerous than red blood cells, involved in body's defense mechanism
- **platelets (thrombocytes);** small, membrane-bound packets of cytoplasm that contain enzymes & other substances important to process of blood clotting; noncellular formed elements

Blood separation. Plasma *versus* serum.

Plasma is the supernatant fluid obtained when anti-coagulated blood has been centrifuged until RBCs settled to the bottom and WBCs settled on top of them forming the "buffy coat". Anticoagulants: heparin, oxalate or ethylenediaminetetraacetic acid (EDTA).



Serum is the liquid fraction of whole blood that is collected after the blood is allowed to clot. The clot is removed by centrifugation and the resulting supernatant is designated serum. Blood serum is blood plasma without fibrinogen or the other clotting factors (i.e., whole blood minus both the cells and the clotting factors).



Collection of blood specimens

1. Venipuncture (figure 4.2) is the process of collecting fresh whole blood from superficial vein, such as median cubital vein on anterior surface of elbow. It is a common sampling technique because superficial veins are easy to locate, walls of veins are thinner than those of comparably

sized arteries and blood pressure in venous system is relatively low; so puncture wound seals quickly.

- Use a tourniquet on the upper arm to produce venous congestion.
- Tell patient to close the fist in the designated arm and select an accessible vein (palpate the vein lightly with the index finger, moving an inch or two above and below the site so that you can determine the size and direction of the vein. The vein should feel like a spongy tube.)
- Disinfect puncture site, dry the site,
- Place the needle bevel up in line with the vein and pierce the skin at approximately a 15 to 45 degree angle. Place the needle bevel up in line with the vein and pierce the skin at approximately a 15 to 45 degree angle.Decrease the angle until almost parallel to the skin surface and direct it toward the vein. Pierce the vein wall.
- Blood will fill vacuum tubes automatically because of negative pressure within the collection tube. Instruct the patient to relax and unclench his fist after the needle has entered the vein.
- Remove the tourniquet before removing the needle from the puncture site.
- Remove needle. Apply pressure to avoid formation of a hematoma and put a sterile dressing strip on the site.
- Dispose of contaminated materials/supplies in designatedcontainers.
- Mix and label all appropriate tubes at the patient bedside.
- Deliver specimens promptly to the laboratory.

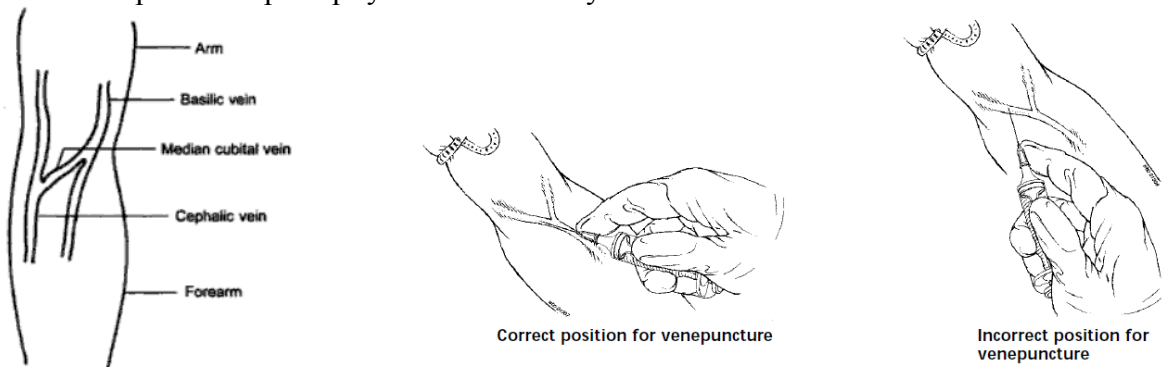


Figure 4.2. Collection of blood by venipuncture

2. Arterial puncture: arterial stick; may be used for checking efficiency of gas exchange at lungs
3. **Capillary (peripheral) blood** is preferred for a peripheral blood smear and can also be used for other hematology studies. Capillary blood is obtained from fingertips or earlobes (adults) or from the great toe or heel (infants).
- Disinfect puncture site, dry the site, and puncture skin with sterile disposable lancet no deeper than 2 mm.
- Wipe away the initial drop of blood.
- Collect subsequent drops in a microtube or prepare a smear directly from a drop of blood.

4.3. Hematocrit (packed cell volume, PCV)

Definition: The **hematocrit** (**Ht** or **HCT**) is the percent volume of the whole blood that is occupied by blood cells.
Principle: The sample of anticoagulated blood is drawn into a hematocrit tube and centrifuged. The Ht is the ratio of the volume of packed RBCs to the total volume (figure 4.3).
Technique: Ht is measured by automated and manual techniques. The two manual methods of direct measurement of PCV are used:
1. Macro method using Wintrobe's tube
2. Micro method using capillary tubes

1. Macro method or Wintrobe's method

Materials required:

- *Wintrobe's haematocrit tube* is a special thick walled glass tube 11 cm long, with an internal diameter of 2.5 mm and a flat inner base. It is calibrated at 1 mm intervals to 105 - 110 mm and holds about 1 ml blood.
- *Venous blood anticoagulated* with EDTA or heparin, freshly or kept at 4° C for up to 24 hours can be used.
- *Technique:*

  Thoroughly mix the blood in the bottle by repeated inversion or by a mechanical rotator.

  Draw the blood into a long Pasteur pipette. Fill the Wintrobe's tube up to the 100 mm mark, starting from the bottom and gradually withdrawing the pipette as blood is expressed. This is to avoid air bubbles, which can get trapped in the column of blood.

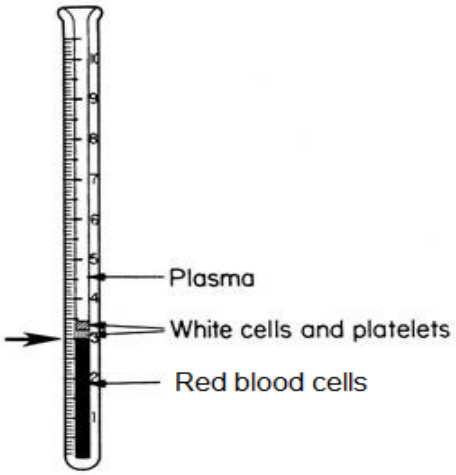


Figure 4.3. Hematocrit

Centrifuge the tube at 2000 – 2300 G for 30 minutes

Reading and interpretation:

- The height of the column of red cells is taken as the PCV i.e., the volume occupied by the red cells expressed as a fraction of the total volume of blood.
- **The buffy coat layer** is an approximate indication of the number of WBCs and platelets. Normally 0.1 mm of this layer corresponds to about 1000 WBC/cu mm. When there is marked leukocytosis (more than 30.000 WBC/mmc) - 0.1 mm then corresponds to about 2000 WBC/ cu mm. Occasionally an increase in buffy coat could be due to marked increase in platelets.
- **Plasma colour:** deep yellow colour of plasma indicates jaundice; opaque plasma is due to lipaemia; pink colour denotes haemoglobinemia.

Normal values:

- Men: 46.5% ± 5 %;
- Women: 42% ± 5 %;
- New born: 56% ± 5 %;
- Children: 40% ± 5 %;

Physiological and clinical significance

1. Ht is a reliable index of RBCs population in the blood. Ht is directly proportional with the number of RBCs and their hemoglobin content. Thus, Ht it is used to detect conditions in which RBCs increases (polycythemia) or decreases (anaemia).

2. The Ht value it is used in determination of **erythrocyte indices**, especially mean corpuscular volume (MCV, mean cell volume of one RBC = HCT/red cell count = 80 -100fL) and mean corpuscular hemoglobin concentration (MCHC, mean hemoglobin concentration in all RBCs = hemoglobin conc/HCT= 320-360 g/L). Erythrocyte indices help in the diagnosis and classification of various type of anaemia.

3. Ht is one of the factors involved in blood viscosity. Ht varies directly proportional with blood viscosity.

Conditions that alter the hematocrit

A. Conditions that decrease hematocrit

Physiological - pregnancy (due to haemodilution)
- excess water intake
- sex predilection (lower in women)

Pathological - various types of anaemia
- conditions associated with haemodilution and increase in plasma volume

(hyperaldosteronism; after massive perfusions – represent un exception since absolute red cell volume may be normal)

B. Conditions that increase hematocrit

Physiological - high altitude (due to hypoxia)
- newborrns
- excessive sweating (due to hemoconcentration)

Pathological - decreased O2 supply to the tissue (congenital heart disease)
- polycytemia
- conditions associated with hemoconcentration (severe vomitind and diarrhea) due to dehydration or loss of plasma / fluid which may occur after severe burns.

C. Normal Ht with decreased absolute RBCs volume in *hemoconcentration after acute hemorrhages before interstitial fluid intravasation.* A Ht that is done immediately after a hemorrhage usually does not show the extent of RBC loss because at the time of the hemorrhage, plasma and RBCs are lost in equal proportions. However, within several hours after hemorrhage, plasma volume increases due to a shift of interstitial fluid into the vascular space. RBCs, however, cannot be replaced quickly, as the bone marrow takes approximately 10 days to produce mature RBCs. As a result, a Ht done several hours after a bleeding episode will be decreased because the plasma volume has compensated for fluid loss while the red blood cells that have been lost cannot be replaced for days.

Sources of Error

A. In centrifugation

Adequate duration and speed are very essential for a tight packing of cells. Error is due to plasma trapped in between the red cells (4-5%).

B. In the sample

· Increased amount of EDTA in the sample can lower the haematocrit due to shrinkage of cells.
· Inadequate mixing of blood.
· Sample left at room temperature for prolonged time.

C. Tubes

· Variation in bore size.
· Improper filling of the tube.

To correct these errors we use corrections factors:

$Ht_{corrected}$ = Ht (f1 x f2), where f1 = correction factor for each anticoagulant
(1.09 for sodium oxalate; 1 for heparine)
f2 = 0.95-0.96 due to plasma trapped in between RBCs

Also, arterial Ht < capillary Ht < venous Ht; Somatic hematocrite = 0.91 x venous hematocrite.

2. Micro haematocrit method

- *Capillary haematocrit tube* about 7 cm long with uniform bore size of about 1 mm is used
- Heparinized capillary tubes are used for capillary blood collection.
- Special centrifuge or centrifuge head to accommodate the capillary tube.
- 2/3 of the tube is filled with blood by capillary action. The empty end is closed by plasticine or moulding clay. The filled tubes centrifuged at the speed of 10,000 to 12,000 G for 5 minutes.

Reading

As the tubes are not graduated, they are measured using millimeter rule. The total length of blood including plasma and the length of red cell column are measured separately and the ratio calculated. A better and convenient way of taking the reading is by using the special chart provided by the manufacturer.

Advantages

1) Only small amount of blood is needed for the test.
2) Even capillary blood can be sampled.

3) Less time is needed for the test.
4) Easier procedure.
5) Amount of trapped plasma is much less.
Disadvantages
1) Ordinary centrifuge cannot be used, needs special and more expensive equipment.
2) Taking accurate reading is difficult as it requires magnifying glasses, special charts etc.
Sources of Error - similar to those of macro method.

3. Measurement of Ht in automated Instruments
This is the method employed in some electronic cell counters. It is calculated by multiplying MCV by red cell count. The values are slightly less than that of the macro and micro methods.

4.4. Erythrocyte sedimentation rate (ESR)
Definition: ESR is the rate at which RBCs settle down when the blood containing an anticoagulant is allowed to stand in a vertically placed tube. This test measures the length of the plasma column (in mm) that is separated from blood cells in a certain period of time, under the influence of gravitation.
Principle: Sedimentation of RBCs occurs due to rouleaux formation that means the tendency of the RBCs to pile one over the other like a pile of coins which is heavier and falling down quickly. Rouleaux formation does not occur in normal circulation under physiological conditions, as the moving cells show little or no tendency to adhere. This is a reversible phenomenon, promotes sedimentation of RBCs and it should not be confused with agglutination where the cells are irreversibly clumped.

Materials required: needle puncture, syringe of 2 ml, anticoagulant - sodium citrate 3.8g%, alcohol, cotton, Westergreen pipettes, stand for the pipettes, timer, and gloves. *Westergreen pipette* is an ended tube. It is 300 mm length with an internal bore of 2.5 mm. It has a scale on it in mm, from 0 to 200 mm. The division of 200 is at the bottom of the pipette and the division 0 is upwards. The graduated volume of the pipette is 1 ml. The pipetts are held vertically in the *Westergreen rack* after filling with blood, and the rack is provided with a rubber pads at the lower end and metal clips at the upper end (figure 4.4).

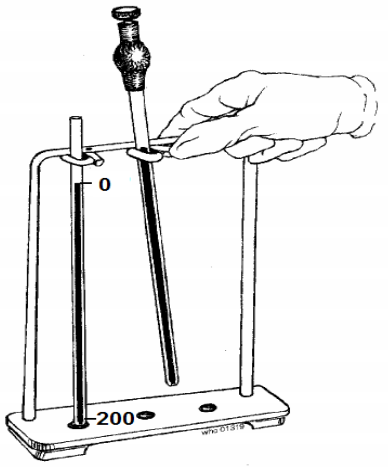


Figure 4.4. Westergreen method

Technique: **Westergreen method**
0,4 ml of a solution of sodium citrate are aspirated into a syringe of 2 ml. By way of a venous puncture 1,6 ml of blood are drawn. The mixture of blood (venous blood) and anticoagulant (Sodium Citrate 3.8%) in the ratio 4:1 is taken in a pipette and left undisturbed in a vertical position (note the time from the moment of aspiration of blood into pipette). The erythrocytes settle to the bottom, leaving a layer of plasma above. The length of the plasma column that is separated from the blood cells is measured after 1, 2, 24 hour(s). The distance is measured from the top of the plasma to the top of the RBCs and is expressed in mm/h.

Normal Values: Men:	2 to 7 mm (after 1 hr);	7 to 15 mm (after 2 hours).
Women:	4 to 9 mm (after 1 hr);	12 to 17 mm (after 2 hours).
In pregnancy:	35 mm (after 1 hr);	
Newborn:	0.5 mm (after 1 hr);	

Discussions:

Factors affecting ESR

1. Erythrocytes factors:

- *size of the rouleaux* – the larger the particle, the faster the fall
- *shape and number of the RBCs* – biconcave discs shape favours rouleaux formation. A change in shape, like in hereditary spherocytosis or sickle cell disease will decrease ESR in spite of anemia. Macrocytosis raises the ESR. Also, ESR is inversely proportional with the number of RBC.

2. Plasmatic factors:

- *albumin tend to low ESR meanwhile fibrinogen and globulins have the opposite effect*. Normally, the RBCs have net negative charges and, therefore, repel each other. Fibrinogen, positively charged, neutralize the charges of the RBCs and favor rouleaux formation and thus would raise the ESR. In some pathological conditions, in addition to fibrinogen, other plasma factors called acute phase-reactants (e.g. C reactive protein, alpha 1 antitrypsin, complement, etc) increase in blood. A high concentration in plasma immunoglobulin and the presence of paraprotein (like in multiple myeloma or Waldenstromís macroglobulinemia) also increase ESR.
- *viscosity*: ESR is increased when the viscosity of blood decrease and vice versa.

Data interpretation/ Clinical significance:

Physiological variations of ESR:

- Age. ESR is less in infants and old people compared to young adults
- Sex. ESR is greater in females compared to males
- Menstruation. ESR is slightly raised during menstruation in females
- Pregnancy. ESR is raised in pregnancy from 3rd month to parturition and returns to normal after 3 to 4 weeks of delivery.

Pathologic variation of ESR:

- ESR is accelerated in acute and chronic inflammatory response, neoplasia, myocardial infarction.
- ESR it is diminished in all situations associated with poliglobulia, decreased fibrinogen levels (disseminated intravascular coagulation)

The erythrocyte sedimentation rate (ESR) is an easy, inexpensive, nonspecific laboratory test commonly used to detect conditions associated with acute and chronic inflammation, including infections, cancers, and autoimmune diseases. ESR is said to be nonspecific because increased results do not tell the doctor exactly where the inflammation is in the body or what is causing it, and also because it can be affected by other conditions besides inflammation. For this reason, the ESR is typically used in conjunction with other tests to aid the diagnosis and follow-up the evolution of different clinical situations.

4.5. Blood cell count

Definition: the determination of the number of each type of blood cell in a given sample of blood blood expressed per mm^3 or per μL of blood.

Principle: blood cell count with a microscope in a known volume of liquid, diluted in a known proportion. Blood dilution is achieved using an isotonic with blood cells counted and lytic for the other formed elements of blood.

The blood cells count represents a common investigation in clinic. It may be achieved by two methods: manual (microscopical) and automated (electronic) methods performed by analyzers. Automated cell count gives higher precision and consumes less time. Manual counting

has the advantage in that it can identify blood cells that may be misidentified by an automated counter. It is, however, subject to human error, and has a much smaller sample size.

Further, it will be presented the manual method for counting of blood cells.

4.5.1.Red blood cell count

Materials required: diluting fluid (Hayem solution), Potain pipette for erythrocytes/trombocytes, Bürker-Türk counting chamber, microscope glass slides, sterile needle, cotton, alcohol, filter paper, gloves (figure 4.5).

Bürker-Türk counting chamber has four longitudinal grooves in the central third of a rectangular and thick base plate made of special optical glass. The grooves are parallel to the short sides of the base plate and the central third has the same size as the coverglass used with the counting chamber. The central support and the two external supports are ground smooth and polished. The surface of the central support is deeper than that of the two external supports. The counting nets are engraved in the central support (chamber base). If a cover glass is placed on the external supports, The depth between the cover glass and the central support is 1/10 mm (depth of the counting chamber). Each counting surface has an area of 9 mm^2 delimited by triple lines and is divided into 9 squares of 1 mm^2 (all delimited by triple lines, too). The square (1mm2) from the middle of the net it is called Thoma grid. Its surface is divided by triple lines in 16 big squares (with the side of 1/5mm and the area of 1/25mm^2). Each of the big square is subdivided by simple lines in another 16 small squares (each with an area of 1/400 mm^2 and the side of 1/20 mm). Thoma grid it is used to count red blood cells and platelets.

Lower and upper squares, right and left sides from Bürker-Türk chamber are divided into 16 squares, separated by double lines, each square with area 1/25 mm^2. These surfaces are used for counting white blood cells.

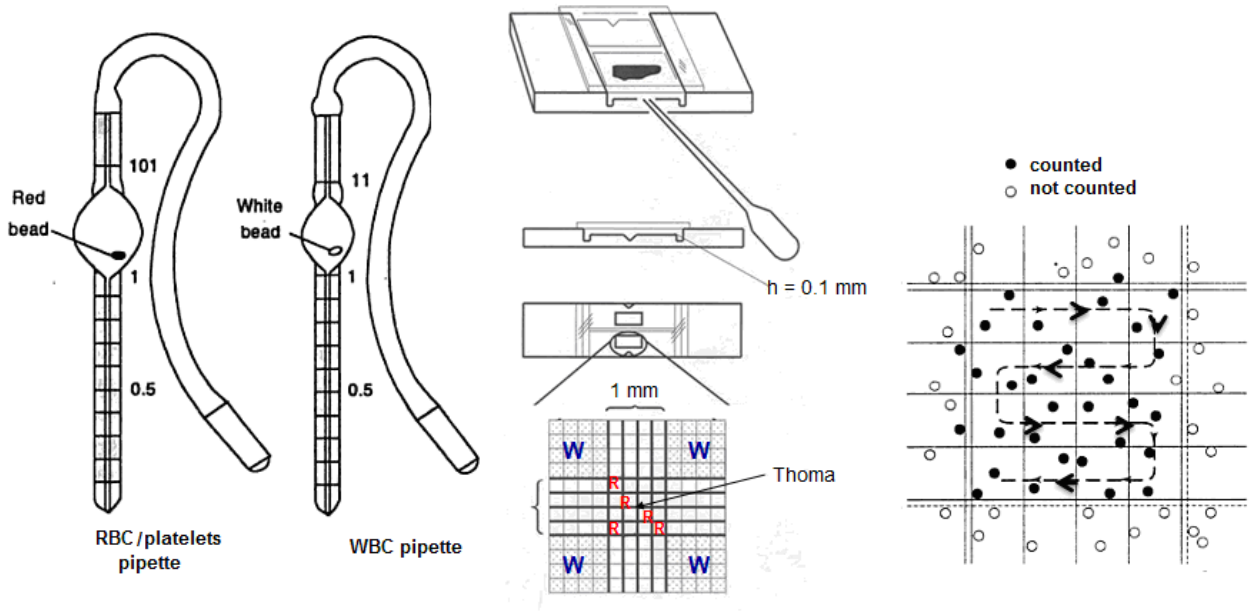


Figure 4.5. Blood cell counting set up

Dilution fluid - Hayem's Solution: (HgCl2, NaCl, Na2SO4, H2O) isotonic with the RBCs, lytic for WBCs and platelets.

The *Potain pipette* is made of a graduated capillary tube, with a bubble of dilution, which

contains a red bead. A rubber tube with a mouthpiece is attached to the top-end of the pipette. The capillary portion is divided in ten units from zero to one and above the bubble the division 101 is marked. The volume of the capillary portion is one hundred times smaller than the volume of the bubble portion. By aspirating the blood until the mark 0.5 or 1 and the diluting solution until the division 101, a dilution of blood of 1/200 and 1/100 is done.

Technique:

Cleanse the counting chamber and cover glass with a piece of cotton saturated with alcohol and let air dry. Prepare the counting chamber by placing the cover glass on its mounting supports. The finger is disinfected with alcohol, let air dry and then is pricked with a sterile needle. The first drop of blood is removed and the second drop of blood is aspirated into the Potain pipette until the mark 0,5 or 1, the remaining volume is occupied with Hayem solution until the mark 101. Thus a 200 or 100-fold dilution is obtained (the volume of the bubble chamber is 101 µl–1 µl=100 µl, containing 0.5 or 1µl of blood). Close the ends of the pipette with your thumb and middle finger and shake the pipette gently for 2- 3 minutes. Throw away the first two drops from the pipette (they come from the capillary part of the pipette and contain only diluting fluid) and with the third drop fill the counting chamber: let the drop fall near the cover glass and the chamber will fill due to capillarity. A properly filled chamber will have diluted blood filling only the space between the cover glass and the counting chamber. No blood should flow out onto the moat. Allow RBCs sedimentation for 2-3 minutes. Place the counting chamber in a light microscope and examine the sample using low-power objective (10 x) and weak light. Count the red blood cells in 80 small squares of 1/20 mm (the five squares from the corners and a square from the middle). To avoid over counting, count the RBCs that touch the lines on the left and top sides only.

Calculation of the RBC number: it is performed according to the following formula:

$$NoRBC = \frac{N}{n \times D \times V} = \frac{N}{80 \times \frac{1}{200} \times \frac{1}{400} \times \frac{1}{10}} = \frac{N}{80} \times 200 \times 400 \times 10 = N \times 10000 \text{, where}$$

N = number of RBCs counted in 80 small squares (surface of 1/400 mm2)

n = the number of small squares = 80

D = dilution of blood (1/100 or 1/200)

V = the volume of a small square (1/400 mm^2 X 1/10mm = depth of the chamber)

Replacing these values into the formula one obtains NoRBC = N x (5000 or 10000)/mm3 (depending on the dilution)

The RBC count is expressed per mm3 or per litre. On most of the bulletins its value is expressed per µL, but 1 mm3 = 1µL.

Normal values:

Men: 4.5 to 5 millions/mm3

Women: 4.2 to 4.5 millions/mm3

Children: 5 to 6 millions/mm3

Higher values – polycythemia:

– physiological: gender, age, high altitude, effort

– pathological: chronic bone marrow, lung or heart diseases

Lower values – anemia

– excessive bleeding

– decreased red blood cell production
– increased red blood cell destruction

4.5.2. White blood cell count

Materials required: dilution fluid (Turk solution), Potain pipette for WBCs, Bürker-Türk counting chamber, microscope glass slides, sterile needle, cotton, alcohol, filter paper, gloves (figure 4.5).

Türk's solution (glacial acetic acid 0.5 ml, gentian violet 1% 1.5 ml, distilled water ad 150 ml) is the diluting fluid. It lyses the red cells and platelets, leaving only the WBC visible.

The Potain pipette for diluting WBCs is similar to the Potain pipette for diluting RBCs. It is made up of a graduated capillary tube, with a bubble of dilution, which contains a white pearl. The capillary portion is divided in 10 units from 0 to 1 and above the bubble the division 11 is marked. The volume of the capillary portion is ten times smaller than the volume of the bubble portion. By aspirating the blood until the mark 0,5 or 1 and the diluting fluid until the mark 11, a dilution of blood of 1/20 or 1/10 is achieved (figure 4.5).

Technique: The finger is disinfected with a cotton swab soaked in alcohol and then pricked with a sterile needle. The first drop of blood is removed and the blood is drawn into the Potain pipette until the mark 0.5 or 1, the remaining volume being completed with Turk solution until the mark 11. Any slight excess of blood, over the 0.5 mark can be withdrawn with a blotting paper. Close the ends of the pipette with your thumb and middle finger and gently shake the pipette for 2- 3 minutes.

The counting chamber and the cover glass must be cleaned. The cover glass is applied on the chamber.

Discarded the first two drops from the pipette (they come from the capillary part of the pipette and contain only diluting fluid) by allowing it to drop onto a piece of paper towelling and with the third drop fill the counting chamber. A tiny drop of blood is deposited on the surface of the counting chamber next to the edge of the cover glass. The blood penetrates by capillarity into the space between the chamber and the cover glass. Pay attention not to let air penetrate the underneath of the cover glass.

Allow WBCs sedimentation for 2-3 minutes and then examine the sample on the microscope using low-power objective (10 x) and weak light. Count the white blood cells in 25 big squares considering the cells inside the squares and from two sides.

Calculation of the WBC number:

$$NoWBC = \frac{N}{n \times D \times V} = \frac{N}{25 \times \frac{1}{20} \times \frac{1}{25} \times \frac{1}{10}} = \frac{N}{25} \times 20 \times 25 \times 10 = N \times 200$$, where

N = number of RBCs counted in 80 small squares (surface of 1/400 mm2)

n = the number of squares = 25

D = dilution of blood (1/10 or 1/20)

V = the volume of a square (1/25 mm^2 X 1/10mm = depth of the chamber)

Replacing these values into the formula one obtains NoWBC = N x (100 or 200)/mm3 (depending on the dilution)

Normal values:

Adult	4000 - 8000/mm3
Newborn	15000 - 30000/mm3
Children up to 1 year	9000 - 15000/mm3
Children up to 6 years	4000 - 10000/mm3

Higher values is termed leukocytosis:

- physiological: after effort, after meals, exposure to altitude, cold temperatures, stress, women: menstruation, pregnancy.
- pathological: infectious diseases, inflammatory diseases, infarction, leukaemia, cancer, intoxications (uraemia, diabetes, Pb poisoning) and bleeding.

Lower values is termed leucopenia:

- physiological: elderly people and starvation.
- pathological: viral infections (hepatitis, chikenpox, small pox, measles, rubeola), typhoid fever, toxoplasmosis, some types of leukaemia, Biermer anaemia, intoxication with benzene, bone marrow failure, metastasis in the bone marrow, after exposure to radiation (X rays) and some drugs;

A WBC lower than 500/mm3 places the patient at risk for a fatal infection; WBCs over 30000/mm3 indicates massive infection or leukaemia.

Functions of leukocytes – see the lecture

4.5.3. Platelets count

Platelets, also called thrombocytes, are counted according to the same method as RBCs and WBCs.

Materials required: thrombocyte diluting solution – Winthrobe solution (containing ammonium oxalate, Na2EDTA, distilled water), Potain pipette for erythrocytes/trombocytes, Bürker-Türk counting chamber, microscope glass slides, sterile needle, cotton, alcohol, filter paper, gloves.

Technique: is the same as for counting RBCs. Prepare the counting chamber and obtain the appropriate blood dilution into the Potain pipette (with red pearl). Fill the chamber and allow platelets to sedimentate for 15 minutes. The chamber is laid on the microscope. Platelets are counted on the same surface as RBCs, namely from 80 small squares (1/400mm2).

The obtained number is multiplied by 1000, if it was drawn up to the mark 0.5 or by 500 if the blood was aspirated until the mark 1 of the Potain pipette.

Normal values: 200000 to 300000/mm^3

Higher values – thrombocytosis:

- physical activity (it is transitory), haemorrhage, bone marrow diseases

Lower values – thrombocytopenia

- bone marrow failure (infiltrative bone marrow disease such as: carcinoma, leukaemia), alcoholic or drug intoxication, vitamin B12 deficiency, increased platelet destruction due to drugs or immune disorders (thrombocytopenic purpura), medication, idiopathic

4.6. Hemoglobin determination

Definition: Estimation of the hemoglobin concentration in a sample of blood expressed in g/dl.
Manual hemoglobin determination by *Sahli method* is a visual method, inexpensive, usually practiced in hematology laboratories in clinical medicine and for performing practicals for students in physiology.
Principle: the hemoglobin contained in a known quantity of blood is converted into acid hematin by means of hydrochloric acid. The colour is then visually compared with a standard tube containing acid haematin of known concentration.
Materials required: Sahli's haemoglobinometer (haemometer), distilled water, 0.2N HCl, dropping pipette, cotton balls, alcohol, sterile needles, rubber gloves (figure 4.6).

Sahli's haemoglobinometer (haemometer) contain a comparator, hemoglobin tube, hemoglobin pipette and stirrer. All the components of the haemometer are named Sahli.
The comparator is a hard rubber or wood stand backed by an opaque white glass plate to provide uniform illumination; the stand holds on the sides, 2 non-fading standard brown tinted glass tubes for colour matching and at the middle of the stand is a slot which accommodates the hemoglobin tube. The colour of the standard glass tubes correspond to the color of a solution of 1% hydrochloride of haematin (acid hematin), equivalent a blood whose hemoglobin content is 16g%.

Hemoglobin tube it is graduated on the side in gram per cent (g%, grams in 100 ml solution, i.e. g/dl) from 2 to 22, and on the other side as percentage (%) from 10 to 140.

Hemoglobin pipette is a glass capillary tube with rubber tubing and mouthpiece and it bears only one mark indicating 20 µL (0.02ml).

Stirrer is a thin glass rod used for stirring the solution in hemoglobin tube.

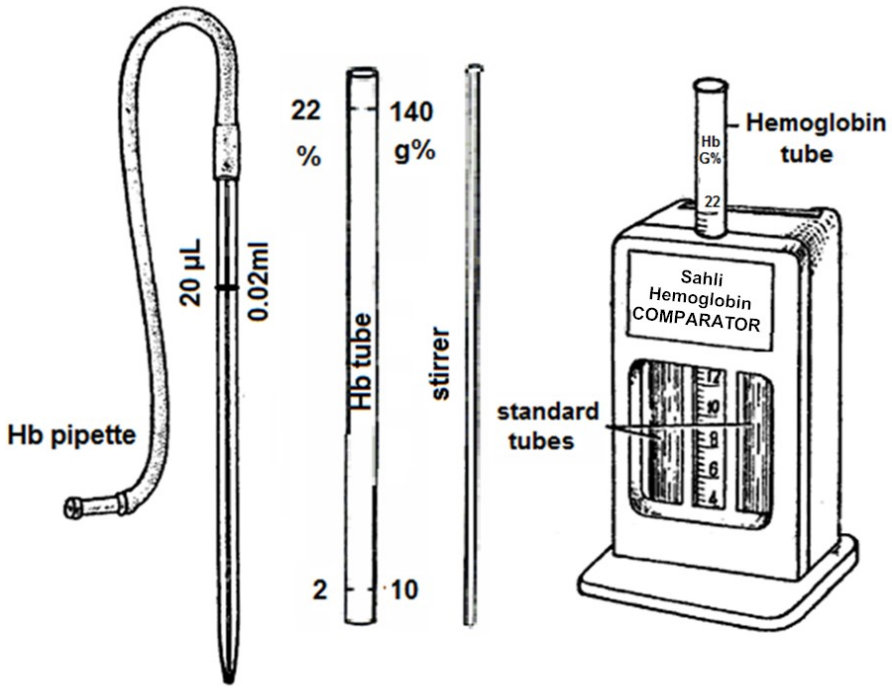


Figure 4.6. Hemoglobin determination kit by Sahli method

Technique:

- Fill the hemoglobinometer tube using a simple glass graded pipette with N/20 HCl up to its lowest mark (10% or 2g%);

- Prick the finger and discard the first drop of blood. Allow a large drop of blood to form on the fingertip and then dip the tip of the Sahli pipette and suck blood up to 20 μL mark of the pipette. Wipe the tip of the pipette.
- Transfer the 0.02 ml of blood from the pipette into the hemometer tube by immersing the tip of the pipette in the N/20 HCl solution and blowing out blood from the pipette. Rinse the pipette 2-3 times by drawing up and blowing out the solution.
- Place the glass tube in the comparator for 7 minutes at 20ºC (for maximum conversion of hemoglobin to acid hematin)
- Add distilled water drop by drop and mix it with the stirrer until the color of the solution exactly matches that of the glass pieces standard. When you compare the colour of the solution in the tube with that of the standard, keep the stirrer above the solution, but do not take it out from the tube because the solution sticking to the stirrer will be lost and will give a false low result.
- Read the scale on the tube at the level of the lower meniscus of the fluid.

Normal values:

Men	14-18 g/dl
Women	12 to 16 g/dl
Newborn	18 to 27 g/dl
Baby up to 6 month	10 to 15 g/dl
Children up to 1 year	9 to 15 g/dl
Children up to 6 years	12 to 17 g/dl

Decrease and increase of hemoglobin concentration usually accompanies higher or lower values of red blood cells. Beside quantitative differences there are important qualitative alterations as well, these are called hemoglobinopathies.

4.7. Blood spectroscopy

Principle: Spectroscopy is based on the difference between the absorbtion spectra of hemoglobin and its related compounds.
Materials required: spectroscope with direct vision, tubes with hemoglobin and derived solutions
Technique and results (figure 4.7):
1. oxyhemoglobin spectra (OHb)

- prepare diluted blood solution (0.2ml blood in distilled water till 10ml)
- in contact with the air the blood turns bright red (Hb gets saturated with oxygen.)
- put the tube with oxyhemoglobin in front of the spectroscope
- the oxyhemoglobin absorbtion spectra has 2 bands:
    - a wide yellow one (577 nm)
    - a narrow green one (536 – 556 nm)

2. Reduced hemoglobin spectra

- put in the oxyhemoglobin solution (10 ml) a couple of crystals of ammonium sulfide.
- the color turns dark red, due to the reduced Hb.
- on the spectroscope observe a single green yellow band = Stokes band (530-548 nm)

3. Carboxihemoglobin spectra

- Carboxihemoglobin is formed by the coordinative binding of CO to hem's iron. It is a reversible
- reaction. The affinity of Hb for CO is 210 times higher then for O2. If we breathe CO will result intoxication: 40% carboxihemoglobin constitutes a lethal value.
- bubble in the tube with oxyhemoglobin solution (10 ml) gas from a Bunsen burner or - put in the oxyhemoglobin solution (10 ml) a couple of crystals of ammonium sulfide.
- with the spectroscope observe 2 absorption bands D and E located on the right of the oxyhemoglobin:
  - A yellow one (564-579 nm)
  - A green one (530-548 nm)
  - both of them have a violet trend.

4. Methemoglobin spectra

- put 5-6 blood drops in 20 ml water.
- add 8-10 drops of saturated potassium fericyanide solution. The solution gets dark brown due to the formed methemoglobin.
- with the spectroscope observe 4 absorption bands:
  - A narrow red one (620-630 nm) – specific for met hemoglobin.
  - 2 pale yellow green bands
  - a wide blue one (486-578 nm)
- Methemoglobin is improper for oxygen transport – a certain degree o hypoxia is reached

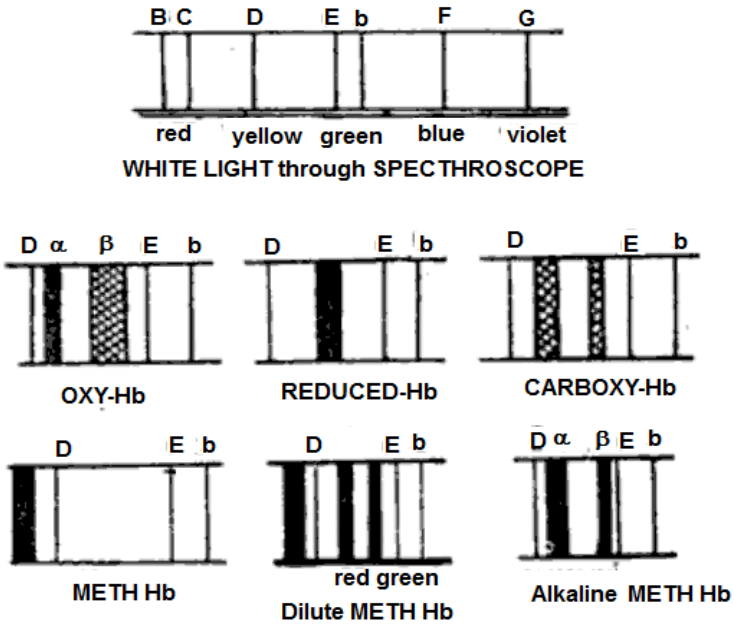


Figure 4.7. Blood spectroscopy

4.8. Blood detection

Definition: Blood detection consists in identification of blood in cases of suspected blood stains or occult haemorrhages.

The tests used for blood identification are:

1. Benzidine test
2. Guaiac rezin test
3. Teichmann's haemin crystal test

1. Benzidine test:

Principle: hemoglobin may act as a peroxidase in the presence of hydrogen peroxide and it give color reaction by oxidation of benzidine.

Materials required: concentrated acetic acid, hydrogen peroxide water 3%, oxyhemoglobin solution, test tubes, benzidine crystals or benzidine solution 0.3%

Technique:

- test tube 1: 2ml acetic acid + benzidine crystals + 2 ml hydrogen peroxide water 3% + 1 drop oxyhemoglobin solution. In the test tube will result a blue or blue-green color.
- test tube 2: 3-4 drops of benzidine solution 0.3% in acetic acid 50% + 3-4 drops hydrogen peroxide water 3% + 1 ml oxyhemoglobin solution. In the test tube will result a blue or blue-green color.

2. Guaiac rezin test

Principle: hemoglobin may act as a peroxidase in the presence of hydrogen peroxide and it give color reaction by oxidation of guaiac rezin.

Materials required: guiac tincture, hydrogen peroxide water 3%, oxyhemoglobin solution, test tubes

Technique: in a test tube add 2ml oxyhemoglobin solution + 15-20 drops guiac tincture + 15-20 drops hydrogen peroxide water 3%. In the test tube will result a blue color.

3. Teichmann's haemin crystal test

*Principle:*in the presence of NaCl and concentrated acetic acid, when heated, hemoglobin transforms into hematin hydrochloride, which crystallizes by cooling that are viewed under a microscope.

Materials required: concentrated acetic acid, crystals of NaCl, oxyhemoglobin solution (diluted blood 1/500-1/1000), microscope slide, glass slide cover, gas burner, microscope.

Technique:

- Put 1 blood drop on the slide, add few crystals of NaCl and cover with coverslip.
- Let a drop of concentrated acetic acid fall on the lateral side of the coverslip and the space between microscope slide and coverslip will fill due to capillarity.
- Heat it until it evaporates and the probe gets a brown colour due to acid hematine.
- Cool it slower in order to get bigger crystals
- Put it under the microscope and examine with 5-7 magnification

Figure 4.8. Haemin crystals

Result: yellow or dark brown rhombic crystals of hemin or hematin arranged single or in clusters are seen if blood is present (figure 4.8).

Clinical significance: Blood identification tests are sensitive, but nonspecific (do not provide information about the species) and they are used in

- forensic medicine in order to distinguish bloodstains from other red color stains-
- clinical medicine to detect occult haemorrhages (!!*see definition*) (e.g. stool guaiac test was originally the principal colon cancer screening technology available)

4.9. Red blood cells parameters

There are two types of RBC indices:
- direct: RBCs count, hemoglobin and haematocrit
- indirect: - they are calculated by the automated analyzer from the first ones
  - mean corpuscular/cell volume (MCV), mean corpuscular/cell hemoglobin (MCH), mean corpuscular/cell hemoglobin concentration (MCHC), color index (CI)

All these parameters are reported as part of a standard complete blood count

1. Mean corpuscular/cell volume (MCV)

- is the average size/volume of an individual RBC
- it is calculated by dividing the hematocrit by the number of RBCs existing in a certain volume of blood, according to the next formula: **MCV = (Ht x 10) / RBCs, where Ht is expressed in % and RBCs in millions / mm^3.**
- the unit of measure of the MCV is the **femtoliter (fL, 1fl = 10^{-15}L) or cubic micrometer (µm^3).**
- normal values: **85 – 95 fL (µm3).**
- *discussions:*
  - lower values (MCV < 80 fL) characterises RBCs called *microcytes* and the condition is named *microcytosis*.
  - higher values (MCV > 95 fL) characterises *macrocytes*, and the condition is called *macrocytosis*.
- *clinical significance:* the MCV allows the morphological classification of anaemias in microcytic or macrocytic. The most common causes of microcytic anaemia are: iron deficiency (due to inadequate iron intake, pregnancy, menstruation loss, grastrointestinal bleeding, cancer), thalassemia. Macrocytosis occurs in: vitamin B12 and folic acid deficiencies, liver diseases and alcoholism

2. Mean corpuscular hemoglobin (MCH)

- is a measure of the average amount of hemoglobin contained in a RBC.
- it is calculated by dividing the hemoglobin level by the RBC count according to the next formula: **MCH = (Hb x 10) / RBCs, where Hb is expressed in g/dL and RBCs in millions / mm^3.**
- the unit of measure of the MCH is the picograms (1pg = 10^{-12}g)
- normal values: **27 – 34 pg**
- the increase of the mass of haemoglobin/cell is possible only by increasing the RBC volume. MCH is diminished in microcytic anaemia and raised in macrocytic anaemia.

3. Mean corpuscular hemoglobin concentration (MCHC)

- represents the average of hemoglobin concentration per unit volume of packed red blood cells
- it is established from the values of hemoglobin and hematocrit according to the next formula: **MCHC = (Hb x 100) / RBCs, where Hb is expressed in g/dL and RBCs in millions / mm^3.**
- the unit of measure of the MCH is the picograms (1pg = 10^{-12}g)
- normal values: **27 – 34 pg**
- the increase of the mass of haemoglobin/cell is possible only by increasing the RBC volume. MCH is diminished in microcytic anemia and raised in macrocytic anemia.

4. Color index (CI)

- represents the amount of haemoglobin in each red cell as compared with the haemoglobin content of a normal red cell.
- is the the ratio of the percentage of hemoglobin (compared to "normal") to the red blood cell count (compared to "normal") : **CI = Hb per cent / RBC per cent**
- the percentage of each is then calculated taking 14.5gm of haemoglobin/dl and 5 million of red cells/cmm as 100 percentage.
- Normal values: **0.85 – 1.10;** CI less than 0.5 indicates hypochromic anemia.

4.10. Differential white blood cell count (leukocyte formula)

Definition: percentage distribution of leukocytes in the peripheral blood.
Principle: the percentage of different types of WBCs (on a peripheral blood smear) is established after counting 200 WBCs.
Materials required: peripheral blood smear stained May Grunwald Giemsa, light microscope with oil immersion objective (90x), immersion oil (cedar tree oil)
Technique:

- examine the blood smear under lower-power objective for general scanning, assessing the quality of the smear and for studying the distribution pattern of the cells.
- place a drop of cedar oil on the slide in the center of the lighted area. Bring the 90x objective and touch with the lower end the drop of oil. Using the fine adjustment screw adjust the objective and focus the cells.
- scan the blood film in crisscross examining both the center and the sides of the smear. Lymphocytes have the tendency to to group toward the center, monocytes and polymorphonuclear cells disperse to the edge and in the fringe
- search for 200 successive WBCs and identify them. Establish the the percentage of different types of WBCs.

Normal values:

- differential WBC count can be expressed in relative (%) and absolute values (/mm3).

	%	**/mm3**
GRANULOCYTES		
Neutrophils	60 to 70%	3000 to 8000/mm3
Eosinophils	1 to 4%	300 to 400/mm3
Basophils	0 to 1%	20 to 80/mm3
AGRANULOCYTES		
Lymphocytes	25 to 35%	1500 to 2400/mm3
Monocytes	4 to 8%	240 to -600/mm3

There are five types of WBCs in the peripheral blood. Three of them (neutrophils, eosinophils and basophils) have granules in their cytoplasm; these leukocytes are designated as granulocytes. The lymphocytes and monocytes lack granules and are called agranulocytes.

Alteration of the ratio of different white blood cells:

cell type	*increase*	*decrease*
neutrophil	neutrophilia	neutropenia
eosinophil	eosinophilia	eosinopenia
basophil	basophilia	basopenia
lymphocyte	lympocytosis	lymphopenia
monocytes	monocytosis	monocytopenia

Clinical significance: Differential count is vital for diagnosis of a number of blood-related diseases involving WBCs and RBCs. Its primary use is to identify changes in number of WBCs and in addition to find morphological abnormalities of blood cells, and the presence of blood parasites.

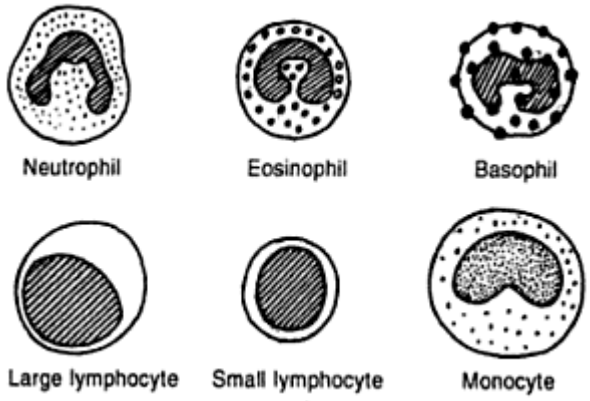


Figure 4.9. WBC (leucocytes) types

Discussions: !!! The morphological characteristics, **the functions of each type of WBCs** and conditions that alters leukocytes formula will be discussed on the lecture.

4.11. Blood typing, blood transfusion

Definition: A blood type (also called a blood group) is a classification of blood based on the presence or absence of inherited antigenic substances on the surface of red blood cells (RBCs).

There are more than 30 blood groups systems containing about 400 antigens. Clinical significance has only 2 systems, the ABO and the Rh system, because are more likely to cause blood transfusion reactions than others.

In the ABO system there are two antigens, also called agglutinogens (A, B) and two antibodies or agglutinins (α, β). In the blood of a person the agglutinogen does not coexist with the homologous agglutinin (rule of Landsteiner). The presence or the absence of the different antigens (Agglutinogens – A, B) determines the blood type (O, A, B, AB). When the type A agglutinogen is not present on the red blood cell membrane, anti-A antibodies (agglutinins alpha) develop in plasma. Similarly, when type B agglutinogen is not present in the red blood cell membrane, anti–B agglutinins (agglutinins beta) develop in plasma.

Blood type	Antigen	Antibody	% population
O	--	--	36%
A	A	β	41%
B	B	α	16%
AB	A, B	--	7%

Principle is based on agglutination reaction that will occur when agglutinogen will react with the corresponding agglutinin when they meet each other.

The determination of blood groups of the ABO system can be done by identification either of agglutinogens from the RBC's membrane, using some test sera (hemotest) whose agglutinins are known (method Beth-Vincent), or of agglutinins from the plasma, using some erythrocytes (test erythrocytes), whose agglutinogens are known (method Simonin).

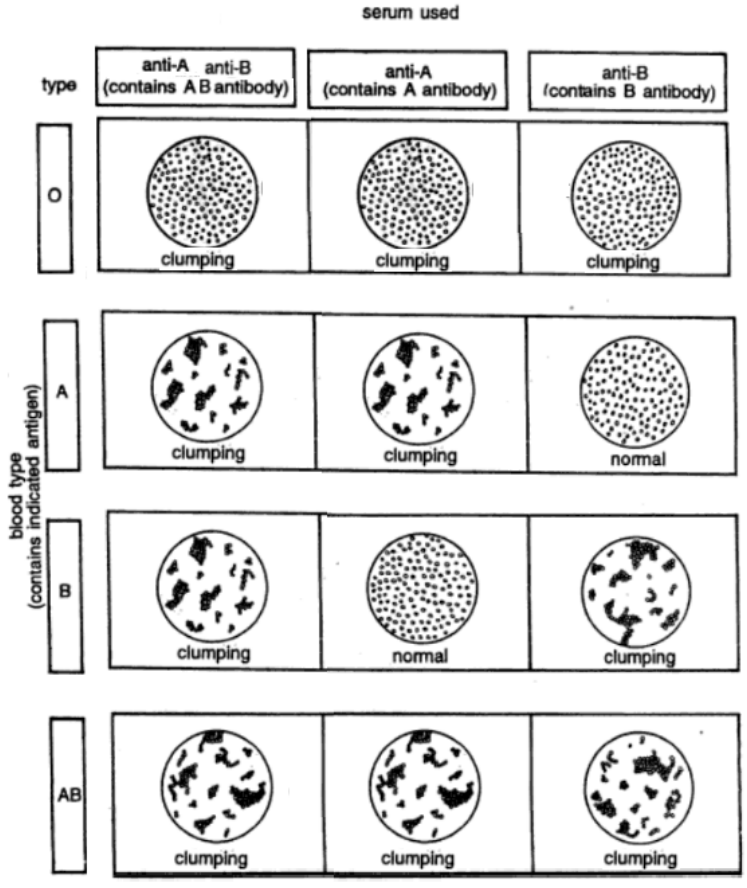
1. Beth – Vincent method (figura 4.10)

Principle: Standard sera (having known agglutinins) are put into contact with a sample of blood, whose agglutinogen is going to be established. In the presence of adequate agglutinins RBCs agglutinate.
Materials required: Slides, sterile needles, cotton, alcohol, test sera: anti-A (it contains agglutinins alpha), anti-B (it contains agglutinins beta), anti-A, B (it contains both types of agglutinins – alpha and beta), microscope slides, blood and surgical gloves.
Technique:

- On a glass slide put 3 drops of different test sera.
- The finger is disinfected with a cotton ball with alcohol, then is pricked and the first drop of blood is removed; the second drop some blood is taken using the corner of a microscope slide.
- That drop of blood is mixed with the first drop of serum (anti-A). The corner of the slide is changed and another drop of blood is taken, which is mixed with the second serum (anti–B). Then the corner is changed again and a drop a blood is mixed with the third drop of serum (anti-A,B). The ratio between the volume of serum and blood must be 20/1 – 10/1.
- According to the drops where the agglutination occurs, the blood group can be established. The reaction can be observed after 2-3 minutes of mixing.

Results:

- agglutination does not appear in anyone of the three drops - the blood does not contain any of the two antigens = blood group O.
- agglutination appears in the drops with anti A and anti A+B agglutinins = blood group A
- agglutination appears in the drops with anti-B and anti A+B agglutinins = blood group B
- agglutination appears in all three drops = blood group AB



Error causes:

- sera stored under inappropriate conditionsor expired can react non-specifically
- the ratio between the drop of serum and blood is not observed
- poly agglutination occurs in the presence of cold agglutinins in the serum and that can cause a false reaction, any blood type can be easily identified as type AB
- when there is a weak A or B antigen subtype, the antigen may not properly be identified, hence A blood type easily seems to be O, similarly AB blood type can falsely be identified as B blood type.

Figure 4.10. Blood groups determination by Beth Vincent method

2. Simonin method

In this method the agglutinins existing in the plasma are unknown and they are going to be determined. Test erythrocytes (having known agglutinogens) are used. The principle is the same as in the previous method.

The determination of blood groups of the Rh system can be done by identification of agglutinogens from the RBC's membrane, using test sera (hemotest) with agglutinin anti-D.

In the Rh system there are six antigens (C, D, E, c, d, e) and there are no antibodies (agglutinins). C, D and E antigens are products of dominant genes; c and e are products of recessive genes.

The most important antigen is antigen D (or Rh antigen from the monkey Macaccus Rhesus), which can be found on the RBC membrane of 85% of the population. Persons having antigen D on the RBC's membrane are named Rh positive (Rh+) and those not having this antigen (even if they have the other antigens of the Rh system) are named Rh negative (Rh-).

In the Rh system, normally there are no plasma agglutinins against antigen D. In Rh negative persons, spontaneous anti-D antibodies (IgG type) do not occur. In order to get that, the person should be first exposed to an Rh antigen (D), which usually happens after immunization through blood transfusion (Rh + blood is transfused to an Rh- person) or during childbirth / abortion when placenta is expelled (Rh negative mothers carried an Rh positive foetus).

Principle: agglutination reaction between an antigen D and the anti-D antibody.

Materials required: specific serum containing anti-D antibodies, microscope slides, for blood sample collection: cotton balls, alcohol, sterile needles, rubber gloves.

Technique:

- a drop of anti Rh serum is placed on a slide
- cleanse the tip of the finger with a piece of cotton saturated with alcohol, puncture and collect a small drop of blood and mixed with the serum.
- move the slide back and forth to see easier the grainy texture of the agglutinated blood.
- the ratio between blood and serum is 1/10- 1/20.

Result: If the blood is Rhpositive, agglutination occurs, if it is Rh negative agglutination does not occur.

Blood transfusion

The clinical importance of blood typing is to set up the compatibility of a blood transfusion. The transfusion is always isogroup (in ABO system) and isoRh. The donor and the receiver must have the same blood groups (ABO) and the same Rh.

A universal donor of blood is a person with O Rh-, but the universal receiver is AB Rh positive.

The general scheme of blood transfusion compatibility is shown in the figure 4.11.

In case of a small volume of transfused blood (up to 500 mL) (emergency), important is the donor`s agglutinogen and recipient`s agglutinis, as the donor`s plasma is diluted in the blood stream of the receiver. When the volume of transfused blood is higher than 500 mL, the transfusion must be isogroup, isoRh, in order to avoid the chance of a transfusion reaction. If incompatible blood is transfused, agglutinins from the recipient`s serum react with the transfused RBCs, producing their agglutination inside the blood stream and finally their haemolysis.

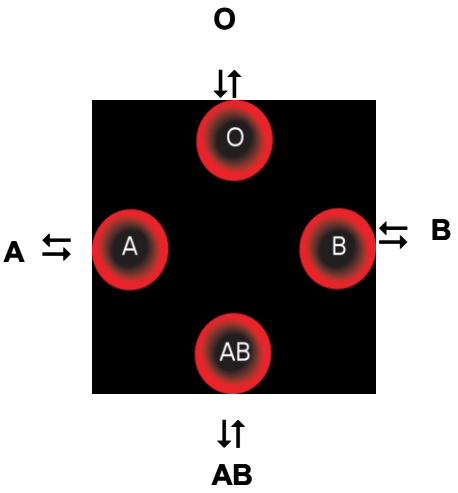


Figure 4.11. Blood transfusion compatibility

4.12. Physiological hemostasis

Hemostasis is a complex process that causes the bleeding process to stop. It can be classify in physiological, surgical and medicamentous hemostasis.
Physiological hemostasis: all mechanisms used by the body to defend against the bleeding from small vessels**.** The process can be divided into *primary and secondary* components and it is initiated when trauma or diseases disrupt the vascular endothelial lining and blood is in contact with subendothelial connective tissue.

1. *Primary hemostasis* is characterized by vascular contraction (decrease the loss of blood as immediate response of small blood vessels to injury) and platelet plug formation (temporary, soft hemostatic plug) occurs due to adhesion, aggregation and granule release reaction. The most used tests which investigate the primary hemostasis involve the the platelet count, test of capillary fragility or by the Rumpel- Leede test (to identify blood vessel wall disorder) and bleeding time test.
2. *Secondary hemostasis* refers to cascade of coagulation that ultimately results in the conversion of fibrinogen to fibrin monomers that are cross-linked into insoluble strands who stabilize the loose platelet clot formed in primary haemostasis. Than the the clot retracts and is slowly dissolved during restoration of the vascular wall. Secondary haemostasis can be explored by global tests (used as screening tests: coagulation time) and tests that explore the intrinsic pathway (partial thromboplastin time) or the extrinsic pathway (prothrombin time) *(for details – see the lecture)*

4.12.1. Bleeding time

Definition: Duration of bleeding induced after dermal puncture with a skin needle.
The bleeding time is measured by Duke method.
Principle: a standard dermal puncture is made and the time the puncture bleeds is measured
Materials required: filter paper, chronometer, cotton balls, alcohol, sterile needle (or lancet), rubber gloves.
Technique:

- cleanse the tip of the finger or ear lobe with a piece of cotton saturated with alcohol. Let the finger air dry.
- make a dermal puncture (~ 3mm deep) on the ear lobe or fingertip in order to cause bleeding (blood should flow freely without squeezing).
- start the chronometer in the moment of puncture
- gently blot the drop of blood (without touch the finger) with filter paper every 30 seconds and place each subsequent drop a little further along the side of the filter paper. Note that the drops become progressively smaller.
- stop the chronometer as soon as bleeding ceases.
- count the number of drops on the filter paper and multiply it by 30 seconds.

Normal values: 2- 4 minutes.
Interpretation / Clinical significance: Cessation of bleeding indicates the formation of the temporary hemostatic plug. Bleeding time depends on the number and function of the platelets, and also by the integrity of the capillaries. Prolongation of bleeding time occurs in: thrombocytopenia (platelet count is less than 50000/mm3 of blood.), platelet dysfunction (Glanzmann's thrombasthenia), vascular defects (allergic, autoimmune purpura), Von Willebrand's disease.

4.12.2. Capillary fragility test (Rumpel - Leede test)

Principle: It measures the resistance or the fragility of the capillary wall at the level of the forearm, by counting the petechiae formed by applying a positive pressure.

The ability of the capillary wall to remain intact under an increased intracapillary pressure is controlled by a blood pressure cuff around the patient's upper forearm.

Materials required: sphygmomanometer to determine patient blood pressure

Technique: Determine the systolic and the dyastolic blood presssure of the patient. Using a pneumatic cuff, a pressure of about 10 mmHg (below the systolic pressure) is applied around the forearm and maintains it for 5 minutes. Then on a circular surface of diameter 2 cm below the edge of the cuff we count the number of red spots.

Results: Simple positive test: 10 to 12 spots (+)

Highly positive test: 20 spots (++)

Pathologic test: 80 spots (+ + +)

Discussions: A positive test indicates an increased fragility of the capillary walls or a platelet defect. It may occur in such conditions as thrombocytopenia, thrombasthenia, von Willebrand's disease, vitamin K deficiency, etc. Conditions unrelated to bleeding defects, such as: scarlet fever, measles, influenza, chronic renal disease, hypertension, and diabetes mellitus with coexistent vascular disease may also increase capillary fragility. An abnormal number of petechiae sometimes appears before menstruation and in some healthy persons, especially in women over the age of 40.

4.12.3. Coagulation time

It is a global test to explore the coagulation. It has relevance when it is prolonged, but one cannot establish where the deficiency is (on the intrinsic, extrinsic or common pathway).

Definition: Coagulation time represents the time between the activation of the coagulation cascade to the formation of insoluble fibrin filaments.

The coagulation time is measured by 2 methods: Lee and White method (in the test tube) and Bazarov method (on glass slide).

1. Lee and White method

Principle: the time taken by the venous blood to clot in a glass tube at 37°C is noted as coagulation time.

Materials required: materials for drawing blood by venipuncture, test tubes, chronometer, waterbath at 37°C

Technique:

- collect 2 mL of venous blood by venipuncture
- remove the needle from the syringe, feel each of the 2 test tubes with 1 mL blood and place them in the waterbath at 37°C
- start the chronometer in the moment of puncture
- every 1 minute remove the first tube from the waterbath and tilt it gently to 45° to check if the blood has clotted. When the blood clots, the tube can be tilted to an angle of 90° without flowing the content. Note the coagulation time for fist tube and than examine the second tube.
- Usually in the second tube the blood clots later compare to the first one. Note the time for the second tube.

Normal values: - in the 1st test tube: 6 to 10 minutes

- in the 2nd test tube: 8 to 12 minutes.

2. Bazarov method

Principle: represents the time from the collection of blood until its complete coagulation.

Materials required: materials for drawing capillary blood, chronometer, glass slide, Petri plate lined with wet filter paper, saline solution 9‰, dropper.

Technique:
- prepare Petri plate: place the glass slide inside Petri plate lined with wet filter paper and let 1 drop of saline solution 9‰ on the glass slide.
- cleanse the tip of the finger with a piece of cotton saturated with alcohol. Let the finger air dry. Using a sterile, disposable needle, quickly make a single puncture on the finger, deep enough so that blood flows freely from the wound. Remove the first drop of blood.
- start the chronometer in the moment of puncture.
- let the second drop of blood fall over the drop of saline solution 9‰ on inside Petri plate
- at every minute the coagulation is check by tilting the slide. When the blood is coagulated, the hemispheric shape of the blood drop is maintained during the tilting of the slide.
- stop the chronometer and note the time

Normal values: 8 to 10 minutes
Interpretation / Clinical significance:
- Coagulation time is prolonged in diseases in which clotting factors are deficient. It signifies a state of hypocoagulability of the blood and a bleeding diathesis.
- Coagulation time is shortened in thrombocytosis, etc. When it is reduced it reflects a hypercoagulability of the blood and the risk of thrombosis.

4.12.4. Prothrombin time (Quick time)

This test evaluates the extrinsic and common pathways of coagulation.
Definition: The prothrombin time (PT) measures the clotting time from the activation of factor VII, through the formation of fibrin clot.
Principle: the test measures the clotting time of plasma in the presence of an optimal concentration of tissue extract (thromboplastin) and calcium.
Materials required: materials for drawing venous blood, chronometer, anticoagulant (sodium citrate), test tubes, pipettes, water bath at 37°C, thromboplastin, calcium chloride solution M/40
Technique:
- Blood is drawn through a venous puncture into a test tube containing sodium citrate (9:1).
- Plasma is separated by centrifugation of the blood for 5 minutes at 1500 rpm
- deliver 0.2 mL of plasma into a test tube placed in a 37°C water bath and add 0.2 mL of thromboplastin. Wait 1 minute to allow mixture to warm.
- add the same volume of pre warmed calcium chloride (0.2 mL) and start the chronometer
- At every two seconds the appearance of white floccules of fibrin is checked.
- The time needed for fibrin formation is recorded.

Normal values: 12 – 16 seconds
Interpretation / Clinical significance:

The prothrombin ratio (PR) represents the patients prothrombin time reported to a control prothrombin time, and is reported in percents: 85-100%

The international normalized ratio (INR) was devised to standardize the results. Each manufacturer assigns an ISI value (International Sensitivity Index) for any tissue factor they manufacture. The ISI value indicates how a particular batch of tissue factor compares to an internationally standardized sample. The INR is the ratio of a patient's prothrombin time to a normal (control) sample, raised to the power of the ISI value for the analytical system used.
$INR = (PTtest/PTcontrol)^{ISI}$. *Normal value = 1*, but in case of *anticoagulation, 2-4*.
PT it is used to monitor oral anticoagulation.

Common causes for **prolonged prothrombin time** are: liver diseases, vitamin K deficiency, hypofibrinogenemia, administration of oral anticoagulant, deficiency of one of the factors of the prothrombinic complex (II, VII, IX, X).

Prothrombin time it is **normal in haemophilia.**

5. EXCRETORY PHYSIOLOGY

5.1. 1. Collection of urine

1. Early morning urine (clean catch midstream collection)

- more concentrated and acidic compared to the later samples, and suitable especially for urinalysis
- commonly obtained by a *spontaneous micturition* into a clean, dry, and capped container performed after thorough washing and wiping of the external urethral orifice. The middle flow of urine is usually for analysis (the initial flow is always contaminated with cells and bacteria from around the external urethral orifice).
- in special situations the urine sample is obtained by *percutaneous suprapubic puncture of urinary bladder, or by urinary bladder catheterisation.*
- it is recommended to examine the urine sample within 2 hours since its collection or the urine should be kept refrigerated or chemically conserved
- Label the container and note the collection date and time on the label.

2. Random urine sample

- used in emergency medicine

3. Time collection of urine

- for quantitative analyses and estimation of clearance of various substances, the urine must be collected during a defined period of time
- *short-term urine collection* (1–3 hours)
- *long-term collection* per 12–24 hours.
- the urine is collected into clean containers, placed in a cool and dark place; a preservative reagent may be added.
- the volume of all urine is measured (with precision to ml), the urine is mixed, and at least 5 ml sample is taken and transported to the laboratory, together with data about the time of start and end of the collection period (with precision to minutes), as well as about the precise volume of urine.

5.1. 2. Physical properties of urine

1. Color (figure 5.1)

- *the color of urine is usually described after visual inspection with common color terms.*
- at emission: pale yellow due to pigments content (urochrome, urobilin, porphyrin). (but darker in the morning – *why?)*
- yellowish brown/orange/green - increased bilirubin, biliverdin or foods (e.g., beets, asparagus, blackberries), ingested dyes (e.g., food coloring) or methylene blue (used as a stain or dye for various diagnostic tests).
- red/brown/black - hematuria; should clear following centrifugation (which may indicate injury to the urinary tract); hemoglobinuria, myoglobinuria (crush syndrome)
- orange urine may be caused by medications (e.g., pyridium [used to treat urinary tract infections], warfarin, laxatives), B complex vitamins, and carotene (found in yellow vegetables).
- milky white, caused by chyle (chyle consists of lymph and droplets of triglyceride, is a milky fluid taken up by lacteal vessels from the food in the intestine during digestion)

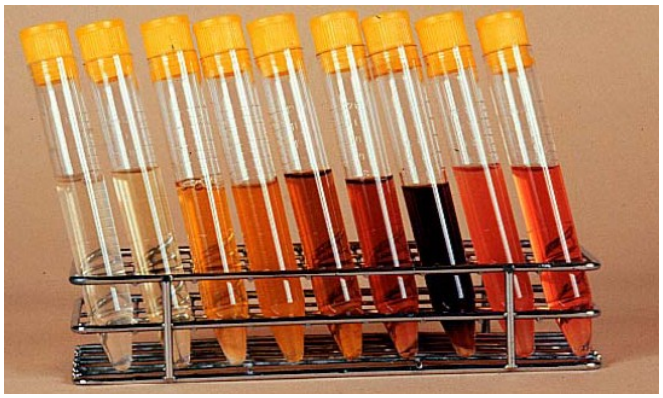


Figure 5.1. Test tubes containing urine of various colous.

2. Transparency / turbidity:

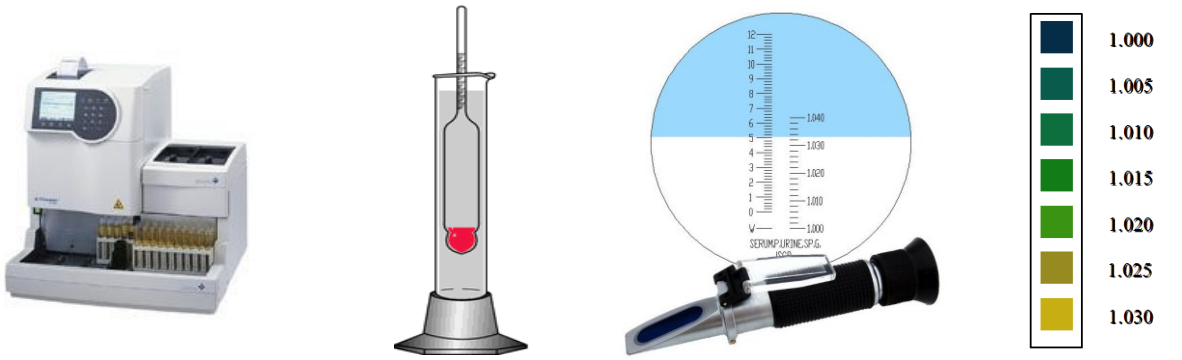
- normally freshly voided urine is clear and transparent.
- when urine is allowed to stand, amorphous crystals may precipitate and **cause urine to be cloudy:** phosphates – clears if 10% acetic acid is added; urates – clears by heating; oxalates – clears if HCl is added
- pathologically, urine can appear cloudy when substances such as excess salts (phosphates, urates, carbonates, oxalates), pus (piuria), lipids (lipiduria) or bacteria are present.

3. **Odor:**

- fresh urine has a distinctive odor due to volatile acids; odor can be strong in concentrated specimens, does not imply infection
- **foul-smelling urine** is a common symptom of urinary-tract infection; **ammoniacal odor** is given by alkaline fermentation
- **fermented apples** (presence of ketone bodies, diabetes mellitus), **fetid** (severe infections of urinary bladder, recto-vesical fistulae), **putrid** (urinary bladder cancer)
- *the odor of the urine is determined by placing ones nose near the orifice of the sample container, moving the air from the container to your nose by gently wafting with your hand, and gently breathing the fumes.*

4. Specific gravity (urine density)

- it reflects the ability of the kidneys to concentrate the urine (conserve water).
- varies directly with the amount of solids dissolved in the urine and inversely proportional with hydration status and dieresis
- normal values: **1.015 to 1.030** g/cm3 during a 24-hour period **(ISOTHENURIA).** Pathologically, isosthenuria is seen in terminal stage renal failure (loss of urine concentration and dilution capacity).
- it will be increased **(above 1.035 g/cm3, HYPERSTENURIA)** when the urine is more concentrated: morning urine, dehydration, but also in persons with diabetes mellitus (due to presence of glucose), persons taking large amounts of medication
- consistently low specific gravity **(1.003 g/cm3 or less, HYPOSTENURIA)** is seen in persons with diabetes insipidus, renal failure (loss of urine concentration capacity), hyperhydration, diuretics use.
- loss of concentrating ability is often one of the earliest signs of kidney disease, clinically evidenced as nocturia (needing to void at night) and polyuria (increased volume of usually dilute urine).
- *is determined* by *direct method* using automated analysers or in manual fashion using an urinometer (hydrometer), refractometer and *indirect method* using urine dipsticks (pads that contains a complex, pre-treated electrolyte that undergoes a pH change based on the ionic concentration of the urine. This change results in a change of color of the pad) (figure 5.2).



automated analyser urinometer refractometer urine dipsticks

Figure 5.2. Different methods for determination of urine specific gravity

5. **Volume**

- Urine volume/ 24 h = **DIURESIS (900-1200 mL/24h)**
- **OLIGURIA** = Urine volume below **500 mL/24h** (low urine production) and may occur in persons who are dehydrated and those with some kidney diseases.
- **POLYURIA = over 2000mL/24h** (excessive urine production); it is common in persons with diabetes mellitus (osmotic diuresis due to glucose presence in urine), nephrotic syndrome (proteins presence in urine), hyperhydration (diabetes insipidus), diuretics therapy.
- **ANURIA = less than 100mL/24H** on the absence of the urinary flow
- It depends on water balance status and ratio between fluid volume intake and water volume eliminated, both on renal and extra-renal pathway (digestive, skin) and renal function, meaning the kidney capacity to secrete and reabsorb water (altered in renal failure).
- Urine elimination is changing in urinary tract infections:
  - inversed urine elimination between day / night = **nocturia**
  - frequent and reduced amount micturitions = **polakiuria**
  - difficult micturition = **dysuria**
- *can be measured volumetrically with a volumetric cylinder.*

6. pH (urine reaction)

- reflects kidney capacity to acidify urine
- in balanced diet - 6.2 to 6.8
- in predominantly proteic diet - 5.2 to 5.3 (more acid)
- in vegetarian diet - 7 to 7.5 (more alkaline)
- *Interpretation:*
  - pH < 5.2 indicates presence of ketone bodies in urine (ketoacidosis in diabetes mellitus metabolic uncompensated, starvation), urinary infection with Escherichia Coli
  - pH > 7.5 is seen in systemic alkalosis (vomiting, prolonged fever), renal tubular acidosis (decreased capacity for urine acidification), urinary infection with Proteus; in contact with air, ammonium fermentation is produced, and urine becomes alkaline
- urinary pH control have *practical importance* in:
  - *renolithiasis* for reducing salts precipitation: calcium/magnesium phosphates, as well as calcium carbonate precipitate in alkaline urine, so that urine should be maintained acid, while uric acid (urates) and calcium oxalates precipitate in acid urine, so that urine should be maintained alkaline
  - *urinary infection* in order to reduce bacterial flora proliferation, e.g.in Escherichia Coli urinary infection, urine should be maintained alkaline, while in Proteus urinary infection, urine should be maintained acid

- *medication* - antibiotics treatment (streptomycin, neomycin, kanamycin) in case of urinary infection is effective if urine is alkaline, while in salycilates intoxication urine should be maintained acid in order to increase salycilates excretion

5.1. 3. Brief urine examination (urinalysis)

Definition: macroscopic (1), chemical (2) and microscopic (3) examination of a freshly urine sample

Components:

1. normally, the urine is pale yellow, transparent, clear	evaluated by physical examination of urine
2. ALBUMIN, GLUCOSE, ketone bodies, biliary pigments and salts ARE ABSENT	evaluated by chemical examination of urine
3. in the sediments will be rare leukocytes, epithelial cells and crystals.	evaluated by microscopic examination of urine

1. Macroscopic examination of urine - see above *physical properties of urine*

2. Chemical examination of urine –

2.1. PROTEINS are NOT found in the FINAL URINE because they are not filtered at the glomerular level due to their large molecular weight (albumins ~ 66000Da).

Physiologically, proteins with small molecular masses (<20 000Da) cross the glomerular membrane, are resorbed by the proximal tube and only small amounts are excreted - 50-150 mg/24h, in which less than 30 mg/24h consists of albumin (proportions of proteins in the urine are 1/3 albumin and 2/3 globulin).

Interpretation:

- normal urine contains small amount of albumins (< 30 mg/day) = NORMOALBUMINURIA – no detectable
- amount of 30 – 300 mg/day = MICROALBUMINURIA – characteristic for diabetic nephropathy, but it cannot be revealed during urinalysis
- quantity > 300 mg/day = PROTEINURIA (ALBUMINURIA) (altered glomerular filtration and/or tubular reabsorption)

Types of proteinuria:

- **physiologic (transient**) - after strong emotions, strenuous physical exercise, cold exposure, prolonged orthostatic position, fever
- **pathologic (persistent)**
  - *renal cause:*
    - increased filtration at the glomerulus (altered permeability of glomerular membrane) lead to selective (albumins) or non-selective (albumins+globulins) proteinuria
    - decreased tubular reabsorbtion lead to excretion of proteins with low molecular weight (Tamm-Horsfal) while the glomerular filtration remaining normal
  - *post-renal* (urinary tract inflammation)
  - *extra-renal* (acute infectious diseases and acute clinical disorders, such as pains, epilepsy, myocardial infarction, stroke, trauma, after surgery)

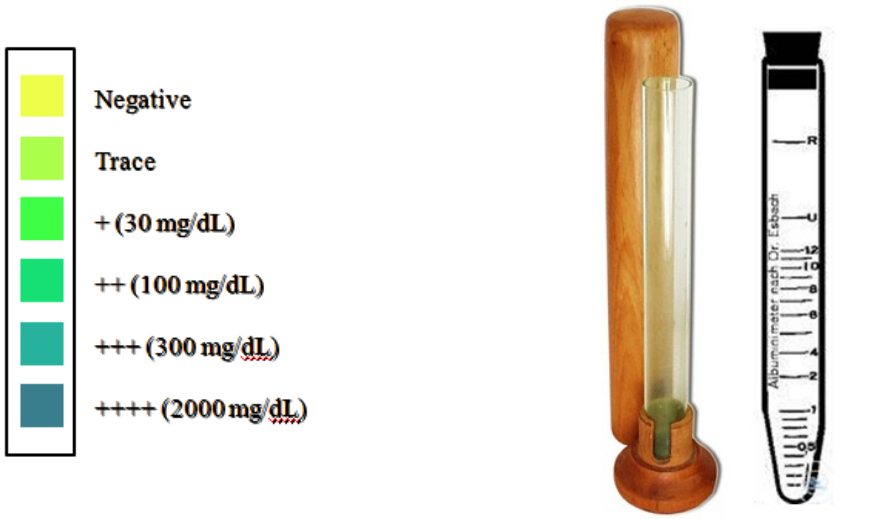
Methods for determination of proteinuria

- *urine dipsticks* (figure 5.3) - semi quantitative screening test; respond best to albumins, so that a negative result can occur when large amounts of other proteins are being excreted. This test is based upon the ability of proteins to change the color of a dye without modifying the pH. When the dye, tetrabromphenol blue, is buffered at pH 3, will result a yellow color if the protein are not present in urine. But in the presence

of protein the color will change to green and then to blue with increasing protein concentrations.

- *qualitative estimation* of protein using the ability of protein to precipitate in the presence of acid solutions (principle of reaction). There are 2 technique:
  - *sulphosalicylic acid method*: add 4-5 drops of sulphosalicylic acid 20% to 4 ml urine. The presence of proteinuria is indicated by the precipitation varying from mild opalescence to real precipitate
  - *Esbach method:* mix 1ml of urine with 1ml of Esbach reagent (10g picric acid + 20 g citric acid dissolved in 1 l of water). Appearance of a white-yellow precipitate indicates the presence of proteins
- *quantitative determination* of protein using *automated analysers* or *Esbach albuminometer*.

Esbach albuminometer (figure 5.3) is a test tube graduated in grams of dried albumin per 1000mll of urine. It has U mark near the middle and another R mark. The test tube is filled with urine till U mark and and the Esbach's reagent is added to the mark R and mixed well. The albumin precipitates and settles down. At the end of 24 hours the level of the precipitate is read against the graduations of the tube. The reading is given in grams per litre and to express it as a percentage, the result should be divided by 10. It can be multiplied by the total 24 hours urine volume to obtain the amount per 24 hours.



albumin urine dipstick Esbach albuminometer.

Figure 5.3. *Methods for estimtion of proteinuria*

2.2. GLUCOSE is NOT found in the FINAL URINE because all glucose filtered at glomerular level is totally reabsorbed on proximal convoluted tubule (by facilitated diffusion).

- in primary urine glucose concentration = blood glucose concentration (glycaemia)
- in final urine glucose concentration = 0 mg/dl

GLYCOSURIA = the presence of detectable amounts of glucose in the urine. It occurs when the blood glucose exceeds the reabsorption capacity of the renal tubules i.e. when the glomerular filtrate contains more glucose than the tubules are able to reabsorb (renal threshold = 180 mg %). Glycosuria can be:

- *physiologic* - pregnancy, ingestion of large amounts of carbohydrates, emotional stress (when usually, blood glucose level is normal, but tubular reabsorption of glucose is below normal, thus allowing certain glucose to escape into the urine)
- *pathologic* - exceeding renal glucose threshold of 180 mg/dl (metabolic uncompensated diabetes mellitus)

Significance:

- Monitor changes as a response to medication
- Used as a screening test to gather information about the physical status

Methods for determination of glycosuria:

- *urine dipsticks* - based on oxidation of glucose to gluconic acid:
  - Glucose +O2(room air) → Gluconic acid + H2O2 *(in presece of glucose oxidase)*
  - H2O2 + Chromogen → Oxidized chromogen (blue) + H_2O *(in presece of peroxidase)*

    - positive test from 1 (+) to 4 (+) (50 – 1000 mg/dl)
- *qualitative estimation* of glucose based on reducing properties of glucose

  - Benedict's test and Fehling's test (reduction of Cu2+). Benedict is a qualitative and semiquantitative test for reducing carbohydrates and is more specific than Fehling's test.

Principle

Cupric ions + Glucose $\xrightarrow[\text{Alkali}]{\text{Heat}}$ Cuprous ions + Oxidized glucose

($CuSO_4$) Blue — (Reducing substance) — (Cu_2O) Orange-red

Technique: Take 5 ml of Benedict reagent (copper sulphate + sodium citrate + anhydrous sodium carbonate in distilled water) in a test tube and add to it 0.5 ml (8 drops) of urine. Mix and boil over a flame or in a boiling water bath for 2 minute and cool the solution. Note the color.

Results:

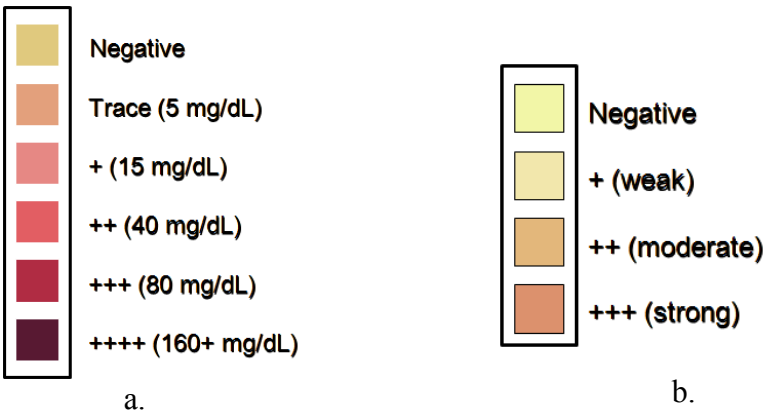
Negative	No change in color	
Trace	Solution appears pale green to slightly cloudy	
1+	Definite cloudy green	0.5% glucose
2+	Yellow to orange precipitate, supernatant fluid pale blue	1% glucose
3+	Orange to red precipitate, supernatant fluid pale blue	1.5% glucose
4+	Brick red precipitate, supernatant fluid decolorizes	2% glucose

- *quantitative estimation* – automated analysers

2.3. KETONE BODIES are acetone, beta-hydroxybutyric acid, acetoacetic acid

Estimation of ketone bodies is made with urine dipsticks (figure 5.4.a) that use the sodium nitroprusside reaction, which detects acetone and acetoacetic acid but not b-hydroxybutyric acid, the primary ketone body:

Acetoacetic acid + Sodium nitroprusside + Glycine $\xrightarrow{\textit{Alkaline pH}}$ *Purple color*



a. b.

Figure 5.4. Estimation of urinary ketone bodies (a) and bilirubin (b) with dipsticks

Interpretation:

- normal urine does NOT contain ketone bodies
- increased amount of urinary ketone bodies = KETONURIA
  - ❑ appears when the body cannot utilize glucose (as in diabetes) and metabolize fatty acids. This catabolism is incomplete, resulting in the formation of ketone bodies.
  - ❑ ketonuria is the most important parameter for monitoring blood glucose level in diabetic subjects, because ketone bodies are detected in urine before they can significantly increase in plasma
  - ❑ ketonuria also occurs in increased vomiting (pregnancy, in children), starvation, lipids-rich diet and low carbohydrates intake

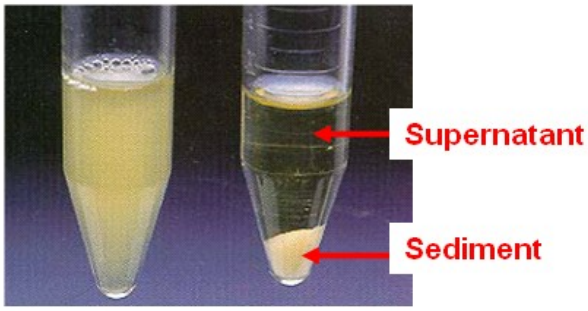
2.4. The pigment **bilirubin** is formed by the degradation of heme. Normally it is not excreted into urine. Tests for detection for bilirubin are:
- *Gmelin's test:* put 3 ml of yellow nitric acid in a test tube and add with equal amount of urine. Result: A play of colored rings, the most distinct being green indicates the presence of bile pigments.
- *colorimetric strip reagent test* (figure 5.4.b) based on the coupling of bilirubin with diazotized 2, 4- dichloroaniline in a strong acid medium to form a brown purple azobilirubin compound. The color ranges through various shades of tan.

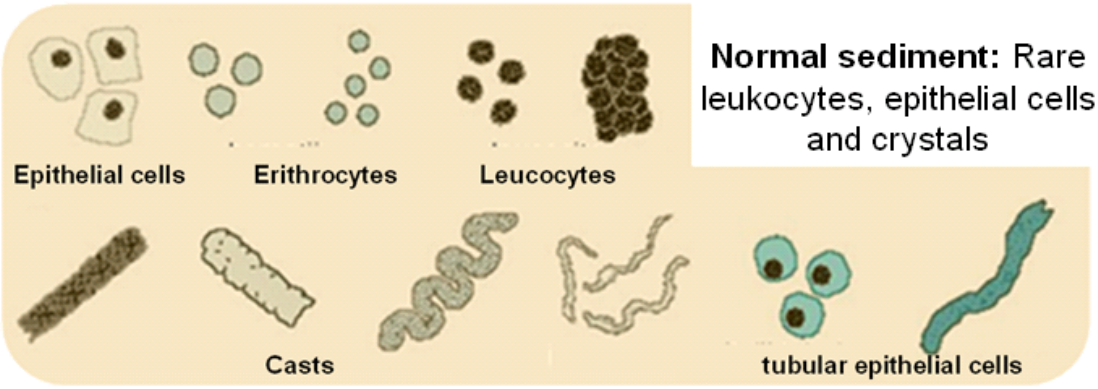
Urobilinogen in urine: Conjugated bilirubin, secreted by the liver into the bile is excreted into the intestinal tract through the bile duct where bacteria converts the bilirubin to urobilinogen. 50% of the urobilinogen formed in the intestine is reabsorbed into the portal circulation and re-excreted by the liver. Small amounts are normally excreted in the urine but the major excretion is in the feces.
Normally 1-4 mg of urobilinogen is excreted in the urine in a 24 hour period. The concentration of urobilinogen in random normal urine is 0.1-1.0 Ehrlich units/dl.

Urine	Normal	Hemolytic Disease	Hepatic Disease	Biliary Obstruction
Urobilinogen	Normal	Increased	Increased	Low or absence
Bilirubin	Negative	Negative	Positive or Negative	Positive

3. Microscopic examination of urinary sediment

- Mix the urine specimen gently, put ~ 5-10 ml into a centrifuge tube and spin at low speed (1000 to 1500 rpm for ~5 minutes)
- Pour off the supernatant by quickly inverting the tube without shaking (may be used for biochemical testing)
- Transfer one drop of the deposit on to a slide using a Pasteur pipette and cover with a coverslip.
- Label the slide with the patient's name
- Examine to the microscope using a big objective

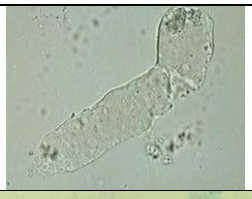
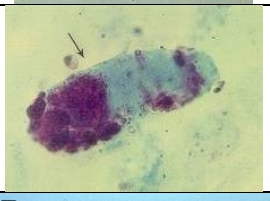
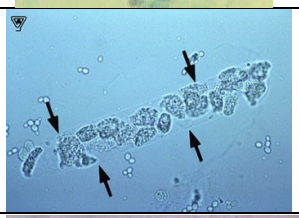
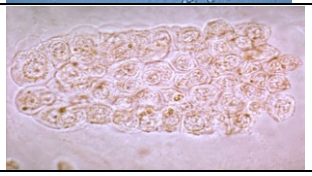


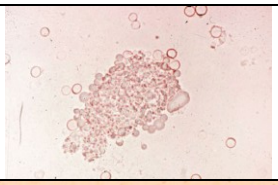
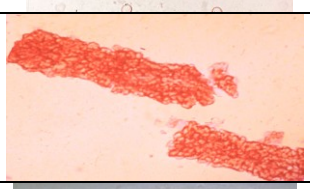
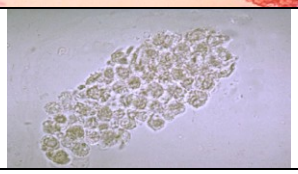


Urinary sediment can be classified as:
1. *organized sediment*, insoluble, including physiologic elements (cells, casts) and pathologic elements (bacterial flora, parasites, neoplastic cells)
2. *unorganized sediment*, soluble, which comprises of crystals.

- Leukocytes - in normal urine < 5 leukocytes/HPF (per high power field). Increased leukocytes count = LEUKOCYTURIA – could be seen in urinary tract infections
- Red blood cells - in normal urine < 5 RBC/HPF. Increased RBC count = HEMATURIA - glomerular nephropathy, kidney stones, renal trauma, anti-coagulant therapy. Hematuria can be macroscopic (red urine) and microscopic (clear urine).
- Epithelial cells – absent or rare in normal urine (1-3 epithelial cells/HPF). Frequent epithelial cells indicate a destruction of the tissue in the urinary tract.
- Casts – represent moulds of renal tubules lumen, formed of proteins (Tamm-Horsfall protein), cellular elements (RBC, WBC, epithelial cells), and tissue detritus. They are washed out into the collecting tubules and the bladder. They require acidic conditions, high salt concentration, reduced urine flow to be formed.

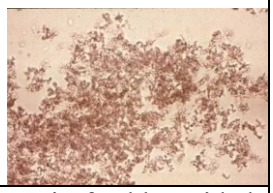
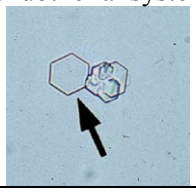
Normally, 1 hyaline cast/HPF is admitted. Increased number of casts = CYLINDRURIA, having pathologic significance only if associated with proteinuria and changes of organized sediment. Urinary casts can be:

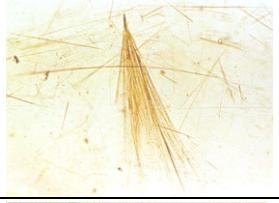
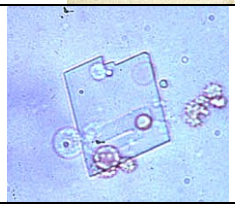
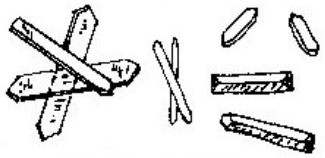
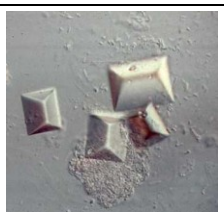
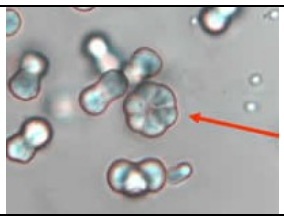
Hyaline (non-cellular) casts are the most frequently occurring casts in urine (acid urine, after physical exercise). They are colorless, homogeneous, transparent, and usually have rounded ends.	
Granular casts – contain large or fine granules embedded in coagulated protein. They are not found in normal urine and their presence almost always indicate significant renal disease. Color: black to pale yellow	
waxy casts: result from the degeneration of granular casts. Appears in severe chronic renal failure, malignant hypertension, and diabetic disease of the kidney. They are short, broad casts, with blunt or broken ends	
Epithelial casts: are formed of fused desquamated tubular cells. They are rare and occurs in renal diseases that primarily affects the tubules	

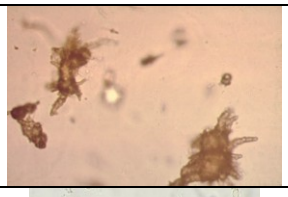
Fatty casts- contain cholesterol and fat globules. They are not normally found in urine and are derived from degenerating cells.	
Red blood cells casts indicates renal hematuria. Hematic casts may appear brown to almost colorless and are usually diagnostic of glomerular disease.	
White blood cell casts – indicates an inflammation or infection of the kidneys	

- **Microbial flora** – in normal urine THERE IS NO bacterial flora. Presence of bacterial flora, estimated as rare, moderate or abundant = BACTERIURIA. This is associated with (+) uroculture in tubule-interstitial nephropathies and common urinary tract infections or (-) uroculture in urinary infection with Chlamydia

- **Crystals** – in normal urine, crystals are rare. Increased number of crystals = CRYSTALURIA. The solutes precipitate and form crystals if the urine pH is increasingly acidic or basic, the concentration of dissolved substances is increased and the urine temperature promotes their formation. The crystals may group together to form kidney "stones" or calculi

Acid urine			
Name	*Color*	*Shape*	*Significance*
amorphous urates	brick-red	granules	disturbances of uric acid metabolism (gout) and in fevers (concentrated urine). 
uric acid	yellow brown	polymorphous: hexagonal plate, whetstones, rosette of prisms, rhombohedral	pathologic only when seen in freshly voided urine; concentration depends on dietary intake of purines and breakdown of nucleic acids (seen in leukemia patients receiving ch emotherapy)  
cystine (rare)	colorless, highly refractile	hexagonal plates in clusters	cystinosis - an inborn error of metabolism in which cystine crystals are found in the urine, reticuloendothelial system and eyes. 
leucine (rare)	yellow or	oily looking	liver disorders

	brown, highly refractile	spheres with radial and concentric striations	
tyrosine (rare)	colorless, yellow	fine silky needles in sheaves or rosettes	severe liver diseases 
Cholesterol (rare)	colorless,	„broken window panes" with notch corners, flat plates	
Acid, Neuthral or Slightly Alkaline Urine			
calcium oxalate	colorless	octahedral, dumbells	in normal individuals with high dietary oxalate ingestion (tomatoes, spinach,  rhubarb), in patients with nephrolithiasis, and in patients with acute renal failure due to ethylene glycol ingestion.
hippuric acid	colorless	rhombic plates	 Hippuric acid
Alkaline, Neuthral or Slightly Acid Urine			
triple phosphate (ammonium magnesium phosphates or struvite)	colorless	„coffin lids", feathery or leaf like forms	In freshly passed urine they indicate stones in the bladder or kidney: 10-20% urinary calculi 
Alkaline Urine			
calcium carbonate	colorless	needles, spheres, **dumbells**	
ammonium biurate	yellow, opaque, brown	„thorn apple" spheres, dumbells,	Rarely seen in freshly voided urine - common in old specimens

		sheaves or needles	
calcium phosphate	colorless	prisms, plates, needles	

5.1. 4. Addis test

Principle: quantitative examination of blood cells and cylinders in the urinary sediment from the urine collected per 24 hours.

Materials required: urine, microscope, centrifuge, counting chamber (Burker - Turk), Pasteur pipettes.

Technique: Collect the urine from 24-hours and mix. Measure the volume and record the diuresis. Take 2 test tubes and pipette into each 10 ml of urine; centrifuge 5 minutes at 1500-1800 RPM. Discard the supernatant and homogenizes the sediment. Using a Pasteur pipette charge a Burker-Turk counting chamber, wait for 5-10 min to sedimentate, put under a microscope, count the RBCs / WBCs on 1mm2 and cylinders on 9 mm2.

Normal values

	Adults	*Children*
Red blood cells	< 500 000/24 h	< 450 000/24 h
White blood cells	1-1.8 millions/24 h	9 000- 720 000/24 h
Cylinders (casts)	<5 000/24 h	< 6 500/24 h

5.1. 5. The testing of urine dilution and concentration abilities of the kidneys

The methods used for this purpose are based on the fact that a healthy kidney is able to produce a more diluted or concentrated urine, depending on the degree of hydration of the body. One of the methods was described by **Volhard:**

Principle: determination of urine volume and density under two controlled conditions: fluid loading and fluid intake restriction.

Technique:
1. The urine dilution test: the patient empties the bladder at 7a.m. and during the next 30 minutes he drinks approximately 1500 ml of water/tea unsweetened. No breakfast is allowed. The bladder is emptied from 30 to 30 minutes during the next 4 hours and urine samples are obtained in separate containers. The volume and specific gravity of each specimen is measured.
2. The urine concentration test: can be done after dilution test or independent. It begins by giving the patient a considerable lunch consisting of proteins and no more than 300 ml (1 glass) of

fluids. After that, no more fluid intake is allowed until the test is over. Every 2 hours for 8 hours, the patient empties the bladder. Urine samples are obtained in separate containers. The volume and specific gravity of each specimen is measured.

Results and interpretation of:
1. dilution test:
- normally, all the water drunk is excreted during the 4 hours and the specific gravity of at least one specimen is 1001-1003
- if the kidney function is impaired, the volume excreted is less and the specific gravity does not fall so low.

2. concentration test
- normally, the volume of urine is 800ml and the specific gravity of at least one specimen is no less than 1025.
- if the specific gravity reached below 1022 it indicates impaired renal function (50% of nephrons are damaged)
- the specific gravity falls nearer to 1010 as the kidney condition become worse (chronic renal failure)
- this test should be avoided on patients with diabetes insipidus, heart failure, renal failure, dehydration.

Clinical significance: in renal or tubular diseases **concentrating capacity impairs sooner than diluting capacity** (the concentration test is more sensitive to impaired tubulus function than the dilution test).

5.1. 6. Renal clearance

Kidneys clear substances from the blood plasma. When a substance is excreted in the urine, a certain volume of plasma is, in effect, freed (or cleared) of that substance.
Definition:

i. It is volume of plasma from which that substance is completely removed (cleared) per unit time.
ii. It is the volume of plasma that is cleared of a substance in one minute by excretion of the substance in the urine

Determination of clearance:
Renal clearance formula: Cx = (Ux•V) / Px where,

x	= substance of interest,
Cx	= clearance of the substance x (ml/min)
Ux	= urinary concentration of substance x (mg/ml)
Px	= plasma concentration of substance x (mg/ml)
V	= urine flow rate (ml/min)
Ux•V	= excretion rate of substance x (mg/min or mEq/24h)

The clearance of a given substance can be determined by measuring the concentration of a substance in urine (Ux), in the plasma (Px) and the volume of urine per unit of tine (V) and substituting this values into the clearance formula.

Since filtration, reabsorbtion and secretion processes participates to urinary excretion (or plasma clearance), the renal clearance of different substances provide useful information about renal function and the way by which the substances are managed by the kidneys.

Principles governing renal clearance of a substance(figure 5.5):

- substances that are freely filtered, but neither reabsorbed nor secreted (e.g. inulin) have the clearance equal to glomerular filtration rate (GFR) and hence are called ideal filtration (glomerular) markers.
- substances that are freely filtered, but are partially reabsorbed (e.g. uree, creatinine) in the tubules have the clearance less than GFR.
- substances that are filtered, but completely reabsorbed (e.g. Na+, glucose, amino acids, Cl^-, $HCO3^-$) have lowest renal clearance.
- substances that are filtered, not reabsorbed, but secreted by the tubules (e.g. para-minohippuric acid, Diodrast) have the clearance high than GFR.

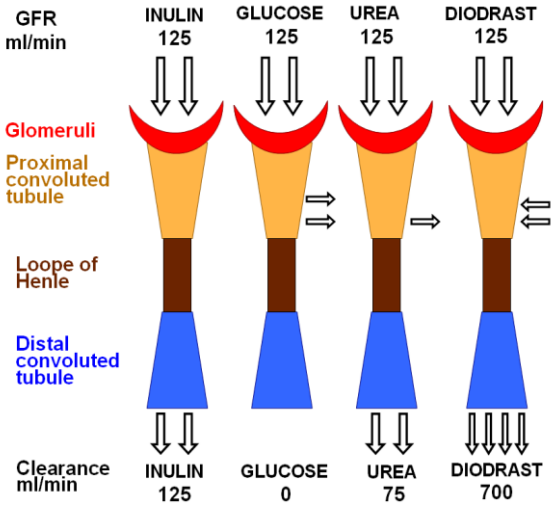


Figure 5.5.Tubules manage various substances differently (after de Vasudevan, 2007)

Measurement of the clearance is mainly a test of glomerular filtration rate (GFR). GFR is the rate at which plasma is filtered by the kidney glomeruli. Measurement of GFR is routinely used to evaluate kidney function. A considerable renal damage occurs before GFR is decreased. Normal, GFR for young adults is 120-130ml/min/1.73m2. GFR decrease with age and more than 25% of people older than 70 years have GFR<60ml/min. GFR cannot be measured directly and it is estimated from the clearance of a filtration marker – ideal marker is inulin, but in practice, endogenous markers are preferred, like creatinine and urea.

Inulin clearance test

Definition: volume of plasma completely cleared of inulin per minute

Inulin is an exogenous fructose polymer and it is considered the ideal substance to measure GFR, because of its following characteristics:

- it is freely filterable by the glomeruli
- it is not reabsorbed or secreted by the kidney tubules
- it is not synthesized, destroyed, or stored in the kidneys
- it is nontoxic
- its concentration in plasma and urine can be determined by simple analysis.

Inulin clearance is a measure of GFR because volume of plasma completely cleared of inulin per minute equals the volume of plasma filtered per minute.

Technique:

A single e bolus dose of inuluin is injected iv, followed by a continuous iv infusion at a rate which compensates its loss in urine (plasma concentration of inulin will be kept constant). The inulin clearance is determined according to the formula: C_{inulin} (or GFR) = $(U_{inulin} \bullet V)/P_{inulin}$, where U_{inulin} = urine concentration of inulin (mg/ml), V = urine flow rate (ml/min), P_{inulin} = plasma concentration of inulin (mg/ml).

Clinical significance:

In addition to its use as a estimation of GFR, the inulin clearance is used as an indicator of plasma clearance processes. A comparison between clearance of a certain substance x (Cx) and the clearance of inulin (Cin) gives information about the renal transport mechanisms used to remove the substance from the plasma (figure 5.6):

- Cx = Cin, clearance ratio is 1, so the substance has the same characteristics like inulin. Examples of such substances are: mannitol, sorbitol, ferricyanide, vitamin B12 and sucrose. The listed substances can also be used to measure GFR.
- Cx < Cin, clearance ratio is less than 1. Such substances are filtered and reabsorbed: glucose, xylose, fructose
- Cx > Cin, clearance ratio is more than 1. Such substances are filtered and secreted: para-aminohippuric acid (PAH) and phenol red.

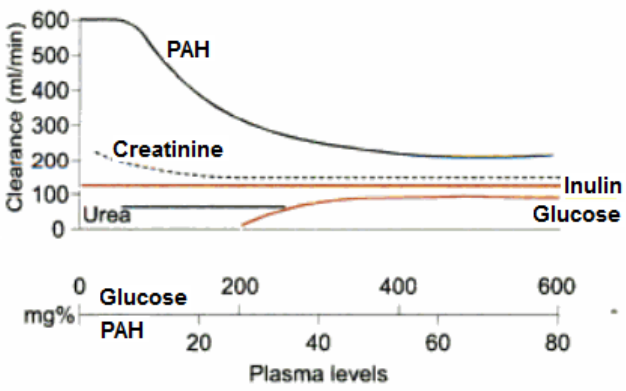


Figure 5.6.Clearance of substances plotted against their plasma concentration (after de Khurana, 2009)

Creatinine clearance test:

Definition: volume of plasma completely cleared of creatinine per minute

Creatinine clearance is preferred to inulin clearance in estimation GFR because:

- it is easier to measure, does not require continuous iv infusion since creatinine is an endogenous substance
- has a fairly constant plasma concentration (0.6 – 1.5 mg/dl) because its formation and release is relatively constant and proportional to the amount of muscle mass of the body. Creatinine is an end-product of muscle metabolism formed from creatine phosphate.

Despite these advantages, creatinne clearance does not indicate very accurately GFR because creatinine it is filtered by the glomeruli and *is secreted by the tubules* to some extent. But the value of creatinine clearance is close to GFR, so its measurement is a fairly good method of estimation GFR.

Principle: is based on determination of plasma and urine concentration of creatinine and measuring of urine volume during a fixed period of time.

Technique:

- at the beginning of the test the patient is asked to completely empty the bladder by voiding the urine and the exact time is recorded. Volume of urine (V1) and urine concentration of creatinine (U1) is measured. Immediately after micturition it is taken a blood sample and it is determined the plasma creatinine concentration (P).
- exactly after 1h, the patient is asked to void again and the volume of urine (V2) and urine concentration of creatinine (U2) is measured.

So, a single blood sample is drawn at the midpoint of a carefully timed urine collection.

Calculation: $C = (C1 + C2)/2$

$C1 = (U1 \bullet V1)/60 \bullet P$ and $C2 = (U2 \bullet V2)/60 \bullet P$,

where 60 is a correction factor for urine volume from hour to minute

Results: 80 -110 ml/min in an adult and decline with age in healthy individuals.

Interpretation:

- a decreased creatinine clearance is a very sensitive indicator of a reduced GFR:
  - physiological – after exercise, cold exposure, hyposodium diet, in conditions of pain, fear, thirst
  - pathological: hypovolemic state (dehydration, hemorrhages), glomerulophaties, renal failure
- the importance of creatinine clearance is in the early detection of renal functional impairment without obvious signs and symptoms
- the inverse relationship between GFR and plasma creatinine concentration allows the use of plasma creatinine concentration as an index of a decrease in GFR of similar magnitude

Some equations have been developed that allow physicians **to estimate GFR from serum creatinine concentration.** These equations take into consideration such factors as age, gender, race, and body size. The equation: **GFR (in mL/min per 1.73 m2) = 186 × (serum [creatinine] in mg/dL)−1.154 × (age in years)−0.203 × 0.742 (if the subject is female) or × 1.212 (if the subject is black)** is recommended by the National Kidney Disease Education Program, which provides a calculator on its web site: www.nih.nkdep.gov.

Ureea clearance test:

Definition: urea clearance is the volume of blood which contains the urea excreted in a minute by the kidney.

Ureea is the end product of protein metabolism. Ureea is freely filtered by the glomeruli, but about 40% is reabsorbed actively by the tubules causing its clearance to be considerable less than GFR, and further it is influenced by the protein content of the diet. For these reasons, urea clearance in not as sensitive as creatinine clearance, for assessing renal function.

The principle, technique and calculation are the same as in creatinine clearance test

Since the urea clearance significantly changes when the volume of urine is less than 2ml/min, 2 indices have been developed:

- maximal urea clearance = (U•V)/P applicable when the volume of urine > 2ml/min
- standard urea clearance = (U•V)/P applicable when the volume of urine < 2ml/min

Normal values of maximal urea clearance = 75ml/min and standard urea clearance = 54ml/min

Interpretation:

- Ureea clearance < 75% of normal value it is considered abnormal. The values falls with increasing renal failure.
- Since it is an indicator of renal damage, *blood urea level* (normal value = 0.2 - 0.4g/l) as such is found to increase only when the clearance falls below 50% normal.

Para-aminohippuric acid clearance test

Para-aminohippuric acid (PAH) is an organic anion that is almost completely cleared from the plasma *in a single pass through the kidney* by means of a combination of glomerular filtration and proximal tubular secretion. Calculated clearance of PAH (C_{PAH}) therefore represents renal plasma flow (RBF). To obtain maximal PAH extraction, a steady state with a low plasma PAH level must be achieved by giving an intravenous loading dose followed by an infusion to maintain plasma PAH concentration of about 2 mg/dL. A carefully timed catheter urine collection is required. Because only 90 percent of the renal plasma flow enters the peritubular capillaries surrounding the proximal tubule, PAH clearance underestimates true renal plasma flow, and it is known as the effective renal plasma flow (ERPF).

$ERPF = C_{PAH} = \frac{U_{PAH} * V}{P_{PAH}}$, where UPAH is the concentration of PAH in the urine and PPAH is its concentration in the plasma. The normal effective renal plasma flow is 660 mL/min/1.73 m^2.

Effective renal blood flow (ERBF) may be derived if the hematocrit level (Hct) is known:

$ERPF = \frac{ERPF}{1 - Hct}$

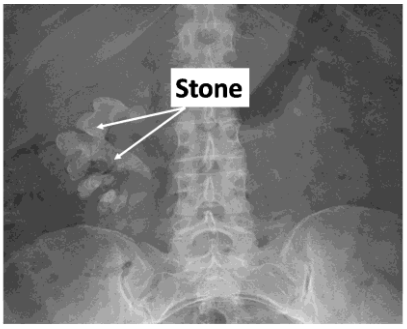
Thus, despite its relative convenience as an experimental tool, PAH clearance may be an unreliable indicator of RBF during the perturbations induced by anesthesia and surgical stress.

Also, a **Doopler ultrasound** can be used to evaluate blood as it flows through the renal arteries.

5.1. 7. Reno-urinary apparatus imaging

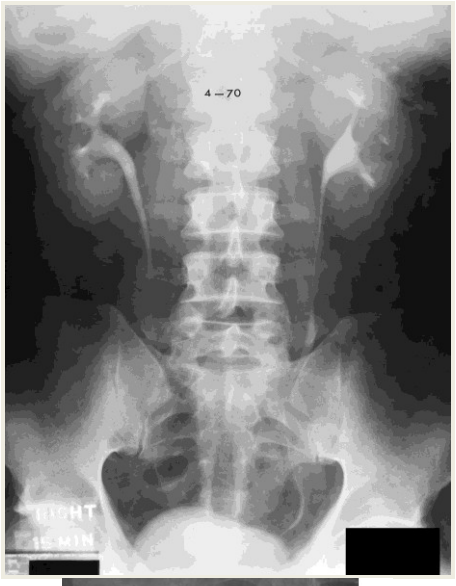
1. Plain X-ray of renal system

- offers information regarding size, shape, position of the kidney
- identifies kidney stones – renal calculi (radiopaque area) localised in the kidney, renal pelvis, ureters or bladder
- is an investigation non invasive, cheap, simple, routine and available in most academic centres.



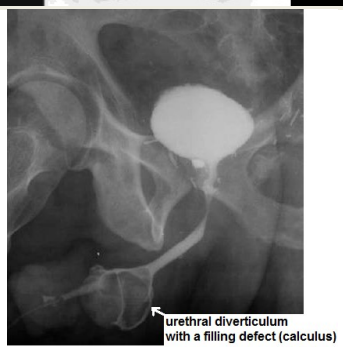
2 . Intravenous pyelography (IVP)

- is an invasive investigation carried out by injecting iv a radiopaque dye and taking X-rays of the abdomen at short intervals (1, 5, 10, 30 minutes)
- the dye is filtered by glomeruli and concentrated by renal tubules amplifying the radiodensity of renal parenchyma (nephrogram), by which size, shape, position of the kidney can be assessed
- than the dye is excreted into the pelvicalyceal system, making their outlines clear to study them for any abnormality.



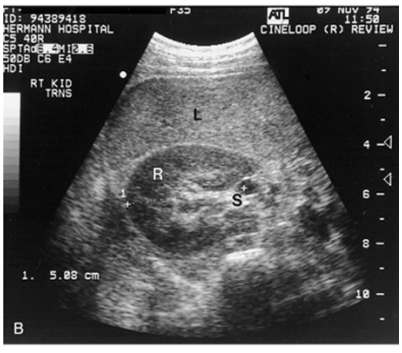
3. Retrograde urography / Cystography / Voiding cystourethrography

- bolus of contrast dye is injected into the urinary bladder through a urethral catheter, followed by a series of X-rays.
- evaluate bladder wall integrity, asses vesico-ureteral reflux, evaluate bladder filling defects (malignancies), lower urinary tracts congenital anomalie, urinary incontinence in women.

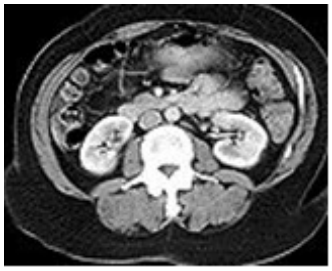


4 . Ultrasonography

- is an investigation non invasive used to measure sizes of parts of renal system (kidney, ureter, prostate, urinary bladder), check for blood vessel diameter, check whether masses are cysts or solid/textured, measure perfusion, check for clots (uses Doppler shift in signal)
- evaluate the bladder wall thickness, stones or emptying.
- disadvantages consist in lack of detail (poor specificity/sensitivity), cannot fully visualise adult ureter, may miss small stones, or those in ureters, reliant on experience and interpretation of the operator

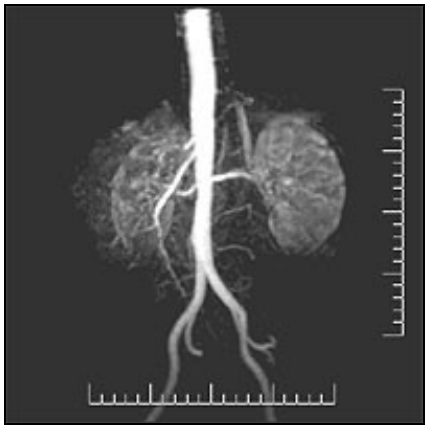


5. Computed tomography (CT)



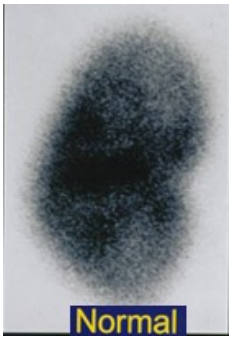
- non invasive technique perform to detect in and around the kidneys: renal cysts and tumors, evaluation of renal trauma, vascular abnormalities, localisation of renal obstruction (e.g. renal calculi), congenital malformations, assess of postoperative complications
- high dose of radiation

6. Magnetic resonance imaging (MRI)



- can image renal masses not identified by previous methods, useful alternative to CT for patients in whom the use of iodinated contrast material is contraindicated (gadolinium it is used instead), can measure from many angles/planes, has good contrast.
- image based on radiofrequency (RF) pulse returned from RF-stimulated protons in magnetised tissues
- can't use near pacemakers and cannot image bone or calcium
- **MR angiography** offers flow-velocity measurements, 3D representation of vasculature, used as guidance for renal artery interventions, evaluation of a living donor before kidney transplantation.

7. Renal scintigraphy



- invasive procedure: a radioactive substance is injected, and radioactivity of the kidney is recorded with a gamma camera during concentration and excretion;
- provide physiologic and anatomic information about renal system because
- allows examination of renal blood perfusion, glomerular filtration, transit through kidney and outflow from urinary tract

8. Transcutaneous renal biopsy

- invasive procedure carried out under ultrasound control with a needle (require anaesthesia)
- examine tissue via histochemistry, electron microscopy and immunofluorescence
- small mortality risk (o.1%); it gives a lot of biochemical and structural information

5.1. 8. Evaluation of urinary bladder function

Definition: Cystometry is a test of bladder function in which pressure and volume of fluid in the bladder is measured during filling, storage, and voiding.

Principle: is based on establishing a relationship between intravesical pressure and volume of urine from the bladder.

Technique: There are described 2 methods:

(i) *voidind cystometry* permits physiologic filing of bladder with urine (disadvantage: bladder volume is inferred from the amount of urine voided, assuming thet there is no residual urine)

(ii) *static cystometry* when the bladder is gradually filled with fluid through a urethral catheter (disadvantage: fluid is introduced at a more rapid rate than occurs naturally through urine, which could affect bladder function). A double lumen catheter is inserted into the urinary bladder. The fluid is introduced through one lumen and pressure changes are recorded by connecting the other lumen to pressure transducer of a polygraph. The blader is completed emptied, then a small known amount of fluid (50 ml saline solution) is introduced at regular intervals and the pressure

is recorded constantly. A graph called *cystometrogram* is obtained by plotting values of volume and pressure. Three phases of filling are observed on the graph (figure 5.7):
Phase Ia – the initial phase of filling when *the pressure rises* (from 0 to 10 cm H2O) *directly proportional with volume* (from 0 to 100 ml fluid) in the bladder.
Phase Ib – *plateau phase* when is recorded a considerable increase in bladder volume (from 100 to 400 ml) due to the infused fluid, but the pressure remains almost unchanged (around 10 cm H2O). As the bladder fills up, it adjust its tone and a large volume of urine can be accommodated without a significant rise in intravesical pressure. This phenomenon is named *adaptation* and occurs (i) because of the intrinsec property of the detrusor called *plasticity* and (ii) because of law of Laplace. According to this law, pressure in a spherical object is equal to twice the wall tension divided by the radius: P = 2T / R, where P = pressure, T = tension, R = radius. When the bladder is distended by urine, wall tension rises, but due to adaptative relaxation of detrusor owing to plasticity, there is in diameter of the bladder. As a result, vesical pressure does not rise and is maintained at the same level (2T/R remains the same).
Phase II – when the bladder volume increases further than 400 ml, the pressure rise markedly, triggering the micturition reflex. Normally, the voidind contraction raises the ntravesical pressure by about 20 to 40cm H2O. If voiding is not initiated, the pressure continues to rise, as shown by dotted lines beyond the phase II in the figure. Beyond 600 ml, the urge of micturition becomes almost intolerable.

Normally, when 100 of urine are collected into the bladder, the first sensation to urinate is felt. An additional volume up to 300 ml (*functional vesical capacity*) constitutes in adults the adequate stimulus for micturition, the sensation of micturition occurs, but it can be voluntarily suppressed till a convenient surrounding is found. Beyond 400 ml, there is a strong urge for micturition accompanied by fear of leakage or pain. Further than 600 to 700 ml collection of urine (*anatomical vesical capacity*), voluntary control starts failing (the detrusor contractions becomes painful).

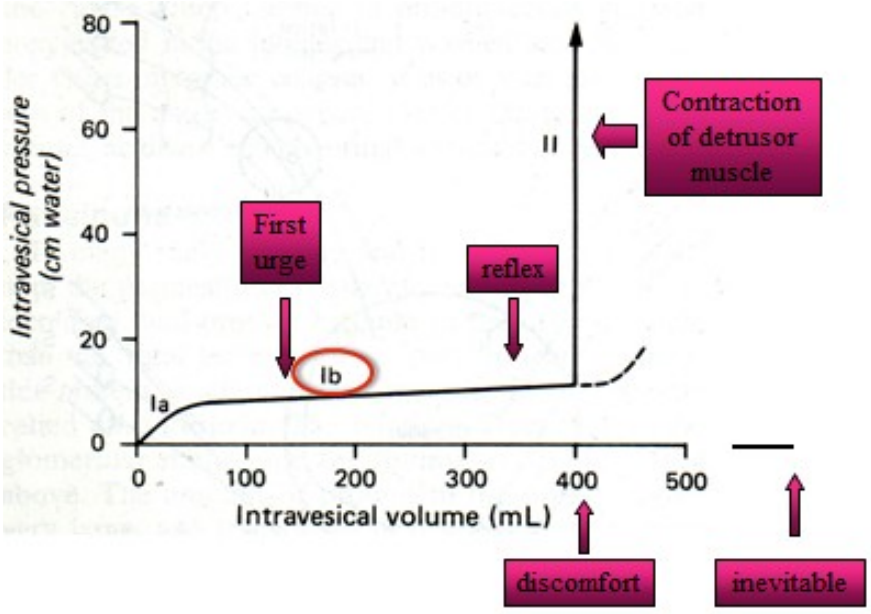


Figure 5.7. Normal cystometrogram

Practical importance: Cystometry is used to determine urinary bladder functions like bladder filling sensation, bladder capacity, accommodation, detrusor contractility, voluntary bladder control, and emptying.

ANNEXE – MINIMUM KNOWLEDGES
(Ist Year)

1. **Osmolarity of internal environment** = 280 – 300 mOsm

2. **Intracellular pH:** 7 (neutral)
   Normal values range: 6 – 7.5 (depend on cell type)

3. **Extracellular pH**:
   pH = 7.4 (arterial plasma)
   pH = 7.35 (venous blood plasma)
   pH = 7.4 (interstitial fluid)

4. **Sodium concentration** in intra- and extracellular medium:
   Intracellular medium: 10 - 37 mEq/l
   Interstitial fluid: ≈ 136 mEq/l
   Blood plasma: 140 ± 5 mEq/l

5. **Potassium concentration** in intra- and extracellular medium:
   Intracellular medium: ≈ 150 mEq/l
   Blood plasma: 3.5 -5.6 mEq/l

6. **Calcium concentration** in intra- and extracellular medium:
   Intracellular medium: ≈ 10 (-7) M
   Blood plasma: 2.1 – 2.6 mEq/l

7. **Magnesium concentration** in intra- and extracellular medium:
   Intracellular medium: 2.6 mEq/l
   Interstitial fluid: ≈ 136 mEq/l
   Blood plasma: 1.5-2.5 mEq/l

8. **Chloride concentration** in intra- and extracellular medium:
   Intracellular medium: 1-3 mEq/l
   Interstitial fluid: ≈ 117 mEq/l
   Blood plasma: 90-110 mEq/l

9. **Total blood plasma protein**: 6-8 g/dl

10. **Blood plasma protein (proteinogram)**

	g/dl	%
Total protein	6-8	100
Albumins	4.5-5.7	55-70
Globulins	1.46-2.54	30-45
Fibrinogen	0.2-0.4	
Fraction albumins / globulins	1.5-2.5	

11. **Globulin fractions** (% of total plasma proteins)
    α_1: 3-7 %; α_2: 7-10 %; β: 9-17 %; γ: 12-20 %;

12. Hematocrit: Definition and normal values.
Definition: It is defined as the ratio between the volume of blood cells and the volume of blood in which they are contained.
Normal values: Men: 46.5% ± 5 %;
Women: 42% ± 5 %;
New born: 56% ± 5 %;
Children: 40% ± 5 %;
Somatic hematocrite = 0.91 x venous hematocrite
(Therefore, somatic hematocrite is lower than venous hematocrite)

13. Red Blood Cells count. Normal values
Normal Values: Men: 4.5 - 5 millions/mm^3
Women: 4.2 - 4.5 millions/mm^3
Children: 5 - 6 millions/mm^3

14. Hemoglobin concentration in RBC's and blood
Normal Values: Men: 14-18 g/dl
Women: 12 - 16 g/dl
New born: 18 - 27 g/dl
Children: up to 1 year: 10 - 15 g/dl
up to l0 years: 12 - 17 g/dl

15. Erythrocyte Sedimentation Rate (ESR): Definition and normal values.
Definition: It is defined as the height of the supernatant column, separated after 1, 2 or 24 hours from the mixture of blood (venous blood) and anticoagulant (Sodium Citrate 3.8%) in the ratio 4:1.
Normal Values: Men: 2 - 7 mm (after 1 hr); 7 - 15 mm (after 2 hours).
Women: 4 - 9 mm (after 1 hr); 12 - 17 mm (after 2 hours).
In pregnancy: 35 mm (after 1 hr);
Newborn: 0.5 mm (after 1 hr);

16. **White Blood Cells Count. Normal Values**:
Adult: 4.000 - 8.000/mm^3
New born: 15.000 - 30.000/mm^3
Children: up to 1 year: 9.000 - 15.000/mm^3
up to 6 years: 4.000 - 10.000/mm^3

17. Differential white blood cell count (leukocyte formula)

GRANULOCYTES

Neutrophils	60 - 70%	3000 - 8000/mm3
Eosinophils	1 - 4%	300 - 400/mm3
Basophils	0 - 1%	20 - 80/mm3

AGRANULOCYTES

Lymphocytes	25 - 35%	1500 - 2400/mm3
Monocytes	4 - 8%	240 - -600/mm3

18. Bleeding Time: definition and normal values: Duration of bleeding induced after dermal puncture with a Frank needle. *Normal Values*: 2 - 4 minutes

19. Coagulation Time: definition and normal values.
Definition: It's the time interval between harvesting of the venous blood until its complete coagulation (disappearance of blood fluidity). There are 2 techniques:
a) Lee and White technique (on test tubes)
- in the 1st test tube: 6 - 10 minutes
- in the 2nd test tube: 8 - 12 minutes.

b) Bazarov technique (on glass slide):
- 8 - 10 minutes.

20. Platelet count: normal values.
Normal Values: 200.000 - 300.000/mm^3

21. Exploration of the capillary fragility using the Rumpel Leeds test.
Using a pneumatic cuff, a pressure of about 10 mmHg (below the systolic pressure) is applied around the forearm and maintains it for 5 minutes. Then on a circular surface of diameter 2 cm below the edge of the cuff we count the number of red spots:
Simple positive test: 10 - 12 spots (+)
Highly positive test: 20 spots (++)
Pathologic test: 80 spots (+ + +)

21. Blood typing. Determination of blood groups.

There are 2 methods:
a) Determination of agglutinogen (Beth-Vincent method):
On a slide we put 3 drops of serum belonging to groups O, A, B. To each of them add a small quantity of blood that has been obtained from venous or capillary puncture. If there is agglutinogen in the blood and if it corresponds with the agglutinin in the serum, then the RBC's will agglutinate (clot) and then precipitate. The result will be a red precipitate. If there is no agglutinogen then the drops will remain as a homogenous mixture with a reddish yellow color without precipitation.

b) Determination of the agglutinin (Simonin method)
On a glass slide we put 3 drops of the patient's blood. On top of them we put a small quantity of standard erythrocyte (corresponding to the groups O, A, B). Then we read the result like in the 1st determination.

22. Normal urine: aspect, pH and diuresis.
Aspect at emission: pale yellow (but darker in the morning), transparent, clear
pH: - in balanced diet - 6.2 - 6.8
- in predominantly proteic diet - 5.2 - 5.3
- in vegetarian diet - 7 - 7.5

Diuresis: volume of urine in 24 hrs is 900 - 1500ml, with physiological variations according to age, sex, environmental temperature and liquid ingestion, etc

23. Components of standard urine examination (urinalysis).
Density: 1.015 - 1.022
pH: 6.2 - 6.8 (in normal diet)
In the normal urine glucose, albumin, ketone bodies, biliary pigments and salts are absent
There will be normal values for urobilinogen.
In the sediments will be leukocytes, epithelial cells and crystals.

24. Addis test: normal values.
Definition: quantitative examination of blood cells and cylinders in the urinary sediment from the urine in 24 hours.

	Adults
Red blood cells	< 500 000/24 h
White blood cells	1-1.8 millions/24 h
Cylinders (casts)	<5 000/24 h

25. Normal concentration of urea in blood plasma
0.2 - 0.4g/l

26. Normal concentration of urea in final urine.
15 - 35 g/l.

27. Normal concentration of uric acid in blood plasma
2 - 5mg/dl

28. Normal concentration of uric acid in final urine
0,5 - 1 g/l.

29. Normal concentration of creatinine in blood plasma
0.6 - l.3 mg/dl

.

30. Normal concentration of creatinine in final urine.
0.75 - 2 g/l.

31. Normal concentration of glucose in blood plasma
0.8 – 1.1 g/l.

32. Normal concentration of glucose in final urine.
0 g/l.

33. Normal concentration of Ca^{2+} in blood plasma
4.3 – 5.2 mg/dl.

34. Normal concentration of Ca^{2+} in final urine
0.14 – 0.20 mg/dl.

35. Normal concentration of PO_4^{3-} in blood plasma
3 - 4.5 mg/dl

36. Normal concentration of PO_4^{3-} in final urine
1.2 – 1.5 mg/dl.

REFERENCES

Berne RM, Levy MN, Koeppen BM, et al: Berne & Levy: *Principles of Physiology*, 4th ed. St. Louis, Elsevier Mosby, 2006

Bullock J, Boyle J, Wang MB: *Physiology*, 4th ed. Philadelphia, Lippincott Williams / Wilkins, 2001

Chadha PV: *Handbook of Experimental Physiology and Biochemistry*, 6th ed. New Delhi, Jaypee, 1993

Chandrasekar M: *Practical Physiology Book*, 1st ed, New Delhi, Jaypee, 2012

Chatterjea MN, Shinde R: *Textbook of Medical Biochemistry*, 8th ed, New Delhi, Jaypee, 2012

Constanzo L: *Physiology*, 4th ed. Philadelphia, Lippincott Williams / Wilkins, 2007

Davila GW, Ghoniem GM, Wexner S: *Pelvic floor dysfunction: a multidisciplinary approach*, London, Springer, 2006

Despopoulos A, Silbernagl S: *Color Atlas of Physiology*, 5th ed. Thieme, 2003

Fox SI: *Human Physiology*, 8th ed. New York, McGraw-Hill, 2004.

Ganong WF: *Review of Medical Physiology*, 22nd ed. New York, McGraw-Hill, 2005

Goldman L, Ausiello D: *Cecil Medicine*, 23rd ed.Philadelphia, Saunders, 2007

Guyton AC, Hall JE: *Textbook of Medical Physiology*, 11th ed. Philadelphia, Saunders, 2006

Joshi VD, Joshi-Mendhurwar S: *Physiology: Prep Manual For Undergraduates*, 3rd ed. Noida, Elsevier, 2009

Khurana I: *Essentials of Medical Physiology*, 1st ed. Noida, Elsevier, 2009

Khurana I: *Textbook of Medical Physiology*, 1st ed. Noida, Elsevier, 2009

Meyer BJ et al: *Human physiology: chemical, physical, and physiological principles*, 3rd ed: Cape Town: Juta, 2002.

Nigam SC, Omkar: *Experimental Animal Physiology and Biochemistry*, 1st ed., New Delhi, New Age International, 2003

Pal GK, Pravathi P: *Textbook Of Practical Physiology*, 2nd ed. Chennai, Orient Longman, 2006

Pocock G: *Human Physiology: the basis of medicine*, 3rd ed. Oxford, Oxford University Press, 2006

Rastogi SC: *Experimental Physiology*, 2nd ed., New Delhi, New Age International, 2005

Ratan V: *Handbook of Human Physiology*, 7th ed. New Delhi, Jaypee, 2004

Rhoades RA, Tanner GA: Medical Physiology, 2nd ed. Philadelphia, Lippincott Williams/Wilkins, 2003

Sherwood L: *Fundamentals of physiology: a human perspective*, 3rd ed. Belmont, Calif., Brooks/Cole, 2006.

Silverthorn DU: *Human Physiology: An Integrated Approach*, 4th ed. Upper Sadle River, NJ, Pearson/ Benjamin Cummings, 2007

Sircar S: *Principles of Medical Physiology*, 1st ed. Stuttgart, Thieme , 2008

Thies R, Barron KW: *Physiology*, revised 4th ed. Springer-Verlag, 1987

Tortora GJ: *Introduction to the human body: the essentials of anatomy and physiology*, 7th ed. New York, Wiley & Sons, 2007.

Vasudevan DM, Sreekumari S: *Textbook of Biochemistry for Dental Students*, 5th ed. New Delhi, Jaypee, 2008

West JB: *Best and Taylor's Physiological Basis of Medical Practice*, 12th ed. Baltimore, Williams and Wilkins, 1991

Widmaier EP: *Vander's human physiology: The Mechanisms of body function*, 11th ed. Boston, McGraw-Hill, 2008.