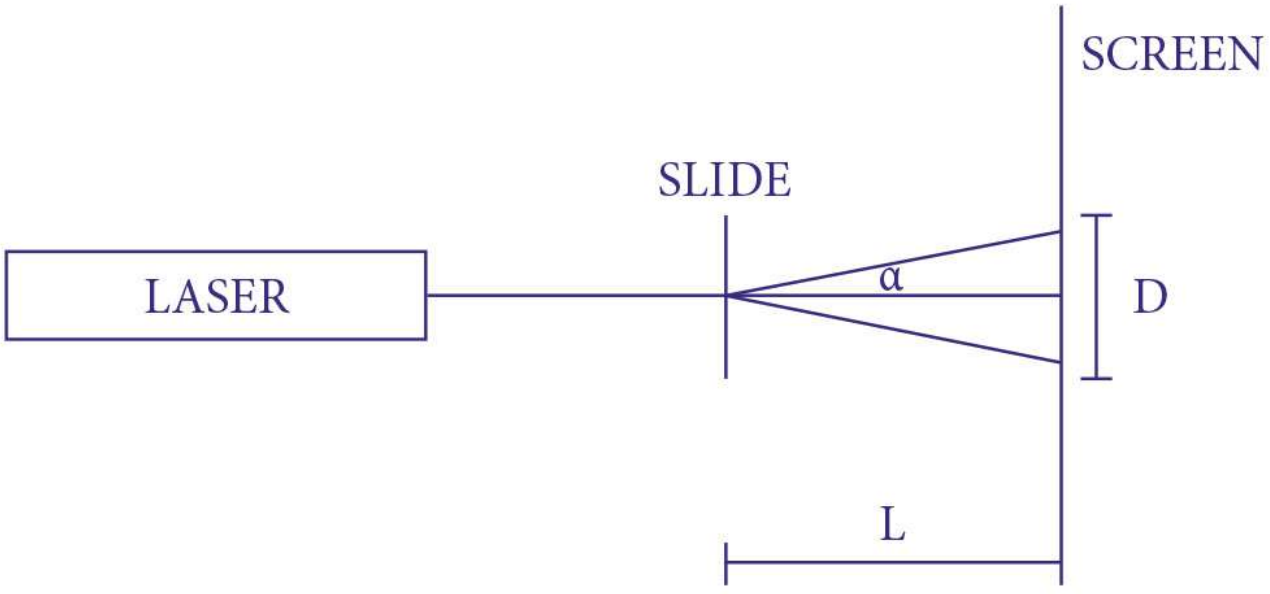
UNIVERSITY OF DEBRECEN
FACULTY OF MEDICINE
DEPARTMENT OF BIOPHYSICS AND CELL BIOLOGY

BIOPHYSICS LABORATORY MANUAL



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Content

1. Absorption photometry I.

The light, like any other electromagnetic wave, can interact with different atomic and molecular systems. One of these interactions is the one when the light as a particle (photon) interacts with the electron system of a molecule (or atom) in a manner that its energy turns into the internal energy of the electron system while the photon "disappears". This kind of an interaction results in the phenomenon of light absorption which means that in general the light intensity decreases when the light is passing through a gas, liquid or solid phase built up by atomic systems capable of such an interaction.

When dealing with the case of a homogeneous and isotropic substance for the light to travel through, one can say that the decrease of the light intensity, dI, is proportional to the light path, dx, and the incident light intensity I (see Fig.1). The proportionality factor in this relationship, k', is characteristic of both the substance the light interacts with (the atomic or molecular structure) and the wavelength (related to the energy content) of the light:

$$dI = -k'I\,dx \tag{1}$$

The negative sign in eq. (1) refers to the fact that the light intensity decreases with the increase of the distance the light travelled through (the increase of x results in a decrease of I).

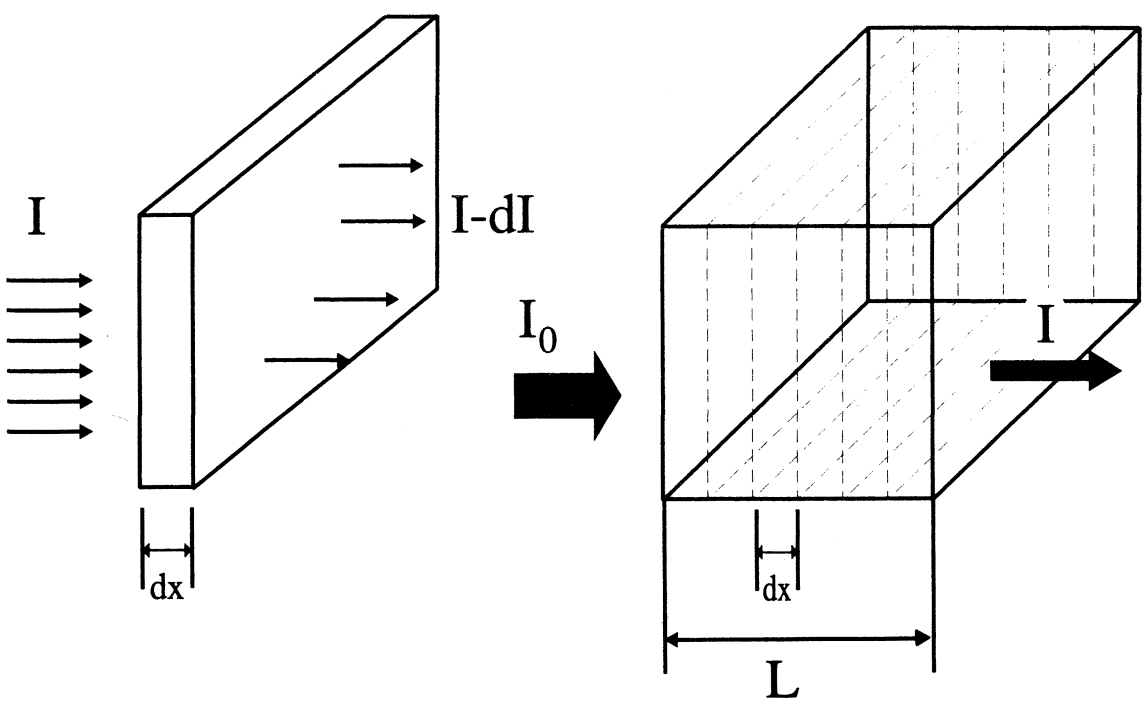


Figure 1

Eq.(1) is a first order differential equation which can be solved by integration. First let us separate the variables:

$$\frac{dI}{I} = -k'dx \tag{2}$$

Integrating both sides of the equation,

$$\int \frac{dI}{I} = -k' \int dx \tag{3}$$

we arrive at the form of

$$\ln I + C_1 = -k'x + C_2 \tag{4}$$

where C_1 and C_2 are arbitrary constants (i.e. eq.(4) corresponds to eq.(3) at any set of the values for the two constants). To obtain the values of the two constants relevant to our case we define I_0 as the intensity of the incident light, i.e. I_0 is the value of I when $x = 0$. Accordingly,

$$\ln I_0 = C_2 - C_1, \tag{5}$$

which results in the form of

$$\ln \frac{I}{I_0} = -k'x \tag{6}$$

We should note that to arrive at eq.(6) we have used the relationship

$$\ln a - \ln b = \ln \frac{a}{b} \tag{7}$$

Taking the exponential of both sides of eq.(6) we obtain:

$$I = I_0 e^{-k'x} \tag{8}$$

Eq.(8) describes the so-called **Lambert-Bouguer** law which tells us that the light intensity decreases exponentially with the distance x travelled by the light in the given (homogeneous and isotropic) substance. k' is the **absorption coefficient** with the unit of cm^{-1} and it gives the reciprocal value of the light path which (in the given case) corresponds to an e (=2.71828...) times decrease of the incident light intensity, I_0, i.e. when $I/I_0 = e^{-1}$. We can change the base of the logarithm in eq.(8) from e to 10 and arrive at the form of

$$I = I_0 10^{-kx} \tag{9}$$

It is to note that due to this change, k' is has to be replaced by other value, k, which is called the **Bunsen's absorption coefficient** (and it gives the reciprocal value of the light path corresponding to a ten times decrease of the light intensity).

Eq.(9) can be used to determine the solute concentration in a solvent provided that the dissolved molecules show absorption. In that case we can compare the absorption of the solvent and the solute (for details see later). The basic assumption here is that the dissolved molecules have no interaction with each other or with the solvent molecules (i.e. the change of concentration does not involve any chemical change). In such a case we can go back to eq.(1) and see that by increasing the concentration by a factor of 2 we also obtain a 2 times increase in dI. (The 2 times increase in the concentration doubles the chance of the photons to meet dissolved molecules). Then k' (and k also) can be dissected into the product of the

concentration of dissolved molecules (responsible for the absorption) and a constant (which is now independent of the concentration):

$$k = \varepsilon c \qquad \textbf{(10)}$$

ε is the **molar decadic absorption coefficient** and eq.(10) is called the **Beer-law.** Inserting it into eq.(9) we obtain:

$$I = I_0 10^{-\varepsilon c L} \qquad \textbf{(11)}$$

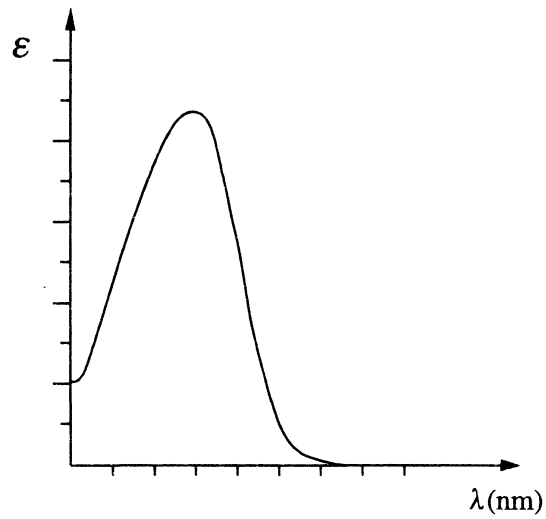


Figure 2.a

which is the **Lambert-Beer law** and is the most frequently used form to describe absorption. In this equation L corresponds to the total thickness of the absorbing medium. The unit of ε is usually given in $M^{-1}cm^{-1}$. Although ε is independent of the concentration of the dissolved molecules it still depends on the wavelength (λ) of the incident light and is characteristic of the (dissolved) material which is responsible for the absorption. Accordingly, the wavelength dependence of ε can be used for chemical analysis (see Figs. 2a and 2b) and its time dependence can be used to follow chemical reactions. The product of εcL is usually called absorbance (A) or extinction (E) or optical density (OD).

An important property of the absorbance is its additivity. This means that the mixture of two kinds of molecules (in a solvent) gives an absorbance value which is the sum of the absorbance values given by the two substances dissolved alone in the same solvent (and, of course, the same light path is used):

$$A = A_1 + A_2 \qquad \textbf{(12)}$$

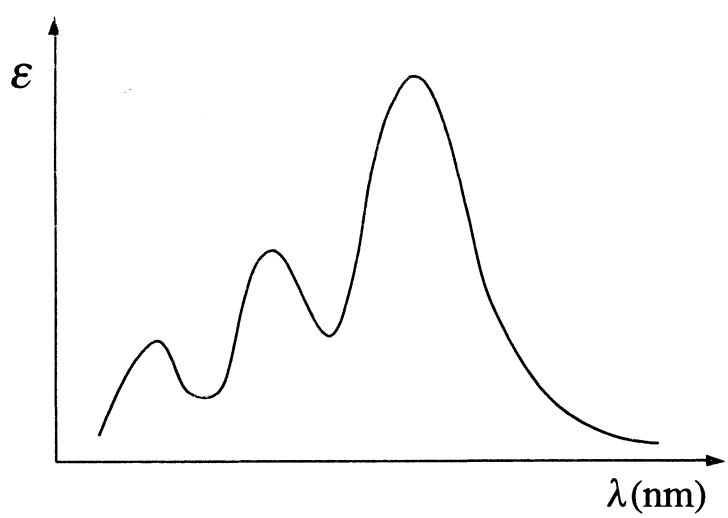


Figure 2. b

To verify eq.(12) let us take two chemicals, with ε_1 and ε_2 molar decadic absorption coefficients, dissolved in a liquid which does not show any absorbance at the applied wavelength. If c_1 and c_2 are the appropriate concentrations, eq.(1) can be rewritten (see eq.(10)) as:

$$\mathrm{d}I = -I(\varepsilon_1 c_1 + \varepsilon_2 c_2)\,\mathrm{d}x \qquad \textbf{(13)}$$

If x is the total distance travelled by the light in the solvent (optical path length) eq.(13) gives the result of

$$I = I_0 10^{-(\varepsilon_1 c_1 x + \varepsilon_2 c_2 x)} \qquad \textbf{(14)}$$

i.e.

$$I = I_0 10^{-A_1} 10^{-A_2} = I_0 10^{-A} \qquad (15)$$

From eq.(15) one can readily see that eq.(12) does really hold.

From the validity of eq.(12) it follows that in a case when both the solvent and the dissolved material have a given absorbance value, the absorbance belonging to the dissolved material can be calculated by subtracting the absorbance of the solvent alone from the absorbance characteristic of the solution.

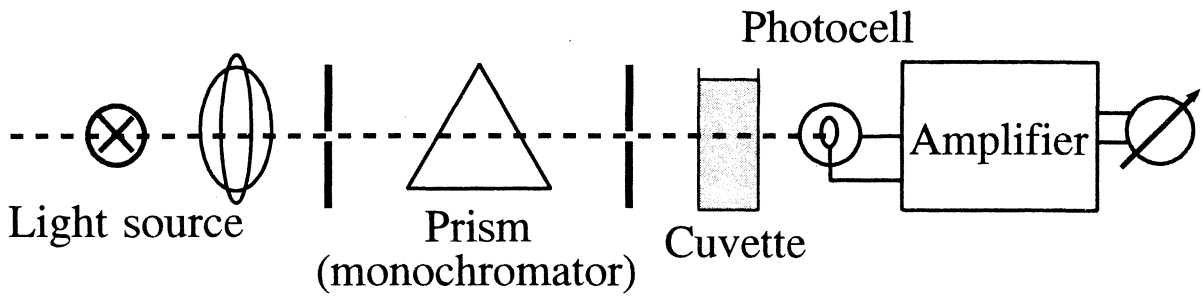


Figure 3

Measurement of absorbance: the spectrophotometer

The main units of a spectrophotometer can be seen on Fig.3. The light emitted by the light source is made parallel by the entering optical system which is also guided by the slits. The monochromator (e.g. a prism) is the optical unit which dissects the white light and separates the different colors to leave at different angles. Accordingly, by turning the monochromator we can select the light component (depending on which wavelength (or color) we want to use) which actually will reach the sample through the slit Nr.2. The photodetector located right behind the cell will measure the light intensity coming from the sample (by giving an electronic signal proportional to the light intensity). This electronic signal enters an amplifier which gives an output either to an analog or to a digital readout system.

It is easy to see, how this arrangement can be used to measure the I value of eq.(11) (or eq.(14)) in a case when the solvent also has an absorption). The value of I_0 is, again, easy to measure. All we have to do is to measure the light intensity (more precisely the electronic signal proportional to it) when only the pure solvent (without the dissolved material) is present in the cell. If the solvent has no absorption (or it is negligible) we

measure the value of I_0. In the case when its absorbance is not negligible we measure the $I_{solvent} = I_0 10^{-A} 1$ value of eq.(15) which can be compared to $I_{solution}$, measured in a separate experiment with the solution, to obtain $10^{-\varepsilon} 2^{c} 2^{x}$ [according to eq.(14) $I_{solution}$ has to be divided by $I_{solvent}$ to obtain $10^{-\varepsilon} 2^{c} 2^{x}$].

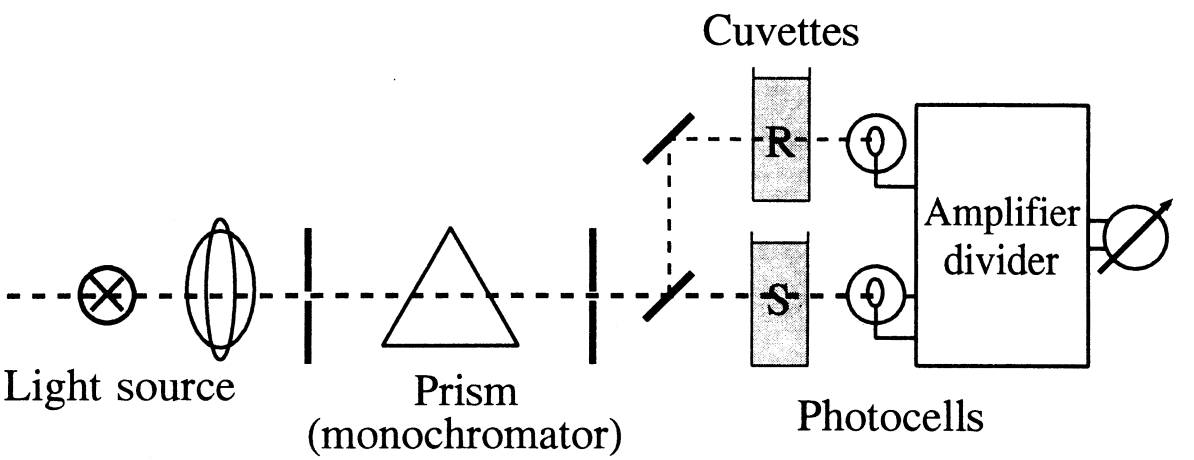


Figure 4

The spectrophotometers described above are the so-called one (light) beam photometers. There are photometers where the light beam coming from the monochromator is dissected into two identical beams (Fig.4). These are two light beam spectrophotometers. In this case one cell (S) contains the solution while the other (R) contains the solvent (reference) and the amplifier electronically divides the two signals (belonging to the two light intensities). The double beam spectrophotometers used to be more accurate but the recent development of electronic circuitry makes it possible to built single beam photometers with the same (or similar) performance parameters like the double beam photometers have.

Besides the use of absorbance, it is also necessary to talk about the **transmittance**, T, which is related to the absorbance as

$$A = \lg\left(\frac{100}{T}\right) = 2 - \lg T \qquad \textbf{(16)}$$

(lg in the above equation is the 10 base logarithm function). The definition of $A = \varepsilon cx$ together with eq.(11) readily gives us that T is nothing but the I/I_0 ratio in percentage (%) units (i.e. 100 I/I_0). The use of transmittance instead of absorbance is primarily related to the single beam spectrophotometers or, on a historic background, to selected topics in spectroscopy (like infrared spectrophotometry). In some cases, mainly when working with single beam photometers (like the ones in our lab) there are analog type output meters which are calibrated in both absorbance (A) (extinction (E)) and transmittance (T) units (Fig.5)

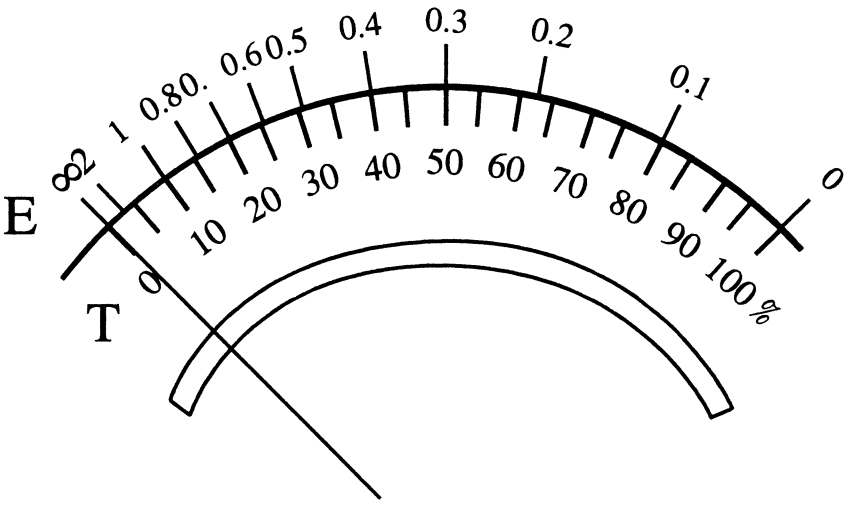


Figure 5

Experiments:

Solutions:

1. Five $CoCl_2$ - solutions (known concentration, solvent: distilled water)
2. Five fluorescein solutions (known concentration, solvent: Phosphate Buffered Saline solution (PBS))
3. Two $CoCl_2$ - solutions (unknown concentration, solvent: distilled water)
4. Two fluorescein solutions (unknown concentration, solvent: PBS)

1./ Record the absorption spectrum of the $CoCl_2$ or fluorescein-solution (measure the extinction in the 410-600 nm wavelength range by 10 nm).

2./ Plot the measured extinction values as a function of wavelength. Read and record the absorption maximum of the examined compound.

3./ Measure the extinctions of the solutions of known concentration at the wavelength of the absorption maximum. Plot the resulting data (the extinction values as a function of concentration).

4./ Determine the molar extinction coefficient (ε) of the compound using the curve obtained in point 3.
(Molecular weights: $CoCl_2$ 237.9
fluorescein 376.3)

5./ Measure the extinctions of the two unknown solutions and determine their concentrations on the basis of the Lambert-Beer law as well as using the calibration curve.

The course of the measurement:

1./ Switch on the instrument and the lamp (20 min for warming up).
2./ Set the wavelength desired.
3./ Set the dark current (when the light path is blocked move the indicator to the $E = \infty$, $T = 0$ position with the button on the upper right side $<0>$, see Fig. 5).
4./ Set $E = 0$ with distilled water (after opening the ligth path, move the indicator to $E = 0$, $T = 100$ position with the button on the lower right side $<100>$, see Fig 5.).
5./ Control the $E = 0$ position at every wavelength.
6./ Replacing the distilled water (solvent) with the samples, read the extinction values.

Warning!

1. The ligth path should be opened during the measurements and the setting of the $E=0$ position!
2. The cuvette should be rinsed with distilled water and kept clean!

2. Absorption photometry II.

As it is known from the preceding section (Absorption photometry I.), the molar decadic absorption coefficient (ε) and its wavelength dependence (spectral characteristics) are characteristic of the chemical structure of the given material. This makes it possible to study the time course and/or the equilibrium properties (e.g., equilibrium constant) of chemical reactions by means of spectrophotometry.

Our aim here is to show how the equilibrium constant (or its reciprocal value, the dissociation constant) of a reversible bimolecular reaction can be obtained by this technique. For this reason let us take a simple bimolecular reaction with the scheme of

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \qquad \textbf{(1)}$$

where A and B are the reactants and X is the product. k_1 and k_2 are the formation and decomposition rate constants of X, respectively (note that k_1 is a bimolecular while k_2 is monomolecular rate constant). For later use we note that there are two kinds of constants characterizing the equilibrium of a reaction: K_d ($=k_2/k_1$) is called the dissociation constant of the reaction and K_a ($=k_1/k_2$) is the association constant (the two are in reciprocal relationship with each other).

When the reaction is in equilibrium the forward and reverse rates of the reaction are, by definition, the same, i.e.:

$$k_1\,[A]\,[B] = k_2\,[X] \qquad \textbf{(2)}$$

where $[A]$, $[B]$ and $[X]$ are the equilibrium concentrations of the appropriate reactants and product, respectively.

In order to determine the dissociation constant in question, the most important condition which has to be satisfied is that the

$$A + B \rightleftharpoons X \qquad \textbf{(3)}$$

transition should result in a change of absorbance (in some wavelength range of the absorption spectrum), i.e. $\varepsilon_A + \varepsilon_B$ should be different from ε_X (can be either higher or lower) where the ε values are the molar decadic absorption coefficients for the appropriate molecules referred to by the indices.

For the sake of simplicity we can assume that the concentration of one reactant, e.g. that of B, is much higher than that of the other (in the practice, this condition is usually easy to satisfy):

$$[B]_0 \gg [A]_0 \qquad \textbf{(4)}$$

$[B]_0$ and $[A]_0$ are the reactant's concentrations when the experiment starts at $t=0$ (provided that $[X]_0 = 0$, i.e. we start the reaction by mixing the two reactants at $t=0$).

To proceed further, we use the mass balances for the two reactants:

$$[A]_0 = [A] + [X], \quad \textit{i.e.} \quad [A] = [A]_0 - [X] \qquad \textbf{(5)}$$

and

$$[B]_0 = [B] + [X], \quad i.e. \quad [B] = [B]_0 - [X] \tag{6}$$

Inserting $[A]$ and $[B]$ from eqs. (5) and (6) into eq.(2) leads us to:

$$([A]_0 - [X])\,([B]_0 - [X]) = K_d\,[X] \tag{7}$$

i.e.,

$$[A]_0\,[B]_0 = [X]\,(K_d + [A]_0 + [B]_0 + [X]) \tag{8}$$

According to the inequality (4), $[B]_0$ is much higher than both $[A]_0$ and $[X]$ (the value of $[X]$ can not be higher, by the definition of the reaction conditions, than $[A]_0$), therefore, $[A]_0$ and $[X]$ can be neglected as compared to $[B]_0$ in the expression on the right hand side of eq.(8). Then, the rearrangement of eq.(8) gives:

$$[X] = \frac{[A]_0\,[B]_0}{K_d + [B]_0} \tag{9}$$

Eq.(9) can be arranged into a linear form,for later use, by taking the reciprocal of both sides:

$$\frac{1}{[X]} = \frac{1}{[A]_0} + \frac{K_d}{[A]_0}\,\frac{1}{[B]_0} \tag{10}$$

Eq.(10) shows that by changing $[B]_0$ at constant $[A]_0$ (while maintaining the $[B]_0 >> [A]_0$ condition) the value of $1/[X]$ will vary in a linear fashion as a function of $1/[B]_0$.

In order to calculate K_d, we have to know the values of $[A]_0$, $[B]_0$ and $[X]$. We know $[A]_0$ and $[B]_0$ since these are from the starting conditions for the mixing. We have, however, no information about the value of $[X]$. To overcome this problem, we can determine, from experiments or by calculation from some given parameters, the total absorbance of the solvent when A and B are present in the concentrations of $[A]_0$ and $[B]_0$:

$$E_\Sigma = \varepsilon_A\,[A]_0\,L + \varepsilon_B\,[B]_0\,L \tag{11}$$

where L is the optical length (usually 1 cm). At the same time, we can measure the absorbance after the reaction has reached the equilibrium (for simple chemical reactions it usually falls into the millisecond time scale):

$$E_{mix} = \varepsilon_A\,[A]\,L + \varepsilon_B\,[B]\,L + \varepsilon_X\,[X]\,L \tag{12}$$

The difference of eqs. (11) and (12), ΔE, can be obtained as

$$\begin{aligned} \Delta E &= E_\Sigma - E_{mix} = L\left\{\varepsilon_A([A]_0 - [A]) - \varepsilon_B([B]_0 - [B]) - \varepsilon_X[X]\right\} = \\ &= L[X](\varepsilon_A + \varepsilon_B - \varepsilon_X) = L[X]\Delta\varepsilon \end{aligned} \tag{13}$$

In deriving the last line, we have used eqs.(5) and (6) to obtain [X] as

$$[X] = [A]_0 - [A] = [B]_0 - [B] \tag{14}$$

The definition of $\Delta\varepsilon$ in eq.(13) is obvious:

$$\Delta\varepsilon = \varepsilon_A + \varepsilon_B - \varepsilon_X \tag{15}$$

which can be either positive or negative depending on the spectral characteristics of the reaction system.

We can rearrange eq.(13) as

$$\frac{1}{[X]} = \frac{L\Delta\varepsilon}{\Delta E} \tag{16}$$

which can be substituted into eq.(10) to obtain:

$$\frac{1}{\Delta E} = \frac{1}{[A]_0 L\Delta\varepsilon}\frac{1}{[B]_0} + \frac{1}{[A]_0 L\Delta\varepsilon} \tag{17}$$

This equation makes it possible to determine K_d from a series of ΔE values obtained at different $[B]_0$ values (while maintaining $[A]_0$ at a constant value): The plot of $1/\Delta E$ versus $1/[B]_0$ gives a straight line (Fig.1) with the slope of

$$\text{tg}\,\alpha = \frac{K_d}{[A]_0 L\Delta\varepsilon} \tag{18}$$

and with the intercept of

$$a = \frac{1}{[A]_0 L\Delta\varepsilon} \tag{19}$$

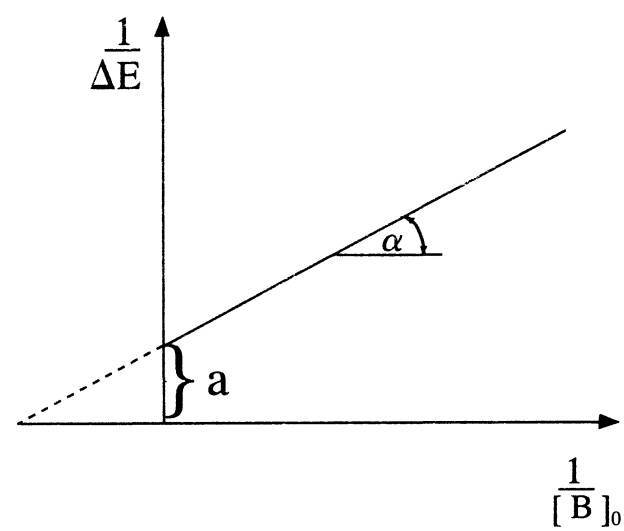


Figure 1

Accordingly, K_d can be obtained as the ratio of the slope and intercept,

$$K_d = \frac{\text{tg}\alpha}{a} \tag{20}$$

without using the values of $[A]_0$, L and $\Delta\varepsilon$.

Experiments:

1./ Measure the optical spectra of $E([A]_0)$ and $E([B]_0)$ over the wavelength range available (at given $[A]_0$ and $[B]_0$ values).

2./ Repeat the above experiment by using the reaction mixture of $[A]_0$ and $[B]_0$.

3./ Plot the above three spectra and determine the wavelength where the absolute value of $\Delta\varepsilon$ has maximum (λmax).

4./ Determine ΔE values at λmax with different $[B]_0$.

5./ Plot $1/\Delta E$ against $1/[B]_0$ and determine the slope and intercept of the linear curve fitted to the experimental data points.

6./ Calculate K_d from the slope and intercept (see eq.(20).

3. Absorption photometry III.

The theory of reaction kinetics relates the dissociation constant of a given reaction, K_d, to thermodynamic parameters like the Gibbs free energy difference between the reactants and product(s). In the case of a simple reaction, like

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \qquad (1)$$

the expression of K_d is:

$$K_d = e^{-\frac{\Delta G}{RT}} \qquad (2)$$

where T is the absolute temperature, R is the universal gas constant and ΔG is the free energy difference between the reactants and product (Fig.1).

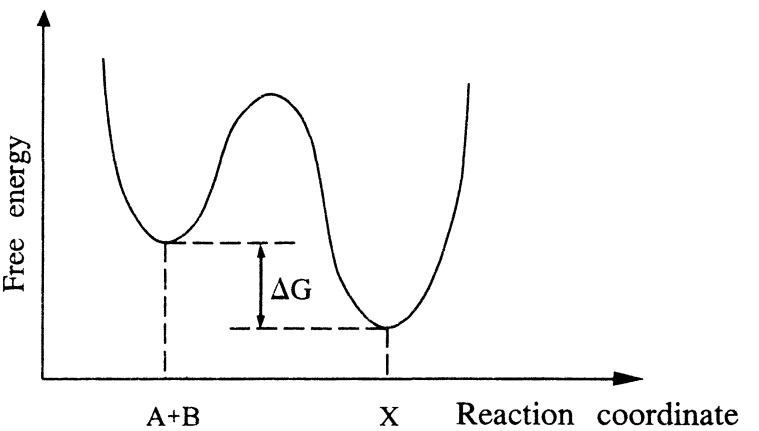


Figure 1

Fig.1 shows how the free energy level changes along the so-called reaction coordinate as the reaction goes on. In the simplest cases the reaction coordinate can be the length of a bond to be formed or cleaved. In a general case, however, it is more difficult to visualize it in a multidimensional phase space. It is also shown on Fig.1 that the reaction component(s) has (have) to overcome a free energy barrier to undergo either the forward or reverse reaction step. The form of eq.(1) is related to the forms of the individual rate constants, k_1 and k_2, which are exponentially related to G_1 and G_2 (due to the Maxwell-Boltzmann distribution).

An other form of eq.(2) can be given as

$$\Delta G = -RT \ln K_d \qquad (3)$$

which, again, is a useful form (see it later).

To proceed further, we have to use the basic thermodynamic equation described as

$$G = H - TS \qquad (4)$$

or

$$\Delta G = \Delta H - T\,\Delta S \qquad (5)$$

Here H is the enthalpy, S is the entropy and ΔH, ΔS are the enthalpy and entropy differences between the two states of the reaction system (i.e. the reactants and product(s), respectively). Then, inserting eq.(5) into eq.(3) we obtain:

$$\ln K_d = \frac{\Delta S}{R} - \frac{\Delta H}{R}\,\frac{1}{T} \qquad (6)$$

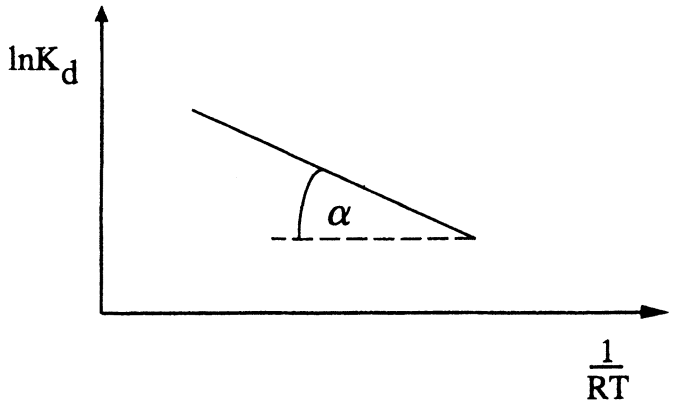


Figure 2

Eq.(6) shows that K_d varies with the temperature. By measuring K_d at different temperatures the plot of $\ln K_d$ against $1/RT$ gives a straight line (see Fig.2) with the slope of

$$\mathrm{tg}\alpha = -\Delta H \qquad (7)$$

(the intercept is rarely used to obtain extra information due to the high uncertainty related to the fact that the $1/RT$ values are usually falling in a range located far away from 0).

The ΔG value itself can be calculated from eq.(3) using a K_d value measured at a given temperature T. Accordingly, ΔS can also be calculated from eq.(6) and this way we can obtain the three thermodynamic parameters, ΔG, ΔH and ΔS, characterizing the equilibrium of the given (reversible) reaction.

Experiments

1./ Determine the K_d values for a given reaction at different temperatures.
2./ Plot $\ln K_d$ as a function of $1/RT$.
3./ Calculate ΔG, ΔH and ΔS values characteristic of the reaction.

4. Spectrofluorimetry: evaluation of the dissociation constant for a reversible reaction

Spectrofluorimetry, just like any other spectroscopic method, can be used to investigate a reversible chemical reaction and determine, e.g., the dissociation constant characteristic of the given reaction.

To monitor a reaction by spectrofluorimetry, the obligatory condition is that at least one of the reactants should be a fluorophore with fluorescence characteristics showing a big enough change while the reaction goes on.

Let us regard the reaction between bovine serum albumin (which is a serum protein) (BSA) and 1,8-anilino naphtalene - sulphonic acid (ANS):

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \tag{1}$$

ANS is a fluorophore which binds to the hydrophobic region of different proteins and its fluorescence yield increases upon this binding (by a factor of about 100). To obtain the value of the dissociation constant, K_d, let us assume that

$$[A]_0 \ll [B]_0 \tag{2}$$

where $[A]_0$ and $[B]_0$ are the concentrations of ANS and BSA, respectively, at $t = 0$ (when we mix the two components). Let us further assume that we measure the fluorescence emitted by the ANS in different reaction mixtures where $[A]_0$ is kept constant and $[B]_0$ varies from sample to sample.

The fluorescence intensity, F_0, of the sample containing ANS only ($[B]_0 = 0$) will be:

$$F_0 = [A]_0 \varepsilon \phi \tag{3}$$

where ε is the molar absorption coefficient of the free ANS at the excitation wavelength and ϕ is its fluorescence yield (quantum efficiency). The fluorescence intensity of the mixture containing both ANS and BSA will be:

$$F = [A] \varepsilon \phi + [X] \varepsilon' \phi' \tag{4}$$

where ε' and ϕ' are the appropriate parameters characteristic of the ANS molecule when it is bound to the hydrophilic region of BSA. Here it is to note that

$$[A]_0 = [A] + [X] \tag{5}$$

which is the equation of the mass balance.

Now we have to calculate the value of [X] by using the condition given by eq.(2) as

$$[X] = \frac{[B]_0 [A]_0}{[B]_0 + K_d} \tag{6}$$

(to see how to obtain eq.(6) the reader is referred to the chapter of Absorption photometry II). Eq.(6) can be inserted into eq.(5) which then can be rearranged to obtain [A]:

$$[A] = [A]_0 \frac{K_d}{[B]_0 + K_d} \tag{7}$$

Having both [X] and [A] expressed by the starting parameters $[A]_0$, $[B]_0$ and the unknown K_d, we can insert eqs.(6) and (7) into eq.(4) to obtain:

$$F = [A]_0 \left(\frac{K_d \varepsilon \phi}{[B]_0 + K_d} + \frac{[B]_0 \varepsilon' \phi'}{[B]_0 + K_d} \right) \tag{8}$$

Subtracting eq.(8) from eq.(3) we arrive at:

$$F_0 - F = \frac{[B]_0 [A]_0}{[B]_0 + K_d} (\varepsilon \phi - \varepsilon' \phi') \tag{9}$$

Then, dividing eq.(3) by eq.(9):

$$\frac{F_0}{F_0 - F} = \frac{[B]_0 + K_d}{[B]_0} \frac{1}{\alpha} \tag{10}$$

where

$$\alpha = 1 - \frac{\varepsilon' \phi'}{\varepsilon \phi} \tag{11}$$

Eq.(10) then can be put into the final form of

$$\frac{F_0}{F_0 - F} = \frac{1}{\alpha} + \frac{K_d}{\alpha} \frac{1}{[B]_0} \tag{12}$$

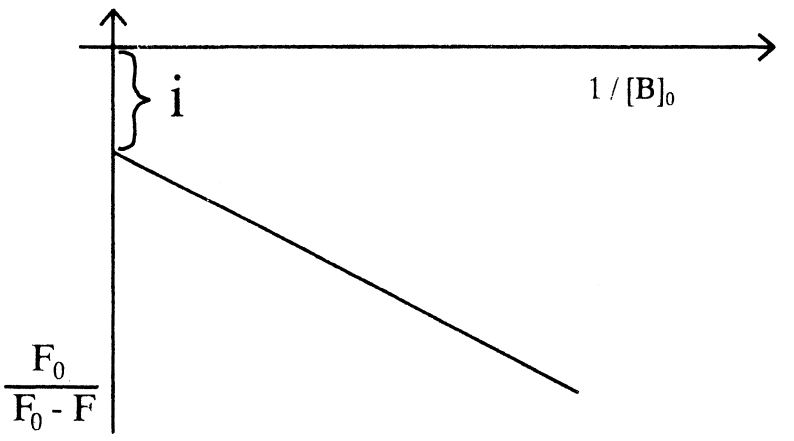


Figure 1

It is easy to see that the plot of $F_0/(F_0 - F)$ against $1/[B]_0$ will give a straight line (see Fig.1) with the intercept of

$$i = \frac{1}{\alpha} \tag{13}$$

and the slope of

$$s = \frac{K_d}{\alpha} \tag{14}$$

The value of K_d can be calculated as

$$K_d = \frac{s}{i} \tag{15}$$

In using eq.(12), however, there is a difficulty originating from the fact that the different concentrations of BSA (in the different samples) result in different optical densities (at the excitation wavelength). This means that the intensity of the excitation light at the middle of the cell, I, will vary from sample to sample according to the Lambert-Beer law (see Absorption photometry I). Therefore, the fluorescence intensities (the F values) will also be affected (actually, F will decrease with the decreasing light intensity since F and I are proportional to each other). To correct for this distortion of the fluorescence intensities it is necessary to use correction factors (c) in eq.(12):

$$\frac{F_0}{F_0 - Fc} = \frac{1}{\alpha} + \frac{K_d}{\alpha}\frac{1}{[B]_0} \qquad \textbf{(16)}$$

where

$$c = 10^{0.5\,\Delta OD} \qquad \textbf{(17)}$$

(ΔOD) is the optical density difference (at the excitation wavelength) between the solvent containing only ANS, and ANS+BSA mixtures (see Table I). 0.5 is the optical length (it is assumed that a 1x1 cm rectangular cell is used and the fluorescence emitted from the middle of the cell is monitored).

Table I. Preparation of the solutions (with the same $[A]_0$ and different $[B]_0$ concentrations) by using stock solutions of: BSA - $4.47 \cdot 10^{-4}$ M; ANS - $2.5 \cdot 10^{-5}$ M and buffer - 50 mM phosphate, pH: 7.04.

Sample number	BSA added in μl	buffer added in μl	ANS added in μl	ΔOD
1	500	500	1000	0.402
2	350	650	1000	0.291
3	250	750	1000	0.213
4	100	900	1000	0.094
5	50	950	1000	0.056
6	35	965	1000	0.041
7	25	975	1000	0.033
8	-	1000	1000	0

Experiments:

1./ Switch on the spectrofluorimeter. After 10 minutes the instrument is ready to operate.

2./ Record the emission spectrum of the solution No. 1 (BSA-bound ANS) in the wavelength range of 520 to 440 nm, in 1 nm steps.

Adjustment of the parameters:

Press keys "2" and "ENTER" sequentially. Program the given values of parameters. Store the programmed numbers with key "ENTER".

EXCITATION	365	(nm)
EMISSION*	520	(nm)
EX.SCAN	OFF	
EM.SCAN	ON	
SCAN RANGE	80	(nm)
SCAN SPEED	20	(nm/min)

(* The scanning starts at higher wavelengths and proceeds to the lower ones)

Return to measuring mode with pressing key "RETURN".

Press keys "3.2" and "ENTER". Program the values of parameters. Store each programmed value with "ENTER".

HIGH VOLTAGE	350	(V)
BLANK	0.0	
FACTOR	1.0	
RESPONSE	1.0	
RATIO*	ON	

(* In RATIO mode there is an automatic correction for sudden intensity changes and wavelength dependence of the excitation light source)

Return to measuring mode. Put the cell containing the sample into the cell holder. Press key "start/stop". Read the fluorescence intensities.

3./ Record the emission spectrum of the solution No. 8. (free ANS dye) in the wavelength range of 520 to 440 nm, as described under point 2.

4./ Plot the measured fluorescence intensities as a function of the wavelength.

5./ Determine the wavelength of the maximum emission and measure the fluorescence intensities of the free ANS and the BSA-ANS solutions given in the table, at this fix wavelength. Use the following instrumental setting:

Enter the values of parameters:

EXCITATION	365 (nm)
EMISSION	wavelength of max. emission
EX.SCAN	OFF
EM.SCAN	OFF

Return to measuring mode with key "return".
Put the cell containing the blank solution into the cell holder. After displaying its fluorescence intensity, press key "f" (auto blank), and this value will be automatically subtracted from all the measured data.

Put the cells containing the samples into the cell holder and read the fluorescence intensities appearing on the display. (Use the "read/print" button)

6./ Correct the measured fluorescence intensities for the absorption-increment caused by addition of BSA using the following expression:

$$F_{corr} = F_{measured}\ 10^{\Delta OD\ L} \qquad \textbf{(18)}$$

where L is the optical pathlength (0.5 cm), the optical density difference (ΔOD) values are given in Table I..

7. Calculate the values of $F_0/(F_0 - F_{corr})$! F_0 is the fluorescence intensity of ANS in the absence of protein and F_{corr} is the corrected fluorescence intensity of the (BSA+ANS) solutions listed in Table I.

8. Plot the values of $F_0/(F_0 - F_{corr})$ as a function of the reciprocal of BSA concentration and determine the dissociation constant of the dye molecule (K_d)! (eqs.(12) - (15)).

NOTES:

The ANS molecule has 3 binding sites of different affinity (K_d) on the Bovine Serum Albumin protein. These binding sites are located at three distinct and highly hydrophobic regions of the protein matrix. This fact results in a nonlinear binding plot (see point 8.). In case of satisfactorily high number of data points one could fit three distinct linear functions with different slopes to the experimental curve. In our case the number of points in the binding plot is enough to distinguish only two distinct binding sites, the "low affinity" and "high affinity" sites by fitting two linear functions to the two ends of the experimental curve. From these two straight lines one can determine the dissociation constants (K_d) for the low and high affinity binding sites, from their slopes and intercepts as described in the theoretical section of the practice.

5. Monitoring conformational changes of proteins by luminescence quenching

Proteins are large, folded polypeptide chains arranged very often to a complex multisubunit structure. Fig.1 shows a typical example of such complex proteins, the Major Histocompatibility Complex (MHC) molecule which is found at the surface of almost all eukaryotes. The protein is an integral membrane protein composed of a "heavy chain" (a polypeptide chain forming three distinct domains) and a non-covalently associated "light chain", the ß2-microglobulin. The different structural motifs, such as α-helices, ß-sheets and random coils are clearly distinguishable on this structural model derived from X-ray diffraction measurements. These macromolecular structures can be characterized by their *"conformational state", which is defined as a certain three-dimensional arrangement of their amino acid residues relative to a coordinate system (x,y,z) fixed to the mass center of the protein.* In Biochemistry, analysis of the conformational changes of proteins, especially of enzyme molecules, is very often restricted to 2 or max. 3 possible conformations of the particular enzyme. These are usually the "active", "inactive" or "intermediate" conformations.

The so called "active" and "inactive" conformations are usually separated by a relatively large free-energy barrier (ΔG^*). Conformational changes are essential in regulation of the function of thousands of cytoplasmic enzymes, membrane-bound enzymes, ion-channels and cell surface receptor or antigen proteins.

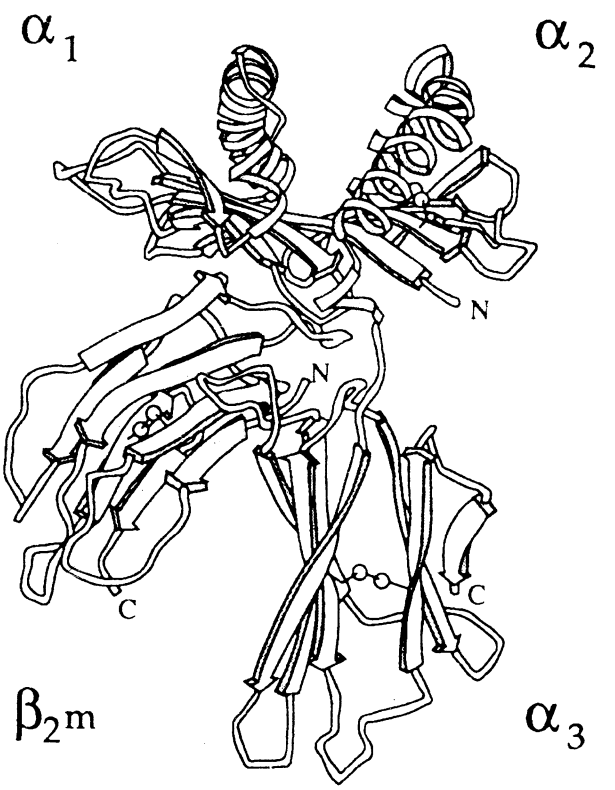


Figure 1

The conformational changes of protein macromolecules can be induced either by interaction with small molecules ("ligands") specific to the particular protein or by interaction with nonspecific, small molecular compounds or with other proteins. Conformational changes may also be induced by changes in the physical parameters of the surrounding microenvironment (cytoplasmic fluid or lipid bilayer), such as temperature, pressure, viscosity, ionic strength, pH, membrane-potential etc. This results in a chance for a "super-fine conformational tuning" of protein function by specific and non-specific ways.

Being "multi-particle systems" from physical point of view, even the smallest globular proteins may exist in a large number of different conformational sub-states separated by small free-energy barriers from each other. In other words, all protein macromolecules are flexible, "**fluctuating**" structures. *The word "structural fluctuation" means temporary changes of spatial positions of different structural elements (atoms, residues, segments, domains, subunits) around their "equilibrium position" on different time scales (ranging from picoseconds to hours).*

The functional importance of protein dynamics emerged in the early 70's when the first dynamic models of the enzymatic action appeared [for review see: e.g. Somogyi et al., 1984 Biochim.Biophys.Acta 768: 81-112]. Later a number of experimental and theoretical works reported on the importance of protein conformational dynamics in *ligand-recognition*, and in the process of *substrate to product conversion.*

The conformation of proteins can be studied by a variety of physical techniques, among others by **NMR**, **ESR** spectroscopies, **X-ray crystallography** or by various methods of **optical (absorption and emission) spectroscopy**. Presently we can say that the technical developments made both NMR spectroscopy (for both solid state and solution studies) and X-ray crystallography (for solid, crystalline state studies) extremely powerful in exploring the three dimensional structure of proteins at atomic (angstrom level) resolution. Besides this unique potential, both techniques, have several drawbacks, as well. X-ray crystallography needs "perfect, error-free" crystals which cannot be formed from all kinds of proteins. This problem is particularly significant for membrane proteins the majority of which can hardly be crystallized or not at all. For NMR spectroscopy a minor drawback is that the time-resolution of the technique (i.e. measurements on protein dynamics) is restricted to a relatively narrow "time-window" (microsecond time scale) and the good spatial resolution is limited only to small proteins.

Luminescence (fluorescence and phosphorescence) spectroscopy offers a large variety of experimental approaches to study protein conformation. Though it has a major drawback of not giving direct information about the three-dimensional structure, it is compensated with the advantages of minimal perturbation of the native structure/conformation by the sample preparation procedure, and it also allows dynamic measurements on a wide time scale (from picoseconds to milliseconds). *Here we will focus on demonstration of the capability of fluorescence spectroscopy (namely the technique of fluorescence quenching) to explore details of protein conformational changes.*

Theory of luminescence quenching

In general any excited state of a fluorophore molecule F^* can decay by a few competing and independent relaxation pathways:

$$F^* \Rightarrow F + h\nu \qquad (1)$$

$$F^* \Rightarrow F + \Delta E \qquad (2)$$

$$F^* \Rightarrow P^* \Rightarrow F + \Delta E \qquad (3)$$

(1): fluorescence emission
(2): radiationless relaxation
(3): singlet to triplet transition followed by phosphorescence

In addition to these natural deexcitation pathways, the fluorophores can also be deexcited upon interaction with small solute molecules (**"quenchers", Q**) which are able to extinguish the fluorescence when they are in contact with the excited fluorophore.
For this case the following reaction scheme need to be considered:

$$F^* + Q \underset{k_{-D}}{\overset{k_D}{\rightleftharpoons}} (F^{*}\cdots Q) \xrightarrow{k_r} F + Q + \text{heat} \qquad (4)$$

where k_D is the bimolecular rate constant characteristic of the formation of the collisional encounter complex ($F^*\bullet\bullet Q$), while k_r and k_{-D} are the monomolecular rate constants characteristic of the quenching (relaxation) process and the decomposition of the encounter complex, respectively.

When continuous illumination is applied to a fluorescent sample, a steady fluorescence intensity is observable. If the light intensity is low enough (true for most of the conventional spectrometer light sources) than only a small fraction of the fluorophore population will be in an excited state and therefore the fluorescence intensity will be proportional to the total fluorophore concentration. This system is in a so called "steady state".

It is obvious from the above reaction schemes that the presence of quencher molecules (see eq.4) results in a decrease in the fluorescence intensity (and also in fluorescence lifetime) of the sample. For steady-state systems Stern and Volmer described the relationship between the extent of the quenching and the quencher concentration. (*It should be noted here that their equation is valid only for the so called collisional (or dynamic) mechanism of quenching. A real experimental system maybe further complicated by appearance of static quenching reaction which is a complexation between the ground-state fluorophore and the quencher resulting in no fluorescence upon illumination. This static quenching, of course, is not accompanied with a shortening of the fluorescence lifetime.)*

The Stern-Volmer equation for dynamic quenching :

$$\frac{F_0}{F} = \frac{\tau_0}{\tau} = 1 + K_{sv}[Q] = 1 + \gamma k_D \tau_0 [Q] \qquad (5)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, τ_0 and τ are the lifetimes in the absence and presence of the quencher, respectively, $[Q]$ is the quencher concentration, K_{SV} is the Stern-Volmer constant, k_D is the bimolecular rate constant (eq.4) while γ is a factor characteristic of the efficiency of the quenching ($0 \leq \gamma \leq 1$). It approaches 1 when $k_r \gg k_{-D}$. At such conditions the Stern-Volmer constant, which can be easily determined from the quencher concentration dependence of the quenching (F_0/F vs. $[Q]$ plot), is characteristic of the relative transport rate (k_D) of the fluorophore and quencher molecules.

A kinetic or thermodynamic analysis of the quenching reaction allows a detailed characterization of the tryptophan accessibility and structural dynamics of proteins.

The fluorescence signal from protein molecules may originate from natural, "intrinsic" fluorophores such as the aromatic amino acid residues **tryptophan (Trp)** and **tyrosine (Tyr)**. (Phenylalanine is only very weakly fluorescent.) The fluorescence of these intrinsic probes can be excited in the UV (excitation max. wavelength: 270-290 nm, depending on the environment) and their emission peaks around 300-310 nm for Tyr and 310-350 for tryptophan. The spectral distribution of the emission is very sensitive to the location of the aromatic residues. An increase in the polarity (dielectric constant) of the microenvironment shifts the spectrum towards longer wavelength (red shift) while location in highly apolar regions result in a blue shift of emission spectrum. Thus, recording of the excitation and emission spectra, itself, may be informative of conformational changes.

Some well defined binding sites of proteins can also be selectively labeled by conjugation with extrinsic fluorescent probes reacting specifically and covalently with certain protein sidechains such as lysine, sulfhydryl, arginine residues or in case of glycosylated proteins, the carbohydrate moieties.

Using these external labels one can get information about the accessibility of the particular binding site by a quenching analysis. Using two covalently attached fluorescent probes at a time, as a pair of energy donor and acceptor, to label two distinct sites of a protein, one can measure the efficiency of Forster-type Resonance Energy Transfer (FRET) (a special case of fluorescence quenching) for determining intermolecular distances (and their changes) between these two sites. The FRET method can also be used for monitoring association of two distinct protein molecules (by measuring intermolecular energy transfer).

Bimolecular quenching reactions also need another molecule, the **quencher**, to proceed. Two family of quencher molecules are used in general for quenching studies in protein solutions or in membranes. The ionic quenchers include cations (e.g. Cs^+, Ag^+, Tl^+, N-methyl-pyridinium cations) and anions (I^-, Br^-, IO_3^-, NO_3^-), as well. These quenchers are mostly used to characterize the solvent-accessibility of protein fluorophores, because being charged they cannot penetrate the hydrophobic interior of proteins therefore they can selectively quench the fluorescence of the solvent-accessible fluorophores.

There are some **neutral, uncharged quencher** molecules which are also often used to quench protein fluorescence such as e.g. **acrylamide ,molecular oxygen (O_2)** or **nitroxide radicals**. These quencher molecules can enter the interior of the proteins through transient pathways opened by structural fluctuations of the protein matrix. Therefore they are able to quench the fluorescence from both "buried" and "surface exposed" fluorophors. Exploiting this feature they can be used to characterize structural fluctuations of proteins having buried Trp residues by means of a kinetic analysis of the quenching, since in this case their "diffusion" rate inside the protein matrix will be mainly determined by the frequency of protein fluctuations. See the example of CO migration in myoglobin governed by the fluctuations of the protein matrix (Fig.2).

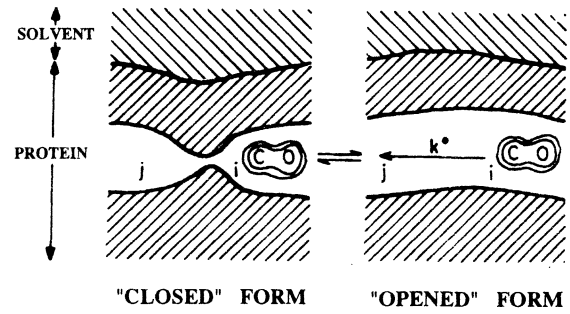


Figure 2

Analysis of solvent-accessibility of Trp residues in proteins

In case of a protein containing more than one Trp sidechain being localized in different microenvironments one can consider, as a simplest approximation, n independent, equally absorbing fluorophors. Although there might be a variation in the individual quantum yields of the Trps, it is reasonable to assume that they all follow the Stern-Volmer law (eq.5).

The protein fluorescence quantum yields in the absence and presence of the quencher, F_0 and F, respectively will be given by

$$F_0 = \frac{\Sigma F_{0i}}{n} \tag{6}$$

and

$$F = \frac{\Sigma F_i}{n} = \frac{1}{n}\Sigma\left(\frac{F_{0i}}{1 + K_{svi}[Q]}\right) \tag{7}$$

where the sums are taken over the n Trp fluorophors of the protein.

The difference between the two intensities is:

$$\Delta F = F_0 - F = \frac{1}{n}\left(F_{01} - \frac{F_{01}}{1+K_{sv1}[Q]} + F_{02} - \frac{F_{02}}{1+K_{sv2}[Q]} + \ldots\right) = \frac{1}{n}\sum\left(\frac{F_{0i}K_{svi}[Q]}{1+K_{svi}[Q]}\right) \quad (8)$$

and

$$\frac{F_0}{\Delta F} = \left[\sum\left(\frac{f_i K_{svi}[Q]}{1+K_{svi}[Q]}\right)\right]^{-1} \quad (9)$$

where $f_i = F_{0i}/F_0$. Equation 9 describes the effect of collisional quenchers on the fluorescence of a multitryptophan containing protein. This equation can be easily converted to a practically useful form allowing determination the fraction of solvent-accessible fluorophors. If we assume that there are m accessible fluorophore with approximately equal K_{sv} and there is n-m inaccessible fluorophors (with $K_{sv} = 0$), than eq.9 becomes:

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_{sv}[Q]} + \frac{1}{f_a} \quad (10)$$

where $f_a = \sum F_i$ summed over m, that is fractional maximum accessible fluorescence. A certain fraction of protein fluorescence $f_b = 1 - f_a$ is associated with the buried fluorophors inaccessible to the quencher.

Using equation 10, a plot of $F_0/\Delta F$ vs. $1/[Q]$ is expected to yield an approximately straight line with a slope of $(f_a \cdot K_{sv})^{-1}$ and an intercept of $1/f_a$. In case if we know the total number of fluorophores, the number of accessible fluorophores can be calculated.

Experiments:

Detection of pH-induced conformational transitions of bovine serum albumin (BSA) by fluorescence quenching

Bovine Serum Albumin is a ca. 69 kD protein (with 3 Trp residues) which is known to undergo 2 major conformational transitions upon a shift of the pH of the medium. One transition (the so called normal to fast , N → F transition) appears between pH:3.5 and 4.5. Another transition appears between pH:6.0 and 9.0 and it proved to be dependent on the Ca^{2+} concentration of the medium, as well. The shift in the pH results in a change in the net protein charge. This is expected to induce domain-rearrangements within the protein resulting in altered exposure/accessibility of certain groups. We will monitor this pH-dependent conformational change by determining the Trp accessibility of BSA with the technique of fluorescence quenching, using an ionic quencher KI. The urea (or guanidinium) induced unfolding (denaturation) of BSA can also be followed by KI quenching of the fluorescence.

1./ Record the tryptophan emission of BSA in solution, at pH:5.0 and pH:9.0 in a buffer of ionic strength of 0.2, maintained by KCl. Record the emission spectrum at pH:9.0 when the KCl is replaced by $CaCl_2$ in the buffer and when the buffer (pH:5.0) contains 8 M urea. Use an excitation wavelength of 295 nm for selective excitation of Trp fluorescence. Record the emission between 300 and 400 nm.

2./ Determine the Stern-Volmer constant for the quenching of BSA fluorescence by KI. Titrate the 2.0 ml volume of protein solution by successive addition of small aliquots of a concentrated quencher stock solution (2.5 M KI). Measure the fluorescence intensity (excited at 295 nm) at the emission maximum before (F_0) and after the addition of the following aliquots of the quencher (10, 20, 40, 80, 120, 150 μl) (F_1, F_2, F_3, etc...) Mix the protein solution by gentle shaking after each sequential addition. (Use parafilm to cover the top of the cuvette).

3./ Plot the F_0/F values vs. quencher concentration [Q] and determine the Stern-Volmer constants (K_{sv}) from the slope of the graphs obtained at both pH:5.0 and pH:9.0. Check if the Ca^{2+}-replacement in the buffer resulted in any change in the K_{sv}.

4./ Calculate the $F_0/F_0 - F$ values and display them vs. $1/[Q]$ to determine the accessible fraction of fluorophores (f_a). Determine the f_a value of BSA at both pH:5.0 and 9.0.

Write a short conclusion about your findings.

6. Measurement of concentration and index of refraction

The electromagnetic radiation travels through a homogenous medium in a straight line. When a ray of light is incident on the interface between two medium one portion of light is reflected by the interface. The angle of reflection (α') is equal to the angle of incidence (α). The light passing in the second medium is refracted by it, if the optical density of these two media is not identical. The angle of refraction (β) is the angle between the normal and refracted ray (Fig. 1.).

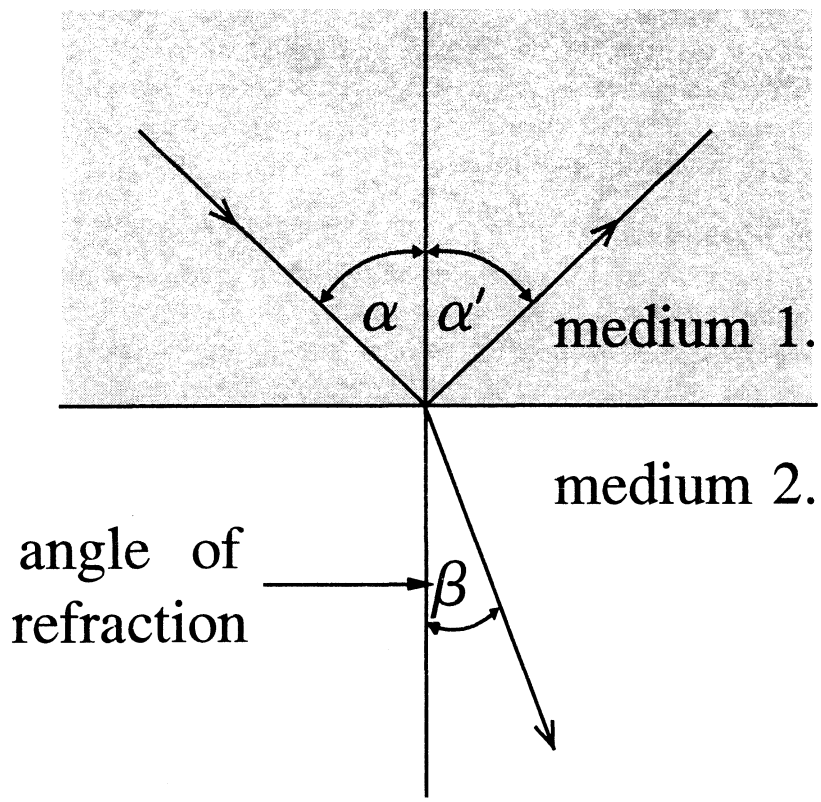


Figure 1

According to the Snell's law

$$\frac{\sin\alpha}{\sin\beta} = \frac{c_1}{c_2} = \text{constant} = n_{21}$$

The quantity n_{21} is called the relative index of refraction of the second medium with respect to the first medium and is equal to the ratio of velocities of light (c_1 and c_2) in the two media.

If the medium of the object space is vacuum (or for practical purposes, air) and that of the image space is some other optically transparent medium, the law of refraction state that $\sin\alpha/\sin\beta = n$. The constant n (for a given wavelength) is the absolute index of refraction of the medium making up the surface and image space; n is a basic physical property. The refractive index of liquid decreases with increase of temperature and varies with the wavelength of the light used. Values are usually quoted for the yellow D-line

(λ = 589.3 nm at 293 K) of the sodium lamp. Values of n_D^{20} for some liquids are: water 1.333; n-hexane 1.375; ethanol 1.362. n is relatively high for the compounds such as aromatic substances having π bonds in their molecules, e.g. n_D^{20} for benzene is 1.501; styrene 1.531; pyridine 1.509; nitrobenzene 1.553. These compounds have delocalized electrons which are comparatively free to interact with light. The refractive index is a useful property for characterizing a pure liquid. It can be measured experimentally by a number of methods. The principles involved in three methods are outlined below.

1. Interference Method

When two beams of light from a common source travel by different paths and are then recombined the intensity of the resultant light is found to vary with the difference in optical path-length of the two beams. Optical path-length may be considered to count the number of wavelength traversed. In the Rayleigh refractometer two interference patterns are formed. The interference refractometer is useful for following small changes in n in chromatography and in reaction rate studies.

2. Hollow Prism Method

The liquid is contained in a hollow prism with thin glass walls. Monochromatic light from a sodium or mercury lamp illuminates a vertical slit. The light from the slit is rendered parallel by a lens and is then focused by another lens on the other side of the prism. The fixed geometry of the apparatus, together with the determination of the position of the slit image, enable the refractive index to be calculated from the bending of the light beam.

3. Critical Angle Method

As the angle of incidence of light at an interface increases, so does the angle of refraction. The maximum value of α is 90^o or $\pi/2$ radian, so the maximum value of β is the value corresponding to $\alpha = 90^o$, $\sin\alpha = 1$. Since $n = \sin\alpha/\sin\beta$, $\sin\beta_{cr} = 1/n$, where β_{cr} is the maximum value of β, called the critical angle of refraction. By determining the maximum value of β, n can be calculated. The Pulfrich and Abbe refractometers are based on this principle; the latter can give accurate values with one drop of liquid.

In these instruments, the interface at which the measurement of critical angle is made, is that between the liquid and glass.

The refractive index of solutions may be measured by a **Pulfrich refractometer** over a range of n = 1.4 to 1.9. Its accuracy is $\pm\ 3\cdot10^{-5}$ and it requires only a few ml of solution for the measurement.

The range of measurement for an Abbe refractometer is from n = 1.3 to 1.7. Its accuracy is $\pm\ 2\cdot10^{-4}$. The Abbe refractometer (Fig. 2.) has a pair of prisms and two scales: one for extract content measurement and one to determine the refractive index. The extract content scale is only valid at 20 °C. If any of these conditions fails we have to make corrections. For measurement we only need some drops of solution. The prism may be thermostated by circulated water. One of the most important parts of this instrument is the double prism made of flint glass (n = 1.75). There is a gap between these prisms. First we put one or two of the solutions here, then set the mirror under the prism to project the light to the prisms. Afterwards we set the cross-hairs of the telescope on the contour line by turning a control knob (it is on the lower left side of the refractometer). When the contour line is not sharp but it seems to be a multicolored band use the compensator on the slight side of the refractometer to eliminate it. If the intersection of the cross-hairs is on the contour line the refractive index and/or the saccharose content can be read by looking into the microscope parallel to the telescope.

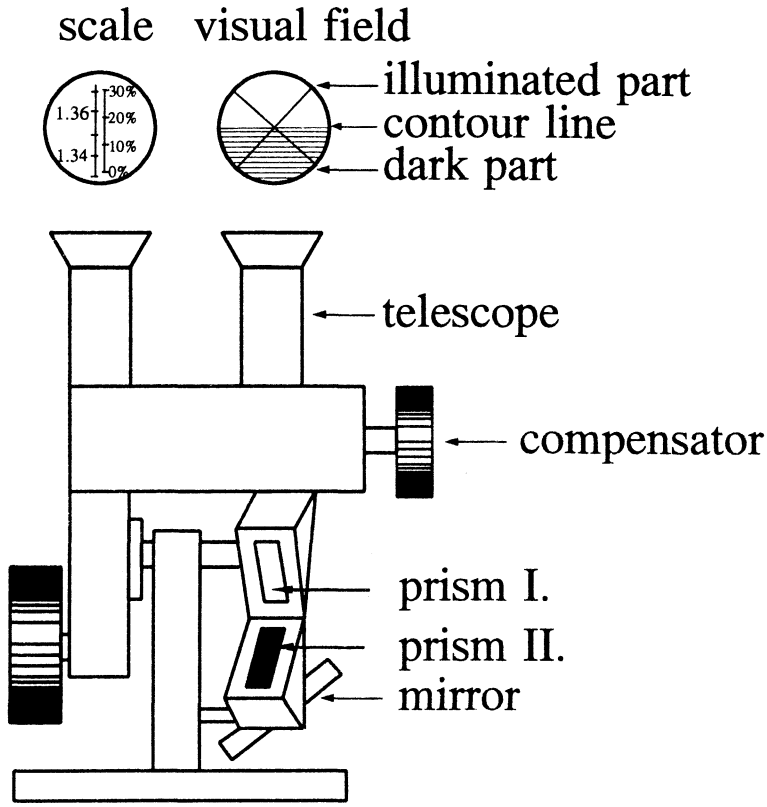


Figure 2

Experiments

1. Calibrate the refractometer with distilled water.
2. Measure the refraction index of five polyethylene glycol solutions of known concentration.
3. Plot the measured refraction index values as a function of concentration.
4. Measure the refraction index of the unknown solutions and determine the corresponding concentration values using the diagram. Test the obtained concentration value with calculation!
5. Centrifuge the given blood sample in order to separate the plasma (R.P.M. 5000; 15 min).
6. Determine the protein concentration of the blood plasma using the enclosed calibration curve.

NOTE: Before measurement open the pair of prism and wash them thoroughly with distilled water and dry their surfaces with soft wiper or filter paper. This must be done before every measurement. Then put some drops of the solution between the cleaned surfaces and close the prism. Do not touch the prism by hand or by any hard object. If the visual field of the telescope is still dark, there is not enough solution between the prisms!

Rinse the dropper thoroughly in distilled water after every usage!

7. Measurement of optical activity

Most of the biological uses of optical activity have been found in the study of polymeric material such as polypeptides and polynucleotides and their natural counterparts, DNA, RNA and proteins. One may question the wisdom of plunging into a study on the optical activity of such enormous macromolecules when, in fact, the optical activity of small molecules, such as glucose, is rather poorly understood. It is considerably easier to interpret the asymmetric interaction of an array of identical amide chromophores in a polypeptide than it is to understand a small and apparently simple molecule such as glucose. When a substance is only optically active in solid state the activity is due to the crystalline structure. The organic compounds, which are optically active in solution, too, have at least one **asymmetric carbon atom** and these atoms are responsible for the optical activity.

Optical rotation and its dependence on wavelength were first described in 1815 to 1817 by Biot. In the latter part of the century the ideas of the tetrahedral carbon atom, on which much of modern organic chemistry is based, were developed with the aid of optical rotation. Studies of the rotation of the plane of polarized, monochromatic light (usually the D lines of sodium spectrum, 589 nm) have been used to investigate many problems of molecular structure. Empirical rules based on studies of the optical activities of large number of chemically related substances have been used in structure determinations.

When the light incident on the material is unpolarized and incident normal to the surface it will be resolved into two components, each following a different path through the crystal with different velocities. One component, call the extraordinary ray, deviates from the normal and is polarized parallel to the axis of the long chain molecules, whereas the other, called the ordinary ray proceeds to the crystal undeviated and is polarized perpendicular to the axis of the long chains. Hence we have two rays polarized in mutually perpendicular planes emerging from the crystal. A crystal of this type is said to be birefringent. When the incident light is already plane polarized the plane of polarization may be rotated about the propagation axis. This is called rotary birefringence or optical rotation. The plane of vibration may be rotated in such a manner that the terminal point on the electric vector describes a corkscrew curve through space after leaving the crystal. If this path is circular in cross section, the light is said to be circularly polarized. If it is elliptical in cross-section (i.e. if the electric vector is less in some direction than in others) the light is elliptically polarized. These variations in activity are illustrated in Figure 1.

A special case of birefringence is the case when incident light is polarized at 45° from the planes of polarization of the two rays in a birefringent crystal. The emergent light is then resultant of two rays, plane polarized at right angles to each other, and displaced in phase. If the phase difference is an integral number of half cycles, the emergent beam is polarized either exactly as the incident ray. If the phase difference is a quarter cycle, or any odd number of quarter cycles, the

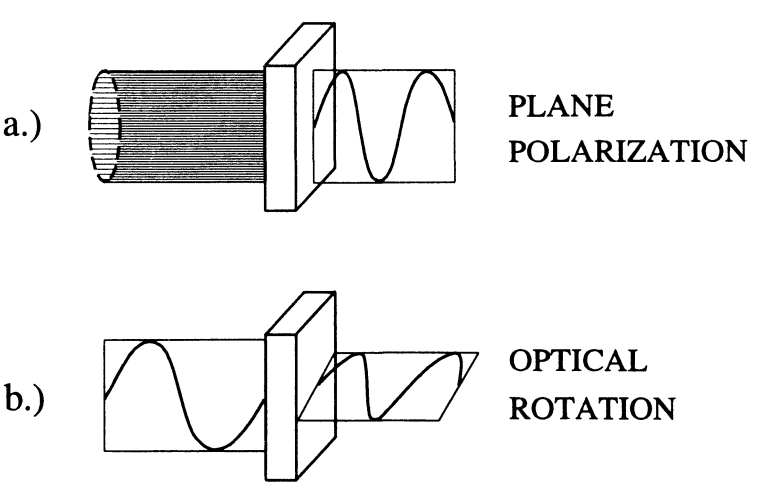


Figure 1

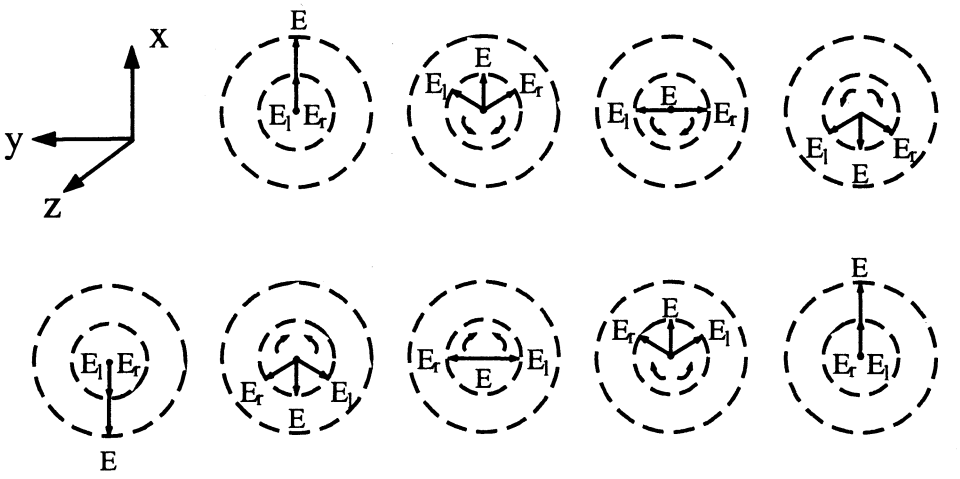


Figure 2

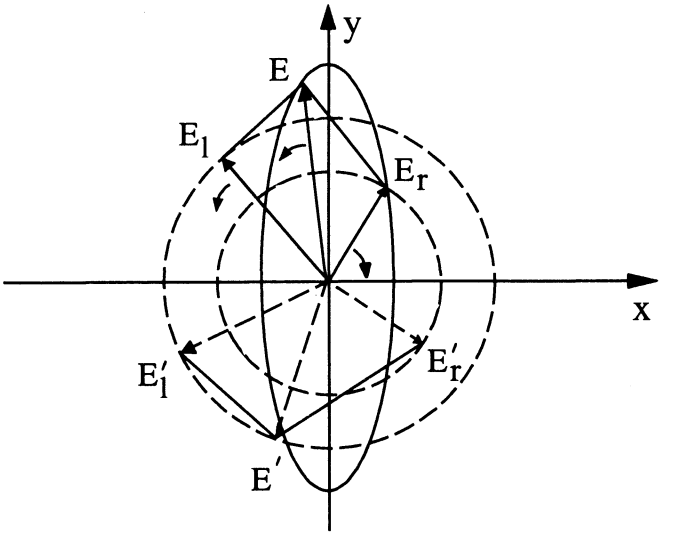


Figure 3

ray is circularly polarized: the electric field (*E*) rotates in space like a corkscrew without change in amplitude (Fig. 2.)

For intermediate phase differences, the light is elliptically polarized, i.e., the electric vector (*E*) rotates but with change in amplitude (Fig.3.). The main axis of the ellipse of polarization and its eccentricity vary together as the phase difference of the two rays increases.

Measurement of optical activity:

Optical rotation is defined as the angle of rotation of plane polarized light by a sample and given the symbol α. For optically active molecules in isotropic solution the angle of rotation depends on the distance through which the light travels in the substance, on the wavelength of the light used, in case of solution, on the concentration and the temperature:

$$\alpha = [\alpha]_{\lambda}^{T} \frac{cl}{100} \qquad (1)$$

The angles are measured in degrees, l is the path length in decimeters, and the concentration is in g/100 cm^3. $[\alpha]_D^{20}$ is the specific optical activity at 20 °C. It follows that the specific optical activity equals to the angle of rotation of the 1 dm thick material of a concentration of 1 g/cm^3 at 20 °C and at a given wavelength. The molecular optical rotation Φ is used correlating optical activities and is defined as $\Phi = [\alpha]_D^{20} M/100$, where M is the molecular mass.

In clinical laboratories the polarimeter, an apparatus capable of measuring optical activity, is used mainly for determining the glucose content of urine but it is suitable to measure the concentration of any optically active substances. The general scheme of the polarimeter is shown on Fig. 4.

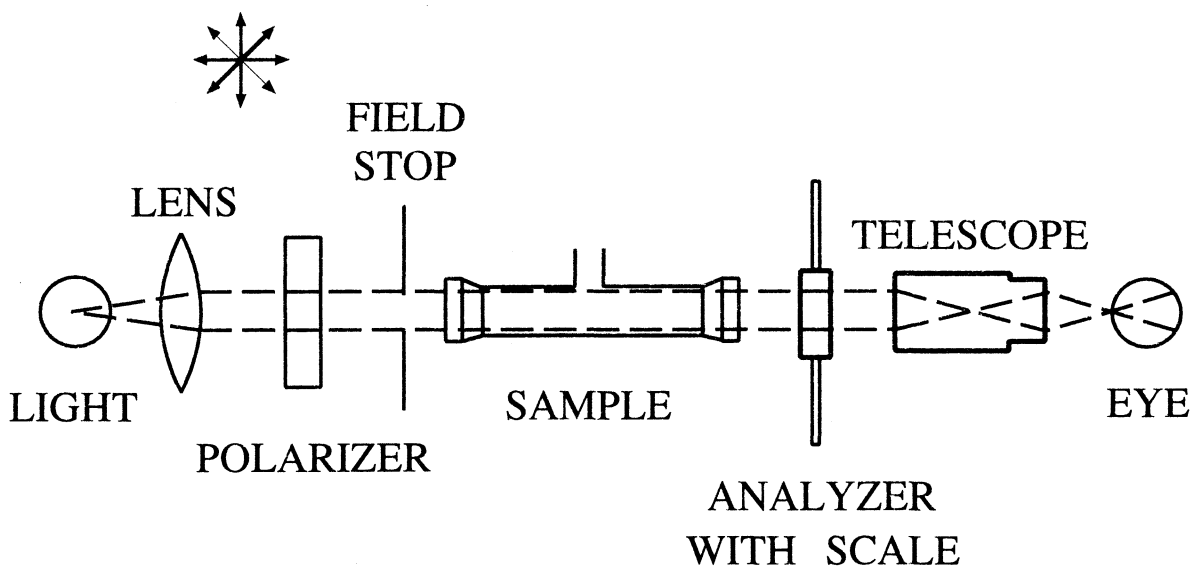


Figure 4

The polarimeter has a monochromatic light source, e.g. a sodium discharge lamp. Due to the polarizer in front of the light source the light passing through the solution is polarized. If the solution is optically active it will rotate the plane of polarization. By the means of a second polarizer (analyzer) we can measure the angle of rotation. Some substances rotate the light clockwise, viewed against the oncoming beam, and are called *dextrorotatory*; others rotate light counterclockwise, and are called *levorotatory*.

The polarizer is made of calcite rhomb that is cut across in a certain direction, as shown in Fig. 5. The cut surface are carefully polished and then the two parts of the crystal are cemented together again with Canada balsam. The calcite splits the light which passes through it into

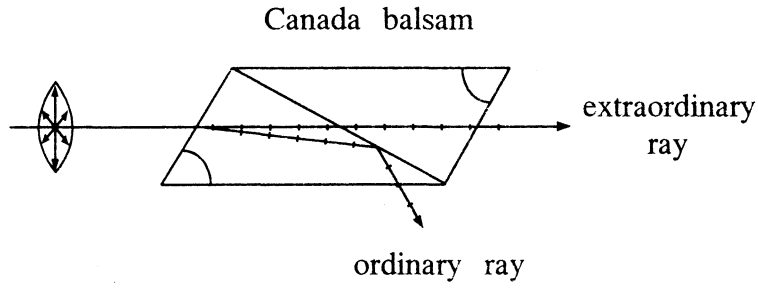


Figure 5

two distinct polarized beams. One of the rays follows the direction to be expected by Snell's law from its index of refraction 1.66. This is the ordinary ray; the other ray with an index of 1.49 is called the extraordinary ray. The ordinary ray is plane polarized and so is the extraordinary ray at right angle to the polarization plane of the ordinary ray.

The refractive index of Canada balsam is between that of calcite for the extraordinary ray (1.66) and that of calcite for the extraordinary ray (1.49). The crystal is cut an angle that the ordinary ray is totally reflected by the layer of the Canada balsam and it is absorbed by the surface of the prism painted black. The extraordinary ray passes through the prism as shown in Fig. 5. In the polarimeter both of the Nicol prisms (the polarizer and the analyzer) are mounted on a common axis in a way that one may be rotated so that the light is entirely cut off, we know that the Nicol prisms are crossed. When a solution of sugar is between the Nicol prisms the polarization plane of the light is not cut off by the analyzer. Now we rotate the analyzer until the light is cut off again and we may read the angle of rotation.

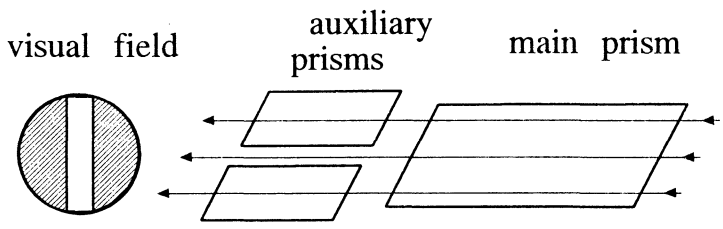


Figure 6

The sensitivity of our eyes of detecting the darkest position is not too good. This is the reason why two auxiliary prisms are used usually. The polarization plane of these two prisms is uniform, and their polarization plane are turned by $\delta = 5^{\circ}$ to that of the main prism (Fig. 6.).

Now the visual field of the analyzer is divided into three parts. The outer parts are always equally illuminated, darker or brighter than the central part depending on the position of the analyzer to the polarization plane of the light passing through the solution. Between these positions there is a special one when the visual field is homogeneous when the angle between the polarization plane of the auxiliary prism and the light is $\pm\delta/2$.

Our eyes are very sensitive in detecting homogeneity of the visual fields and this is the reason why this half-shade type of polariscope is generally used.

The polarimeter is equipped with an auxiliary vernier scale. In this scale we usually divide 9 units of the main scale into 10 parts. The main and the auxiliary scales may be shifted to each other. We read the integer part on the main scale and the tenth of the angle on the vernier scale (Fig. 7.). First we read the smaller main scale value (5) next to the 0 of the auxiliary scale, then the auxiliary scale value (4) which is coincident with that of the main scale. Therefore the result is 18.4°.

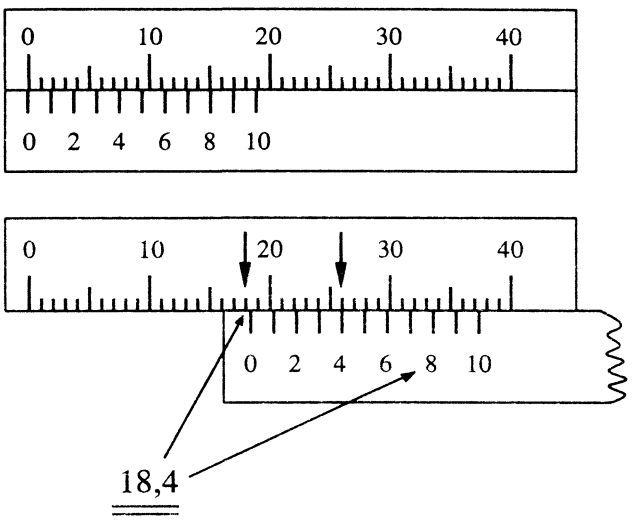


Figure 7

The polarimeter we use has a vernier scale where 19 division of the main scale is divided into 20 parts. Therefore the accuracy is 0.05 division. To make easier the reading there is a magnifying glass in front of the vernier scale.

Experiments:

NOTE: First switch on the polarimeter. Wait for a few minutes while the Na-lamp warms up. Then take the polarimeter tube out of the apparatus and remove the cap and the glass cover plate. Fill the tube with distilled water. Bubbles must be avoided! Close the tube with the cover plate and the cap. Dry the window of the cover plate and put back the tube into the polarimeter. Turn the ring next to ocular to have a sharp picture in the visual field of the telescope. Turn the analyzer slowly to look for the homogeneous visual field (the darker one!). When you have found it read the scale of the polarimeter. This is the zero position. Now change the distilled water for a sugar solution, repeat the whole process and look for the homogeneous visual field again. Read the scale and correct for the zero position. The angle of rotation can be used for the determination of the specific optical activity or the concentration of the sugar.

After the measurement remove the solution you have used and wash up the tube of the polarimeter.

1./ Determine the null position of the polarimeter with distilled water. Fill the optical tube completely with the liquid. (Avoid bubbles in the tube). Read the angle of rotation on the rotating scale. Calculate the mean of 4 independent measurements.

2./ Measure the angle of rotation of the given sugar solutions (glucose, fructose, or galactose) at different concentrations. Calculate the mean of 4 independent measurements at each concentration .

3./ Identify the type of the sugar and direction of rotation by calculating the specific optical activity.

4./ Determine the concentrations of "unknown solutions" by measuring their optical activity.

Note: give all the results together with estimated errors!

a, Calculate $\Delta\alpha$ and $\Delta\alpha_0$ the standard deviations of the rotation angles around the mean value.

$$\Delta\alpha = \sqrt{\frac{\Sigma(\alpha_i - \bar{\alpha})^2}{n(n-1)}} \quad \textbf{(2)}$$

$$\Delta\alpha_0 = \sqrt{\frac{\Sigma(\alpha_{0,i} - \bar{\alpha}_0)^2}{n(n-1)}} \quad \textbf{(3)}$$

α_i and $\alpha_{0,i}$ are the angles obtained in each individual reading for the sugar solutions and water (optically inactive material), respectively, and n is the number of measurements.

b, Calculate the specific optical activity ($[\alpha]_\lambda^T$) and the error of the specific optical activity ($\Delta[\alpha]_\lambda^T$) for the solutions of known concentrations:

$$[\alpha]_\lambda^T = \frac{100}{cl}(\alpha - \alpha_0) \quad \textbf{(4)}$$

$$\Delta[\alpha]_\lambda^T = \frac{100}{cl}\sqrt{(\Delta\alpha)^2 + (\Delta\alpha_0)^2} \quad \textbf{(5)}$$

c, Calculate the mean and the error of the specific optical activity using the Gaussian error propagation function

$$[\bar{\alpha}]_\lambda^T = \frac{\Sigma[\alpha]_\lambda^T}{n} \quad \textbf{(6)}$$

$$\Delta[\bar{\alpha}]_\lambda^T = \frac{1}{n}\sqrt{\Sigma\left[[\Delta\alpha]_\lambda^T\right]^2} \quad \textbf{(7)}$$

d, Using these variables calculate the error of the determination of the concentration of unknown solutions by the use of Gaussian error propagation function:

$$\Delta c = \frac{100}{l\,[\bar{\alpha}]_\lambda^T}\sqrt{\frac{(\bar{\alpha} - \bar{\alpha}_0)^2}{([\bar{\alpha}]_\lambda^T)^2}\left[\Delta[\bar{\alpha}]_\lambda^T\right]^2 + (\Delta\alpha)^2 + (\Delta\alpha_0)^2} \quad \textbf{(8)}$$

8. Centrifugation

In biology, the range of substances analyzed varies enormously in size from a molecule to a cell. Particles include molecules, macromolecules, subcellular components, viruses and cells. If particles are suspended in a solvent and the resulting solution is left to stand, large particles will tend to move under the influence of gravity. This process is called "sedimentation". For a given particle its sedimentation velocity is proportional to the force applied so that the particle sediments more rapidly than under the Earth's gravitational force only. This is the basis of centrifugation: to exert a larger external force for increasing the sedimentation rate of the particles. The force of sedimentation for particles suspended in a liquid is:

$$F_x = Vg_c(\varrho - \varrho_0) \tag{1}$$

where V is the volume of a particle, ϱ is its density, ϱ_0 is the density of the solvent, g_c is the centrifugal acceleration. The centrifugal acceleration is proportional to the radial distance (r) (in meters) and the square of the angular velocity (ω).

$$g_c = r\omega^2 = 4\pi^2 n^2 r = \frac{4\pi^2 N^2 r}{60^2} = 0.011\ N^2 r \tag{2}$$

where n is the number of revolutions per second, N is the number of revolutions per minute (r.p.m.) and r is the radius of the revolution path measured from the rotation axis. If the g_c is divided by the acceleration of gravity, the relative centrifugal force (R.C.F.) is obtained.

The sedimentation coefficient is defined as a sedimentation rate in a certain centrifugal field.

$$s = \frac{v}{\omega^2 r} = \frac{\frac{dr}{dt}}{\omega^2 r} \tag{3}$$

For macromolecules, s is in the order of 10^{-13} seconds, so a new unit is defined, the Svedberg (S) which is equal to 10^{-13} s.

There are many types of centrifuges but they may be broadly classified into five categories.

1./ **Bench centrifuges** create low centrifugal field (up to R.C.F.=600 g). They are mostly portable, usually without temperature control system.

2./ **Large capacity centrifuges** are capable of centrifuging large volumes, up to 6 litres. The R.C.F. ranges around max. 2000 g. They usually have temperature control system.

3./ **Laboratory centrifuges** are equipped with a wide range of interchangeable rotors. R.C.F. goes up to 40000 g. Temperature control system is always installed.

4./ **Ultracentrifuges** are quipped with vacuum pump system to avoid the frictional resistance of air during the spinning which may result in significant warming. The temperature control system is very accurate.

5./ **Continuous flow centrifuges** are equipped with rotating vessels having inlet and outlet tubing. The solution is continuously entering the vessel and the particles are precipitated on the wall of vessel and the excess medium overflows. This type has no interchangeable rotors.

In the first four types of centrifuge the following rotors can be applied.

a./ **Fixed angle rotors** In angle sedimentation particles move only a short distance through the liquid before reaching the outer wall of the container where they join the stream of particles travelling towards the bottom of the tube.

b./ **Vertical tube rotor** is a zero angle fixed rotor. The vertical tube presents the shortest possible path length for the particle.

c./ **Swing out rotors** has buckets which start off in a vertical position but during acceleration swing out to the horizontal position.

d./ **Zonal rotors** have coupling at the top of the rotor thus the rotor can be filled and emptied while at speed avoiding this way the convection effects that occur during the acceleration and deceleration.

Biological materials may be fractionated using the following techniques.

Differential pelleting may be used when components are widely spaced by factor of 3 or more. For fast pelleting fixed angle rotors or vertical tube rotors are used most frequently.

Rate zonal centrifugation technique involves layering in the sample onto the top of a medium so that during centrifugation each component migrates as a band. Good separation can be achieved even when the difference in "s" values of particles is fairly small. This technique is normally performed in swing out rotors.

Isopycnic centrifugation is used to separate particles of different density. In this case the centrifuge tube is filled with a solution the density of which increases along the tube. In the density gradient the particles sediment at different depths according to their mass density.

Separation and purification of blood cells is necessary for diagnosis and monitoring of certain diseases in the everyday clinical routine and research. The simplest method for the separation is the density gradient centrifugation. The physical basis of the method is the following:

Let us consider a vessel filled with a suspension of cells with different mass (m) and density (ϱ) (e.g. blood diluted with isotonic solution) where density of the solvent is ϱ_0. The vessel is rotating around an axe with angular velocity ω. In such a system the following forces act on a particle with mass m, at a distance r from the axe:

- centrifugal force:

$$F_c = ma = mr\omega^2 \tag{4}$$

- buoyant force:

$$F_b = ma = V\varrho_0 r\omega^2 = \frac{m}{\varrho}\varrho_0 r\omega^2 \tag{5}$$

- frictional force

$$F_f = fv \tag{6}$$

where f is the so-called form factor, v is the velocity of the sedimenting cells.

The resultant force (F_e) acting on the particle:

$$F_e = F_c - F_b - F_f = mr\omega^2 - \frac{m}{\varrho}\varrho_0 r\omega^2 - fv = mr\omega^2\left(1 - \frac{\varrho_0}{\varrho}\right) - fv \tag{7}$$

It follows from the equation (7) that sedimentation of the particle stops when $\varrho = \varrho_0$. Creating density gradient in the centrifuge tube by careful layering of solutions with different densities on top of each other, the cells (particles) sediment until they reach the layer the density of which is identical to their density. If such a layer does not exist ($\varrho \neq \varrho_0$) the sedimentation ends at the bottom of the centrifuge tube (i.e. if $\varrho > \varrho_0$) or the cells (particles) enrich at the border of the layer with higher density (if $\varrho < \varrho_0$).

The tissues of the body have different densities within a relatively narrow range, with the two extremes of fat and bone tissues. This is true for the cell components of the blood, as well. Lymphocytes and monocytes have the lowest density ($\varrho < 1072$ kg/m^3) the granulocytes (neutrophil, basophil, eosinophil) have an intermediate density (ϱ is ca. 1095 kg/m^3), while erythrocytes have the highest density ($\varrho > 1095$ kg/m^3). If we layer a mixture of blood cells on top of a separation solution of an approximately identical density to that of the cell mixture, and let the cells to sediment - or accelerate the sedimentation by applying a centrifugal field - then only those cells enter the separation solution which have a higher density while cells of lower density remain on the surface on the separation solution.

According to these principles the density of the I. separation solution of our experiment is chosen as $\varrho = 1072$ kg/m^3, the centrifugal acceleration: 800 g, sedimentation time: 20 min. Using this protocol we can separate lymphocytes and monocytes from the other blood cell components.

The density of the II. separation solution is chosen as $\varrho = 1095$ kg/m^3, centrifugal acceleration: 200 g, sedimentation time: 15 min. In this case the density of the granulocytes is approximately identical or only infinitesimally larger than that of the II. separation solution. Therefore, they can enter the II. separation solution, though significantly slower than in case of I. separation solution. At the applied experimental condition (centrifugal acceleration, time, sedimentation path), however, the cannot pass this layer. At such conditions the granulocytes are located inside the II. separation solution, the red blood cells pass the separation solution, while the lymphocyte-monocyte layer remains on the top of the separation solution.

Experiments:

1./ Measure the values of r_{min}, r_{aver}, r_{max} for the given centrifuge and rotor. By using the above r values calculate the centrifugal acceleration for 1000, 2000 and 3000 revolution per minute (r.p.m.) values. Plot the calculated centrifugal acceleration values as a function of number of revolution for the different r values.

2./ Dilute the blood sample with physiological sodium chloride solution to a ratio of 1:1.

3./ Add 1 volume of the I. separation solution (ϱ = 1.072) into the centrifuge tube. Layer carefully 2 volumes of the diluted blood onto the top of the solution and then perform the centrifugation at 800 g for 15 min.

4./ Repeat the centrifugation experiment in the same way. New conditions: 1 volume of the II. separation solution (ϱ = 1.095) + 2 volumes of the diluted blood layered carefully; 200 g, 20 min.

5./ Compare the results of the two centrifugation experiments and make notes.

NOTE:

It is very important to balance the tubes being in opposite positions in the rotor, otherwise the axle of centrifuge would be damaged. After you had placed the tubes in proper positions in the centrifuge head, **close the lid**. Set the time of centrifugation on the timer. Start the engine and **gradually** increase the speed until the required number of r.p.m. is reached. When the time of centrifugation expired the engine is switched off automatically. Let the centrifuge stop. Do not open the lid until the rotor had stopped and never brake it by hand, it is dangerous and a sudden stop would mix up the sediment.

9. Measurement of nuclear radiation

Various devices have been developed for detecting radiation. These devices are used for a broad variety of purposes, including medical diagnoses, radioactive age determination, the measurement of background radiation, etc.

The measurement of radiation is always based on the interaction of the radiation with the medium (detector material). The Geiger-Müller counter is one of the most common devices used to detect radiation. It can be considered as the prototype of all detectors that make use of ionization of a medium, as the basis of detection. Using the Geiger-Müller counter single particles of α, β and γ radiation can be detected. Therefore it is also used for quantitative measurements. The device consists of a cylindrical metal tube (or glass tube coated with metal inside), filled with gas at low pressure and a long wolfram wire along the axis of the tube. The filling gas is 90% argon and 10% alcohol having 13 kPa pressure (Fig.1). The wire is maintained at high positive potential (about several hundred V) compared to the tube. The voltage is just slightly lower than the ionization potential of the gas atoms. When a charged particle or a γ photon passes through the thin window at the end of the tube, it ionizes a few atoms of the gas. The freed electrons are attracted toward the positive wire and as they are accelerated, they strike and ionize additional atoms. An "avalanche" ionization produces a substantial amount of ion pairs. As the ions move towards the appropriate electrodes a voltage pulse appears on the output of the tube.

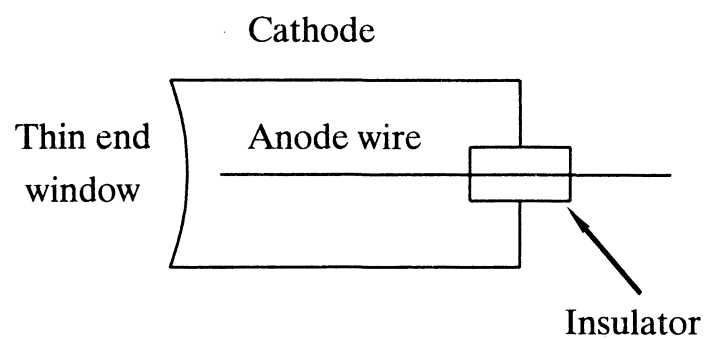


Figure 1

The negative ions (electrons) owing to their high mobility reach the wire within 10^{-8} second. During this time the positive ions moving with a relatively smaller velocity make a shielding effect decreasing the field strength. However, this decrease itself might not be enough to brake the "avalanche" self-discharge, because the photons emitted during the discharge process may induce the emittance of photoelectrons from the metal wall of the cathode. These photoelectrons cause a continuous discharge in the counter tube. Alcohol is applied to stop this unwanted process in the tube. The vapour of the alcohol absorbs the photons, and breaks the continuity of the discharge. Tubes operating on this principle are called "self-quenching" tubes. The life time of the quenching is around 200 μs. The electrons reaching the anode cause a short (10^{-4} s) current pulse, resulting in a few hundred millivolt voltage pulse while flowing through a 1 Mohm resistor. The number of pulses reports on the number of particles detected (Fig.2). The pulses also can be sent to a loudspeaker and each detection of a particle can be heard as a "click".

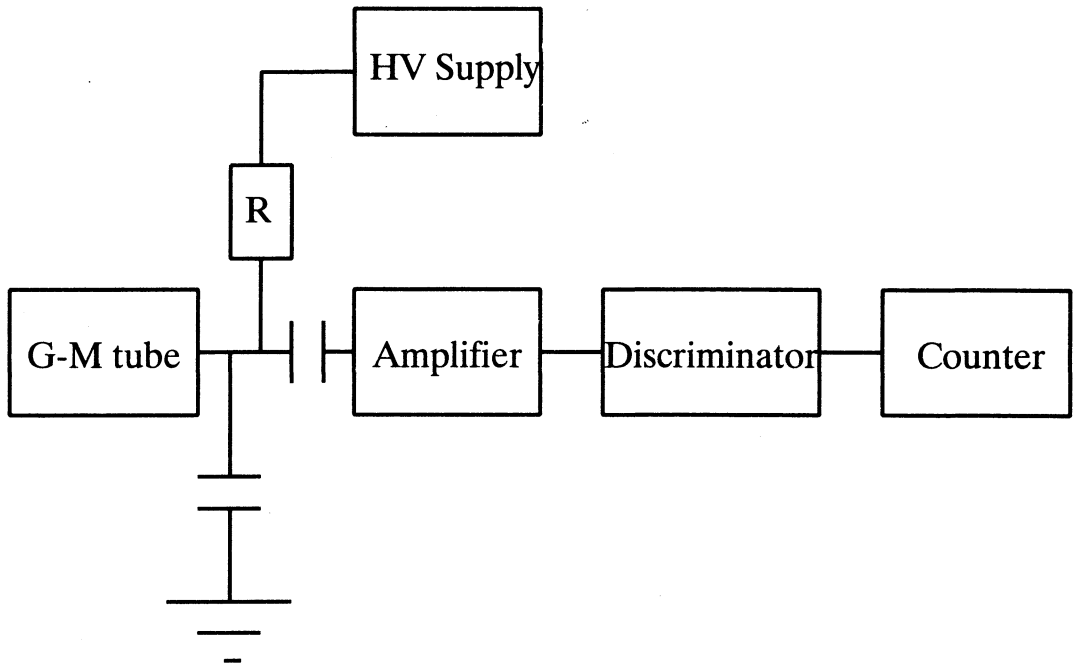


Figure 2

All pulses from a Geiger tube are of the same amplitude regardless of the production of the original ion pairs that initiated the process. The Geiger-Müller tubes can therefore be used as counters of radiation induced ionization but cannot be applied in direct radiation spectroscopy. The information on the amount of energy of the particle that induced the discharge is lost.

Measurement of the characteristics of the Geiger-Müller tube

The threshold potential of the G-M tube (U_0) depends upon the type of the tube (in the case of the argon-alcohol counter it is about 300-500 Volts). If the voltage applied between the anode and cathode is less than the threshold potential the avalanche process does not occur. By increasing the voltage, ion multiplication takes place and the process spreads over larger and larger portion of the tube. If the discharge spreads over the entire volume of the tube identical pulses are detected on the output. However the counting efficiency of the tube does not reach hundred percent because (due to the not high enough field strength)

the ionization might remain local under unfavourable geometry of the entering particle. A further increase in the voltage results in a near to hundred percent efficiency making the complete discharge practically independent from the entering point of the incident particle. This is manifested in a very small slope portion of the count rate versus voltage characteristic called plateau. Additional increase of the applied voltage eliminates more and more completely the residual dependence on the geometry. The plateau region (the length of which depends on the construction of the tube) is continued by an abruptly increasing curve indicating the transition to a self-maintained discharge region. Figure 3. displays a typical count rate versus voltage characteristic.

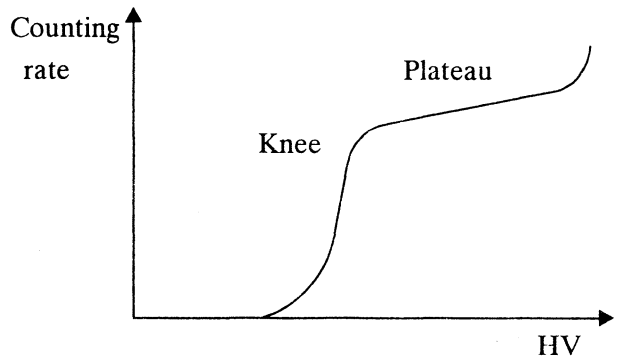


Figure 3

Experiment:

1./ Turn the G-M counter on. (The switch is on the back side of the device!)

2./ Increase the voltage by turning the resistor on the right upper side of the equipment by 25 V steps!

3./ Define the G-M threshold potential!

4./ Determine the length and the rise of the plateau using the following formulae:

$$S = \frac{N_2 - N_1}{N_1} 100$$

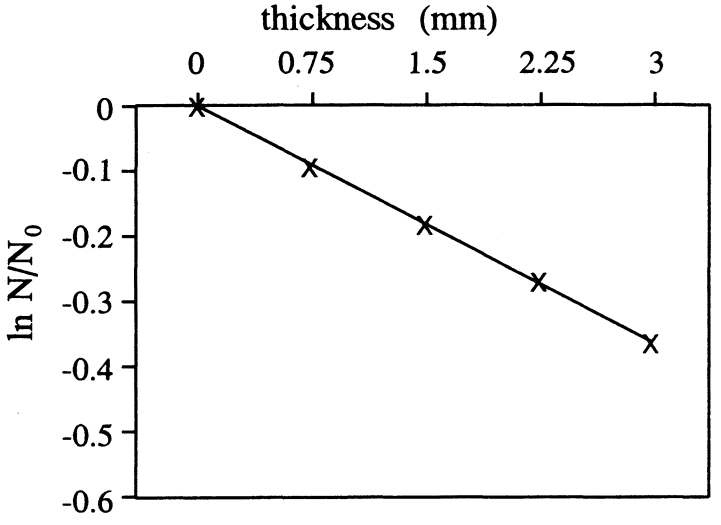
Where S = the slope of the plateau
N_2 = the number of pulses per unit time, counted at the end of the plateau
N_1 = the number of pulses counted per unit time at the beginning of the plateau

5./ Place a paper disk on the input window of the tube and determine the ratio of the β radiation. (The applied isotope has γ as well as β radiation. The β radiation is absorbed by 1 mm of paper).
Repeat the experiment three times and calculate the mean and the standard deviation.

6./ Place one, then two, three and four aluminium disks on top of the radioactive material and determine the attenuation coefficient of the aluminium using the following formulae (Fig. 4.):

$$N = N_0 e^{-\mu x}$$

$$\ln \frac{N}{N_0} = -\mu x$$



Where:

N_0 = number of pulses before placing the disks

N = number of pulses after placing the disks

x = thickness of the disks

Plot the natural logarithm of the ratio of pulses against the thickness of the aluminium disks (Fig.4).

Make three measurements and calculate the mean and the standard deviation.

Figure 4

7./ Repeat the experiment No. 6 with copper disks.

8./ Turn the voltage to zero before switching off the counter.

Labnote (sample)

1./

Voltage	No. of counts per unit time					Mean	SD
	1	2	3	4	5		
100	0	0	0	0	0	0	0
125	0						
...							
...							
600	766	756	766	699	758	749	28,3
...							
700	950	873	938	912	961	926	35,1
...							
800	921	1005	929	889	922	933	24,3
...							
900	940	976	908	933	939	939	24
...							
1000	1176	1132	1156	1174	1163	1160	17,6

2./ The threshold potential is :.....Volts

3./ The plateau is from ..... V to .....V.

4./ Working point (The middle value of the plateau) is:....V.

5./ The slope of the plateau is S = ...%.

6./

Number of measurements	Number of counts				
	isotope	isotope + paper disk	isotope + paper disk + 1 Al disk	isotope + paper disk + 2 Al disks	isotope + paper disk + 3 Al disks
1	1176	1136	565	295	116
2	1132	1118	560	254	109
3	1163	1195	593	240	122
4	1156	1035	597	298	113
5	1174	1132	567	234	166
mean	1160	1103	572	264	125
SD	17.6	41.3	13.3	30.4	23

$$SD = \sqrt{\frac{\Sigma (x_i - \bar{x})^2}{n-1}}$$

10. Computertomography (CT)

1., Theoretical introduction:

Wilhelm Roentgen was awarded the Nobel prize for physics in 1901 for exploring the so-called X- or Roentgen radiation. Since then X-ray has been exploited in several scientific fields. The X-ray machine was already used for composing the two dimensional image of the 3-dimensional human body at the beginning of the century. The disadvantage of this process is that we lose the third dimension, because the composed picture doesn't provide information concerning the place of the extinction of the radiation. We can evaluate only the sum of the absorbing capacity of the volume units along the way of the radiation (summation picture). In Figure 1 square A, B and C can't be distinguished based on their projection on axis y. It is equivalent of the situation when we take an X-ray picture of the human body from one direction. If we take the projection on axis x into consideration too (e.g. we make a two-directional examination), we can unequivocally draw the image of square C, but square A and B still seem to be identical. Based on the projection on line z (that is at 45 degree with line x and y) we can unambiguously distinguish square A and B. Furthermore, based on the above ideas, we can determine the inner structure of any square composed of 4 little ones.

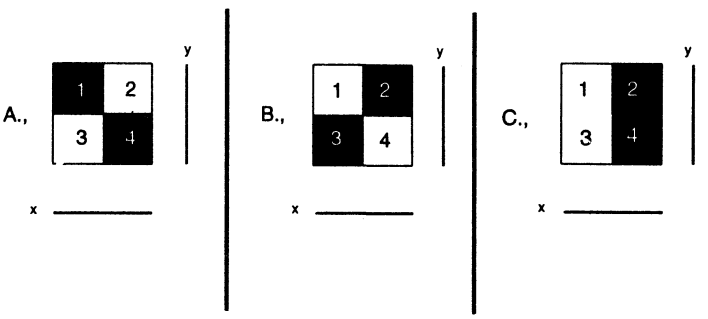


Figure 1

Let us suppose that we move a detector and a source of radiation parallel to each other on two opposite sides of a square (Figure 2). When we move the detector and the source along line x, we obtain intensities I_A and I_B, which depend on the X-ray absorbance of squares 1, 2 and 3, 4, respectively. The attenuation of X-ray is described by the following equation:

$$I_x = I_0 e^{-\mu x} \qquad (1)$$

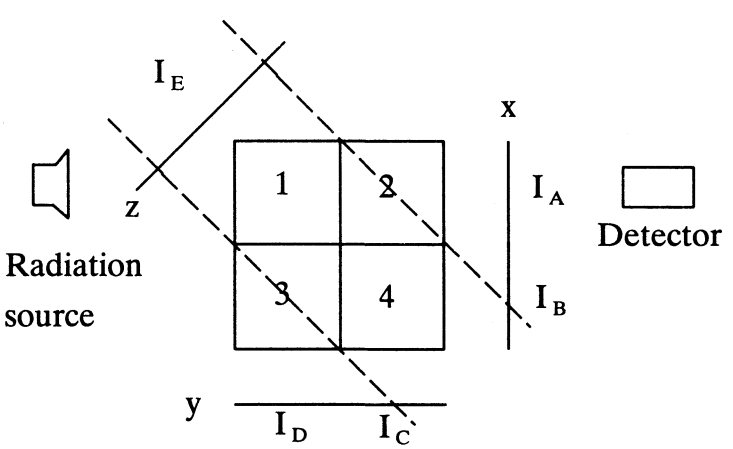


Figure 2

where I_0 and I_x are the intensity measured before and after the radiation passed through a material with a thickness of x and absorbing coefficient of μ. It is conceivable that I_A and I_B equal

$$I_A = I_0 e^{-(D_1 + D_2)} \qquad (2)$$

$$I_B = I_0 e^{-(D_3 + D_4)} \qquad (3)$$

where $D_k = \mu l$ (l is the length of the little squares).

If we move the detector and the source along line y, we obtain quite similar equations for intensities I_C and I_D:

$$I_C = I_0 e^{-(D_2 + D_4)} \quad (4)$$

$$I_D = I_0 e^{-(D_1 + D_3)} \quad (5)$$

We have four unknown quantities in the set of equations (D_1, D_2, D_3 and D_4), and we need four independent equations to be able to solve it. But any three equation from the above four determine the fourth one. That's why we need an examination from an additional direction that gives I_E. The set of equations can be solved exactly without approximation in this way. We obtain the values of D_k giving the X-radiation absorbing capacity of each unitary point (the little squares) of the big square. $D_{1..4}$ give the density matrix of the body. These values are counted by a computer and are displayed on a screen in a color-coded fashion or any optional output device of the computer. This way we get the CT picture, the densitogram.

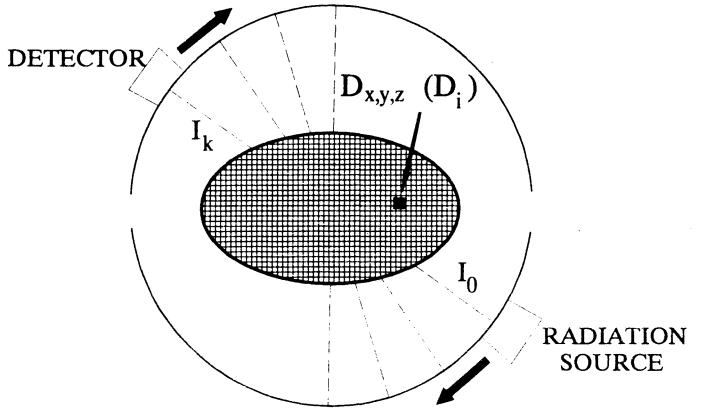


Figure 3

It's easily conceivable that in the case of a real three dimensional body, the situation is much more complicated. To obtain high resolution pictures the measures of the unitary squares, the so-called pixels should be chosen as little as possible. Because of the above mentioned, the number of the I intensities determining the unknown $D_{x,y,z}$ values (the density of the pixel characterized by coordinates x,y and z) grows so excessively that the set of equations determining the density matrix cannot be solved even with very fast computers within reasonable times. So the density matrix is reconstituted from the I_k intensity values using the so-called Fourier-transformation that gives a good approximation. The I_k values are provided by a radiation source and a detector going around the body (Figure 3). ($I_k = I_0 e^{-\mu l}$, where I_0 is the intensity entering the body, l is the distance travelled by the radiation in the body, μ is the average absorption coefficient along this distance. $\mu = (\Sigma \mu_i \Delta l)/n = \Sigma D_i/n$, where n is the number of the pixels along the way of the radiation.) The smaller the angle between two measurement sites is, the better the resolution is, but the time needed for computation grows quickly, too. So a compromise has to be reached in each case considering the desired resolution and the computation time.

In modern CT devices the detectors aren't moved, but are placed along a semi-circle around the body. In the newest devices not even is the radiation source moved, but the radiation emitted from a fixed X-ray tube is diverted to the desired direction by a wolfram ring. These machines can take an image in 30-50 msec, so it becomes possible to follow the movement of the heart. To enhance the information content of the image, it is usual to give radiopaque material to the patient. Generally horizontal sections of the body are examined, but the computer is capable of creating sagittal and vertical sections, too.

Introducing CT technic made a great leap forward in the area of medical imaging processes. A lot of pathologic states (tumors, bleedings) change the X-ray absorbance of a certain tissue, and are detectable with CT, but not with conventional X-ray technic, because

in the case of the latter one, the density of each point of the picture is the sum of the absorbing effect of all the pixels along the way of the radiation in question. So little differences in density cannot be seen because they get averaged out. CT devices can distinguish very little differences in density, and although they are left behind the conventional X-ray technic in resolution, they make quick diagnosis possible concerning i.e. pathologic processes in the scull.

2., Measurement:

In the experiment we use the γ radiation of ^{137}Cs instead of X-ray. The radiation detector is a scintillation counter equipped with NaI(Tl) crystal. We use the detector in INTEGRAL discriminator mode. The major goal of using a discriminator is to get rid of the noise that is generated within the photomultiplier. In this mode voltage pulses exceeding the discrimination level are considered as the detection of a particle and a pulse is sent to the counter. Pulses below the discrimination level are not counted. The discrimination level is proper if the discrimination voltage is set according to the voltage-pulse amplitude produced by the particle to be detected.

We make the densitogram of a model head: it's composed of a bearly absorbing wooden box with some objects of equal density in it. The center of the 16 mm wide stripes on the box should be adjusted to the mark painted on the frame containing the radiation source. (The sketch of the model head can be found in the protocol.)

3., Tasks:

1./ Turning on and adjusting the device:
Turn the HV CONTROL button to the left completely. The device can be switched on with the button on the rear side.
The voltage of the detector (HV CONTROL): 1 kV (showed on the analog display). (If you cannot increase power upto 1 kV, turn the HV control button to the left completely, and push the RESET button on the rear side of the device, and try to increase the power again.)
CHANNEL WIDTH: 0
Discrimination level (DISC LEVEL): to be determined
INPUT: NEG
GAIN: 5
MODE: INT
PRESET: TIME
Time of measurement (SEC): 10 s
RATEMETER/SCALER= SCALER
RECYCLE/MANUAL= MANUAL

2./ Determination of the discrimination level:
Put the isotope into the frame (with the plexiglass facing the detector). Change the discrimination level from 0 to 200 with steps of 20. Measure the number of impulses over 10 seconds at each discrimination level 4 times. Calculate the means. Put the isotope back into the lead holder. Measure the background under the same conditions at the same discrimination levels. Calculate the means and the following ratio: mean of the number of impulses (background) / mean of the number of impulses (isotope). Plot this ratio against the discrimination level. Adjust the discrimination level to the minimum site of the curve.

3./ Measure the radiation intensities crossing the sections marked A..E and 1..5 on the model head 3 times.

4./ Calculate the mean. Subtract the value of the background from the means. Calculate the densities of the sections according to the following formula:

$$D = \sum \mu_i \Delta x \lg e = \lg \frac{I_0}{I} \qquad (6)$$

The biggest intensity is denoted I_0. (We need lg e because of the conversion from natural logarithm to base 10.)

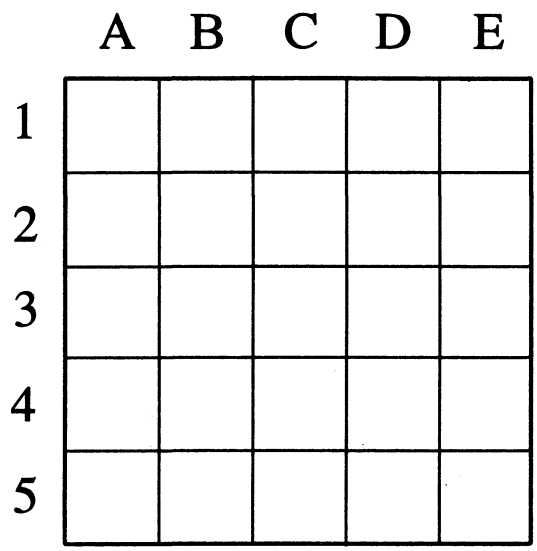
5./ Try to infer the densitogram of the model from the above intensities.

Labnote (sample):

Disc. level:	Background:				Mean:	Isotope:				Mean:	Ratio :
0											
20											
40											
60											
80											
100											
120											
140											
160											
180											
200											

	A	B	C	D	E	1	2	3	4	5
Meas. 1										
Meas. 2										
Meas. 3										
Mean										
Mean - background										
Density:										

Section of the model:



11. Measurement of surface tension

In liquids there are cohesive forces between molecules within a certain distance. These forces can be classified into three groups: orientation effect exists between molecules with permanent dipole moment; induction effect between molecules co-polarizing each other and dispersion effect between molecules of apolar substances. In certain cases the H-valence bond is of great importance.

A molecule of a liquid located well below the surface is attracted by all the neighboring molecules and these attraction forces counterbalance each other. But in the case of a molecule in the surface layer of the liquid the resultant of these forces is not zero, because there is a molecular attraction only downward but no forces are pulling the molecule upward. The result is that the molecules in (or near) the surface of the liquid are pulled toward the interior, consequently this molecular attraction decreases the liquid surface to the possible minimum. The resulting contractile force is called **surface tension** and defined as the force per unit length exerted in the surface. The unit of the surface tension is newton meter^{-1} /Nm^{-1}/. It may be expressed in other terms: the surface tension can be defined as the work exerted in increasing the surface layer by unit area. This amount is also called **free surface energy**:

$$\gamma = \frac{W}{A} \qquad (1)$$

where W means the work and A the area. The unit of free surface energy is Jm^{-2} = Nm·m^{-2} = Nm^{-1} and is the same as that of the surface tension.

The surface tension depends, among others, on the composition of the liquid. Addition of so called capillary inactive substances (e.g. salts) to water increases slightly the surface tension, whereas the capillary active substances (e.g. proteins, fatty acids, detergents, i.e. amphoteric substances) in relatively low concentrations cause a considerable decrease in the surface tension. Since the free surface energy must be at the minimum, the concentration of the capillary inactive substances in the surface layer is smaller than that in the interior of the water solution. On the contrary, molecules of capillary active substances tend to cumulate in the surface layer decreasing the surface tension and hereby producing a lather.

The surface tension is present on all boundary surface and prevents the penetration of the molecules of the dissolved substances from one phase into the other. In the case of biological membranes the surface tension at the membrane-liquid surface is small enough (ca. 1 mJ/m^2), therefore the transport of various substances across them is only slightly limited.

The magnitude of surface tension must be taken into account in dosing drugs in droplets. The size of a droplet is determined by the radius of the medicine dropper and the surface tension of the solution containing the drug. The surface tension of water (73 mJ/m^2 at room temperature) is greater than that of ethanol (22,7 mJ/m^2 at room temp.) therefore using the same dropper larger drops are formed from the water solution. Various methods are known to measure the surface tension of liquids, two of which are summarized below.

1. Capillary tube method

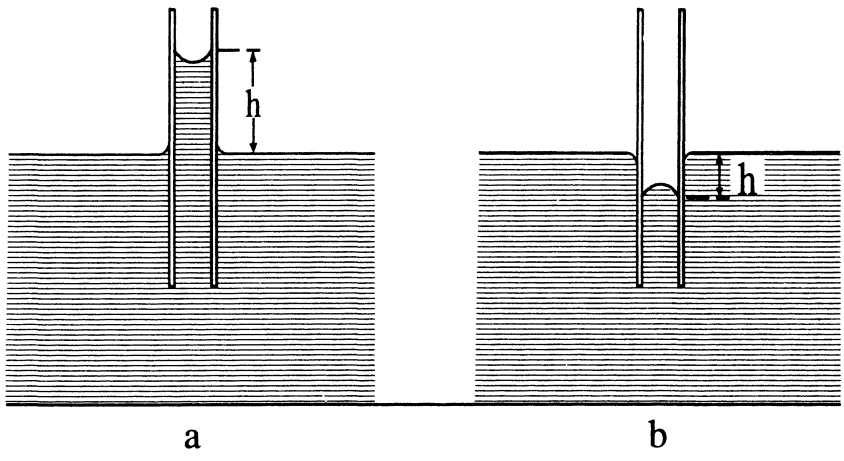


Figure 1

This method is based on the capillary action. If a narrow tube is dipped into a liquid and the level of a liquid in the tube will be higher than the original one, the liquid is called wetting while in the opposite case, when the level of the liquid is depressed in the tube, it is called non-wetting liquid. Examples for the wetting and non-wetting systems are shown on Fig. 1, where a narrow glass tube is immersed into water (Fig. 1a) and liquid mercury, (Fig. 1 b) respectively.

The surface tension results in a force F lifting or depressing the liquid surface in the tube:

$$F = 2r\pi\gamma \tag{2}$$

where r is the internal radius of the tube, γ is the surface tension, $2r\pi$ is the circumference of the capillary. F counterbalances the weight of the liquid column in the tube. The volume of the column of liquid up to the curved liquid surface (meniscus) is $V = r^2\pi h$, h is the height of the column liquid. The weight of the column of liquid equals to $W = V\varrho g = r^2\pi h\varrho g$, where g is the gravity constant, ϱ is the density of the liquid.

The condition of balance:

$$F = W \tag{3}$$

$$2r\pi\gamma = r^2\pi h\varrho g \tag{4}$$

$$\gamma = \frac{1}{2}rh\varrho g \tag{5}$$

Determining h, the rise of the liquid in the tube with known values of r and ϱ, the value of γ can be calculated. If the level of the liquid in the tube is lower than the original one, h has to be taken as a negative amount. If the exact value of γ is not required, this method can be used for measuring the relative surface tension. In this case it is not necessary to determine the exact value of the radius, r. With known density values, ϱ_1, ϱ_2, measuring the height of the columns of the two liquids, the relative surface tension, γ_{rel} can be calculated as follows:

$$\gamma_{rel} = \frac{\gamma_1}{\gamma_2} = \frac{h_1\,\varrho_1}{h_2\,\varrho_2} \tag{6}$$

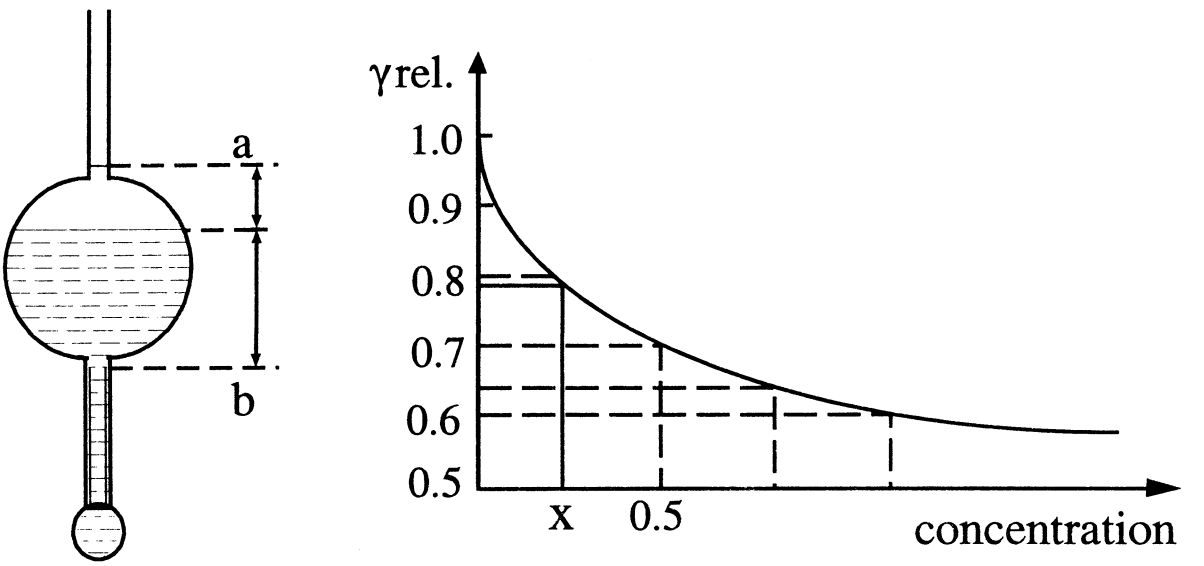


Figure 2

2. Dropping method

The tool of this method is called stalagmometer (Fig. 2). It is a capillary tube with thick walls from which the liquid drips out very slowly. In the first moments the force due to the surface tension counterbalances the weight of the liquid drop and prevents it from falling down. But when the diameter of a drop is increasing, the weight of the drop will increase. When it surpasses the force exerted by the surface tension, the surface layer will be broken and the drop falls down. The circumference of the "neck" of the drop at the end of a capillary is $2r\pi$, where $2r\pi$ is the effective diameter of the capillary (Fig. 3).

The force due to the surface tension is (see Eq.(2)):

$$F = 2r\pi\gamma$$

In the moment of the break of the surface layer:

$$F = G_{drop} \qquad (7)$$

(G_{drop} is the weight of the drop.)

To determine the weight of a single drop would be a very difficult task, so we determine an average weight value instead of a

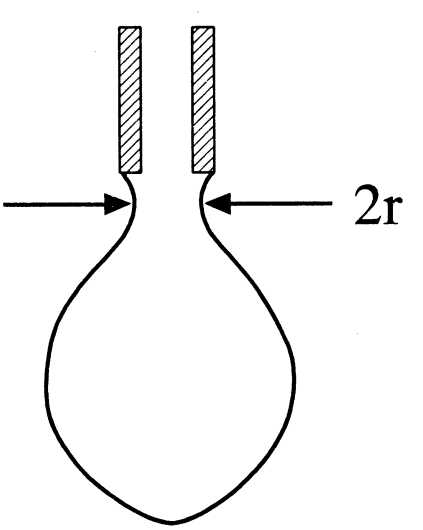


Figure 3

direct measurement. The stalagmometer is filled up with liquid. The volume V of the stalagmometer bulb between the two marks is given. Let flow out the liquid very slowly, drop by drop and count the number of drops n while the meniscus sinks from the upper mark to the lower one. The weight of the liquid in the bulb is $V\varrho g$, and the weight of a single drop is

$$G_{drop} = \frac{V\varrho g}{n} \tag{8}$$

From $F = G_{drop}$

$$2r\pi\gamma = \frac{V\varrho g}{n} \tag{9}$$

and

$$\gamma = \frac{V\varrho g}{2r\pi n} \tag{10}$$

Since the effective radius, r, is smaller than the geometric radius of the capillary and it is not easily measured, usually we make a relative determination of the surface tension of a liquid.

First we use a liquid of known surface tension γ_0. We count the number of drops n_0. The known density of the liquid is ϱ_0. We do the same with the other solution of known density ϱ, but having an unknown surface tension γ. The number of drops is expressed by n. The surface tension of the known and unknown liquids are:

$$\gamma = \frac{V\varrho g}{2r\pi n} \tag{11}$$

and

$$\gamma_0 = \frac{V\varrho_0 g}{2r\pi n_0} \tag{12}$$

The relative surface tension can be calculated as follows:

$$\frac{\gamma}{\gamma_0} = \frac{\varrho n_0}{\varrho_0 n} \tag{13}$$

From this equation:

$$\gamma = \gamma_0 \frac{\varrho n_0}{\varrho_0 n} \tag{14}$$

However, if the radius of the capillary is given, the value of surface tension of a liquid may be determined by direct measurement.

Experiments:

Determination of surface tension with a stalagmometer

Solutions: 5.0 - 30.0 % propylalcohol solution
1 - 5 propylalcohol solution with different unknown propylalcohol content

1./ Determine the drip number of distilled water.

2./ Determine the drip number of six solution of known concentration.

3./ Determine the drip number of two solutions of unknown concentration.

4./ Calculate the surface tension of known solutions.

5./ Plot the relative surface tension of known solutions as a function of the concentration.

6./ Calculate the surface tension values of the unknown solutions and determine their concentration using the above curve.

Notes:

1./ The density values of the different solutions are given on the bottles.

2./ Before measurement wash the stalagmometer at least twice with distilled water and then with the liquid to be measured.

12. Measurement of internal friction of liquids

Real fluids in motion always exhibit some resistance to motion. This phenomenon shows that there are **frictional** or **viscous** forces in liquids, which are the result of the mutual attraction of liquid molecules. This internal friction influences flow conditions. We consider the flow conditions for the case of the so called **laminar flow**, when the flow rate is low enough for the liquid layers to move parallel with the surface at different velocities on each other.

At high rates the flow becomes turbulent. If two flat parallel liquid layers are moving on each other, the frictional force acting against the displacement can be given as

$$F = \eta A \frac{\Delta v}{\Delta x} \tag{1}$$

where Δv is the relative velocity and A is the area of the fluid layers, Δx is their distance. The proportionality constant, η, is called the viscosity coefficient (in short, viscosity), and its reciprocal is the fluidity Its value depends on the composition of the liquid and is given in units of Pascal·second (Pas) in the SI system.

The temperature-dependence of viscosity can be expressed as follows:

$$\eta = A e^{\frac{U}{RT}} \tag{2}$$

where U is the activation energy, R is the universal gas constant and T is the absolute temperature. The viscosity decreases with increasing temperature, the degree of this change depends on the substance. For example, the viscosity of strongly associated liquids changes significantly with temperature.

The viscosity of dilute solutions is usually related to the viscosity of the pure solvent (at the same temperature). This gives the **relative viscosity**:

$$\eta_{rel} = \frac{\eta}{\eta_0} \tag{3}$$

where η and η_0 is the viscosity of the solution and the solvent, respectively.

To express the viscosity changes caused by the dissolved agent we can use, besides the relative viscosity, the **specific viscosity**. This is the difference in viscosity values of the solution and solvent divided by the viscosity of the pure solvent:

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0} = \eta_{rel} - 1 \tag{4}$$

The specific viscosity divided by the concentration of the dissolved material gives the **reduced viscosity**:

$$\eta_{red} = \frac{\eta_{sp}}{c} \tag{5}$$

As there is an interaction between macromolecules in solution, to describe their solutions usually the so called **intrinsic viscosity** is used, which is the reduced viscosity

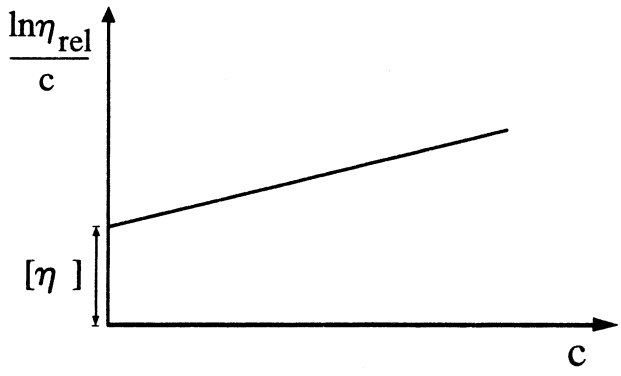


Figure 1

extrapolated to 0 concentration (it is characteristic of the infinitely diluted solution):

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c} \tag{6}$$

For dilute solutions

$$[\eta] = \lim_{c \to 0} \frac{\eta_{rel} - 1}{c} \cong \frac{\ln \eta_{rel}}{c} \tag{7}$$

The limit value is determined by graphical extrapolation (Fig. 1). In the case of linear macromolecules, this limit value is proportional to the molecular weight:

$$[\eta] = kM \tag{8}$$

where M is the molecular weight of the polymer in question and k is a constant depending on both the solvent used and the nature of monomers constituting the polymer.

According to Kuhn, the following expression is more accurate:

$$[\eta] = kM^{\alpha} \tag{9}$$

where the value of α is between 0.5 and 2, depending on the given polymer (Table 1).

Determining k and α for different polymer - solvent pairs, the above relationship makes it possible to calculate the molecular weight of a polymer and, knowing the monomer, the degree of polymerization.

Polymer	Solvent	^{0}C	Range of molecular weight·10^{-3}	k·10^{-4}	α
Cellulose-acetate	Acetone	25	11-130	0,19	1,3
Cellulose-nitrate	Acetone	27	15-400	0,38	1,0
Nylon	90 %Formic acid	25	5-25	11	0,72
Polyvinyl-acetate	Acetone	20	43-640	2,76	0,66
Polystyrene	Benzol	30	10-600	1,7	0,72
Poly(methyl-methacrylate)	Chloroform	20	56-980	0,49	0,82
Natural caoutchouc	Toluol	25	40-1500	5,02	0,667

Table I.
k and α values of some polymer - solvent pairs

Applying equation (9), the molecular weight of a polymer can be determined in the following way:

$$\lg[\eta] = \lg k + \alpha \lg M \tag{10}$$

Plotting the values of lg [η] against the lg$M$ values of polymers of known molecular weight (Fig. 2), α and k can be determined graphically.

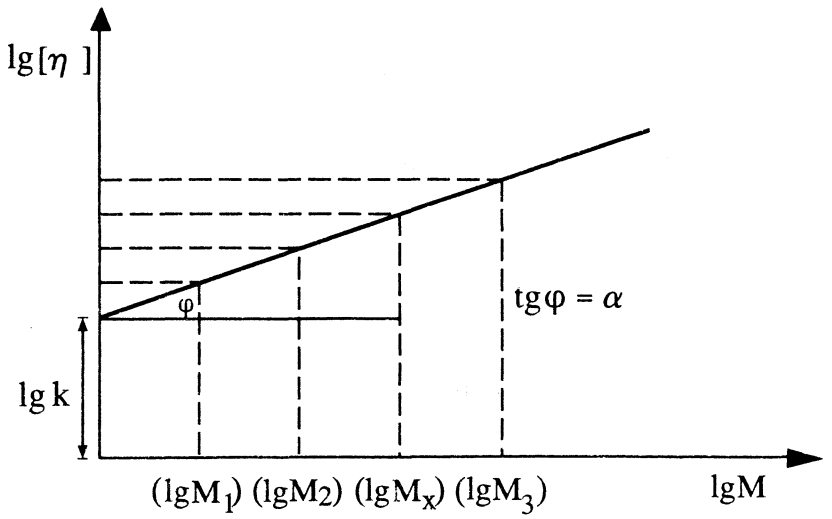


Figure 2

Measuring viscosity we can determine by relatively simple methods the molecular weight of macromolecules quite precisely, what is impossible by classical methods. There are particularly good results for molecules with long rod-shape. Processes decreasing the chain length can be followed excellently by viscosity measurements.

Viscosity measurements can be carried out by checking the flow rates of liquids in a capillary tube. According to Hagen and Poiseuille the volume of a liquid (V) flowing through a cross section of a capillary during time (t) is proportional to the pressure difference between the ends of the capillary (p'-p) and the fourth power of the radius (r^4) and inversely proportional to the length of the capillary (l):

$$V = k \frac{r^4 (p' - p)}{l} t \qquad (11)$$

This equation was adjusted to laminar flows by Stokes and Helmholtz where the flow occurs in concentric layers and the flow rate is the highest along the axis and the lowest along the wall of the tube:

$$V = \frac{\pi}{8} \frac{r^4}{l} \frac{p' - p}{\eta} t \qquad (12)$$

In our experiments we use the Ostwald's viscosimeter to compare the viscosity of liquids. It is an U-shaped glass tube with two bulbs and a capillary with a length of l and a radius of r (Fig.3).

The liquid to be measured is introduced at the left side tube. It is drawn by suction through the capillary into the bulb of a volume of V until the meniscus is above the mark "a"; then the flow time t, necessary for the liquid level to fall between the fixed marks "a" and "b" is measured. The viscosity of a liquid may be determined by comparing the flow times of the same volumes of liquids with known and unknown viscosities, since the flow times will be proportional to the viscosities of the liquids:

$$V = \frac{r^4 \pi}{8} (p' - p) \frac{t}{\eta} \qquad (13)$$

$$V = \frac{r^4 \pi}{8} (p' - p) \frac{t_x}{\eta_x} \qquad (14)$$

where the index x refers to the unknown liquid.

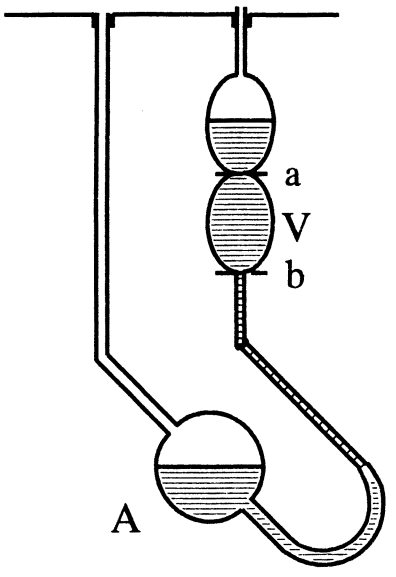


Figure 3

The pressure differences can be expressed (neglecting the effect of capillary forces) as follows:

$$p' - p = hg\varrho \qquad (15)$$

$$p_x' - p_x = hg\varrho_x \qquad (16)$$

Replacing the above pressure differences into the equations (13), (14) and taking their ratio, we arrive:

$$\eta_{rel} = \frac{\eta_x}{\eta} = \frac{\varrho_x t_x}{\varrho t} \qquad (17)$$

If η_{rel} and η is known, η_x can be calculated. No specific parameters of the instrument (r or l) is required for this measurement and calculation. Usually, the liquid of known viscosity is water, its viscosity is tabulated as a function of temperature. We take the density of water at room temperature as 1 g/cm^3 or 10^3 kg/m^3.

Experiments:

1./ Determine the flow of distilled water and polyethylene glycol solutions made from polyethylene glycol of different molecular weights (Fig. 3.).

2./ Make three measurements in each case and calculate the average.

3./ Calculate the relative viscosity values of the solutions.

4./ Plot the values of $(\ln\eta_{rel})/c$ as a function of concentration and extrapolate the curve for the $c = 0$ value (read the value of $[\eta]$, Fig 1.). Make these curves for each polyethylene glycol solution having different molecular weight.

5./ Plot the values of $\lg[\eta]$ against the value of $\lg M$ and determine the unknown molecular weight (M_x) using this curve (Fig. 2.).

13. Biocalorimetry

In living organisms the body-temperature is one of the most important factors controlling physiological processes. Animals are often classified into two major classes according to their thermal regulation behaviour related to the outside environment. The body-temperature of the so called "cold blooded" organisms (e.g. invertebrates, fishes, reptiles, amphibians) can very flexibly accommodate to the environmental temperature. A smaller subpopulation of the animals, the so called "haematotherma" (e.g. birds, mammals) maintain a relatively constant body-temperature through a very complex mechanism of thermoregulation. A third group occupies an intermediate position between the previous groups. They are called heterotherm organisms and can function as coldblooded ones at extremely high environmental temperatures. In mammals the constant body temperature is assured by a complex thermoregulation which is based on the balance of heat generation, heat uptake and heat loss of the given organism. Heat uptake in mammalian organisms takes place by two major mechanisms:

1./ accumulation of radiation energy of the sun

2./ utilization of the heat produced in the metabolic processes of the organism.

The mammalian organisms supply their energy-demand through nutrition, in form of chemical energy. According to the second law of thermodynamics this energy is converted to useful work (e.g. mechanical work in the muscle; glandular secretion work, etc.) only partially, while a major portion of this energy traffic of living organisms can be described with the following simplified scheme:

energy uptake (nutritional + environmental) =
useful work + chemical energy stored in organs + heat-production.

During heat loss mammalian organisms utilize almost all kind of mechanisms of direct and indirect heat-transport. The direct heat-transport is usually realized through the following transport mechanisms:

a./ conduction

b./ convection

c./ radiation

In the heat loss of haematotherm organisms the conduction mechanism is usually negligible due to the weak conductory properties of the air and the tissues. The heat energy lost by convection mechanism is more significant, it takes about 10-15% of the total heat energy lost by the organism. Moreover, this percental ratio may increase with the decrease of environmental temperature or in the case of powerful air-convection. The dominating form of the heat loss is the radiation mechanism, which in haematotherma takes about 50-60% of the total heat energy.

It is noteworthy that in haematotherm organisms a significant portion of the heat energy to be lost is transported by indirect mechanisms. For example, about 20-25% of the total heat energy is lost by evaporation of a significant amount of steam from the skin-surface or through the respiratory organs. There are two other important modes of indirect heat loss. These are heating of the food and air taken up by nutrition and breathing to the body-temperature, and giving off warm excrement and urine.

Let us examine more deeply the most significant mechanisms of the heat loss, the radiation. From this point of view the skin surface covering the mammalian organisms can be regarded as a "black body" with about 310 K temperature and ca. 95% absorbancy. This "black body," depending on its actual temperature, can emit heat-rays (covering the infrared

frequency domain) heating this way its environment. The basic properties of the heat-radiation can be characterized using the empirical Stefan-Boltzmann law as follows:

$$\Delta E = \sigma (T_s^4 - T_e^4) F t \qquad (1)$$

where $\sigma = 5.67 \cdot 10^{-9}$ $Wm^{-2}K^{-4}$, F is the surface of the body, t is the time interval. This way one can estimate the radiative heat loss (ΔE) of a body with T_s surface temperature in an environment of a T_e temperature. For instance, the radiative heat loss of an average human organism is approximately 2.73 kJ/m^2h in the case of 298 K environmental temperature. These data are valid for resting organisms located in an environment having near room temperature In the case of intensive muscle-work or when the environmental temperature is high the extent of the heat loss is increased and depending on other environmental factors (e.g. degree of humidity) the ratio of the different heat loss mechanisms can also be shifted.

Biocalorimetry is a widely used method for measuring heat generation and exchange of animals. In general the calorimetric method is based on direct or indirect determination of the heat energy taken up or lost by a given body. Let us take a body with a mass of m and a temperature of T_1. If the temperature changes from T_1 to T_2 the heat energy exchanged between the body and its environment:

$$Q = c\, m\, (T_2 - T_1) \qquad (2)$$

where c is the specific heat measured at constant pressure (c_p) or at constant volume (c_v) and it is characteristic of the chemical composition of the body.

Calorimetric determination of the heat energy exchanged is based on the following principles:

A./ in thermodynamically closed systems the summed heat energy is constant.

B./ when two bodies with different temperature contact, their temperature will be equalized while heat energies are exchanged between them.

Changes of heat energies accompanying vital processes can be measured in biocalorimeters. A typical biocalorimeter has to satisfy the following requirements:

1./ It should be isolated from the environment (i.e. from the air-space of the lab) from the point of view of heat-conduction.

2./ The inner wall and other accessories of the calorimeter should be made from a material of negligibly small heat-capacity (i.e. these elements should not take up a significant amount of heat).

3./ The basic life-conditions for the animal (e.g. the necessary amount of oxygen) have to be guaranteed in the closed inner space of the biocalorimeter.

4./ The possibility for carrying a definite amount of heat (by an electric heater of known power) into the inner space of the calorimeter should be assured.

At these rigorous experimental conditions it is possible to follow the heat energy changes accompanying vital processes of the animal even for a longer time interval. The heat loss or heat uptake of the mammalian organism can be determined by measuring the exchange of the heat energy between the body of the animal and its environment (i.e. the air space of the inner part of the calorimeter). Let us examine the details of the heat-exchange processes involved in biocalorimetric measurements. The inner air-space of the calorimeter is warmed up by the heat lost by the animal through different mechanisms. The major mechanism is the heat radiation and a part of the heat energy is lost by evaporation or convection (e.g. giving off excrement). At a certain time after placing the animal into the closed inner space of the calorimeter the system comes into the stage of thermal equilibrium, when temperature of the inner space (T_1) does not increase further. At this point one should

measure the constant temperature difference between the inner space ant the outside air-space,, (T_1 - T_0). Of course, a constant environmental temperature (T_0) should also be assured during the experiment.

This constant temperature difference (T_1 - T_0) in the equilibrium stage is a result of the heat generation of the animal and proportional with his/her heat loss.

Experiments:

The biocalorimetric measurements are carried out according to the following experimental description.

1./ Open the calorimeter and allow it to achieve a thermal equilibrium with the air space of the lab. Put the experimental object (animal) into the inner space of the calorimeter and close the calorimeter carefully. Switch on the oxygen pump to assure the basic life-condition for the animal. Immediately after closing the calorimeter read the temperature of the inner space displayed on a digital multimeter (using a semiconductor as temperature-detector). Follow reading of the temperature (in every minute) until its increase becomes steady. This is the so called "pre-period". It usually takes about 8-10 minutes (Fig. 1.). The main period starts from this point, therefore note the temperature value (T_p). In the main period read the temperature in every minute during about 30 minutes. After the main period also note the temperature corresponding to the end-point (T_f). The constant temperature difference observed in the main period (T_f - T_p) is proportional to the heat generated by the animal during the given time-interval.

2./ Remove the animal from the calorimeter, clean it carefully and allow the calorimeter's air space again to equilibrate with the air space of the lab. Then close the calorimeter again, switch on the oxygen pump and warm up the inner air-space of the instrument using an electric heater of known power. The rate of the warming can be regulated by changing the voltage or the current of the electric heater. Set the rate of warming to a value close to that of obtained in the case of animal-experiment. Read the temperature in the main period (after a similar "pre-period") in every minute. (The voltage and current values **should not** be changed during the main period!) If the rate of the warming by the electric heater was set properly during the "pre-period" then starting (T_p) and the final (T_f) temperature values corresponding to the main period will be close to those obtained in the animal-experiment (o - o) (Fig. 1).

3./ Plot the temperature data obtained under point 1./ and 2./ as a function of time. Determine the temperature inrease during the main period caused by the animal and the electric heater as well.

4./ The heat energy introduced by the electric heater can be calculated from the experimental data. This heat energy is related to the basic electric characteristics of the heating apparatus as follows:

$$Q = I^2Rt = UIt \qquad (3)$$

where I is the current, U is the voltage of the heater while t is the time interval of the main period. This expression can be used to estimate the heat energy generation (heat

loss) of the animal during the main period, since in the case of biological objects equation (2) cannot be used directly due to the unknown specific heat.

5./ Determine the heat quantity produced by the animal during the main period. Determine heat quantity produced by a unit of mass (kg) during a unit of time (s).

If the warming-rate is nearly equal in the case of biological and electrical heat generation , and the constant temperature difference achieved in the main period (T_f - T_p) are also close to each other in the two cases, then the heat energy calculated by the use of equation (3) is a good estimate of that generated by the animal during the given time interval.

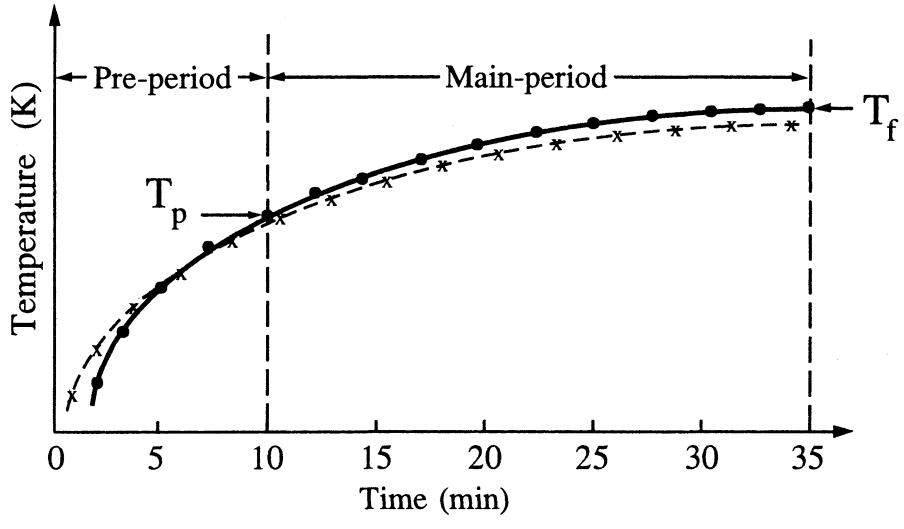


Figure 1

In the case of different warming rates observed in biological and electrical heating, respectively, the calculation procedure is somewhat different from the latter one. In such a case one should calculate the electrically introduced heat energy required for a 1 K increase in the inner temperature during the main period. This value (expressed in Joule units) should be multiplied with the constant temperature difference (T_f - T_p) (expressed in degrees) observed in the main period of the animal-experiment. This way, one can get the amount of heat energy lost by the animal in a certain time interval.

Appendix: Diagram of the electric heating apparatus (Fig. 2.)

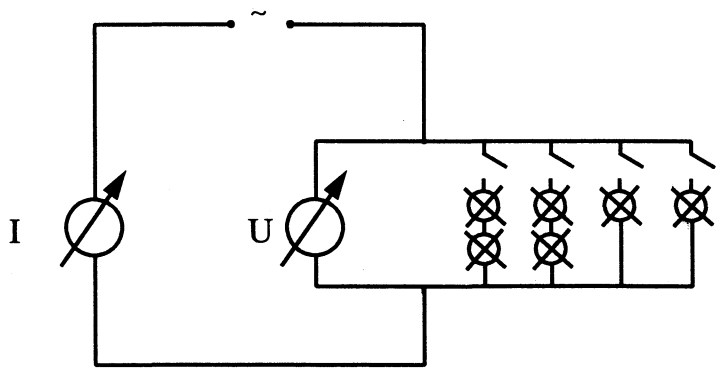


Figure 2

14. Measurement of diffusion constant

Of fundamental importance in the understanding of biological systems is a knowledge of the processes by which chemical materials are distributed within a cell or tissue. It is often true that the rate of a reaction governing the behavior of a system depends not only on the system itself but rather on the speed by which the essential materials are transferred into the active cells and the speed by which the end-products are removed. The basic process involved in material transfer is "diffusion".

In its simplest form, diffusion is the passive physical transfer of particles from a region of higher concentration to another one of lower concentration. This is an irreversible process involving therefore a decrease of free energy at constant T and p.

For the investigation of laws describing diffusion a simplified model can be considered: Let us imagine a tube with a cross-section A (cm^2), containing a solute concentration of c (Fig. 1). The concentration is decreasing parallel to the arrow ($dc < 0$), and is the same perpendicular to the arrow, so the solute diffuses in the direction parallel to the arrow.

The diffusion occurs only in one direction in the tube. The driving force (F) is the chemical potential gradient ($d\mu/dx$) where x is the axis along the tube.

$$F = -\frac{\partial \mu}{\partial x} = -kT\frac{\partial \ln c}{\partial x} = -\frac{kT}{c}\frac{\partial c}{\partial x} \qquad (1)$$

where $\mu = \mu^0 + kT\ln c$, k is the Boltzmann constant, T is the absolute temperature and μ^0 is the standard chemical potential.

This force producing diffusion is counterbalanced by the frictional force between the diffusing molecules and the solvent. So the average velocity of molecules (v) is given as:

$$\bar{v} = \frac{F}{K} \qquad (2)$$

where K is the frictional constant and can be expressed from the Stoke's law as :

$$K = 6\pi\eta r \qquad (3)$$

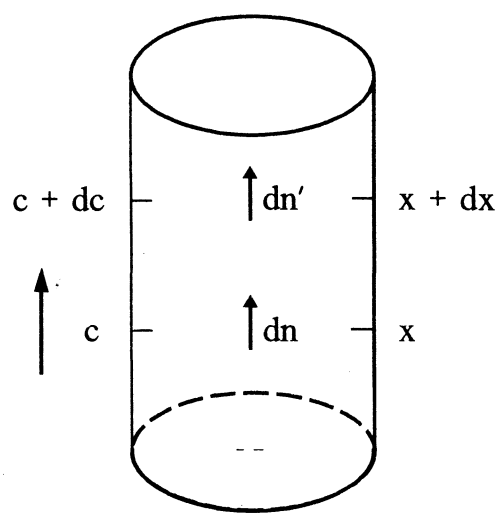


Figure 1

where η is the viscosity and r is the radius of the diffusing molecule. The amount of the material (dn) (in moles) diffusing across the plane of area A, during dt time is given as:

$$dn = cA\bar{v}dt \qquad A\bar{v}dt = \text{volume, } V \qquad (4)$$

Substituting equations (1)-(3) into equation 4. gives

$$dn = -\frac{kT}{6\pi\eta r} A \frac{\partial c}{\partial x} dt \tag{5}$$

Or in another form:

$$dn = -DA \frac{\partial c}{\partial x} dt \tag{6}$$

where

$$D = \frac{kT}{6\pi\eta r} \tag{6.a}$$

is the diffusion coefficient. The equation 6. expresses the Fick's first law of diffusion.

This law is valid only if the concentration gradient is constant. If the concentration gradient is a function of the distance the concentration of different layers will change in the course of time.

To calculate the concentration change in the course of time, let us take the flow rate at x distance and at time t as:

$$\frac{dn}{dt} = -DA\left(\frac{\partial c}{\partial x}\right)_t \tag{7}$$

at the same time the flow rate at $x+dx$ distance is:

$$\frac{dn'}{dt} = -DA\left(\frac{\partial (c+dc)}{\partial x}\right)_t \tag{8}$$

Since the concentration is the function of space and time:

$$c + dc = c + \left[\frac{\partial c}{\partial x}\right]_t dx \tag{9}$$

$$\frac{dn'}{dt} = -DA\left[\left(\frac{\partial c}{\partial x}\right)_t + \left(\frac{\partial^2 c}{\partial x^2}\right)_t dx\right] \tag{10}$$

In the layer with dx thickness the amount of the diffusate has been changed by the value of $(dn\text{-}dn'/dt)$. Since the volume of the layer is Adx the rate of increase in concentration with time at a fixed value of x is:

$$\frac{dn - dn'}{dt A\, dx} = \left(\frac{\partial c}{\partial t}\right)_x \tag{11}$$

Substitution of equations (7) and (10) into the equation (11) results in Fick's second law:

$$\left(\frac{\partial c}{\partial t}\right)_x = D\left(\frac{\partial^2 c}{\partial x^2}\right)_t \tag{12}$$

For the determination of diffusion constant the differential equation (12) has to be solved at specific conditions so the resulting equation should contain parameters which can be measured easily (e.g. concentration, time)

The boundary conditions defining the integration of equation (12) are:

a./ The diffusion process is taking place through the surface of a homogenous cylinder with radius r_0, which contains a solute of c_0 initial concentration.
b./ The area of the circular bases is negligible comparing with that of the curved surface of the cylinder.
c./ The concentration is always zero outside of the cylinder
d./ The diffusion process is observed at a time point (t) sufficiently far enough from the beginning.

Without giving details the resulting equation is:

$$\frac{c}{c_0} = Ae^{-\left(BD\frac{t}{r_0^2}\right)} \tag{13}$$

where c is the average concentration inside the cylinder at t time point. A (=0.693) and B (=5.78) are constants.

According to the condition d./ the equation (13) can not be used for obtaining exact results at t=0 time point. However, the diffusion constant can be determined at sufficiently far enough time points, at t_1 and t_2 with corresponding c_1 and c_2 values, using equation below:

$$c_1 = c_0 Ae^{-\left(BD\frac{t_1}{r_0^2}\right)} \tag{14}$$

and

$$c_2 = c_0 Ae^{-\left(BD\frac{t_2}{r_0^2}\right)} \tag{15}$$

Subtracting the above two equations from each other, (t_2 - t_1), and taking the logarithm of the resulting equation the following relationship is obtained:

$$\ln c_1 - \ln c_2 = (t_2 - t_1)\frac{BD}{r_0^2} \tag{16}$$

Expressing the diffusion constant from equation (16):

$$D = \frac{r_0^2(\ln c_1 - \ln c_2)}{B(t_2 - t_1)} \qquad (17)$$

Using equation (17), the simplest way for the determination of the diffusion coefficient is to plot the natural logarithm of concentration against time. The slope of the resulting straight line equals with BD/r_0^2.

The diffusion constant depends upon the concentration since the solute molecules interact with each other. Therefore the diffusion constant extrapolated to zero concentration is usually determined. To accomplish this extrapolation the diffusion constants (D_i)have to be determined at different concentration (c_i) and then the D_i values have to be plotted as a function of c_i. At $c_0=0$ point the ordinate of this function is D_0, the diffusion constant of infinitely diluted solution. The cylindric geometry is guaranteed by enclosing the solute under investigation in a gel forming long and thin cylinder.

The concentration of the diffusing material can be determined by photometry when the solute is chromophore or by conductometry in case of electrolytes.

Determination of diffusion constant of potassium chloride using conductometry:

Conductivity (G_e) is the inverse of resistance (R_e), and its unit is Siemens (S = Ω^{-1}). For characterization of the conductivity of a given material the specific conductivity (g_e) is generally used. (Its unit is $\Omega^{-1}cm^{-1}$).

Besides their material characteristics, the conductivity of diluted solutions of strong electrolytes depends upon the concentration, too. The conductivity of electrolytes is increasing with the elevation of the concentration because of the number of ions per unit volume is also increasing. In concentrated solution the conductivity is decreasing after surpassing a maximum value since the dissociations of electrolytes is repressed at higher concentrations.

At low concentration there is a linear relationship between the conductivity and the concentration of KCl so this linear phase can be used for the conductometric determination of KCl concentration after appropriate calibration.

In the laboratory the conductivity of solutions will be measured by a Radelkis conductometer (type OK 102/1). The conductivity values can be read directly on the scale of the instrument in μS or mS units. The schematic representation of the conductometer is shown on Fig.2.

Experiments:

For the determination of the diffusion constant KCl solution of 0.1 N, enclosed in a cylindric agar-agar gel with the diameter of few mm, is used. The agar gel has open pores, so the diffusion to K^+ and Cl^- ions is not hindered significantly, these ions move almost freely in the gel. The gel cylinder is provided in the laboratory. (Caution! The gel may become dry easily causing significant shrinking of the gel.)

1./ Calibrate the instrument using the following procedure (Fig. 2): While depressing the calibration switch (4) the needle has to be moved to the calibration point (6) by turning the calibration potentiometer (3). Determine the conductivity of the deionized water!

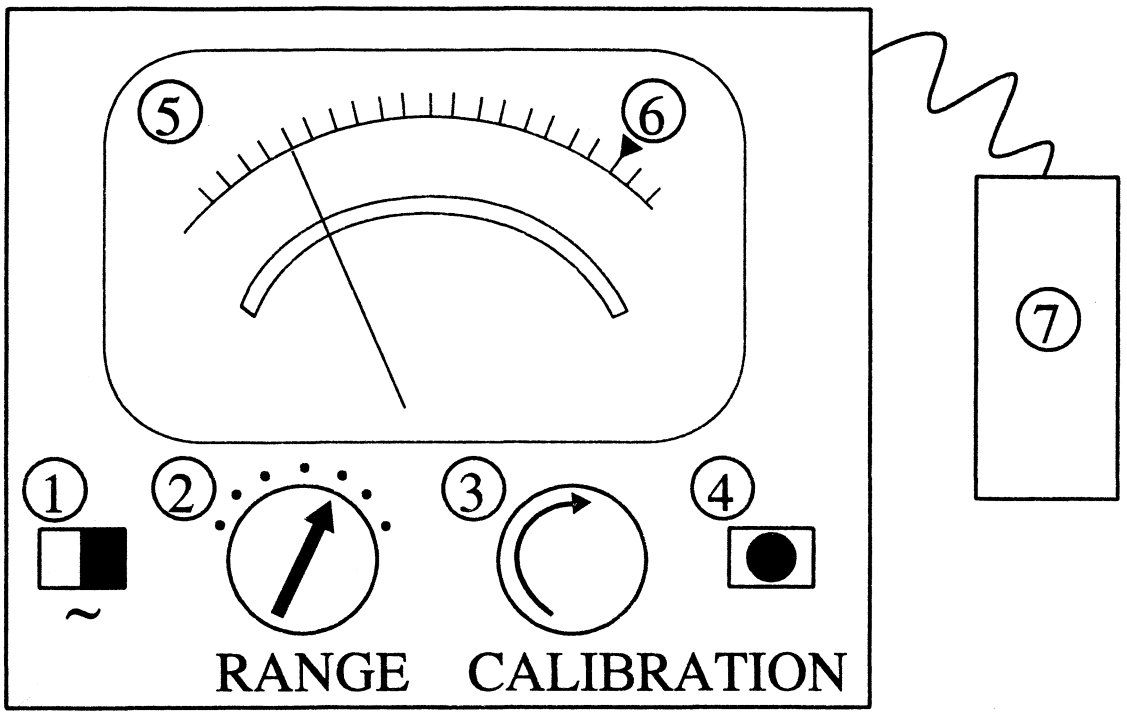


Figure 2

1. power switch; 2. range switch; 3. calibration potentiometer; 4. calibration switch; 5. scale; 6. calibration point; 7. measuring cell

2./ Add 25 ml of deionized water to each of 6 Erlenmeyer flasks. Place the gel into the beaker, pour the content of the first Erlenmeyer flask onto the gel. Incubate for 0.5 min. Shake the Erlenmeyer flasks occasionally during the incubation time! at the and of the incubation pour back the solution into the Erlenmeyer flask (#1 Erlenmeyer flask) while keeping the gel in the beaker. Then add the content of the next Erlenmeyer flask to the gel. Incubate again for 1 min and pour back the solution into the Erlenmeyer flask (#2). Repeat the process with incubation time 2.0, 5.0, 10.0 and 15.0 min.

3./ Measure the conductivity of the effluxed KCl. Subtract the conductivity of dH_2O from each value measured (corrected conductivity). Determine the concentration of the effluxed KCl from the measured data using the enclosed calibration curve.
You can calculate the actual concentration of KCl in the gel (z_i) using the following formula normalizing mass changes to the volume of the gel:

z_0=0.1 [M] (concentration of KCl in the gel before diffusion)
$z_1=z_0$ - (volume of the water/ volume of the gel)$\cdot u_1$
$z_i=z_{i-1}$ - (volume of the water/ volume of the gel)$\cdot u_i$

Meanings of the u and z are given in the table.

4./ Summarize your data in Table!

5./ Plot the natural logarithm of KCl concentration remaining in the gel against the time of diffusion! The slope of the resulting straight line equals with DB/r_0^2 (r_0=radius of the gel, D= diffusion constant, B= 5.78).

6./ Determine the diffusion constant (D) from the linear part of the curve applying equation (17).

NOTE:

height of the gel:	2.5 cm
diameter of the gel:	0.7 cm
concentration of KCl in the gel:	0.1 M

	inc. time [min]	diff. time [min]	measured conductivity [μS]	corr. conductivity [μS]	[KCl] outside of the gel ($u_0 \ldots u_i$)	[KCl] in the gel ($z_0 \ldots z_i$)	ln[KCl] in the gel
	dH_2O	0			0	0.1	-2.301
1.	0.5	0.5					
2.	1.0	1.5					
3.	2.0	3.5					
4.	5.0	8.5					
5.	10.0	18.5					
6.	15.0	33.5					

15. Measurement of membrane permeability

Several important functions can be rendered to biological membranes. They are **structural** (mechanically holding the cell together); **compartmental** (within the cell it may separate substrates from the products of an enzymatic reaction or create ion concentration gradients); **selective** (e. g. glucose molecule of appropriate configuration penetrates cell membrane); **adherent** (cells stick together to form tissues); **kinetic** (membrane participates in cell mobility); **recognitory** (membrane elements are responsible for special recognition important in cell-cell communication); **catalytic** (arrangement of enzymes in membranes provides effective rates for sequences of reactions).

Transport of different molecules are taking place through the membranes. Permeability of a membrane for a given solute is given as:

$$\Delta m = -k q \Delta c \Delta t \tag{1}$$

where Δm (mole) is the amount of the solute passing through the membrane during Δt (min) time, Δc (mole/cm^3) is the concentration difference between two sides of the membrane, q (cm^2) is the area of the membrane, k is the permeability coefficient.

On the scheme shown on Fig. 1 the volumes separated by a membrane are designated as V_2 and V_1, respectively. V_1 contains the solvent, V_2 is the volume of the solution of c_0 concentration. Because of the solute molecules are in constant motion by virtue of their kinetic energy, the concentration difference will cause flow of the solute molecules across the membrane until the original c_0 concentration difference is ceased.

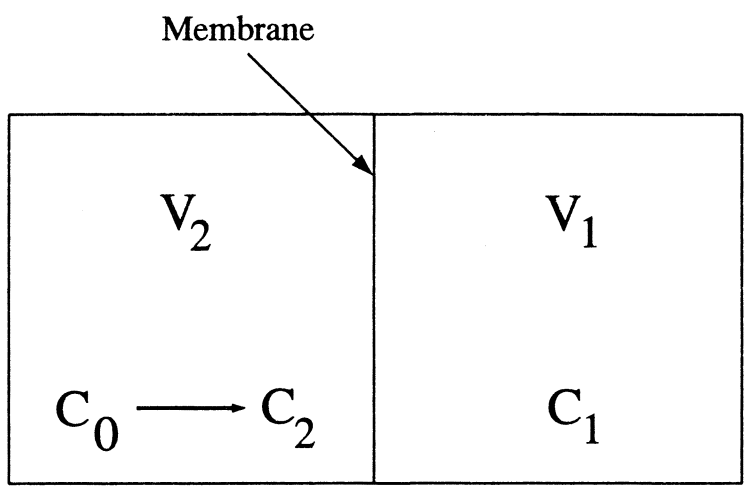


Figure 1

At the beginning the kinetics of the process (when c_1 is negligible) is described by the following equation:

$$\frac{dc}{dt} = -k q c_2 \frac{1}{V_2} \tag{2}$$

therefore

$$\frac{1}{c_2} dc = -k q \, dt \frac{1}{V_2} \tag{3}$$

Performing the integration between t and t_0:

$$\ln c_2(t) - \ln c_2(t_0) = -\frac{k}{V_2} q (t - t_0), \tag{4}$$

if $t_0 = 0$ and $c_2(t_0) = c_0$,

$$\ln\frac{c_2}{c_0} = -k\,q\,t\,\frac{1}{V_2} \tag{5}$$

When ln (c_2/c_0) values are plotted against time the slope of the resulting straight line will give the $-k \cdot q/V_2$ value. This simplified model can be used for the determination of k when the whole process is divided into short periods (i.e. in V_1 volume the concentration of solute molecules is kept low by refreshing the solvents in V_1 volume from time to time).

When c_1 is not negligible (there is no solvent refreshing in volume V_1) the phenomena is described by the following set of differential equations.

$$\frac{dc_2}{dt} = -\frac{k\,q\,c_2}{V_2} + \frac{k\,q\,c_1}{V_2} = k(c_1 - c_2)\,\frac{q}{V_2}$$
$$V_2\,\frac{dc_1}{dt} = -\frac{dc_2}{dt}\,V_1 \tag{6}$$

The solutions for $c_1(t)$ and $c_2(t)$ are:

$$c_1(t) = \frac{c_0}{2}\,e^{-\frac{2kqt}{V_2}} + \frac{c_0}{2} \tag{7}$$

$$c_2(t) = -\frac{c_0}{2}\,e^{-\frac{2kqt}{V_2}} + \frac{c_0}{2} \tag{8}$$

Subtracting the above equation from each other:

$$c_1(t) - c_2(t) = c_0\,e^{-\frac{2kqt}{V_2}} \tag{9}$$

and then

$$\ln\frac{c_1(t) - c_2(t)}{c_0} = -\frac{2kqt}{V_2} \tag{10}$$

c_0 is the initial concentration, $c_1(t) - c_2(t)$ is the difference between the concentrations in the two compartments at time t. Plotting the $\ln(c_1(t) - c_2(t))/c_0$ against time the slope of the resulting straight line is $-2kq/V_2$. In the knowledge of q the k can be calculated. This procedure gives accurate result for k, if even concentration distribution is maintained within volumes separated by the membrane.

In our experiments the permeability of a dialysis tube for acridine orange will be determined using the simplified model described previously. The concentration of acridine orange is to be measured spectrophotometrically using a calibration curve established earlier. The dialysis tube containing V_2 volume of acridine orange solution is placed in V_1 volume of solvent (deionized water) for a short period (e.g. 2 min). The tube is placed in a new V_1 volume of fresh solvent for a longer period (e.g. 4 min). This procedure is repeated for 6-7

times. The concentration of acridine orange effluxed across the membrane into the V_1 volume of water is determined photometrically. The concentration changes ($c_2(t)$) inside the dialysis tube have to be determined from time to time by subtracting the amount of acridine orange effluxed across the membrane from the initial concentration of acridine orange. Plotting $\ln(c_2/c_0)$ as a function of elapsed time the k permeability coefficient can be determined from the slope of the curve.

Experiments:

1./ Switch the spectrophotometer and set the position of the 0 and 100 transmission, according to the description in Absorption photometry I. (solvent: distilled water).

2./ Make a series of dilutions from the dye stock solution (0.1 % = 0.1 g/100 ml) given in the laboratory (50,100, 200, 400x dilution).

3./ Determine the absorption maximum by recording the spectrum between the wavelength interval of 420 - 550 nm in 10 nm steps according to the description in Absorption photometry I.

4./ Measure the absorption of different acridine orange solutions at the absorption maximum determined in the previous step. Make the absorption - concentration calibration curve.

5./ Rinse the outside of the dialysis tube with distilled water and place it successively into 6 beakers containing 20 cm^3 of distilled water and leave it for 2, 4, 6, 8, 10 and 12 minutes. During dialysis move the dialysis tube in order to mix the effluxed dye with water. The dialysis tube contains approximately 1 ml of 0.1% acridine orange solution, its length is 8 cm, its diameter is 0.6 cm.

6./ Determine the concentrations of the 6 solutions using the calibration curve and calculate the permeability coefficient regardless the reverse diffusion according to eq.(5). [Plot $\ln(c_2/c_0)$ as a function of total dialysis time, and calculate the permeability constant from the slope.]

Caution!
The solution of acridine orange is toxic!
Throwing away of the dialysis tube is prohibited!

Labnote:

No. of test tube		1	2	3	4	5	6
Incubation time (min.)	0	2	4	6	8	10	12
Dialysis time (min.)	0	2	6	12	20	30	42
Absorption	0						
[AN] (c_1) in the test tube (V_1)	0						
[AN] (c_2) in the dialysis tube (V_2)	c_0=0.1						
c_2/c_0	1						
ln (c_2/c_0)	0						

[AN]=acridine orange concentration (%)

16. Examination of semiconductor devices

Biological systems and the "amorphous semiconductors" show numbers of similar characteristics. Phenomena in excitable tissues (thermoelectricity, frequency transformation, role of trace elements) suggest that the semiconductive properties of these tissues may have an important role in stimulation and transfer of stimuli. Rapid and effective energy transfer in macromolecular level is realized by biological semiconductors (proteins, nucleic acids, lipids).

We aim at demonstrating with the aid of the simplest semiconductor devices (germanium diode, transistors) some semiconductive properties occurring in biological systems.

Metals are characterized by the presence of many so called "free" electrons, whereas good insulators have virtually none - all their outer electrons are tied up in interatomic bonds. Semiconductors are solid materials which are neither good conductors nor good insulators. Mainly elements in the 4. column of the periodic table have semiconductive properties (germanium, silicon). The main characteristic of the structure of energy bands in pure (free of impurities) semiconductors is that the forbidden band separating the valence and conducting bands is of small width, so it is between the widths of metals and insulators (Fig. 1). As a consequence, the valence electrons can move easily from the valence band to the conducting band already at room temperature.

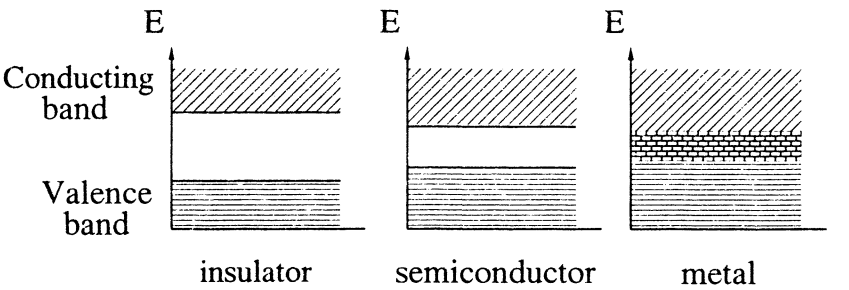


Figure 1

Introducing contaminants, the band structure can be greatly modified, therefore the conductivity of contaminated crystals can surpass that of pure semiconductors. In semiconductors containing four valence electrons and contaminated with elements of five valence electrons (P, As), a so called donor band is formed near the conducting band. The transition of electrons from these donor levels to conducting levels requires only a small energy, so the electron conduction is ensured at room temperature. The resulting electron conduction is called n-type.

When atoms with three valence electrons (B, Al, Ga, In) enter the lattice of elements containing four valence electrons, the lattice bonds are unsatisfied and an acceptor band is formed near the conducting band resulting in holes (electron deficiency) in the valence band. The holes act as positive charges and the conduction is p-type. The band structures of semiconductors of p- and n-type are shown in Fig. 2.

Diodes

When semiconductors of p- and n-type are joined, a significant diffusion current flows in the first time. The reason for this is the very different concentrations of electrons and holes in the two parts. A flow of holes from p-direction and that of electrons from n-direction takes place across the junction area. After a short time, however, this diffusion current will be stopped,

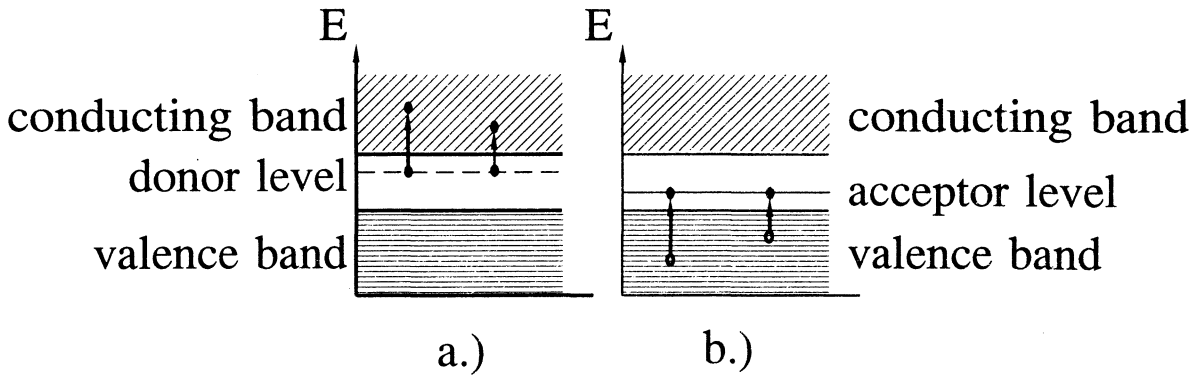


Figure 2

because the crystal regions on the two sides of the junction area becomes electrically charged, and a closing layer is formed. When the p-type region is made positive with respect to the n-type region, holes and electrons are driven toward each other for any time. High currents may flow, and the voltage needs only be enough to keep the holes and electrons moving toward each other. This is called the forward direction (forward-biased diode).

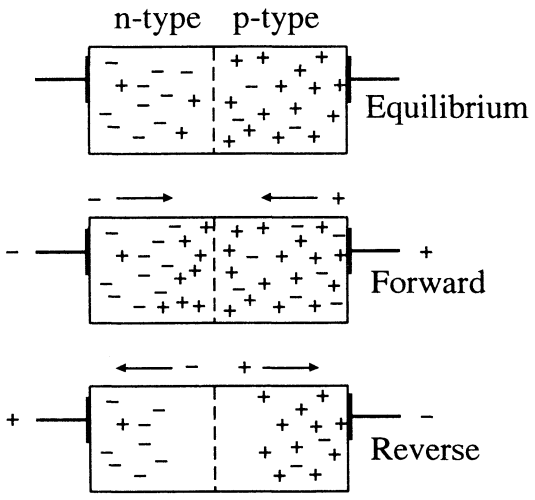


Figure 3

Current carriers in a p-n diode

On the other hand, if the p-type region is made negative with respect to the n-type region, the holes and electrons are moved away from each other leaving the junction region devoid of charges carriers. This is called the reverse direction (see Fig. 3). Here a large voltage can be maintained with essentially no resultant current flow (reverse-biased diode). As a result, diodes are characterized by a low resistance in the forward direction and a high resistance in the reverse direction, permitting rectification. They are applied as rectifiers in power supplies to convert mains AC (alternating current) to DC (direct current).

Transistors

The ordinary junction transistor is a combination of three differently contaminated semiconductive layers. To first approximation, it can be considered as a combination of two p-n junctions back-to-back in a "sandwich" arrangement, as represented in Fig. 4. The figure shows a p-n-p transistor. The thin common layer is called base, the diode biased forward is the emitter and the second one biased reversely is the collector.

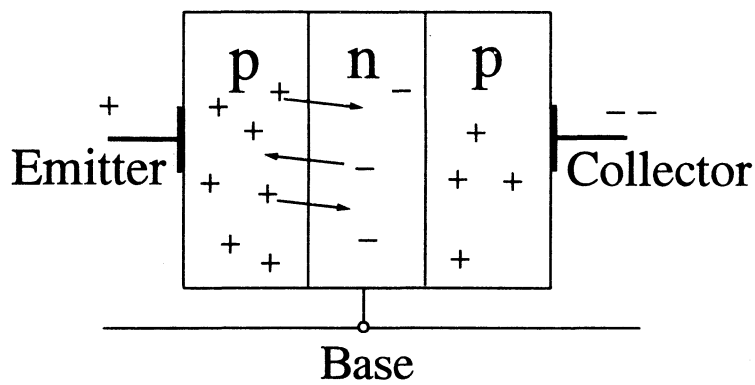


Figure 4

p-n-p transistor

In the emitter-base diode biased forward a great number of holes pass through from the emitter to the base, this is the emitter current (I_E). But in the base, due to its small size and low degree of impurities, there are only few electrons for recombination, the rest electrons necessary for recombination are provided by a power supply connected to the base and emitter, respectively. This is the base current (I_B). The collector is biased negatively with respect to the base, so it is biased in forward direction for the holes. The main rest of the holes is attracted to the collector, increasing the collector current (I_C).

The collector-base diode with its own power supply constitutes a circuit, in which the current intensity (I_C) is influenced by another circuit (emitter-base diode). This is the control process of transistors. If a current of alternating magnitudes gets to the base, this signal is amplified by the greater alteration of collector current. The degree of amplification can be expressed by the current amplification factor (ß). It shows that changing the base current by unit, how great will be the change in the collector current at constant collector voltage:

$$\beta = \left.\frac{\partial I_c}{\partial I_b}\right|_{U_{CE}}$$

The measurement of ß is accomplished according to the circuit given by Fig. 5. The collector current-base current characteristic at constant emitter-collector voltage is determined by changing the base current. The current amplification factor is obtained as the slope of the curve in a given point.

Experiments:

1./ Measure the characteristic of two different diodes biased forward using the connection for diode investigation [I_D=f(U_D)].

2./ Determine the break voltage of Zemer-diode by measuring the characteristic of it in reverse direction.

3./ Measure the

a, $I_B = f(U_{BE})$

b, $I_B = f(I_B)$

characteristics of two different transistors at different collector-emitter voltage values using the connection for transistor investigation (Fig. 5) and plot them in the

a. I_B - U_{BE}

b. I_B - I_B

coordinates, respectively.

4./ Determine the $I_B = f(U_{CE})$ characteristics of transistors at different base currents. Change the base current by values of 0.1 mA and the collector-emitter voltage by values of 1.5 V, respectively.

5./ Determine the current amplification factors of the transistors

$$\beta = \frac{\partial I_c}{\partial I_b}\bigg|_{U_{CE}}$$

using the collector current - base current characteristics.

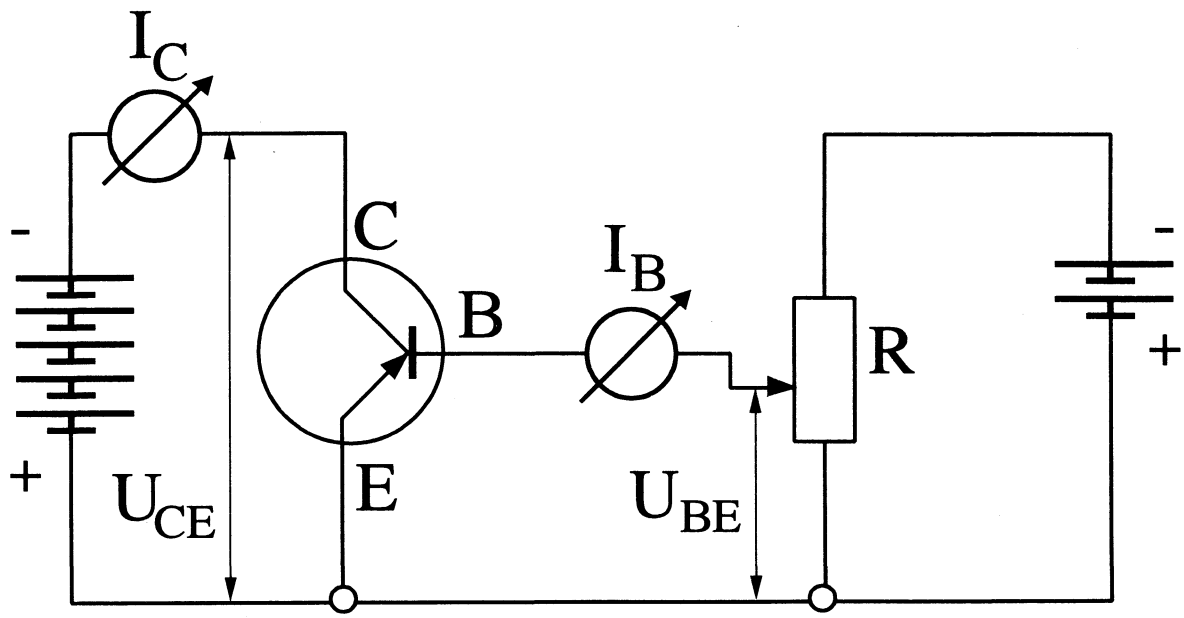


Figure 5

17. Simulation of electric activity of the cell membrane

Cells continuously interact with their environment, "messages" are transmitted back and forth, thus promoting normal functioning of cells, tissues and the whole organism.

In the process of communication the cell membrane plays a central role. Stimuli reaching the cell are transformed into processable signals by membrane proteins. These signals evoke responses in the cell, often manifesting in a change of enzyme activities. This process is commonly termed transmembrane signalling.

Ionic channels in the cell membrane are important elements of transmembrane signalling. Ionic channels are transmembrane proteins, supramolecular structures made up of several subunits. Ions trespass the membrane, otherwise hardly permeable to them, through pores formed by these subunits. Ionic channels are capable of producing specific responses to certain stimuli, i.e. voltage gated channels respond to a change of transmembrane potential, ligand operated channels to chemical triggers. The response of channels is very simple: opening or closing (Fig. 1a: single K^+ channel currents in a lymphocyte held at membrane potentials indicated). Open channels exhibit a special property, selective permeability: depending on the type of channel usually only a certain type of ion is able to pass through according to its electrochemical potential gradient (Na^+, K^+, Ca^{2+} channels). When more than one channel is activated, the current passing through all activated channels is cumulated. If one observes such a cumulated current (Fig. 1b: whole cell K^+ currents of a lymphocyte recorded at membrane potentials indicated at each trace), opening and closing of individual channels is not distinguishable any more, however, the time course of the observed current is still dependent on the type of channels activated and the parameters regulating channel opening.

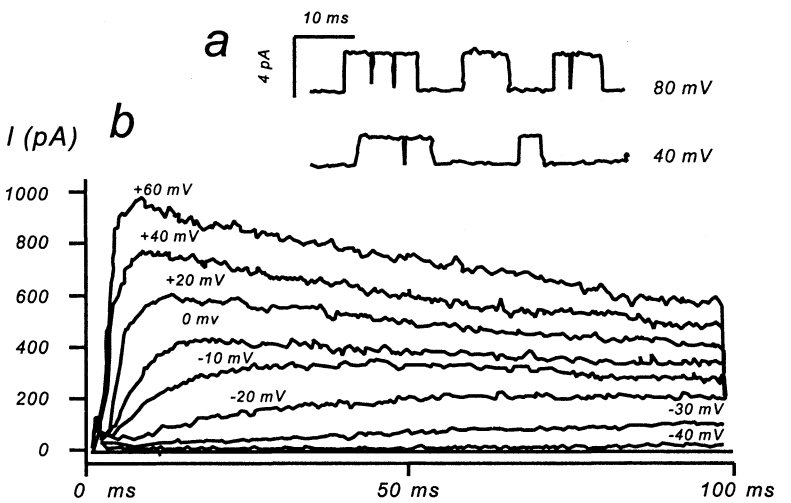


Figure 1

In general, distribution of ions on the two sides of the cell membrane is uneven. The Nernst equation can be used to calculate the equilibrium potential of a given ion based on its concentration. (The equilibrium potential is defined as the electric potential balancing the chemical potential. For details see your current textbook.)

$$E_x = \frac{RT}{z_x F} \ln \frac{[x]_o}{[x]_i} \qquad (1)$$

For example, the equilibrium potential of K^+ would be E_K = -98 mV from this equation using K_o^+ = 4 mM, K_i^+ = 155 mM, and T = 25°C.

The net electric driving force acting on a given ion when the membrane potential is E_m can be expressed as $E_n = E_m - E_x$, where E_x is the equilibrium potential for the given ion. Based on Ohm's law we can calculate the ionic current I_x according to

$$I_x = G_x E_n = G_x(E_m - E_x) \tag{2}$$

where G_x is the conductance of the membrane specific to ion "x" ($G=R^{-1}$). $I_x = 0$ when $E_m = E_x$. The membrane potential, where the current carried by a given ion is zero, is called the reversal potential of the given ion, since the current changes its direction at this potential value.

Based on the Goldmann-Hodgkin-Katz equation a given ion contributes to the membrane potential according to its permeability. (See textbook for details!)

$$E_m = \frac{RT}{zF} \ln \frac{p_{Na}[Na^+]_o + p_K[K^+]_o + p_{Cl}[Cl^-]_i + \ldots}{p_{Na}[Na^+]_i + p_K[K^+]_i + p_{Cl}[Cl^-]_o + \ldots} \tag{3}$$

Accordingly, activation of channels carrying certain ions, and the consequently evolving current is measurable only if E_m is kept constant, independent of changing in permeability (which under normal circumstance causes a change in E_m, see Goldmann-Hodgkin-Katz equation above). The so-called **Voltage-Clamp** technique can be used to set and maintain (=clamp) E_m at the required value. When investigating voltage gated channels, this technique permits detection of the voltage dependence of the evoked current.

Voltage clamping has already been introduced in the 1940s using intracellular microelectrodes pricked through the cell membrane. Owing to the relatively large size of microelectrodes, this method was only useful in the case of larger cells such as neurons and muscle cells.

The introduction of the **Patch-Clamp** technique greatly improved the investigation of smaller cells (Fig 2.). The basic principle behind this method is the following: The cell membrane is touched with a special glass micropipette and a weak vacuum is generated inside of the pipette to promote the formation of mechanical contact having high electrical resistance ("giga-seal", 1-100 GΩ) between the membrane and the pipette. The high resistance seal insulates the inside of the pipette from the buffer in which the pipette and the examined cells are immersed. Thus, the current passing through the pipette is identical to the current passing through the membrane patch covered by the pipette tip itself. The membrane potential can be set to any given value using a current injecting circuitry based on negative feedback. At the same time the current passing through the patch pipette is converted to voltage in special amplifier. The magnitude of the input current is in the order of pA (pico=10^{-12}), so the amplifier has to be based on the following principle: the small current is passed through a high resistance (R_f = 500 MΩ) resistor. The voltage drop across this resistor is relatively

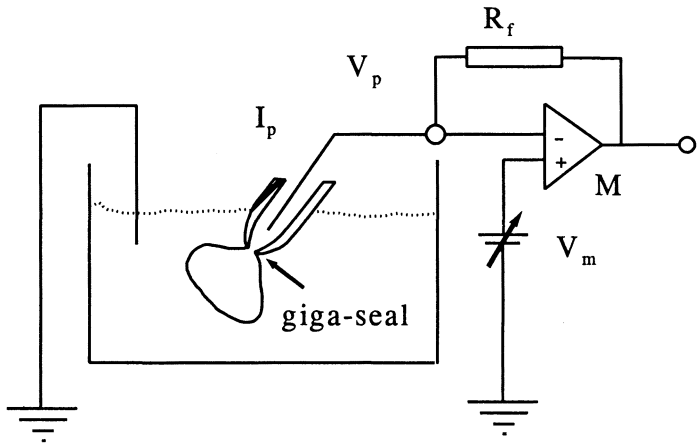


Figure 2

large and thus measurable in spite of the small current. Knowing the resistance and the voltage drop, the current can be calculated.

The membrane patch covered by the pipette tip is approximately of 1 μm in diameter and usually contains only a few channels. The activity of these channels can be studied as the current passes through them (Cell Attached Patch configuration, Fig. 3a).

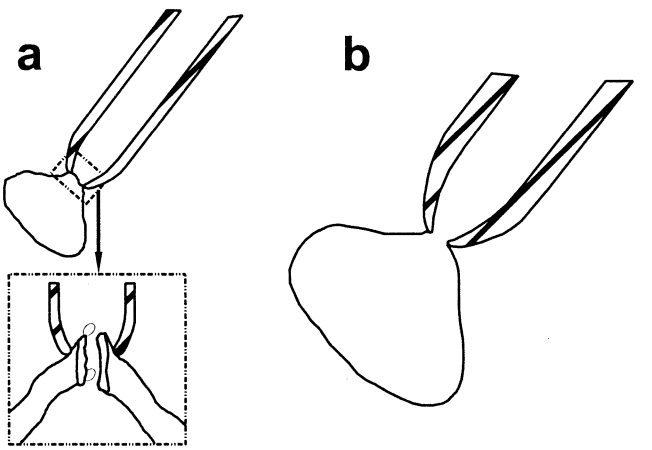


Figure 3

When stronger suction is applied in the Cell Attached configuration, the membrane patch under the pipette tip is disrupted and the interior of the pipette becomes contiguous with the intracellular space. At this point we can adjust the membrane potential of the entire cell using our voltage clamp feedback device, and the current detected is the cumulative sum of the individual currents passing through the active channels. (Whole Cell Recording configuration Fig. 3b).

There are further important physical parameters besides membrane potential, channel current, etc. that can be used to characterize the cell. The cell membrane can be considered as a capacitor, in which the lipid bilayer as a thin insulator separates the intra- and extracellular space containing different ions at different concentrations.

The capacitance of such a capacitor built of two parallel planes (the two sides of the cell membrane) can be calculated as

$$C = \varepsilon_0 \, \varepsilon \, \frac{A}{d} \tag{4}$$

where ε_0 is the dielectric constant of vacuum, ε is the relative dielectric constant of the insulator (in this case the lipid bilayer), A is the surface area of the capacitor and d is the thickness of the insulating layer. Due to variance in the surface area of cells, the capacitance of the membrane is usually given as specific capacitance for unit area. Its usual value is 1-10 μF/cm^2.

When setting E_m to a certain value during voltage clamping in order to detect ionic currents at that potential, the cell membrane capacitance has to be charged. This needs a "passive" charging current which may disturb the detection of the "active" channel currents.

The capacitance (C) of a capacitor is defined as the amount of charge (Q) necessary to generate a potential difference (U) across the plates of the capacitor:

$$C = \frac{Q}{U} \tag{5}$$

Consequently, it is inversely proportional to the rate at which the potential difference is built up:

$$\frac{\mathrm{d}U}{\mathrm{d}t} = \frac{I}{C} \tag{6}$$

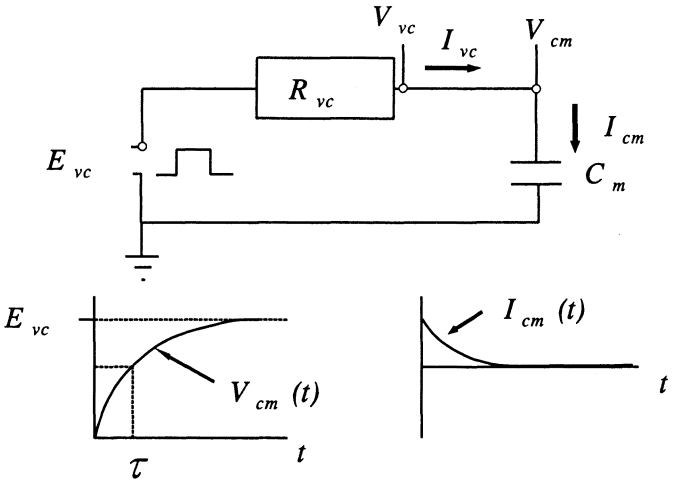


Figure 4

On Fig. 4 E_{vc} is the potential difference to which the membrane as a capacitor has to be charged in Voltage Clamp (VC), C_m is the capacitance of the membrane, R_{vc} is the output resistance of the power supply, V_{vc} is the output potential of the power supply. The relationship of E_{vc} and V_{vc} is the following:

$$V_{vc} = E_{vc} - IR_{vc} \qquad (7)$$

If we take I_c as the charging current,

$$I_{vc} = I_{cm} \qquad (8)$$

according to Kirchoff's law. Taking V_{cm} as the actual potential difference across the capacitor and using Ohm's law and equation (6) we get

$$\frac{E_{vc} - V_{cm}}{R_{vc}} = C_m \frac{\mathrm{d}V_{cm}}{\mathrm{d}t} \qquad (9)$$

This differential equation describes the rate at which V_{cm} changes at given values of E_{vc}, V_{cm} and C_m. Solving for the differential equation (9) gives the time dependence of V_{cm}.

$$V_{cm}(t) = E_{vc}\left(1 - e^{-\frac{t}{\tau}}\right) \qquad (10)$$

$$\text{with} \quad \tau = R_{vc} C_m$$

where τ is the time constant of the RC (resistance-capacitance) circuit, and gives the time required for V_{cm} to reach 63.2% of E_{vc}. Upon applying a voltage step from zero (at $t = 0$) towards E_{vc}, $V_{cm}(t)$ will change from $V_{cm0} = 0$ (at $t = 0$) to $V_{cm\infty} = E_{vc}$ (at $t = \infty$) with a rate determined by $\tau = R_{vc}C_m$. Thus, the smaller R_{vc} and C_m, the faster will V_{cm} reach E_{vc}. Increasing R_{vc} (e.g. inserting another resistor (R_i) into the upper branch of the circuit in Fig. 4), the time constant (τ) increases, and becomes $\tau_1 = (R_{vc}+R_i)C_m$.

The capacitive current (the current charging the membrane, $I_{cm}(t)$) can be measured if we insert an R_i resistor into the upper branch of the circuit in Fig. 4, and measure the voltage drop (U_{ri}) across it. Using $U_{ri}(t)/R_i = I_{ri}(t)$; $I_{ri}(t) = I_{vc}(t) = I_{cm}(t)$ can be calculated.

$$\frac{U_{ri}(t)}{R_i} = \frac{V_{vc}(t) - V_{cm}(t)}{R_i} = \frac{E_{vc} - I_{ri}(t)R_{vc} - V_{cm}(t)}{R_i} = I_{ri}(t) \qquad (11)$$

from which

$$I_{ri}(t) = \frac{E_{vc} - V_{cm}(t)}{R_{vc} + R_i} \qquad (12)$$

substituting (8) into (12) we get for $I_{ri}(t)$:

$$I_{ri}(t) = \frac{E_{vc} e^{-\frac{t}{\tau_1}}}{R_{vc} + R_i} \qquad (13)$$

$$\tau_1 = (R_{vc} + R_i) C_m$$

On the basis of (13) one can understand that the maximum $I_{ri}(t) = I_{cm}(t)$ is at $t = 0$, and its maximum value is $E_{vc}/(R_i+R_{vc})$. The decay of $I_{cm}(t)$ from this maximum value is characterized by τ_1. On the basis of (6), $I_{cm}(t)$ directly affects dV_{cm}/dt, and thus regulates the rate at which the potential difference across a capacitor-like membrane is built up to a new value.

If we want to set the membrane potential to a given value (Voltage Clamp), and want to measure ionic currents at that potential, we have to take into consideration that the cell membrane doesn't reach its desired potential value immediately, but with a certain time kinetics given by equation (10). Charging the "membrane capacitor" requires a time dependent charging current, called "capacitive current" (see eq.(13)), which may disturb the detection of the "active" current under investigation.

Since the lipid bilayer is not an insulator having infinitely high resistance, the core of the equivalent electric circuit of the cell membrane consists of a resistor R_m and a capacitor C_m connected in parallel, as if passing through the membrane between the extracellular fluid and the cytoplasm. As mentioned in the introduction, the cell membrane contains ionic channels, and therefore this simple "passive" model should be supplemented with them. In our model the ionic channels are not simple resistors, rather, they are associated with a voltage source (E_{ch}), which represents the equilibrium potential of the permeable ions. Since the membrane conductance for a given ion usually depends on activating signals (e.g. depolarization activated voltage gated channels), there is a switch S in the ion channel branch in Fig. 5, which allows one to simulate the activation/inactivation of the conductance (opening or closing of the channel). In a living cell during activation or inactivation of the channels the conductance of the membrane for a given ion is changing as a function of the number of opened channels with fixed conductance.

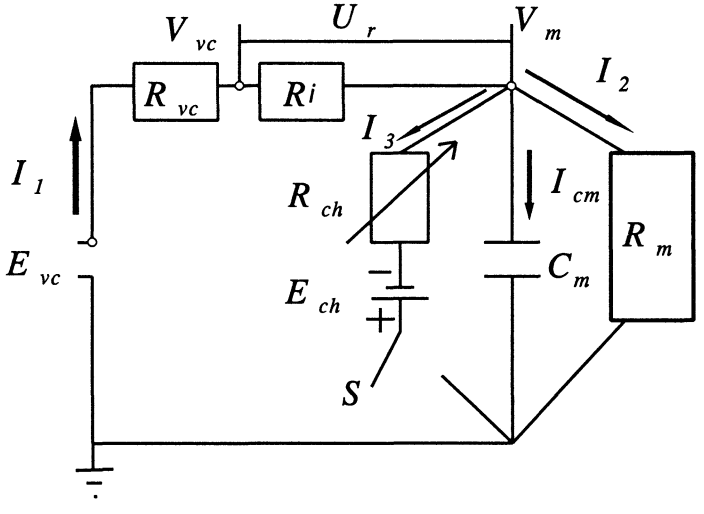


Figure 5

At the level of ionic channels the probability of finding the channel it open state is changing upon stimuli. If we measure the cumulative channel current through the whole of the cell membrane, these changes in opening probability result in changes in the magnitude of the measured current. For simplicity, ionic channels of the membrane, specific for a given ion are substituted with one "big" channel, symbolized by R_{ch}. Variation of the R_{ch} means that the membrane conductance for a given ion is changing.

Connecting the above described circuit with a voltage source we get the model of the Whole Cell Recording technique: the pipette connected with the intracellular space means the upper "intracellular" branch of the circuit. Resistors situated there are the followings: R_{vc} means the output resistance of the voltage source, R_i means the summed resistance of the pipette and the cell membrane-pipette connection (series resistances). R_m, C_m and R_{ch} - E_{ch} are in parallel, but connected in series with the above mentioned resistors. The Voltage Clamp potential (E_{vc}), Voltage Clamp output potential (V_{vc}) and the membrane potential (V_m) indicated in Fig. 5 means potentials relative to the zero point of the circuit (relative to ground).

According to Kirchoff's law, the sum of the current flowing through the elements of the model membrane (R_m, R_{ch}, C_m) equals to the current flowing in the pipette (R_i), which can be measured after current-voltage conversion (so the principle of the measurement applied here is the same as the principle used in patch-clamp amplifiers). In our model, the measured voltage drop U_{ri} across R_i can be used for the *I-V* conversion. Since we know the exact value of R_i, the current can be calculated. (In a real experiment neither the voltage drop on the pipette and on other series resistances can be measured, nor their value is known).

$$U_{ri} = V_{vc} - V_m = I_1 R_i \tag{14}$$

If the S switch in Fig. 5 is opened, the $V_m(t)$ takes its value belonging to $t=\infty$ similarly to that described in equation (10), and this potential is called V_m. After $V_m(t)$ reaching V_m, the membrane charging current diminishes to zero ($I_{cm}=0$).

According to Kirchoff's and Ohm's law:

$$\begin{aligned} I_2 &= I_1 \\ \frac{V_m}{R_m} &= \frac{E_{vc} - V_m}{R_{vc} + R_i} \\ V_m &= \frac{E_{vc} R_m}{R_{vc} + R_i + R_m} \end{aligned} \tag{15}$$

If $R_m >> R_{vc}+R_i$, then:

$$\begin{aligned} E_{vc} &\approx V_m \\ I_1 &\approx I_2 \approx 0 \end{aligned} \tag{16}$$

So at rest (with switch S opened) the current flowing across the membrane (R_m) is approximately zero, therefore using $U_{ri} = IR_i$, the voltage drop across the resistance R_i is also close to zero.

If the operation of voltage gated potassium channels is to be demonstrated, switch S has to be closed when E_{vc} is changed in the direction of depolarization, e.g. from -8 V to -3 V, which in our case represents the activation threshold of potassium channels.

At this point, using $R_i+R_{vc} = R_o$, equations in (15) take the following form:

$$\begin{aligned} I_1 &= I_2 + I_3 \\ \frac{E_{vc} - V_m}{R_o} &= \frac{V_m}{R_m} + \frac{V_m - E_{ch}}{R_{ch}} \\ V_m &= \frac{E_{vc} R_m R_{ch} + E_{ch} R_m R_o}{R_m R_o + R_m R_{ch} + R_{ch} R_o} \end{aligned} \qquad (17)$$

Supposing that $R_m >> R_{ch} >> R_o$, the right hand side of the equation can be divided by $R_m \cdot R_{ch}$. After neglecting values close to zero, we get

$$V_m \approx E_{vc} + E_{ch}\frac{R_o}{R_{ch}} \approx E_{vc} \qquad (18)$$

Using the above circuit, the membrane potential of the model cell can be clamped at the required value, and keeping a constant $V_m \approx E_{vc}$ the total current passing through the membrane can be examined. However, we would like to observe the activation of the channel and the consequent current (I_3) flowing through it. Taking a large value for R_m, from (17) and (18) we can calculate

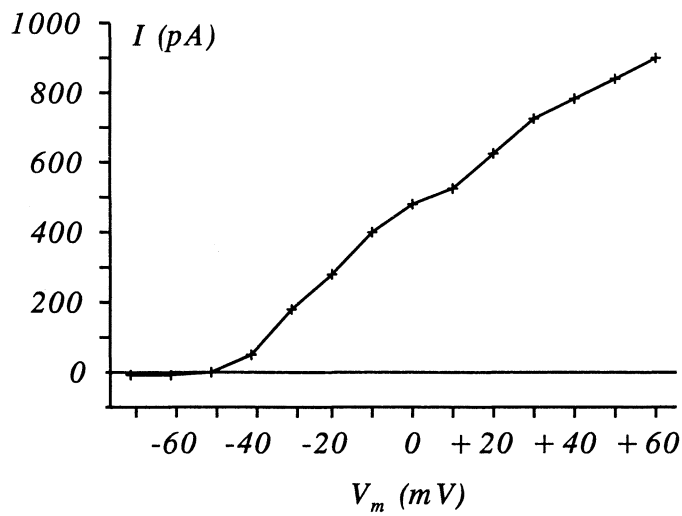


Figure 6

$$I_2 = \frac{V_m}{R_m} \approx 0, \quad \text{thus}$$

$$I_1 \approx I_3 \approx \frac{V_m - E_{ch}}{R_{ch}} \approx \frac{E_{vc} - Ech}{R_{ch}} \qquad (19)$$

This means that by measuring I_1 we gain a good approximation of I_3. From $I_3 (= I_1)$ the conductance G can be calculated using eq.(2), if $E_{vc} = V_m = E_m$ and $E_x = E_{ch}$ is known. As has been pointed out at (14) I_1 can be deducted from measuring U_{ri}.

As a summary, it should be pointed out that current flowing through an R_{ch} resistor symbolizing ionic channels of a model cell can be measured quite reliably under appropriate circumstances. Knowing how in the case of voltage gated channels R_{ch} changes with E_{vc}, setting the known values of E_{vc} and R_{ch} it is possible to obtain the current-voltage (I_{ch}-E_{vc}) diagram of the model cell. Differences of *I-V* diagrams measured on living cells (Fig. 6) carry information about the tested cell. Effects of drugs, hormones, neurotransmitters etc. can be monitored by observing changes in the *I-V* diagram for a given cell. The pathomechanism of such diseases

as diabetes and cystic fibrosis was revealed by results gained with the patch-clamp technique. This is a promising method in other fields of biomedical science too. Professors Neher and Sakmann, who elaborated the patch-clamp technique at the Max Planck Institute of Göttingen received the Nobel prize in 1991.

Practical Instructions

PART I.

Registrating $V_{cm}(t)$ and $I_{cm}(t)$, measuring τ, calculating C_m.

1. Build a circuit according to Fig. 4 using the following values:
   C_m = unknown value, indicated with C_m1 on the board
   E_{vc} = 4 V (to be adjusted using the Amplitude button on the function generator)
   R_{vc} = 50 Ω, this resistor is already in the circuit, inside the function generator, being the "internal resistance" of the device.

Settings on the pulse generator:

	Push-button position
-Count: int	**out**
-Range (Hz):	100 Hz **in**
-function:	square-shaped pulse **in**
-Z_0:	50 Ω **out**
-TTL/CMOS:	pull (out)
-frequency:	100Hz (digital readout on the display)

Connections to the oscilloscope:
-from the function generator (TTL/CMOS) to the **ext. trigger in**put using a BNC connector
-from the model board (OUTPUT) to the **CH1** input.

Connections to the model board:
-from the function generator OUTPUT to INPUT on the model board (BNC connector)
-From model board OUTPUT to the CH1 input of the oscilloscope (BNC connector)

2. Observe and draw $V_{cm}(t)$ when C_m is in the circuit. Remove C_m and observe the shape, length and height of the square pulse, measured from the same socket as previously. Draw your observation.
Settings on the oscilloscope: 0.5 V/DIV
2 ms - 0.1 ms/DIV

3. Determine the value of τ according to equation (10), following the next scheme: Measure - on the scope - the time necessary for $V_{cm}(t)$ to reach 63.2% of E_{vc}. Using $\tau = R_{vc} \cdot C_m$ calculate C_m.
Settings on the oscilloscope: 0.5 V/DIV
0.1 ms/DIV

4. Place an R_i=75Ω resistor into the upper branch of the circuit between the power supply and the capacitor and measure anew τ, then check the value of C_m using $\tau = (R_i + R_{vc})C_m$.
Settings on the oscilloscope: 0.5 V/DIV
0.1 ms/DIV

5. Transfer the R_i resistor to the lower branch of the circuit, representing the extracellular

part, between the ground and the capacitor. Draw $U_{ri}(t)$. $U_{ri}(t)$ measured at this position of R_i is identical to $U_{ri}(t)$ in equation (11). This can be proved using Kirchoff's and Ohm's laws. (Transferring the resistor is only necessary in order to perform the measurement in an easy way. The oscilloscope is only capable of visualizing the potential difference between the ground and a given point of the circuit. Thus the voltage drop across R_i can only be measured if one leg of R_i is grounded. In this case the other leg, connected to C_m is at a potential equal to U_{ri}.

Draw $U_{ri}(t)$, compare its shape and maximum value with data derived from equation (13) describing the capacitive transient $I_{cm}(t)$.

Settings on the oscilloscope: 0.5 V/DIV
0.5 ms/DIV

PART II.

Simulation of the operation of voltage gated potassium channels using the model cell.

1. The circuit according to Fig. 5 is already built in the model board:

C_m = 2.2 mF
R_{vc} = 75 Ω
R_m = 75000 Ω=75 kΩ
R_i = 75 Ω, placed between C_m and the ground, into the "extracellular branch", for reasons explained in part I. of the exercise.
R_{ch} = 0-1 kΩ variable resistor with calibrated scale.
E_{ch} = -9 V (the battery's negative pole is at the cytoplasmic side of C_m. -9 V is approximately 100-fold multiple of the K^+ equilibrium potential).
Push-switch S is open.

Use a D.C. power supply to apply the desired voltage (E_{vc}) to the model cell. To reverse the polarity of the voltage source switch the banana plugs on the output terminals of the power supply.

2. Change E_{vc} and R_{ch} according to the table below, and measure U_{ri} using a digital multimeter connected to the legs of the resistor R_i. After selecting the desired (E_{vc}) "activate" the "potassium channels" with the push-button switch S. When U_{ri} (to which $I_{ri}=I_{ch}$ is proportional, see introduction and equation (19)) reaches its steady value, note this value and calculate I_{ri}. Summarize your rersults in a table.

3. Plot I_{ri} as a function of E_{vc}, compare the plot with Fig. 6, where an *I-V* diagram of a living cell is shown.

IMPORTANT: For simplicity, the circuit for Part II. is built on your model board. You only have to make the connections to the power supply and the digital multimeter. To measure U_{ri} you have to connect the YELLOW socket and its pair to the multimeter.

E_{vc} (V)	R_{ch} (Ω)	S*	U_{ri} (V)	I_{ri} (mA)
-8	1000	0		
-7	1000	0		
-6	1000	0		
-5	1000	1		
-4	740	1		
-3	540	1		
-2	400	1		
-1	300	1		
0	220	1		
1	180	1		
2	160	1		
3	150	1		
4	150	1		
5	150	1		
6	150	1		
7	150	1		

* Open and closed positions of switch S are represented by 0 and 1, respectively.

APPENDIX- description of the instruments

The oscilloscope (scope for short) is the most useful and versatile electronic test instrument. As usually used, it lets one to measure potentials in a circuit as a function of time. The potential measured is the potential of the "measuring point" relative to ground which is an universal zero point of many circuits. The principle of its operation if the following: the vertical deflection of an electron beam is regulated by the potential of the input signal, while the horizontal deflection is controlled by an internal signal forcing the electron beam to sweep horizontally from one edge of the scope screen to the other at a constant speed. The electron beam is visualized on the scintillating screen. The scope consists of three main parts: vertical, horizontal and triggering units.

1. *Vertical*: The input signal (to be measured) is connected to one signal input of the scope (the two almost identical signal amplifiers are called channels). The vertical displacement of the electron beam upon a certain input signal is set by the **calibrated gain** switch in Volts/division. Setting 1 V/DIV means, that the horizontal displacement is 1 division on the display upon 1 V input signal. Warning: when making voltage measurements be sure that the

variable gain knob (the knob concentric with the gain switch) is turned fully clockwise to the end point, otherwise the gain switch is out of calibration. The vertical position of the electron beam on the screen can be set by the "**position** ↕" knob. In our experiments we will use 0.5 V/DIV gain. With the **vertical mode selector** one can display the signal connected to channel 1 (CH1), channel 2 (CH2), both channel 1 and channel 2 (DUAL), or the sum of these two signals (ADD). The AC/GND/DC selector must be in DC position.

2. *Horizontal*: The sweeping speed of the electron beam is set by the calibrated TIME/DIV switch. Setting 1 s/DIV means, that the horizontal deflection of the electron beam is 1 division on the display in 1 sec. For the **variable gain** (situated right to the Time/DIV selector) knob the same warnings can be stated as earlier. The horizontal position (the starting and end point of the sweep) can be adjusted with the **position** ↔ knob. In our experiments we will use 2 ms/DIV through 0.1 ms/DIV gain.

3. *Triggering*: To start the sweep of the electron beam a trigger signal is needed. The trigger can origin from many sources, indicated at the **Source** selector switch. In our case, to synchronize the scope with the pulse we want to study, a trigger signal is sent from the function generator (in our experiment the TTL/CMOS output of the function generator) to the **external trigger input** of the scope. If the source selector is in external triggering position, and the trigger signal reaches the triggering level set by the **trigger level** knob-pair, a sweep is started. Adjustment of the trigger level is sometimes needed in order to "catch" the trigger signal (e.g. to make the triggering level lower than the amplitude of the trigger signal itself). The triggering **Mode** selector must be in NORM position.

The model table: Resistors, capacitors and other elements can be connected to form circuits using banana plugs. The voltage source (E_{vc}) is connected to the table with standard BNC connector. The "command" potential and the "ground" is available from the banana sockets connected to this BNC connector. The banana socket connected to the pin of the CH1 BNC connector is used for the measurements, the ground socket should be connected to the E_{vc} ground socket. The CH1 BNC connector is to be connected to the oscilloscope input. **Part II.** In order to decrease the number of connections to make, you get the whole circuit (Fig. 5) built up and you only have to make the connections to the power source and the digital multimeter.

18. Electrocardiography

Each cycle of cardiac activity is accompanied by electrical potential changes which can be detected at the surface of the body and a record of such alterations in electrical potential is called an electrocardiogram. It is not possible, however, to determine from the record the actual potentials in the cardiac muscle because the heart lies in an irregularly shaped body containing organs which differ in their electrical properties; for example the conductivity of liver and chest wall is relatively greater than that of the lungs. The interpretation of the electrocardiogram is, therefore, to some extent empirical, that is, it must be based on experience and experiment.

Matteucci (1844) was the first to detect electric current from an active heart. Then in 1877 Marchand proved that the excited part of an intact heart became negative with respect to its resting part. The first electrocardiogram was reported in 1903 by William Einthoven who used a string galvanometer. Fig. 1.

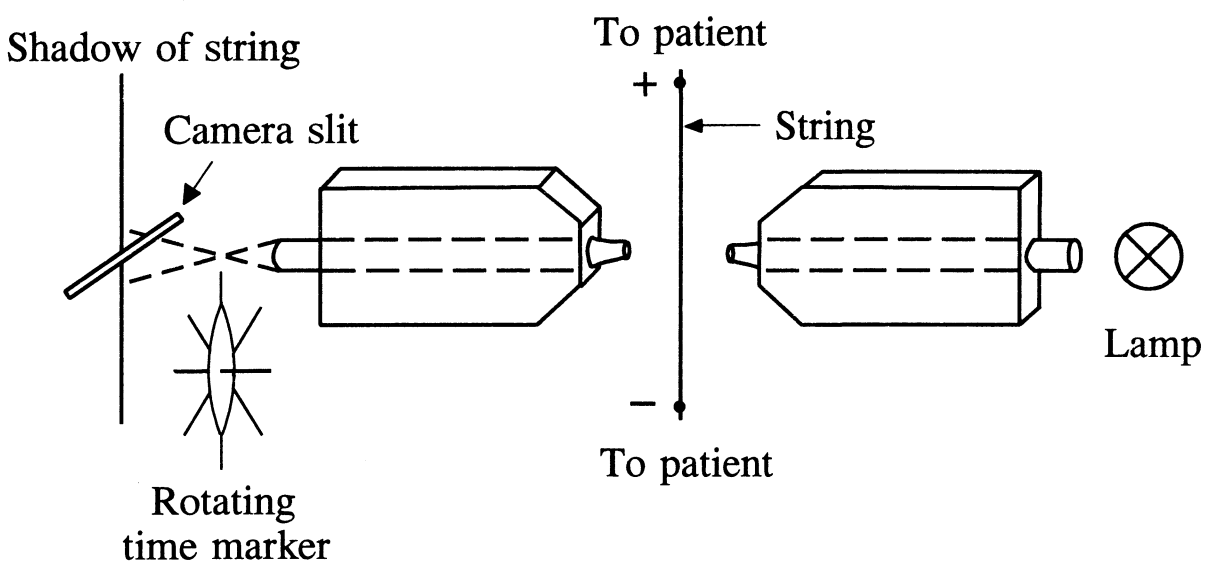


Figure 1

The electrocardiography (ECG) is of great importance because it reveals cardiac disorders which would not be diagnosed otherwise. The electrocardiogram gives invaluable information about the functional state of the heart, on one hand, and the abnormalities of the cardiac rhythm including disorders in the generation and conduction of excitation, on the other hand.

The general form of the electrocardiogram is derived by considering the series of events described in Fig.2. At rest each heart cell is bounded by a polarized membrane having negative ions on the inside and an equal number of positive ions on the outside.

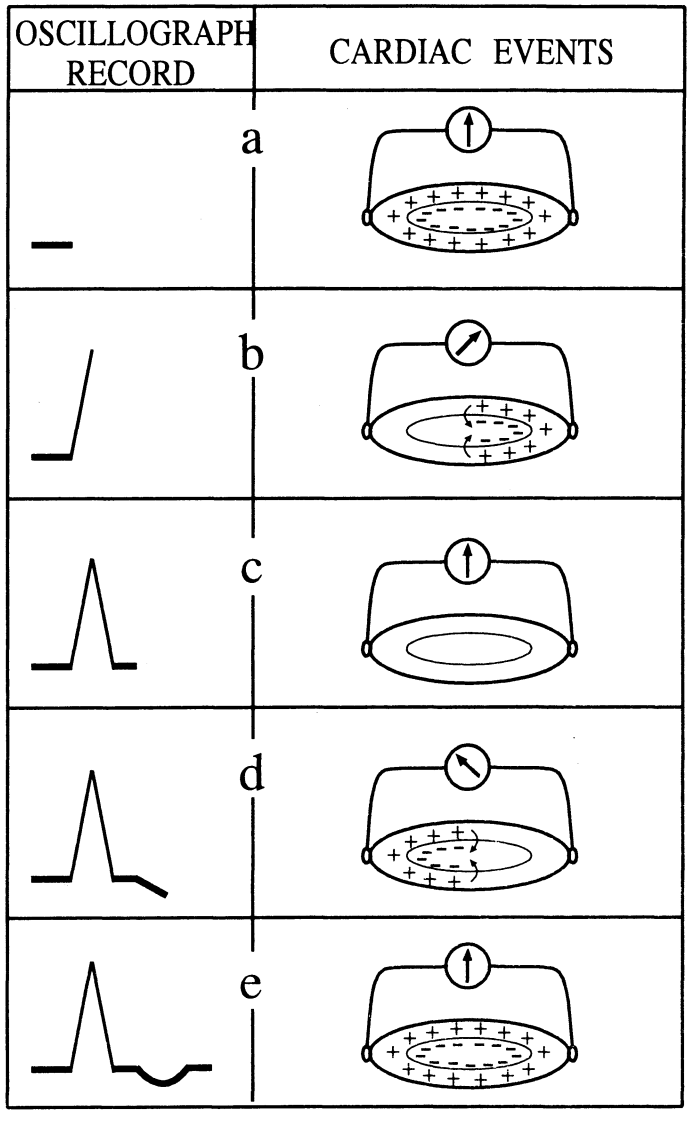


Figure 2

Theoretical derivation of the ECG deflections. a./ At rest: charged membrane, equal numbers of positive and negative ions. No difference of potential at surface; b./ At the beginning of activation part of the cell is depolarized. There is a flow of ions as indicated by arrows and a potential is set up across the electrodes; c./ When all parts are depolarized there is no difference of potential; d./ Beginning of restitution of depolarized area; e./ Restitution or repolarization complete. No flow of current.

The heart, because of the intimate connections between its cells, can be regarded as a large syncytium in the middle of a mass of tissue. In the resting state, that is during diastole, as at Fig. 2a. no current flows and the galvanometer is not deflected, so that its record of a moving surface s a horizontal line. When the cell is activated the polarized membrane is broken down as in Fig. 2b. The electrode to the right is still connected to the layer of positive ions on the surface, while the electrode on the left is now connected to the negatively charged interior of the cell. The galvanometer is therefore deflected and a upright wave is recorded. When all parts of the cell are depolarized (Fig. 2c) the galvanometer returns to zero. As recovery occurs the membrane becomes repolarized (Fig 2d) and current flows in the opposite direction, the record falls below the isoelectric line, returning to it once more when repolarization is complete(Fig.2e). The two parts of this biphasic wave, though opposite in sign, are not, as might be expected, equal in size and similar in shape. In the first place the process of repolarization is a slower one than the initial depolarization, and secondly the order in which the cells of the heart depolarized depends on the spread of the excitatory process from cell to cell, whereas recovery depends on the activity of the individual cells. Thus some parts of the heart are more quickly restored to the resting state than others. For these two reasons the second part of the biphasic wave is smaller in amplitude and different in form from the first part. This theoretical derivation should be regarded as a much simplified account of the actual events.

The electrocardiogram is essentially the time record of electrical process in the heart. Electrical activity precedes mechanical activity by a short interval. The locus of origin of the excitatory process and its speed of travel through the heart can be obtained from the electrocardiogram, which in special circumstances may also provide us with information about the state of cardiac muscle. The electrical record does not, however, indicate the vigour of the heart, its efficiency, or its ability to perform the work demand of it.

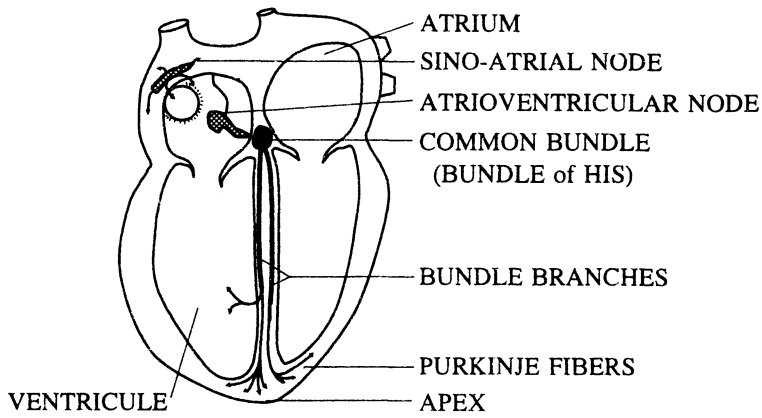


Figure 3

In the case of mammals, each heart beat originates from the sinoatrial node in the dorsal wall of the right atrium. The stimulus generated here elicits an excitation spreading over the atrial muscle and reaching the atrioventricular node, Then, the excitatory wave is conducted through the bundle of His, the bundle branches, and the Purkinje fibers to the apex of ventricles. Finally, the excitatory wave spreads over the whole ventricular myocardium. The atria and the ventricles are separated from each other by the fibrous septum isolating the muscle of atria from that of the ventricles (Fig. 3).

The conduction time from the atrioventricular node to the ventricular muscle is much shorter than in the ventricular muscle because the action potential is conducted in a fast pathway by modified muscle fibers.

The action potential travelling along the cardiac muscle fibers has a constant amplitude but a varying direction. Under the effect of the potential differences generated in the heart electric currents are flowing all over the body. The potential difference determined by the current intensity and the resistance of the tissues can be recorded by means of electrodes in contact with the surface of the body.

The electrocardiogram may also be recorded with limb leads (Fig. 4.). In this case, the electrodes are applied to the left arm (yellow plug), right arm (red plug) and left leg (green plug):

Lead I.	right arm - left arm	(U1)
Lead II.	right arm - left leg	(U2)
Lead III.	left arm - left leg	(U3)

A fourth electrode (attached to the black plug) is placed onto the right leg (indifferent or neutral electrode), functioning as the zero potential reference.

Potential differences recorded between right arm - left arm (U1), right arm - left leg (U2) and left arm - left leg (U3) are designated I., II. and III. bipolar (Einthoven) leads, respectively. Einthoven's rule: the amplitudes of these potential differences are independent,

$$\text{II.} = \text{I.} + \text{III.}$$

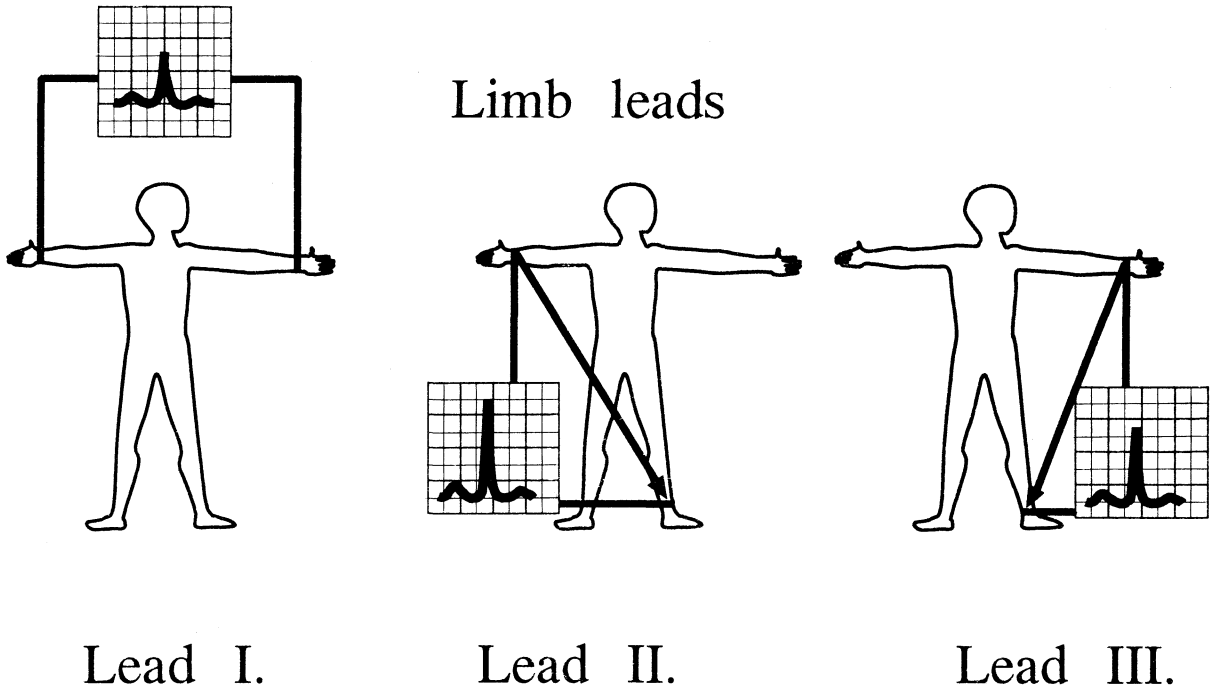


Figure 4

Alternatively, unipolar (Goldberger) leads are applied. In this case the potential of the R (right arm), L (left arm) and F (foot) electrodes are compared to the mathematical average of the other two electrodes (e.g. R compared to the average of L and F). These signals are designated aVR, aVL, aVF, respectively ("aV" stands for augmented voltage, to distinguish these signals from the weaker signals of the also unipolar Wilson-leads).

The normal electrocardiogram consists of various waves with different direction, amplitude and duration (Fig. 5.). Under Einthoven's suggestion (1895) these waves are called P, Q, R, S and T waves.

The P-wave is usually positive in all the three leads and it corresponds to the spread of the excitation from the sinoatrial node over the atrial muscle.

The P-Q interval gives the conduction time of excitation from the sinoatrial node to the ventricular muscle through the bundles of His, the bundle branches, and the Purkinje fibers.

The Q-wave takes its origin from the excitation generated in the apex of the heart. This wave is negative because the apex becomes negative with respect to the base during this period of time. The q-wave is rather small and it may be missing even in a normal subject.

The R-wave corresponds to the ventricular excitation. The synchronous activity in the ventricular muscles results in a wave of high amplitude. The R-wave is followed by the small negative S-wave.

At the end of excitatory process taking place in the ventricles, there is no potential difference in the heart. This period of time is the S-T isoelectric interval on the base lie.

The excitation of heart comes to the end gradually, and the T-wave probably shows that the area adjacent to the base remains to be excited for the longest while. In the normal case the T-wave is always positive at leads I and II. However, it may be negative, positive, or at certain times biphasic in lead III.

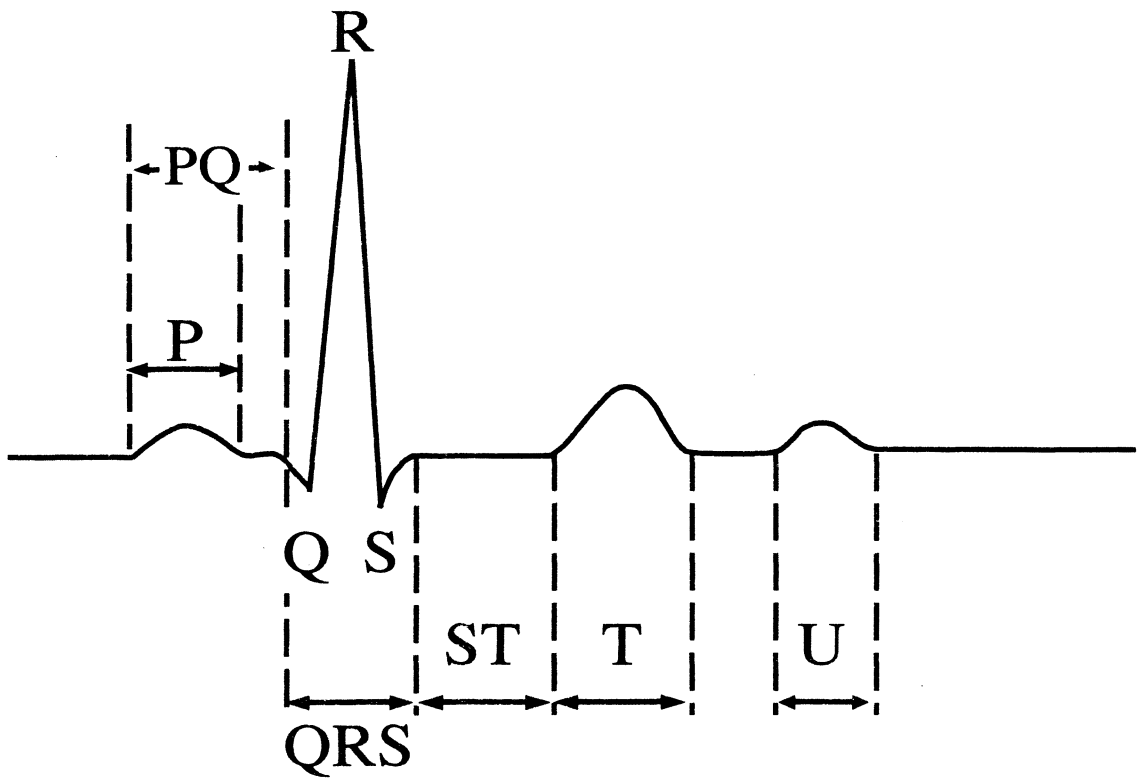


Figure 5

The electric activity of the heart may be represented by an electric vector of changing length and direction. At the height of the R-wave it is directed from the atrium to the apex of the heart. Einthoven used a triangle to determine the axis (position of the electric vector) in the heart (see Fig. 6.). Nowadays the axis can be calculated by plotting the vectors on a triaxial reference grid (which actually is a rearranged Einthoven triangle).

The electrocardiogram can be recorded with precardiac (or chest) leads, too. In this case, the projections of electric vector on the horizontal plane are recorded.

Damage of the heart muscle may bring about alterations in the propagation of the cardiac impulse. Under these circumstances, changes appear in the electrocardiogram. The damaged area can be localized and its extent determined by combined evaluation of the limb and chest leads. The importance of the electrocardiography is confirmed by the fact that the time course and convalescence of the cardiac diseases can be followed by the electrocardiographic method. The analysis of the abnormal ECG is treated in textbooks of pathophysiology, pathology and internal medicine.

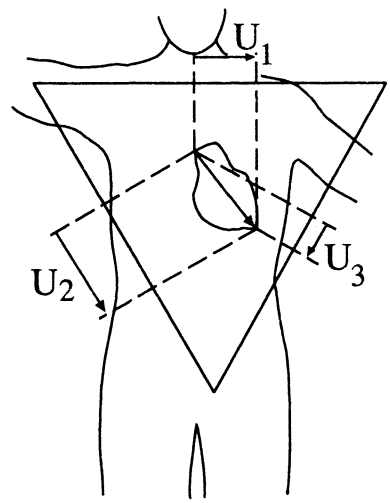


Figure 6

Experiments

The patient is lying relaxed on his back, on a coach. The skin of the wrists and ankles is cleaned. To ensure electric contact, the electrodes are covered with gauze sheets wetted with physiological salt solution. The electrodes are fastened with rubber bands. The electrodes are color coded: red (R), yellow (L), green (F) and black (B) (right leg, indifferent electrode).

1./ Place the ECG electrodes onto your partner's limbs. Remember to use electrode gel or a piece of gauze wetted with physiological saline.
2./ Switch on the ECG instrument and set the speed to 25 mm/sec.
3./ Perform the calibration: 1 mV should correspond to 1 cm.
4./ Record the ECG in the Einthoven leads (I, II, III) for 2-3 cardiac cycles (2-3 seconds).
5./ Repeat the measurement after 20 squats. (For the time of exercise disconnect the leads from the instrument)
6./ Determine the following parameters: RR, PQ, QRS, ST, TP and compare the level of ST to the baseline.
7./ Determine the pulse frequency before and after squats using the paper speed and the distance between the R waves.
8./ Construct the electrical integral vector (R) of the heart according to the drawing below using the R waves in the 3 leads. First check the validity of the Einthoven-Waller rule. Then use leads R_I and R_{II}, which are projections of the integral vector, to obtain the R vector.

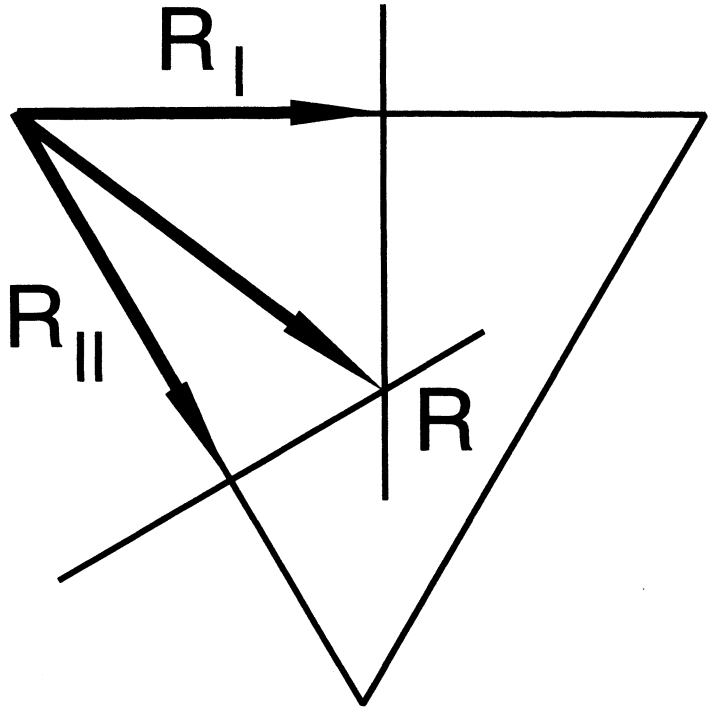


Figure 7

19. Blood pressure measurements

Flowing of liquids is maintained by pressure gradient. The circulating blood, on the one hand, exerts pressure in the direction of flow due to its kinetic energy (dynamic pressure), and on the other hand, exerts pressure on the wall of blood vessels (static pressure).

Blood pressure is expressed relative to ambient atmospheric pressure. In this case atmospheric pressure is taken as the zero pressure for arterial measurements. So the blood pressure values are not given as absolute pressure but as gauge pressure. The anatomic reference point for arterial pressure is the level of the heart. Arterial pressure measurements should be corrected or measured at the level of the heart to diminish the height of the blood column above or below the heart decrease or add to the observed pressure.

The pressure of blood can be measured directly (invasive way) or indirectly (non-invasive way).

Direct Arterial Blood Pressure Measurement

The arterial pressure may be measured directly by coupling the inside of an artery to a manometer. Accurate dynamic recording of arterial pressure requires an electronic pressure transducer. With this direct method the dynamic and static pressure can be measured separately by turning the catheter toward the flow or perpendicular to the wall of blood vessel. The periodic rise and fall of blood pressure within large arteries can easily be followed with the electronic pressure transducer connected to the catheter.

Indirect Blood Pressure Measurement

Human arterial pressure is usually estimated indirectly, because direct measurement requires arterial puncture. A sphygmomanometer is used to determine arterial pressure in the limbs - usually the arm.

The most standard technics employ either the palpation or audible detection of the pulse distal to an occlusive cuff. Palpation detects only the systolic pressure, auscultation detects both systolic and diastolic pressure. The so-called sphygmomanometer consists of a cuff and a manometer to detect pressure (Fig.1.).

The principle of the technique is that a pneumatic cuff is inflated encircling the arm so that the cuff pressure is transmitted through the tissue to compress the brachial artery. A stethoscope is used to listen to the artery distal to the cuff. The cuff is pumped up above systolic pressure and then slowly lowered. Distinctive sounds are heard when the cuff pressure equals to the systolic or the diastolic pressure.

When cuff pressure exceeds systolic pressure, no sounds are heard distal to the cuff. When the cuff pressure is just below systolic pressure, an intermittent surge of blood flows past the cuff with each systole. This can be heard with a stethoscope as a sharp tapping sound. As the pressure in the cuff is reduced further, the systolic sound becomes louder until the diastolic pressure is reached. Here the sounds become muffled and then disappear. Whether the point of muffling or disappearance is the better indicator of the diastolic pressure is unknown. Usually the muffling point is 5-10 mmHg above the disappearance pressure level. However, in normal, healthy persons after exercise or in abnormal persons at rest, the disappearance of the sound is rather unreliable both on the basis of theory and on the basis of direct measurements by catheterization. The disappearance of the sound signifies that

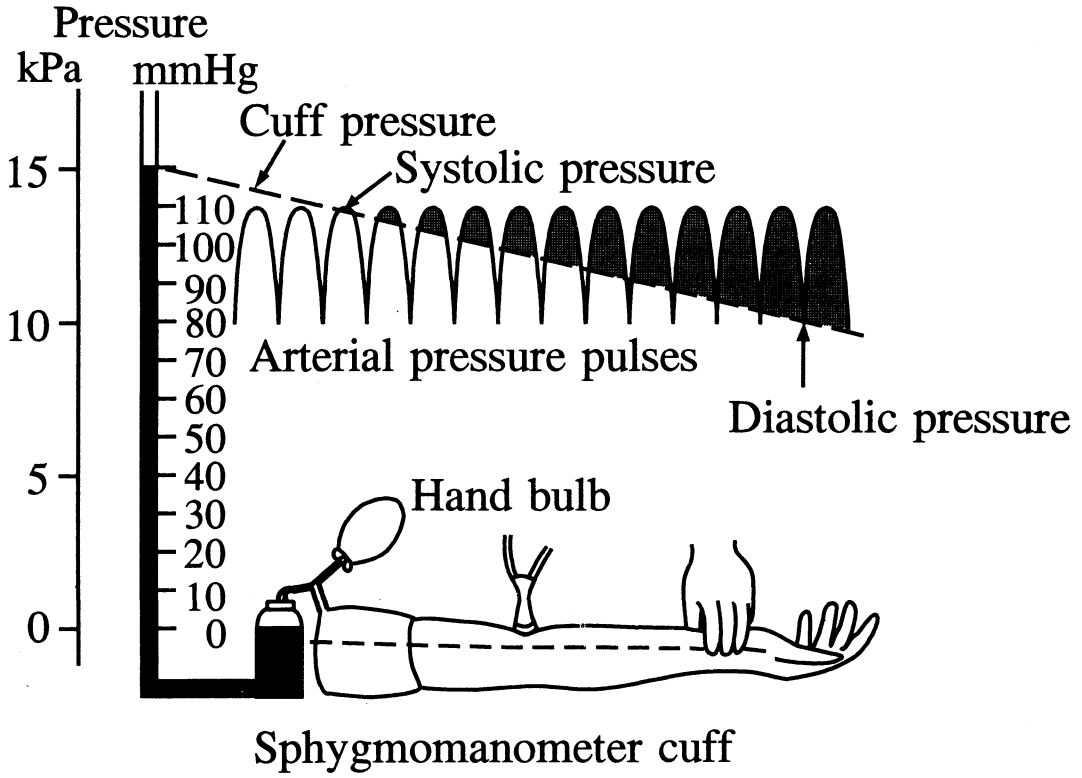


Figure 1

turbulence, or vibration of the vessel wall, caused by the constriction of the artery under the cuff, has disappeared. This has nothing to do with diastolic pressure but depends on the velocity of flow and the diameter of the vessels. The "muffling" follows from the fact that when the cuff pressure falls to diastolic pressure, there will no longer be any period when the artery is momentarily closed. Thus, the noise becomes "continuous" instead of "staccato". Both the muffling and the disappearance of the sound should of course be recorded, but if disappearance is regarded as the more reliable value of the two, many subjects with normal cardiac function will be put under suspicion of having aortic incompetence.

The height of the blood pressure depends on the cardiac output and the peripheral resistance to blood flow. An increase in cardiac output causes a rise in the mean blood pressure with the systolic being more affected than the diastolic pressure. The rise in blood pressure occurring during exercise or with emotion is predominantly due to rise in cardiac output. This may often be associated with a fall of peripheral resistance. In essential hypertension, however, the cardiac output is normal and the elevated blood pressure is due to increased resistance to blood flow.

Another factor which modifies the blood pressure is the elasticity of the arteries. In youth, when the arteries are elastic, the pulse pressure tends to be low, even in the case of a high peripheral resistance.

With increasing age the arteries become less elastic and the pulse pressure increase and may result in the development of so-called benign systolic hypertension of the aged, with a blood pressure in the region of 200/90 mmHg. This type of raised blood pressure may be of less significance than that due to an increased peripheral resistance.

The blood pressure is most often expressed in mmHg and sometimes in kPa. The conversion factors:

1 mmHg = 0.133322 kPa
1 kPa = 1.500638 mmHg.

Experiments:

1./ Determine the systolic and diastolic blood pressure values for every member of your group (including those doing other experiments) using a sphygmomanometer and a phonendoscope. Determine the pulse rate as well.

2./ Repeat the measurement after the person has made 20 squats.

3./ Prepare a histogram of the frequency distribution of both systolic and diastolic pressures. (The distribution of blood pressure being a continuous one, you have to set intervals to be able to treat your data as discrete variables!)

4./ Calculate the mean and S.D. of the distributions

5./ Determine, using self-controlled Student's t-test, whether 20 squats significantly increase the blood pressure or the pulse rate.

Collect your data in a table like the one below.

	in rest			after squats			difference		
No.	Systolic (Hgmm)	Diastolic (Hgmm)	Pulse rate	Systolic (Hgmm)	Diastolic (Hgmm)	pulse rate	Syst.	Diast.	pulse rate
1									
2									
..									
..									
..									
..									
Ave									
SD									

Draw the histograms based on a table like the one below.

No. of individuals with B.P. in a certain interval						
		blood pressure intervals (Hgmm)				
		45-54	55-64	65-74	...	...
In rest	systolic					
	diastolic					
After squats	systolic					
	diastolic					

20. Audiometry

Acoustics is the branch of physics that describes sound, i.e. mechanical vibrations propagating as waves. The most important terms of acoustics are the following ones:

1./ **Sound pressure** (p) is defined as the pressure that is generated by a propagating sound wave at a particular point. Its unit: Pascal (Pa).

$$1\ \text{Pa} = \frac{1\text{N}}{1\text{m}^2}$$

2./ **The intensity** (I) **of sound** is defined as the energy flowing across a unit area per unit of time.

$$\text{Its unit: } \frac{\text{Watt}}{\text{m}^2}$$

3./ **Threshold of hearing** is the minimum intensity or pressure of a sound of a distinct frequency that is just detected by the average human ear. Its conventionally accepted value is

$$I_0 = 10^{-12}\ \frac{\text{watt}}{\text{m}^2} \quad \text{or} \quad p_0 = 2\cdot10^{-5}\text{Nm}^2 \quad \text{at} \quad 1000\ \text{Hz}$$

4./ **Threshold of pain** is the intensity or pressure of a sound with distinct frequency causing pain in the ear. It limits the maximal sound intensity that can be tolerated by the ear. Its generally accepted value is (at 1000 Hz):

$$I_{\text{max}} = 10\ \frac{\text{watt}}{\text{m}^2}$$

The corresponding sound pressure:

$$P_{\text{max}} = 2\cdot10^{8}\ \frac{\text{N}}{\text{m}^2}$$

The human ear is an amazingly sensitive instrument. Measurements show that in terms of frequency the audible range extends from 20 cycles per second (cps, or Hz in Europe) to 16,000 cps, a ratio of 1:800. In the dynamic range, the ratio of intensities from threshold of hearing to the threshold of pain is 1:1 000 000 000 000. The range of human hearing is shown in Fig. 1.

The relation between sensation and stimulus level is the expression of what is known in physiology as the Weber-Fechner law, and applicable to all forms of sensory activity.

$$\text{Sensation lev.} = \text{const.} \cdot \log \frac{\text{actual lev. of stimulus}}{\text{reference lev. of stim.}}$$

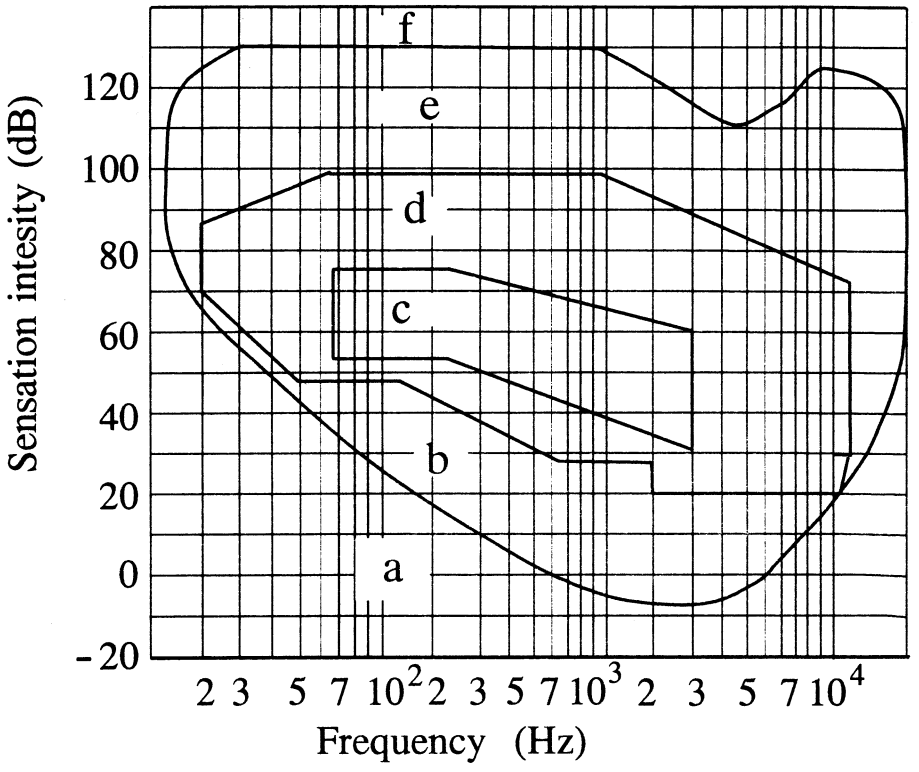


Figure 1

a./ threshold of hearing; b./slight noise; c./speech; d./ music; e./ loud noise: f./ pain limit

In audiometry the **decibel scale** is used for characterization of sensation intensity (*H*).

$$H = 10 \lg \frac{I_1}{I_0} \qquad (\text{dB})$$

where I_0 is the threshold of hearing. I_1 is the actual intensity of the sound. The disadvantage of the decibel scale is that the sensation level of the same sound intensity at various frequency is quite different because of the hearing threshold being different at these frequencies. To eliminate that drawback the **phon scale** was introduced. Here the reference intensity to any sound intensity is the hearing threshold at a frequency of 1000 Hz.

$$H = 10 \lg \frac{I_1}{I_0} \qquad (\text{phon})$$

The sensation level of a sound at any frequency can be determined in phone scale experimentally. Sitting in a "silent cabinet" (a chamber well insulated from any noise) we have a headphone on and we hear in one of the speakers a sound of 1000 Hz of a given intensity. In the other speaker we hear a sound of another frequency, the intensity of this latter may be controlled. Now we change the intensity of the sound of this particular frequency till we feel it to be as loud as the sound of 1000 Hz. In this case the intensity of sensation in phons is the same for both sounds. We repeat this procedure over the audible frequency range and we plot the received intensity levels against frequency, this is a

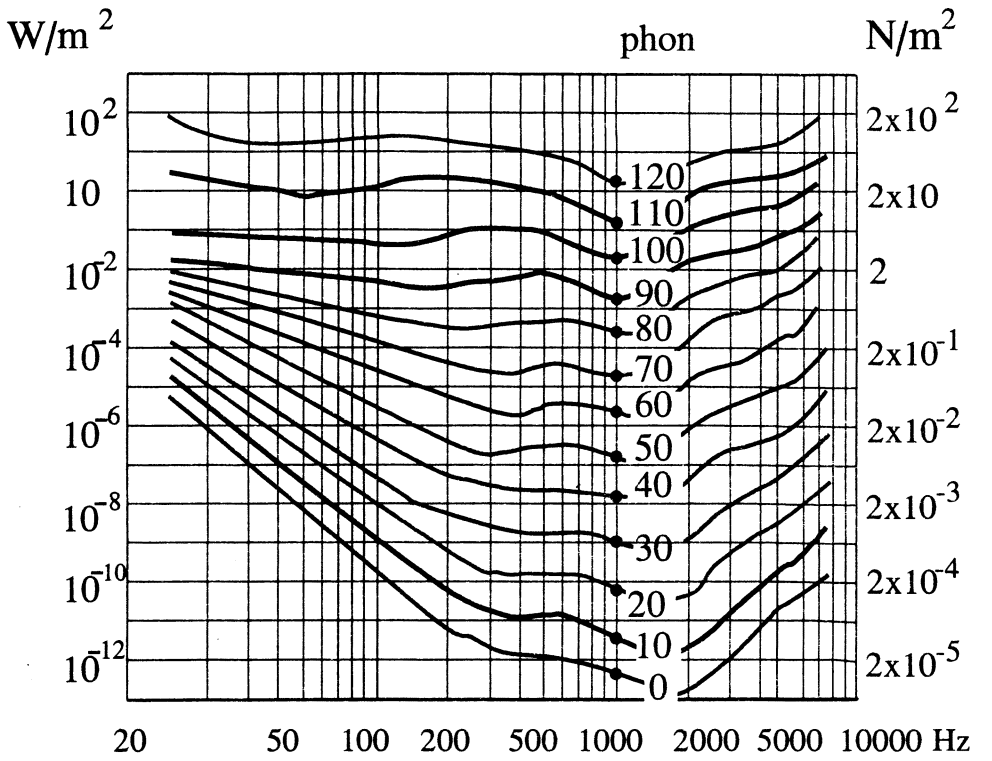


Figure 2

Figure legend: The reference intensities are marked with dots. In this figure each curve represents a special loudness over the audible frequency range. Our auditory response is worse over the higher and the lower frequency range than over the medium range. Therefore we have to set a greater intensity to feel the same loudness as over the medium range. The frequency dependence of hearing is less pronounced at greater intesities.

Fletcher-Muson curve. Then we change the intensity of the sound of 1000 Hz and we repeat the whole procedure. Again, we have obtained another curve. Scanning the intensity range from the threshold of hearing to the threshold of feeling we get a family of curves as shown in Fig. 2.

When $I = I_0$, the conventional threshold at 1000 Hz, its intensity level:

$$H_1 = 10 \lg \frac{I_0}{I_0} = 10 \cdot 0 = 0 \text{ phon}$$

If I_2 is the threshold of feeling, its intensity level:

$$H_2 = 10 \lg \frac{10 \dfrac{\text{watt}}{\text{m}^2}}{10^{-12} \dfrac{\text{watt}}{\text{m}^2}} = 10 \lg 10^{13} = 130 \text{ phon}$$

The intensities of a few sound sources are summarized in table 1.

Table 1

	phon	Watt/m^2
threshold of hearing	0	10^{-12}
whisper	20	10^{-10}
speech	40-60	10^{-8}-10^{-6}
shout	80	10^{-4}
car noise	90-120	10^{-3}-1
threshold of pain	120-130	1-10

The medical practice setting up a diagnosis for the ear function usually starts with the investigation of hearing. Unfortunately more and more noise disturbs people in factories and on the streets. Under the effect of loud noises hearing deteriorates . During hearing there are two ways of conduction of the sound waves. In the case of air conduction, sound waves are transmitted through the external canal of the ear, through the middle ear to the cochchlear fluid of the inner ear. Hearing can occur in the total absence of the middle ear mechanism, provided the inner ear is functioning and the energy of the vibration is great enough. In general, the threshold for bone conduction is about 40 dB higher than threshold for air conduction. Comparing the results of air and bone conduction test, the malfunctions in the middle ear and in the inner ear can be revealed.

The accurate measurement of the ability of the human to hear is performed with the aid of the audiometer. This measurement is actually made by determining the minimum intensity of sound of various frequencies which are just discernible by the ear.

The results of audiometric tests are plotted on the audiogram. Threshold curves are represented with solid line for bone conduction. Green color is used for the right, red for the left ear. In the case of air conduction, the actual points measured are labeled with circles for the right side and by crosses for the left side, while in the case of bone conduction, " < " and " > " symbols are used for the left and right ear, respectively.

Threshold measurements for air conduction:

Start the investigation by the sound of 1000 Hz (cycles per second, cps). Every sound must be demonstrated for the patient with the intensity 20-30 dB higher than the expected threshold, so that the patient can recognize the sound he or she has to pay attention to. Then the threshold can be determined by slowly elevating the intensity of the sound starting from the lowest intensity provided by the instrument. Application of interrupted sound is preferred to steady sound because of a better recognition. After determination of the threshold at 1000 cps, the same measurements are to be carried out at 2000, 4000 and 8000 cps frequencies. Then the results obtained at 1000 cps should be checked again. If there is no deviation from the previous results thresholds should be determined at 500, 250, 125 cps frequencies as well. (The long usage may cause the tiredness of the ear which change the threshold of hearing.)

Threshold measurement for bone conduction:

The vibrator should be placed on the *processus mastoideus* of the skull. The same procedure should be applied for the determination of the threshold of hearing as in case of air conduction. Measurement at 125 cps frequency should not be performed for bone conduction.

Experiments:

1./ Measure the threshold for air conduction at different frequencies (125 Hz to 8000 Hz) for both ears. Change the frequency with the frequency select dial. The tested ear can be changed with the output select (left side air conduction / right side air conduction / bone conduction). Use the impulse mode of the device (the indicator should be flashing), since it is easier to recognize relative than absolute intensities. Mode control should be on "sinus sound". First show the given frequency at reasonable intensity to the "patient" so that he/she knows what to expect. Than turn the intensity control to zero and gradually increase the volume until the examined person (who should turn his back to the instrument) signals by lifting a hand that the sound is audible. At that point mark the registration paper with your pencil through the appropriate hole. Start at 1000 Hz and than proceed upwards and downwards, while regularly checking that the threshold of the 1000 Hz sound is still the same. Should this value increase, give the tired ear a break.

2./ Repeat the same procedure for bone conduction. Output select is on bone conduction, the vibrator should be placed on the mastoid process, first on one side and then on the other. Do not test at 125 Hz, that can be extremely painful. (Actually the device does not permit the operation of the vibrator at that frequency.)

Be sure to mark clearly in your log the different signs (and/or colours) you used for registrating thresholds for different ears and types of conduction. At this point it does not matter whether you follow the convention described in your textbook, but anyone should be able to figure out the results from your records.

3./ Ask someone to read a text with steady intensity. Using the audiometer, gradually increase the intensity of a continuous sound in both ears. Note (in your log) your experience.

21. Optical measurements

For physicians it is necessary to know the basic concepts of optics to understand the image formation in the eye and the principles applied in the operating optical instruments. Only the methods of determination of focal lengths of lenses will be discussed here.

The image formation of lenses are described by the lens equations:

$$\frac{1}{f} = \frac{1}{o} + \frac{1}{i} \tag{1}$$

where (o) is the object distance, (i) is the image distance and (f) is the focal length. The ratio of the image distance to the object distance is called the linear magnification (M):

$$M = \frac{i}{o} = \frac{I}{O} \tag{2}$$

where I and O are the image and object size, respectively.

In the case of thin lenses the focal length can be determined by measuring the image and object distances, according to the equation (1). These distances with thick lenses cannot be measured exactly, therefore we choose such methods which do not require to know the exact values of the image and object distance.

1. Bessel-method

The object and the screen are fixed on the optical table. Moving the lens first the sharp, magnified image of the object (I. state) and then the sharp, reduced image of it (II. state) are produced on the screen. Using converging lenses, this can be always carried out, if $o+i>4f$. The image formations in the two lens positions are shown in Fig. 1.

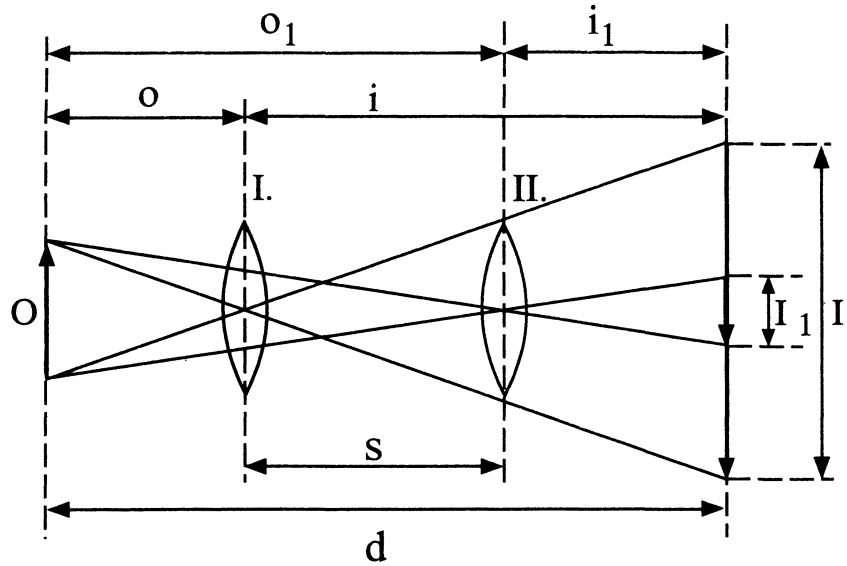


Figure 1

I.	state	$f<o<2f$	$i>2f$	(magnified reversed image)
II.	state	$f<i_1<2f$	$o_1>2f$	(reduced reversed image)

From the principle of the reversibility of rays comes, that

$$o = i_1 \qquad \text{and} \qquad i = o_1$$

It can be seen on the figure, that

$$d = s + 2o \tag{3}$$

$$d = o + i \tag{4}$$

From Eq. (3)

$$o = \frac{d-s}{2} \tag{5}$$

replacing it into Eq. (4):

$$i = o_1 = d - o = d - \frac{d - s}{2} = \frac{d + s}{2} \tag{6}$$

From the above we arrive, that

$$\frac{1}{f} = \frac{1}{o} + \frac{1}{i} = \frac{2}{d - s} + \frac{2}{d + s} = \frac{4d}{d^2 - s^2} \tag{7}$$

$$f = \frac{d^2 - s^2}{4\,d} \tag{8}$$

Both d and s can be measured exactly, so the focal length can also be determined exactly.

2. Abbe-method

This method has something in common with the Bessel-method. Here the magnified and the reduced image of the object is produced and the distance between the two lens positions is measured, too. But in calculations the linear magnification is used instead of the distance d. The magnification is determined by measuring the object and image size (O and I). As

$$M = \frac{i}{o} = \frac{I}{O} \tag{9}$$

$$\frac{1}{f} = \frac{1}{o} + \frac{1}{Mo} \tag{10}$$

From this expression

$$\frac{o}{f} = 1 + \frac{1}{M} \tag{11}$$

Similarly, shifting the lens by s away:

$$\frac{o + s}{f} = 1 + \frac{1}{M_1} \tag{12}$$

Since

$$M = \frac{i}{o} = \frac{o_1}{i_1} = \frac{1}{M_1} \tag{13}$$

$$1 + M = \frac{o + s}{f} \tag{14}$$

Subtracting equation (14) from equation (11):

$$\frac{s}{f} = M - \frac{1}{M} = \frac{M^2 - 1}{M} \tag{15}$$

$$f = \frac{s\,M}{M^2 - 1} \tag{16}$$

So the focal length can be calculated from the distance between the lens positions and the linear magnification. The diverging lenses do not produce a real image, consequently their focal length cannot be determined by the above described methods. However if we prepare a lens system by combining a diverging lens with a converging lens, they may be treated as a single lens whose focal length can be given as

$$\frac{1}{f} = \frac{1}{f_1} + \frac{1}{f_2} \tag{17}$$

where f_1 and f_2 are the focal lengths of the lenses in combination. (The focal length of the diverging lens is of course negative.) The resulting focal length (f) can be measured on the basis of either methods, if the lens power of the converging lens is greater than that of the diverging lens.

3. Calculation

The focal lengths of lenses can be calculated using the refraction index of the lens material (n) and the radii of curvature (r_1, r_2):

$$\frac{1}{f} = (n - 1)\left(\frac{1}{r_1} + \frac{1}{r_2}\right) \tag{18}$$

If the given lens has the same radius of curvature on both sides ($r_1 = r_2$), then the radius of curvature can be determined from the diameter and the thickness of the lens (Fig. 2).

It is apparent from the figure, that

$$r^2 = l^2 + (r - a)^2 = r^2 - 2ar + a^2 + l^2$$

from which r can be calculated as:

$$r = \frac{a}{2} + \frac{l^2}{2a} \tag{19}$$

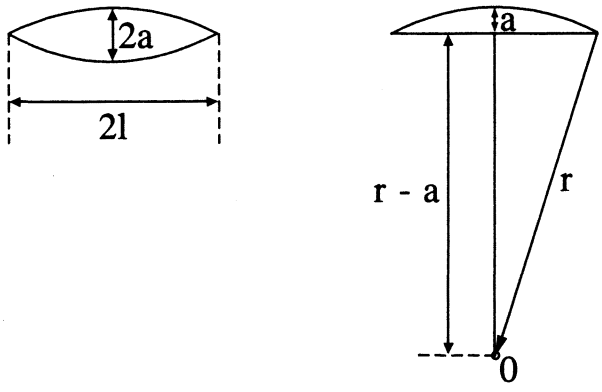


Figure 2

Experiments

1./ Determine the focal length of a converging lens by calculation, make five measurements and take the average!

2./ Determine the focal length of a diverging lens (five measurements!).

3./ Calculate a focal length of a lens using the data provided in the lab! Compare the result of calculation with the result of your measurements!

22. Measurements with a microscope

Structures in which the biologist is interested cover a variable range of sizes: macroscopic organisms, individual cells (a few microns in diameter), parts of cells (fractions of a micron in diameter) and individual large protein molecules (several angstroms in length).

If the objects to be seen are 25 cm away (the distance of the closest approach which is comfortable), they must be at least 0.07 mm apart if they are to be resolved. If we wish to see objects whose dimensions are smaller, the image of the object must be made to subtend an angle greater than one minute of an arch (the smallest visual angle which can be resolved by the eye). This can be achieved with a simple magnifying glass, or if higher magnification is desired, with a compound microscope.

The microscope consists primarily of two systems of converging lenses: the objective placed close to the object and the ocular placed near the eye. The general arrangement is shown in Fig. 1. (For simplification we consider the objective and ocular as single lenses.)

The objective forms the real, magnified, and reversed image (A'B') of the object (AB) placed outside of its focus but very close to it. This real image is inside the focus of the ocular and becomes the object for the ocular which forms the virtual, magnified image (A''B'') of A'B'.

The total magnification of the microscope (M) is the ratio between the apparent size of this virtual image and the actual size of the object and may be derived from the magnification of the objective (M_1) and ocular (M_2), respectively:

$$M = M_1 \cdot M_2$$

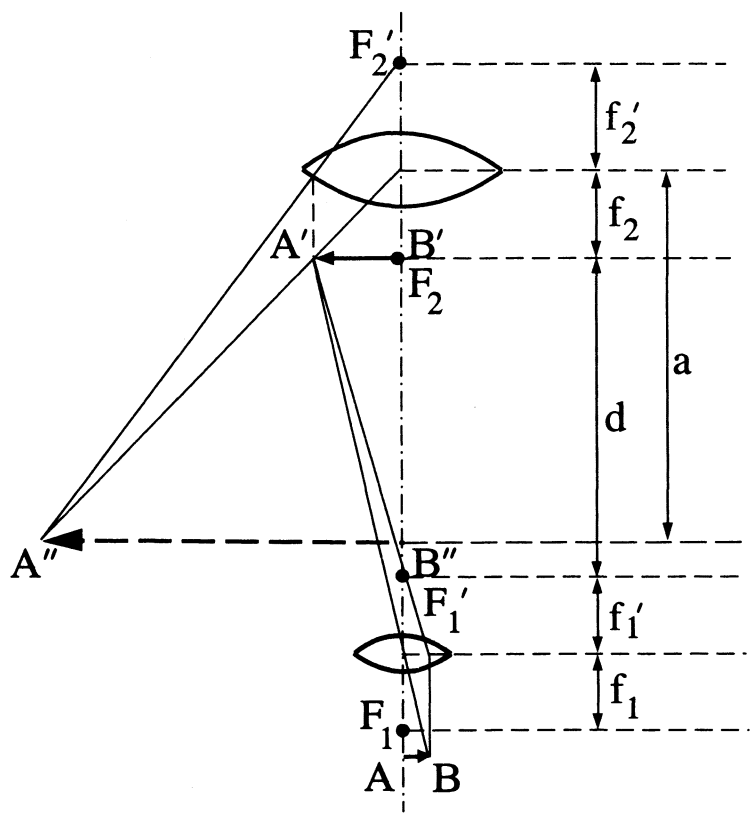


Figure 1

The object distance of the objective equals approximately its focal length (f_1), the image distance equals the optical tube length of the microscope (d). That is, M_1 equals the ratio of d/f_1. The image distance equals $-a$, where $a = 25$ cm, the length of discernment. So the magnification of the ocular: $M_2 = -a/f_2$. The total magnification:

$$M = M_1 M_2 = -\frac{da}{f_1 f_2}$$

The resolving power of microscopes:

The magnification of the microscope cannot be increased without limitation. The principal limit is provided by the wave nature of light (diffraction). If we have two light sources (i. e. two object points), each source will give a rise to its own diffraction pattern (alternate enhancement and extinction of light intensities at the margin). The resolution of a lens or lens system is expressed in terms of its ability to separate two light sources which are close to each other. The criterion proposed by Rayleigh is that two light sources shall be considered as resolved if the central maximum of the diffraction pattern of one coincides with the first minimum of the other. The distance of these two points (the minimum resolvable separation) can be given as

$$d = \frac{\lambda}{n \sin \phi}$$

where λ is the wavelength of the illuminating light, (ϕ) is the half angle, (n) is the refractive index of the medium between the objective and the cover slip. The quantity $n \cdot \sin\phi$ is known as the numerical aperture of the system. The reciprocal of d is the resolution power of the microscope:

$$f = \frac{1}{d} = \frac{n \sin \phi}{\lambda}$$

The resolution power can be increased according to the above expression as follows:

a) decreasing the wavelength of the illuminating light
b) increasing the refractive index (applying for example cedar oil (n = 1.51) or bromonaphtaline (n = 1.66))
c) increasing the total angle that may be practically maximum 140^o

On the basis of this, the possible smallest resolvable separation (d) for visible light is around 200 nm (average wavelength: 530 nm, total angle: 140^o, using immersion oil).

Determination of the magnification of the microscope:

The magnification can be calculated from the individual magnifications of the objective and ocular as mentioned before. These data are given on these units. The magnification can be determined in practice the following way:

The image of a micrometer placed in the microscope is viewed by the left eye, while a millimeter scale paper, placed at the length of discernment in parallel with the objective table of the microscope, is viewed by the right eye. The two images shall be moved until

they coincide with each other and you can determine the number of divisions of the micrometer object corresponding to a given number of divisions on the mm scale paper. The magnification can be calculated on the basis of the $M = K/T$ relationship, where K and T refer to the number of units of the mm scale paper and the micrometer object, respectively.

Calibration of the ocular scale:

For measurements carried out by a microscope, a so called ocular scale is placed into one of the oculars. The calibration of the ocular scale is provided by the micrometer object. The images of the micrometer object and ocular scale shall be moved to coincide with each other and the number of μm-s corresponding to one of division on the ocular scale shall be read. With the application of the calibrated ocular scale the sizes of objects in microscopical range can be determined directly.

Estimation of the average diameter of red blood cells:

1./ Calibrate the scale of the ocular-micrometer at the highest magnification of the microscope! The total length of the bar on the calibration slide is 1 mm.

2./ Determine the diameter of 30-30 red blood cells (RBC) on *A* and *B* smears, respectively! Summarize these data on separated tables as shown below!

classes (size ranges)	diameter of RBC-s in scale units (x_i)	N° of RBC-s in different classes (n_i)	$n_i\, x_i$	x_i^2	$n_i\, x_i^2$
1.					
2.					
. .					
m.					
Σ	—			—	

Calculation:

$$\bar{x} = \frac{\Sigma n_i x_i}{n}$$ (sample mean)

$$SD = \sqrt{\frac{\Sigma (x_i - \bar{x})^2}{n-1}} = \sqrt{\frac{\Sigma n_i x_i^2 - \bar{x}\,\Sigma n_i x_i}{n-1}}$$ (sample standard deviation)

$$SEM = \frac{SD}{\sqrt{n}}$$ (standard error of the mean)

Presentation of the measurements (transform the result to μm):

$$d = \bar{x} \pm \text{SEM } \mu\text{m}$$

3. Calculate the sample mean ($\bar{x}$) and the sample standard deviation (SD) in case of *A* and *B*! Graph the frequency distribution of two population on histograms!

4. Determine the diameter of red blood cells by diffraction method in *A* and *B* smears. The wavelength of the laser is 632.8 nm.

23. Estimation of the average diameter of red blood cells using laser diffraction

The sizes of red blood cells from one individual are not all equal but are distributed about a mean value, hence the average size has to be determined. The average size of red blood cells is important in research and clinical practice in differentiating abnormal from normal cells. For example, in the case of pernicious anaemia, the mean size of red blood cells is greater than that of the normal.

The microscopic examination of red blood cells, besides being tedious, can not be extended to a large number of samples. As an alternative, it is possible to measure the same by Young's eriometer. This method produces Fraunhofer diffraction pattern on the retina of the observer. The method of computing the average size of the diffracting particles is based on the measurement of the angle of diffraction. This involves the adjustment of the distance of the sample from a central hole in the device so that the diffraction ring on the retina of the observer has the same diameter as that marked on the eriometer. This method is always used in finding the diameter of spherical particles, but can not be employed in the case of elliptical objects.

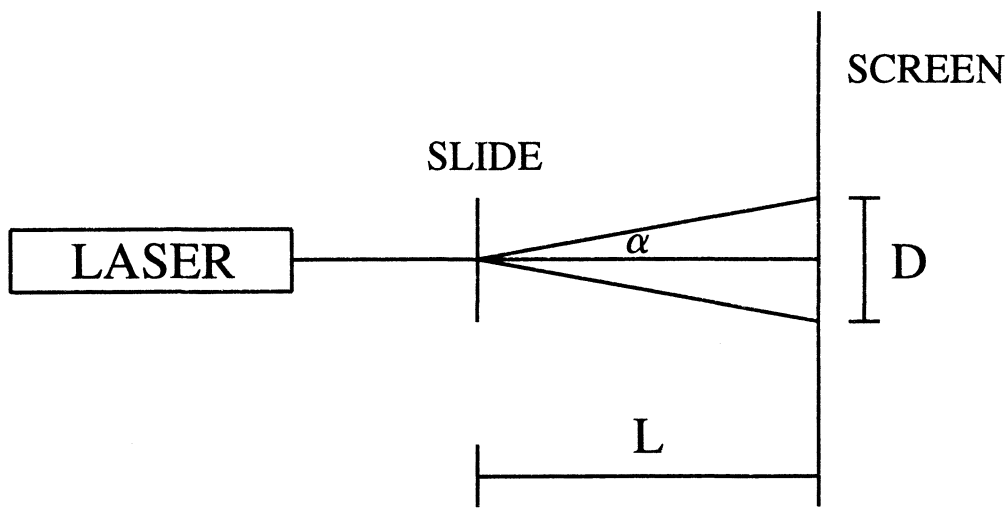
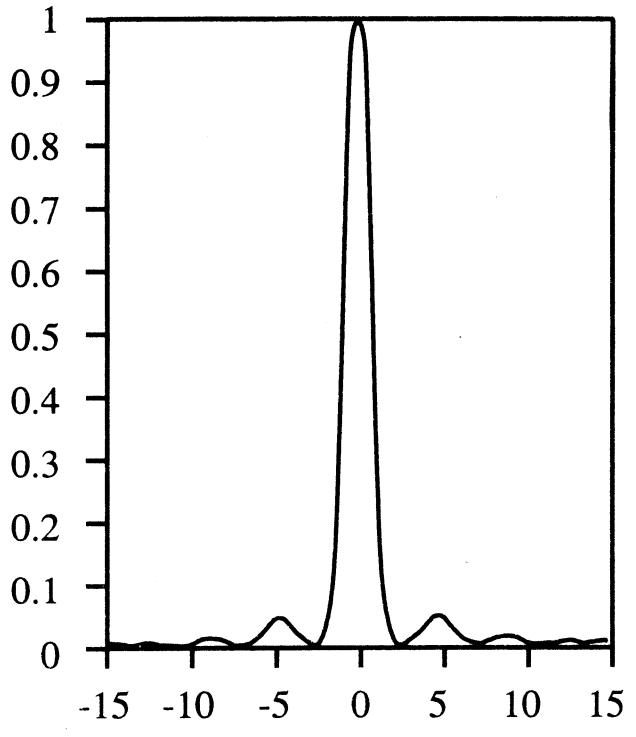


Figure 1

Of the numerous other diffraction methods we will discuss the laser diffraction method. Diffraction effects are traditionally classified into either Fresnel or Fraunhofer types. Fresnel diffraction is primarily concerned with what happens to light in the immediate neighbourhood of a diffracting object, with the object illuminated by a point source usually at a finite distance. Fraunhofer diffraction is primarily concerned with diffracted light sensed at infinite distances from the diffracting object with the illumination usually coming from an infinitely distant point source. Diffraction by erythrocytes applies for the latter one. Figure 1 shows the schematic experimental arrangement. The sample is prepared by smearing a drop of fresh blood uniformly on a microscopic slide and then it is introduced in between the



laser and the screen with the smeared surface facing the screen, which gives the diffraction pattern. Diffraction is caused by the sides of the circular objects, e.g. red blood cells. The diffraction pattern resulting from the red blood cells consists of a central bright region, known as the Airy Disc, surrounded by a number of fainter rings. Each ring is separated by a circle of zero intensity. The irradiance distribution profile in this pattern is shown in Figure 2 and can be described by

$$I_x = I_0\left[\frac{2J_1(x)}{x}\right]^2 \qquad (1)$$

where I_0 is the peak irradiance in the image.

Figure 2

$$J_1(x) = x\sum_{n=1}^{\infty}(-1)^{n+1}\frac{x^{2n-2}}{(n-1)!\,n!\,2^{2n-1}} \qquad (2)$$

$J_1(x)$ is the Bessel function of the first kind of order unity and

$$x = \frac{\pi d}{\lambda}\sin\alpha \qquad (3)$$

Here λ is the wavelength of illumination (in our case it is 632.8 nm since we use a He-Ne laser), d is the diameter of the diffracting object and α is the angular radius from pattern maximum.

The maximum value of the intensity function described by Equation 1 is at $x = 0$. The values rendered to other maxima and minima can be determined using the conditions given below:

$$\frac{d}{dx}\left[\frac{J_1(x)}{x}\right] = 0 \qquad (4)$$

$$J_1(x) = 0 \tag{5}$$

Solving Equations 4 and 5 is rather difficult, therefore we summarize the most important solutions in Table 1. For convenience we give the position expressed as x/π (k_i)

Ring or band	position ($x/\pi=k_i$)	relative intensity (I_x/I_0)	energy in ring (%)
Central maximum	0.00	1.0	83.8
First dark	1.22	0.0	
First bright	1.64	0.0175	7.2
Second dark	2.23	0.0	
Second bright	2.68	0.0042	2.8
Third dark	3.24	0.0	
Third bright	3.70	0.0016	1.5
Fourth dark	4.24	0.0	
Fourth bright	4.71	0.0008	1.0
Fifth dark	5.24	0.0	

When light is diffracted by several (N) objects, the x/π values remain the same, only the intensity will be enhanced N times. The angle of diffraction for the i^{th} minimum (dark ring) or maximum (bright ring) can be determined as follows, where D_i is the diameter of the particular ring:

$$\sin\alpha = \frac{D_i}{2L\left[1 + \frac{D_i^2}{4L^2}\right]^{\frac{1}{2}}} \tag{6}$$

By rearranging Equation 3 and substituting $x/\pi=k_i$ and Equation 6 for sin α we get the average diameter of red blood cells (d):

$$d = \frac{2L\lambda k_i}{D_i}\sqrt{1 + \frac{D_i^2}{4L^2}} \tag{7}$$

Frog erythrocytes are elliptical cells. The laser diffraction method is able to measure both axes, since in the case frog red blood cells we can get an elliptical diffraction pattern, indicating that the diffraction pattern is a function of size, shape and orientation. Microscopic methods prove that the orientation of the frog red blood cells is random on a slide. Despite that, regions of relatively similar orientation can be found in each slide, thus they should be scanned carefully to get a sharp elliptical diffraction pattern corresponding to

the maximum alignment of the cells in a particular direction. As the size of the diffraction pattern is inversely proportional to the cellular size, it follows that the major and minor axes of the elliptical pattern are due to the minor and major axes of the cells respectively.

Table 2 summarizes a comparison of the result gained with different methods:

Animal	Dimension	Microscope method	Eriometer method	Laser diffraction	Angle of diffraction minima	
					I. order	II. order
sheep	diameter	5.5	5.5	5.5	8.0°	14.2°
mouse	diameter	6.5	6.3	6.4	6.85°	12.4°
human	diameter	7.0	6.9	7.2	6.1°	10.9°
frog	minor axis	10.0	12.1	11.3	3.8°	7.2°
	major axis	15.0	15.9	15.2	2.9°	5.3°

EXPERIMENT:
Determine the average diameter of red blood cells of different species using the given slides. Use the first minimum and compare the result with the one gained by using the second minimum. For each measurement use at least three different L values. Plot the diameter of the dark ring as a function of L as shown in Figure 3. Determine both axes of the frog red blood cells. Summarize your data in a Table.

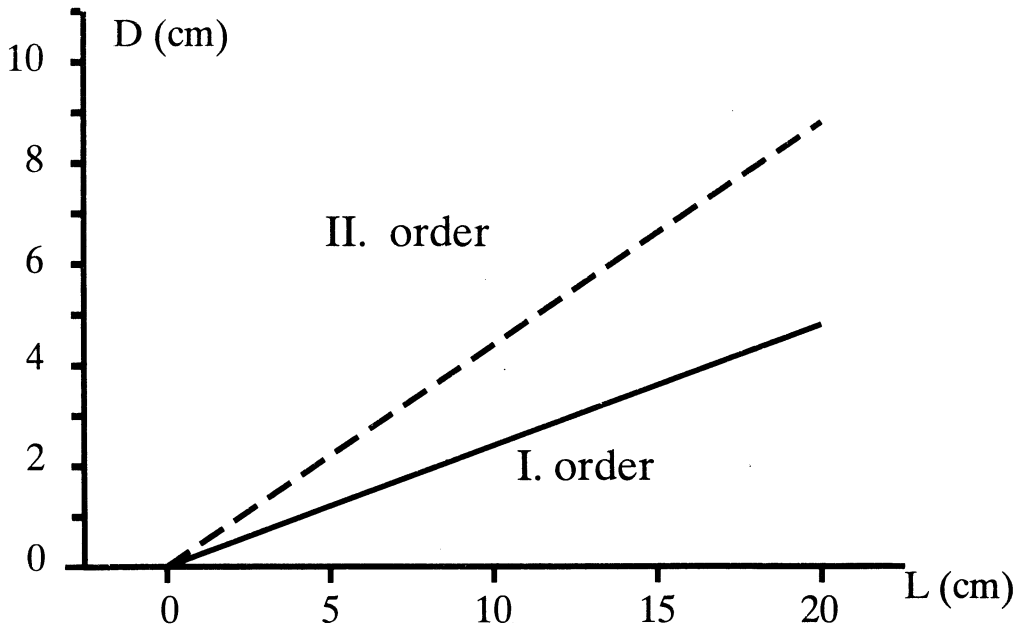


Figure 3

Sample	Dimension	D (cm)	L (cm)	d (μm)	Angle of diffraction minima	
					I. order	II. order
human	diameter		5			
			10			
			15			
frog	minor axis		5			
			10			
			15			
	major axis		5			
			10			
			15			

24. Simulation of biological processes by statistical games

In nature every phenomenon occurs in connection with other phenomena. It is an essential step in getting to learn them that the fundamentally important has to be separated from the less important. The task of every science is not only to provide scientific facts and data, but to interpret them, to find the relationships and laws between them. This phase of scientific investigation is not only observation itself and cannot be considered independently from the abstraction of the examined phenomenon.

In the course of abstraction we cannot take into account the side effects that play an unimportant role in the production, development and outcome of the examined phenomenon. This abstraction process leads to the simplification of the phenomenon without changing its basic characteristics. A model is a logical or mathematical formula or representation of a phenomenon or a system describing the main characteristics of them. Modeling is a useful tool of getting better knowledge and providing more exact interpretation of phenomena in the field of biology and medical sciences.

Some statistical games can be used as a models of the most complex phenomena in nature (e. g. the development of the material world, the evolution, the origin of the genetic code, the development of languages). In this relation the game has a much broader and general meaning: here the game is defined as such a sequence of events, in which prior to each step a great number of equal decision possibilities are available for selecting the next step. According to Eigen and Winkler (Manfred Eigen, Ruthild Winkler: The Laws of the Game, Gondolat, Budapest 1981): "Play is a natural phenomenon that has guided the course of the world from its beginning. It is evident in shaping of matter, in the organization of matter into living structures, and in the social behavior of human beings."

For modeling physical, chemical and biological processes, many games were created, whose principal background was summarized in the above mentioned book of Eigen and Winkler. The following ideas and games are cited from this book. Each game consists of two basic elements: the chance and the law. The chance element comes under the control of a statistical law only if there is a great number of individual events. This law is at work, for instance, when chance fluctuations undergo self-regulation in equilibrium or when they are amplified in the evolutionary process. The games described in detail below are different just in the point of the laws determining the individual events. The equivalent of the laws in nature are the interactions and the initial and boundary conditions of a process or phenomenon.

Small squares in different colors represent different kinds of species: atoms, molecules, numbers or organisms. A square playing board represents the limited area within which various processes can take place. All the squares in the playing area are identified by coordinates. Each square has an equal chance of being selected by the roll of the dice. The x and y coordinates are designated by different colors, and a pair of dice belongs to the game, supplying the element of chance. Depending on the size of the board, the coordinate dice have to be tetrahedrons (4·4), cubes (6·6), octahedrons (8·8), etc. The rules of the game affect the chance decisions of the dice and thereby determine the behavior pattern of different populations: stable balance or catastrophic decline or growth.

In our case, each of the game can be played on a 18·18 square board that means a playing area containing 324 squares identified by coordinates. These games can be simulated by computer by appropriate algorhytm; the advantage of the computerized game over the real dice game is that a larger playing area and a significantly greater number of dice rolls, thereby a behavior pattern of greater populations can be examined in this way.

The game "Equilibrium"

Primarily it can simulate the approach and the temporal changes of a chemical and physical equilibrium. Two different colors are present (for example red and green), and at the beginning the board is completely filled with one of the colors. The only rule of the game is that a square selected by the dice is replaced by a square of the opposite color. If there is a red square in the selected square, then it is replaced by a green one, and reversely.

After a suitable number of dice rolls the game will approach an equilibrium state, that is an approximately even distribution of both colors will be present on the board. Although the individual events contain a certain chance element, the result is a stabilization.

There is a "regulating factor" in this kind of statistics that account for the stable behavior. The greater the excess of any color is, the greater the probability becomes that the dice will select this color and thereby reduce the excess. The increase and decrease of a color is regulated by the distribution on the board at any given moment, and this leads to the stabilization of the equilibrium. This equilibrium game is an example for the negative feedback mechanism.

It can be proved mathematically that the expected value of an average deviation from the equilibrium state equals to the square root of number of squares of one color (in our case /two colors, 18*18 squares/ this is $(18 \cdot 18/2)^{1/2} = 12.73$). In natural processes the number of particles involved is great, so deviations from equilibrium are immeasurable even with the most sensitive methods. For example, the deviation compared with the quantity to be measured is $n^{1/2}/n = 1/n$. Even the measurement of the equilibrium of substances in picomolar quantity needs a relative accuracy better than 10^{-6}.

The game "Catastrophe"

Two colors (e.g. red and green) take part in this game. At the beginning they are present on the board in approximately equal quantity in a random distribution. The only rule that differs is the one affecting the exchange of the squares. The rule here is the opposite of that one, which applies to the game "Equilibrium". The selected square is doubled at the expense of the other color. For example, if the dice pick a square with a green square on it, any red square is removed from the board and replaced with a green one (the original green square remains). At the end of the game, either green or red squares occupy the board completely (that is the process results in the dying out, "catastrophe" of one of the colors).

Many examples can be mentioned for illustrating this statistical process: autocatalytic chemical processes or chain reactions in nuclear explosions. This phenomenon can be observed in a "less clear form" when the reproduction equilibrium is disturbed. Similar laws describe some stages of a demographic explosion.

While the games "Equilibrium" and "Catastrophe" are characterized by a stabilization and a destabilization, respectively, the following game "Selection" is a model for natural processes controlled by the combination of laws of both strategies.

The game "Selection"

Four colors take part in the game (red, blue, green and pink), and the rules provide different selection advantages for the colors (the average birth and death rates will vary from color to color). At the beginning all squares on the board are occupied by different colors in a random distribution. A pair of dice used for selecting the coordinates and a value die determining the outcome belong to the game.

The game consists of successive growth and declining periods; the selection advantages are valid for both periods. The game starts with a declining period: the first step is to designate the coordinates of a square by dice. This step is followed by the roll of the value die. Whether the square on the picked square will be removed from the board or not, it is determined by both the color of the square and the roll of the value die according to the following rules:

a. If the selected square has a pink color, it is removed at any values of the value die.
b. The green color is removed, when the value die shows the values of either 1, 2 or 3.
c. The blue color is removed, when the values of the die are 1 or 2.
d. The red is removed only, when the value of the die is 1.

If, applying these rules, the selected square remains on the board, then the designation of coordinates and the roll of the value die are repeated. These steps continue until one square is removed from the board. Then this is followed by a growth period, which is also determined by the coordinate and value dice.

The empty square is filled with that color, which is in the selected square, depending on the value of the value die. The replacement of the square means actually the doubling of the selected square according to the following rules:

a. If the selected square contains a red square, then this is doubled independently of the roll of the value die (i. e. a red square is placed to the empty square).
b. Blue is doubled when the value die shows the numbers 1, 2 or 3.
c. Green is doubled when it is 1 or 2.
d. Pink is doubled only when it has a value of 1.

If, applying these rules, the doubling of the selected color does not occur (i.e. empty square remains on the board), both the selection of coordinates and the value determining the color to be added should be repeated until the empty square is filled. The growing and declining periods succeed alternately, the rules affecting them are summarized in Table I.

If the square selected by the coordinate dice is	Removal	Doubling
	according to the following numbers of the value die	
	squares will be removed	squares will be added
red	1	1, 2, 3, 4, 5, 6
blue	1, 2	1, 2, 3
green	1, 2, 3,	1, 2
pink	1, 2, 3, 4, 5, 6,	1

Table I.

Red has the highest selective value, pink the lowest. For the red the average birth rate is six times as great as the average death rate. For the pink the reverse is true.

This game provides a proof for that, that Darwin's principle of natural selection can be considered on physical basis, it can be described exactly by settling the initial and boundary conditions. However, at the level of our present knowledge these conditions can be established for the simplest living organisms. The ideal situation presented by the game cannot come about in nature at all. The natural selection is influenced by the available energy

sources and changes in living space. The selection in nature is built up from stabilization as well as destabilization steps. Destabilization results in the incredibly great variety of living organisms, while stabilization ensures the existence of some selected species (at least for a certain time). In the course of evolution, an enormous amount of genetic systems have been realized. The existence of species can be identified with the temporary stabilization of genetic systems.

The game "Struggle"

This game closely simulates the changes that populations of different species undergo in the course of time and in a closed ecological environment. The game is played with three colors (green, grey, red). At the beginning several red and about twice as many grey are present on the board in a random distribution. The other squares remain empty. The rules relating to the behavior and alteration of the colors can be described as follows:

Within a closed area (represented by the 18·18 board) the grass begins to grow.

A green square is placed on the empty square selected by the coordinate dice and on the empty squares next to it.

Rabbits eat the grass, and the rabbit population grows.

If the selected square is green and it has at least one grey adjacent then the green changes to grey. If it has no red adjacent then all the neighboring greens turn also into greys.

Foxes eat the rabbits, and the fox population grows.

If the picked square is grey and one red adjacent exists, then the grey and the greys next to it turn into red.

Foxes begin to die.

If the selected square is red, the square is removed and the square becomes empty again, and if it has no grey adjacent then the neigh boring reds are also removed.

adjacent square	selected square			
	green	green	grey	red
all empty	green	-	-	removed
green	green	-	from green to grey	removed
grey	green	from green to grey	-	removed
red	green	-	from grey to red	removed

Table II.

Thus, the colors must be varied according to the present new relations. The rules are summarized in Table II. If a green-grey and grey-red neighborhoods exist simultaneously, then the transformation of the grey-red relation occurs (the fox eats the rabbit and not the rabbit eats the grass). Similar games with a certain modification of the rules can represent several biochemical phenomena in addition to the mentioned ecological example. Let us take a chemical chain reaction with the reagents A (green), X (grey) and Y (red). A is the initial substance, X and Y symbolize reagents with an autocatalytic effect. The game can be a model for the following reaction scheme:

$$\rightarrow A$$
$$A + X = 2X$$
$$X + Y = 2Y$$
$$Y \rightarrow$$

The fundamental condition of completing the reaction is the continuous replacement of A and the continuous removal of X from the reaction space.

After accomplishing a great number of steps the ratio of the colors shows a time periodicity, but the game results often also in a periodic spatial pattern. This temporal and spatial arrangement can be established only at constant external energy intake (taking up substances rich in energy), that is at continuous energy dissipation. These dissipation processes and forces have a definite function in the shape formation processes of the living world.

However, besides these dissipation forces there are present also conservative forces (forces determined by spatial conditions, such as physical or chemical forces) in producing biological formations. For example in a unicellular living organism the structural stability of molecules or even certain cell organelles is ensured by the conservative forces, but the dissipation forces (metabolism, biochemical reactions) are responsible for the formation of the conservative structure elements determined by the genetic code of the cell. The spatial and temporal changes of the structure of a living organism are determined by these two kinds of forces.

Hence the morphogenesis has two fundamental principles: the conservative and the dissipation principle. The former involves the static and the latter the dynamic interactions. The complex phenomenon of morphogenesis can be also simulated by computer; the game "Struggle" represents a prototype of one of the simplest pattern formation reactions.

Instruction manual to "Simulation of biological processes by statistical games" program.

Device: IBM AT 286 compatible personal computer.

To start the program: press [F3] button

HOW TO USE THE PROGRAM:

i: After the program starts a menu appears at the top of the screen. You can select the desired game by the arrow keys. Press [ENTER] to select.

ii: A pull-down menu appears. This menu contains the parameters and commands of the selected game:

Equilibrium & Catastrophe

1. *Size*: if you want to adjust the number of the boxes on the board press [ENTER] and type in the desired number (8-25) then press [ENTER]
2. *Graph*: select the number of displayed "colors" on the graph (press [ENTER] 1 or 2 then press [ENTER])
3. *Run*: you can start or stop the game if you press [ENTER]
4. *Demo*: if the game runs press [ENTER] if you want an explanation of the process.
5. *Print**: after stopping the game you can print the graph (left-bottom corner) with a dot matrix printer

Selection

1. *Size*: if you want to adjust the number of the boxes on the board press [ENTER] and type in the desired number (8-25) then press [ENTER]
2. *Red*: you can change the amount of the red squares (press [ENTER] [4] ,means 4% of the squares)
3. *Blue*: you can change the amount of the blue squares (press [ENTER] [4] ,means 4% of the squares)
4. *Green*: you can change the amount of the green squares (press [ENTER] [4] ,means 4% of the squares)
5. *Pink*: you can change the amount of the pink squares (press [ENTER] [4] ,means 4% of the squares)
6. *Run*: you can start or stop the game if you press [ENTER]
7. *Demo*: after when you start the game press [ENTER] gives you a description of the processes
8. *Print**: after when you stop the game you can print the graph (left-bottom corner) on a matrix printer

Struggle

1. *Size*: if you want to adjust the number of the boxes of the board press [ENTER] and type in the desired number (8-25) then press [ENTER]
2. *Rabbit*: you can change the amount of the rabbits (press [ENTER] [4] ,means 4% of the squares)
3. *Fox*: you can change the amount of the foxes (press [ENTER] [4] ,means 4% of the squares)
4. *Grass*: you can change the amount of the grass (press [ENTER] [4] ,means 4% of the squares)
5. *Run*: you can start or stop the game if you press [ENTER]
6. *Demo*: after when you start the game press [ENTER] gives you a description of the processes
7. *Print**: after when you stop the game you can print the graph (left-bottom corner) on a matrix printer

*After stopping the game by pressing [ENTER], go to the print option press [ENTER] then press [P] twice then wait. **!!!! Before printing please check the paper in the printer and check the ON LINE option.!!!!** When the printing is finished PRESS [Q] and [Y].

Exercise

1./ Read the relevant chapter of Laboratory Practices and make notes before running the program.

2./ Play the games using different parameters.

3./ Print the graphs (3 graphs/1 page).

4./ Write your conclusion and stick the graphs into your notebook.

25. Measurements with a microscope II.

Basic principles of microscopy

Microscopes are compound optical systems that are used to magnify the image of small objects. They are made of an objective facing the object of observation and an ocular, or eye-piece, facing the observer. To refresh memories of high school physics, the object has to be located in between the focus (F) and twice the focus (2F) of the objective, and its image, magnified, real and inverted, is on the other side of the objective is outside the 2F distance. The eye-piece is placed so that this real image is inside the focus of the eye-piece. Looking into the ocular we see the virtual, upright, magnified image of this first image. In the end, we see the doubly magnified, inverted, virtual image of the object. If we want to make a picture in the microscope, we normally take the first real image created by the objective, by projecting it onto photographic film, or a the chip of a digital camera.

The parts of the microscopes can be classified into (1) optical parts serving illumination and magnification, and (2) mechanical parts that hold and move the former. The names of the major parts are indicated on the first figure of the section outlining your actual experiments. The optical parts of the traditional light microscope are comprised of the condenser (for illumination), the objective and the ocular. For looking at the image with both eyes, a binocular tube is employed, which doubles the first image by means of a prism onto two separate oculars.

Magnification, resolution and visibility

The **magnification** of the microscope is defined by the product of the magnifications of its optical elements. These are the following: the objective (typically between 10x and 100x), ocular (typically between 5x and 20x), and sometimes the prism in the binocular tube (generally 1,5x). The magnification of individual optical elements is the ration of the image distance to the object distance.

The **resolution limit**, that is, the *smallest* distance at the end of which two points are still conceived as two separate points is given by the Abbe formula

$$D = 0.61 \cdot \lambda / NA$$

where $NA = n \cdot \sin\varphi$ is the numeric aperture of the objective, n is the diffraction index of the medium between the objective and the sample, and φ is the angular aperture (1/2 of the angle of light rays the lens can take in when focussed). **Resolving power** is defined as the reciprocal of the resolution limit, R=1/D. When the object is illuminated from the outside, the NA of illumination is also to taken into consideration as it limits the resolving power. The NA in the Abbe formula then must be estimated as the average of numerical apertures for the illuminating and detecting optics. Thus, using green light of 550 nm wavelength, an objective of NA=1.25 and a condenser of NA=0.9, the resolution limit is 250 nm.

The resolving power often limits the usefulness of magnification. If, for example, we use a magnification of 1000x (e.g., objective 100x and ocular 10x), the smallest resolved distance in the above case will be magnified to 0.25 mm. Our eye is capable of resolving 10 separate points

on a 1 mm line, so in this magnified 0.25 mm line we will be able to distinguish 2 points, and hence will clearly see the two points separated by the 0.25 mm line. Now suppose we increase our magnification 3-fold by using a 20x ocular and a 1.5x binocular prism. The images of the best-resolved two points will be separated by 0.75 mm, in which we could in principle distinguish 7 individual points, but the resolving power of the objective only allows for 2 points. Thus we are wasting magnification power. The rule of thumb is not use a magnification exceeding 1000x the NA of your objective.

As it can be deduced from the above, the resolving power can be improved in the following ways (see the Abbe formula):
- decrease the wavelength
  e.g. UV microscope, or, as an extreme example the electron and the X-ray microscope
- increase the NA
  e.g. by increasing the angular aperture (better design of objective, 2 or three objectives) or by increasing the refractive index (use of immersion oil with n=1.5, rather than n=1.0 for air. Immersion oil should be used only with the appropriate objectives marked with a black ring around their body, or labelled HI.

One should not confuse resolving power with **visibility**. The latter is not related to the separability of two point, rather to a single point far away from all other, and states the minimum conditions of size, brightness and contrast against background under which this point is still visible. Seeing this object does not mean we know anything about its size or shape.

Aberrations

Objectives and lense therein have aberrations that impair image quality. Regrading the theoretical background, we refer to the Biophysics lecture notes provided by the department. The main aberrations are:

1. Spherical aberrations occur because light rays passing through the edge of the lense are refracted more than those through the centre. Thus the image of a point will in itself be blurred. Overall this also results in an image that is either in focus at the centre or at the edge of the field, but never both at the same time. Correction for this results in a "flat" image, at the price of some loss in resolving power. Corrected objectives bear the forename "*plan-*".
2. Chromatic aberrations are caused by the wavelength dependence of the refractive index of any material. Henceforth a single lens will have different focal lengths for different colours. This can be corrected for by making compound lense of single lenses from diverse materials that compensate for each others chromatic aberrations. Objectives corrected for two colours are called *achromat,* those corrected for 3 are called *apochromat.* Apochromats are further corrected for the fourth colour of the visible spectrum (the 4 being blue, green yellow and red) by a special compensating ocular.

Illumination in the microscope

Once we have a high quality microscope and a good sample, the most common errors that hinder image quality are problems with adjusting the illumination. The following generic points should be considered.
1. The intensity of illumination should provide and optimal balance of brightness and contrast.
2. The light ray should be symmetric, centered at the optical axis.

3. The field of vision should be illuminated evenly, and no light should fall on points outside the field, as photons scattered from there ruin image quality.
4. The NA of illumination should match that of the objective. Thus, for high NA objectives, the condenser should be high and its diaphragm open, for low NA objectives vice versa.

Some important types of microscopes

Bright field: This microscopy applies white light to illuminate the object that is required to be studied. Both unstained and stained cells can be visualized with this instrument. In the case of the former ones the different parts of cells are distinguished from protoplasm as darkened objects as the result of the light absorption and the scattering away from the objective lens. The latter ones are stained with various dyes (red, blue, green etc.) which bind to a certain organelle or to molecules in the cell (e.g., the nucleus). These dyes absorb all wavelengths except that region of the spectrum that corresponds to their colour. Reducing the intensity of light illuminating the specimen can improve contrast and depth of field. However, this will result in the decrease of effective numerical aperture and resolving power.

Koehler illumination

To see sharp images, the microscope must be equipped with Koehler illumination. In the case of microscopy using external light source this operation provides more intensive and even illumination without decreasing the resolution. It is achieved by focussing the light source onto the substage (condenser) iris diaphragm which is at the same time the rear focal plane of the condenser lens. From every point of the image of the light source formed on the iris diaphragm, a parallel ray is projected towards the sample. This results a nearly uniform bundle of parallel rays impinging on the specimen and hence a more precise and detailed image with less artifacts.

Dark field: Dark field illumination involves the technique of blocking out the direct light completely and viewing an object by means of diffracted light. A special condenser prevents the direct light from reaching the objective but light from rays that are scattered on objects will still enter the objective. This technique enables us to study sudden changes in refractile properties at the edges or discontinuities in an object, subtle colouration of objects. This method can also reveal the presence of objects below the resolving power of the same lens system because of the sensitivity of human eye to the intensity difference. (Dark background - bright object)

Phase contrast: It was developed in 1932 by F. Zernike.(Nobel Prize in 1953) The phase contrast microscope enhances the contrast of the unfixed and unstained cells. Most cell components are transparent to light and have a high refractive index. Light transmitted through these structures is withheld a bit, resulting approximately 1/4 wavelength "out-of phase" shift relative to the light passing through the clear medium. The phase microscope transforms this phase difference to difference in brightness in the following way: separating the diffracted from the undiffracted light, it retards one or other by an additional 1/4 wavelength and afterwards the two beams recombine in the final image. The interference of the "in-phase" (not diffracted) or the "out of phase" (diffracted) beams produces exaggerated contrasts of objects. The beam separation is achieved at a phase plate in the objective's rear focal plane according to the following method: (1) The direct light is made into a narrow cone with a help of a substage transparent annulus (normally built into the condenser). (2) The image of the annulus is adjusted to coincide with the a ring in the phase plate. (3) The remainder of the phase plate is illuminated with the diffracted beam. There are two strategies of phase contrast microscopy:

Positive (dark) phase: It is applied when the more refractile object is desired to make darker than its surroundings. The diffracted light is retarded by an additional 1/4 wavelength on the phase plate, i.e. by using a magnesium fluoride layer on the phase plate except the ring. Eventually, this light beam becomes "out of phase" with direct rays so their interference results a dark spot at the eyepiece.
Negative (bright) phase: The direct light is retarded by a layer at the phase plate ring as a result of which the diffracted and the direct beam come into phase again, yielding a brighter image in contrast to the background.

To enhance the contrast effect, the direct beam must be attenuated by the means of absorbing layer at the annual phase ring. This absorption has efficiency of 75-95%, therefore only a little fraction of the original intensity can be observed. This problem can be avoided by either stronger illumination, or by longer exposure (in photomicrography). A practical problem in phase microscopy is the wavelength-dependence of refractive index: to avoid this, frequently yellow-green filters are used with maximum transmission around 550 nm. (The human eye is very sensitive to this region of spectrum.)

Fluorescence microscopy: In contrast to the previously mentioned microscopic techniques where the specimen is illuminated externally, the fluorescent microscopy makes use of the self-luminescence of objects. In this type of microscopy samples (e.g. cells) are stained (e.g. study of viability) or labeled (e.g. various antigens) with fluorescent dyes and are illuminated with a wavelength of light that corresponds to the excitation (absorption) maximum of the dye. For light sources, mercury or xenon lamps are used to ensure wide excitation spectrum. With application of proper filters, the desired wavelength of light can be selected. The remitted fluorescence light at longer wavelength is detected. To observe only flourescent light, filters and dichroic mirrors are placed between the objective and the eyepiece. (The dichroic mirrors are transparent to a certain wavelength and the remainder of the spectrum is reflected.) The filters absorb the exciting beam of light so only the emitted light rays reach the ocular. The fluorescent objects appear as bright spots (with color coming from their emission spectrum) in the dark background. It is thus easier to study fluorescent images in a darkened room. By applying different types of dyes, many parameters of the cell can be studied: vitality, membrane potential, ligand-receptor bindings, conformational changes of proteins etc..

Experiments with the light microscope:

1. Draw schematically the path of image forming rays in a conventional compound light microscope.

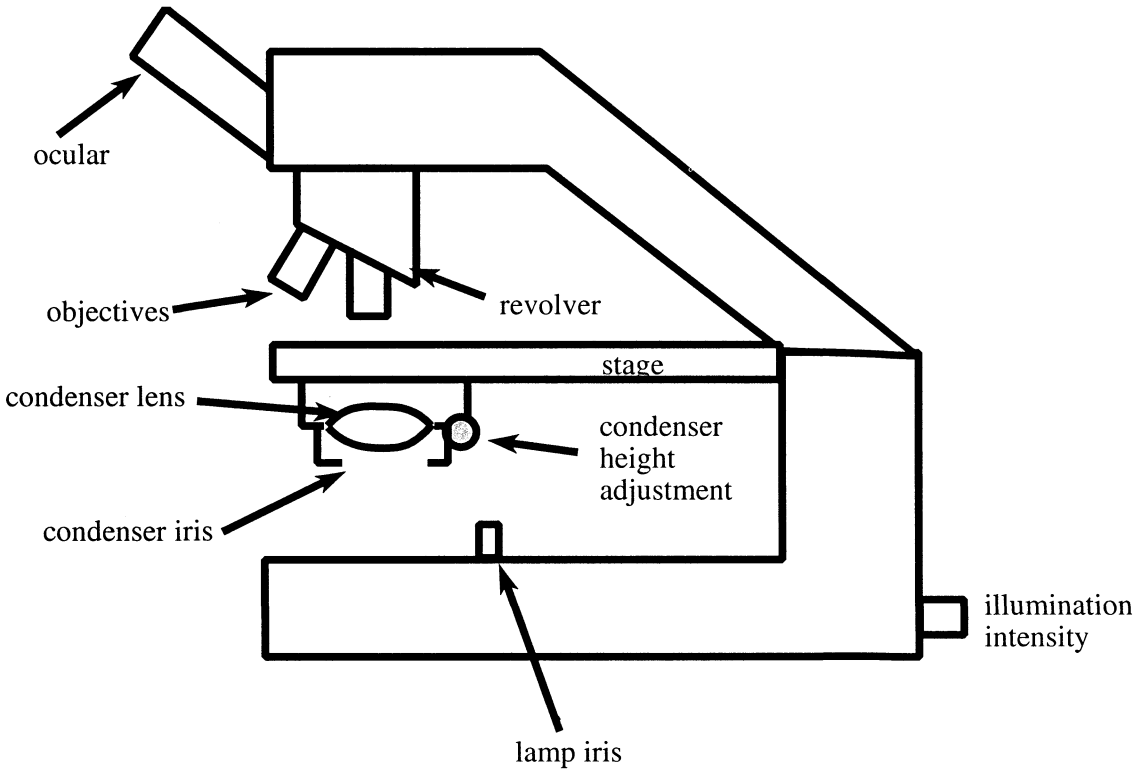


Figure 1. Schematic drawing of a light microscope

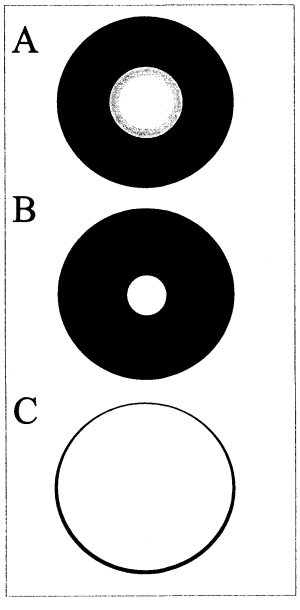


Figure 2. Adjustment of the lamp iris (illuminated area as viewed in the ocular)

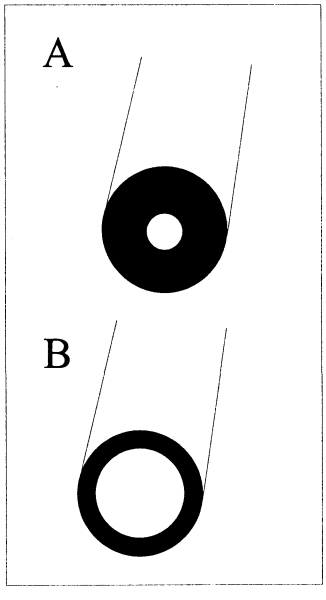


Figure 3. Adjustment of the condenser iris (illuminated area with ocular removed)

Adjust the light microscope according to Koehler illumination. ***Always start adjusting the microscope with the lowest magnification objective.*** It is usually easier to focus on a specimen with a low magnification objective, and the risk of breaking the specimen is lower, if a low magnification objective is used, since the focal distance of a low magnification objective is longer than that of a high magnification objective. ***While focusing on a specimen (especially in the case of high magnification objectives) always watch from the side while slowly approaching the objective to the slide to avoid breaking it. Then slowly move the objective away from the slide*** until the object comes into focus in the eyepiece.

After finishing using the microscope always wipe off immersion oil from the lens using a soft, lens cleaning paper.

Open the lamp iris completely, and focus on a clearly visible specimen. Then close the lamp iris completely (Fig 2A), and bring the edge of the lamp iris into sharp focus by changing the height of the condenser (Fig 2B). Open the lamp iris until the field is illuminated to the edges (Fig 2C). Remove an eyepiece, look down the body tube, and adjust the condenser iris (not the lamp iris) until about 3/4 of the area is illuminated. It is advisable to close the condenser iris completely first (Fig 3A), and then open it until about 3/4 of the field of view is illuminated (Fig 3B). ***View the body tube from far away, because of strong illumination intensity.*** Replace the eyepiece. This adjustment can later be modified, since by adjusting the condenser iris in a Koehler illumination system only the intensity of illumination is altered without changing the area of illumination. However it is easier to change the illumination intensity by changing the power of the lamp with the button on the back of the microscope. Repeat this adjustment from the beginning whenever you change an objective during the experiment. Always use immersion oil with the 100x objective.

Practice adjusting the microscope. Experiment with different lamp and condenser iris settings and condenser height, and determine how these parameters affect image quality. Write your conclusions.

2. Measurement of the diameter of red blood cells

Determine the average diameter of red blood cells in a blood smear according to both of these methods. Use the 100x oil immersion objective.

a. Measurement with an object micrometer. Calibrate the ocular scale with the object micrometer. The distance between two tick marks on the object micrometer scale is 10 μm.

1 unit on the ocular scale=..... μm

Measure the diameter of 10 red blood cells in ocular scale units. Calculate the mean, and using the above calibration give the average diameter of a human red blood cell in μm.

diameter of red blood cells (in ocular scale units)			

Average diameter of red blood cells:.....ocular scale units=.....μm

Give the standard deviation (SD) and standard error of the mean (SEM) of your measurements in μm.

SD= SEM=

b. Hold a graph paper at a distance of 25 cm from your eye, and observe the graph paper with one of your eyes and the ocular scale with the other. The eye piece forms a virtual image of the object in the microscope at a distance of 25 cm from your eye. Therefore the lens of your eye magnifies both the graph paper and the image of the object in the microscope to the same extent. Give the distance on the graph paper to which one unit on the ocular scale corresponds:

1 unit on the ocular scale=..... mm
Take into consideration that the graph paper is viewed at a magnification of 1, while an object in the microscope is viewed at a magnification of 1000.

1 unit on the ocular scale=..... μm

The average diameter of red blood cells using the second calibration:.....μm

Experiments with the fluorescence microscope:

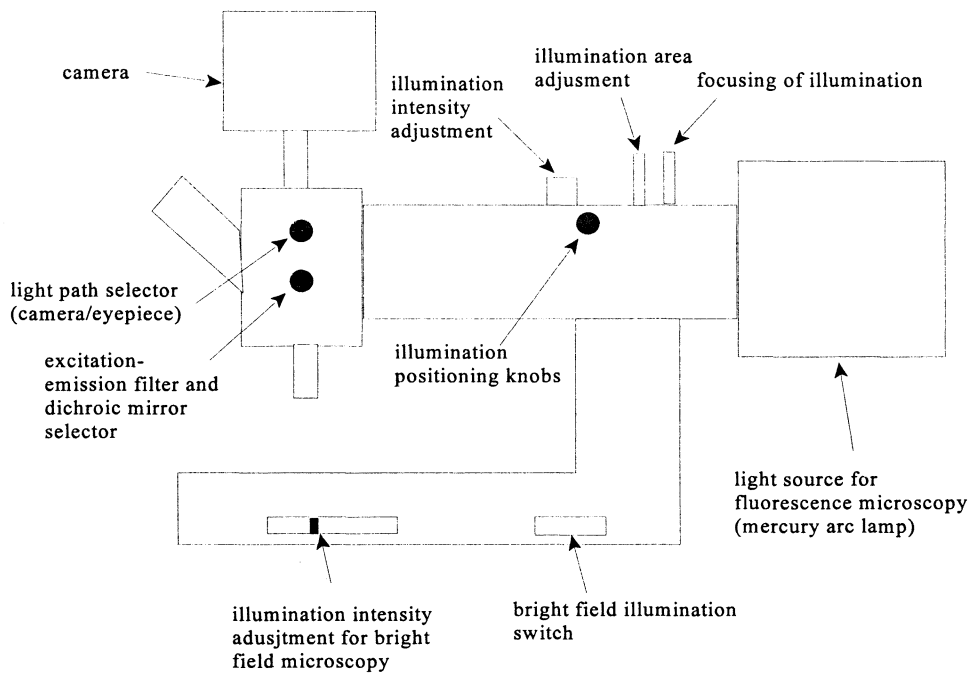


Figure 4. Schematic drawing of a fluorescence microscope

1. Observation of fluorescent beads:

Consult Figure 4. for a schematic drawing of the microscope to be used during the measurements. Place a slide with fluorescent beads into the microscope. Adjust the microscope using the 25x objective (intensity and position of exciting beam, height of the condenser, condenser iris; it is not possible to adjust the lamp iris on this microscope). Observe the slide using both bright field and epifluorescence illumination systems:

– in bright field illumination block the light coming from the lamp for fluorescence

illumination (mercury arc lamp). Do not turn the mercury arc lamp off, since it is not possible to switch it off and on in succession. Adjust the illumination intensity so that the highest contrast is achieved.

- in fluorescence microscopy mode turn the bright field illumination off. Use blue excitation light (and the corresponding excitation and emission filters, and dichroic mirror/beam splitter).

Take an image of the fluorescent beads first with the 25x objective, then with the 100x objective. Use immersion oil for the 100x objective. Image acquisition is done with a computer. The program which has to be run is Olympus C-W95. Under CAMERA>CAMERA CONTROL adjust image quality (HQ=high quality), then press TAKE PICTURE. Afterwards the image is displayed on the screen. Save both of the images with different names in BMP format (FILE>SAVE).

2. Comparing the optical and digital resolution powers of the microscope

Calculate the optical resolving power of the microscope for the 25x and 100x objectives. The wavelength of illumination is about 480 nm.

d_{25x}=

d_{100x}=

The photosensitive detector of the camera is in the image plane of the lens system, which in this case does not include the ocular. The images recorded by the camera contain 1280x1024 pixels, e.g. the camera is able to resolve 1280x1024=1310720 pixels. The smaller these pixels are, the better the digital resolution of a camera is. However, if the width of one such pixel is much smaller that the diameter of the image of a point light source imaged by the objective, the resolution of the digital image is not enhanced further, and the image size (that is the amount of computer memory necessary to store the image) is increased unnecessarily. If, on the other hand, the pixel size is much larger than the diameter of the image of a single point light source, the resolution power of the digital image is inferior to the resolving power permitted by the optical elements of the microscope.

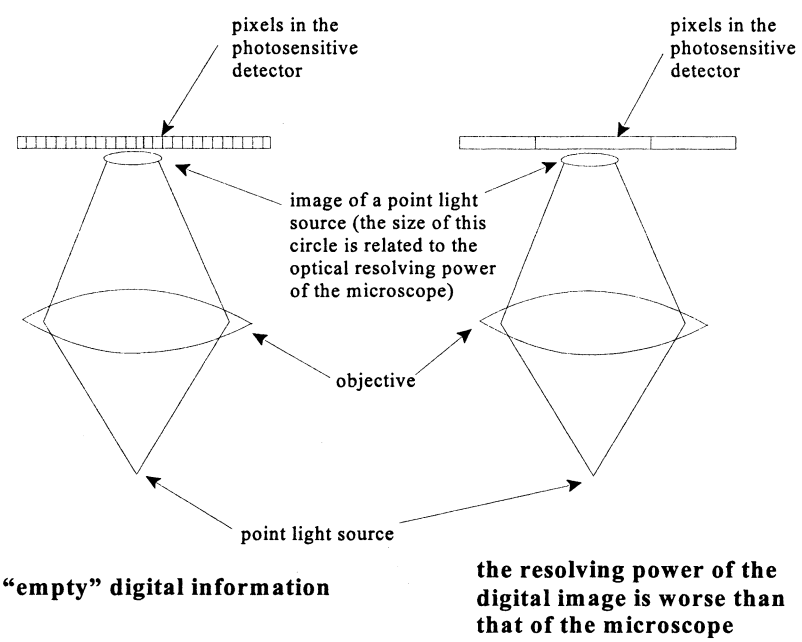


Figure 5. Comparison of digital and optical resolution

Determine the width of the area imaged by the objective onto a single pixel in the photosensitive detector. Make your calculations using both of the methods below:

1. The area of the photosensitive detector is known to be 10x8 mm. Using the total number of pixels (1280x1024) determine the physical size of a pixel, then with the magnification of the objective in mind determine the size of an object whose image size is equal to the physical size of a pixel.

   d_{25x}=

   d_{100x}=

2. The average diameter of the fluorescent beads is known to be 8.5 μm. Using a computer program, ScionImage, determine the number of pixels corresponding to the above diameter.
   Open one of the saved images (FILE>OPEN), then resize the image so that the whole image area is visible (OPTIONS>SCALE TO FIT WINDOW). Increase the contrast of the image PROCESS>ENHANCE CONTRAST), then measure the diameter:
   - under ANALYZE>OPTIONS select "perimeter/lenght"
   - ANALYZE>RESET
   - ANALYZE>SHOW RESULTS
   - choose line measurement (in the TOOLS window it is the fourth icon from the top in the right column)
   - place the ends of the line on opposing points of a bead with the mouse, then measure the distance in pixels (ANALYZE>MEASURE)
   - calculate the mean of 10 measurements
   - carry out the following procedure for both of the images (recorded with the 25x and 100x objectives)

Bead diameters in the case of images recorded with the 100x objective	Bead diameters in the case of images recorded with the 100x objective	Bead diameters in the case of images recorded with the 25x objective	Bead diameters in the case of images recorded with the 25x objective

Average beam diameter in the case of image recorded with the 100x objective:.....pixel
Average beam diameter in the case of image recorded with the 25x objective:.....pixel

Calculate the distance in the object plane corresponding to a single pixel for both objectives:

d_{25x}=

d_{100x}=

Compare the values obtained by the two different approaches, and correlate the digital resolution power of the microscope with the optical one. Write your conclusions.