Task XVII

PREPARATION OF LIPOSOMES. THE ELECTROLYTE INFLUENCE ON LIPOSOME SUSPENSION STABILITY

I. Aim of the experiment

The purpose of this task is the preparation of liposome suspension, its characterization and electrolyte influence on liposome suspension stability.

II. Introduction

1. Characterization of phospholipides. Biological membranes.
2. Classification of liposomes.
3. Methods of liposome preparation.
4. Application of liposomes.
5. Characteristics of colloidal systems. Nephelometric method.

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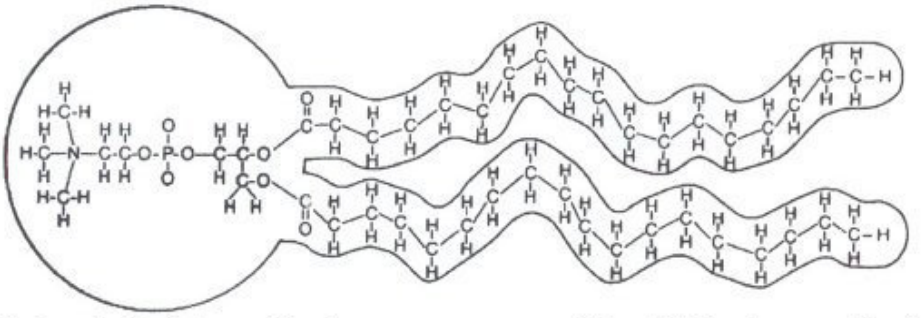
III. Theory

III. 1. Characterization of phospholipids. Biological membranes

Phospholipids are a class of lipids and a major component of all cell membranes. Most phospholipids contain diglyceride, phosphate group, and simple organic particle such as choline. Phospholipids are amphiphilic with the hydrocarbon tail of the molecule being hydrophobic and a hydrophilic head. The hydrophillic part contains the negatively charged phosphate group, and may contain other polar groups. The hydrophobic tail usually consists of long fatty acid hydrocarbon chains.



Hydrophilic **Hydrophobic**

Fig. 1. Structure of phospholipid molecule.

When placed in water, phospholipids form a variety of structures depending on their specific properties, which are presented in Fig.2. Phospholipids can form a phospholipid bilayer with the hydrophobic tails facing each other. The degree of amphiphilic character is the hydrophilic-lipophilic balance of surfactants or biosurfactants (*HLB*). *HLB* is determined by calculating the values for different regions of the molecule giving a result in the scale 0 - 20:

$$HLB = \Sigma W + 7$$

The *HLB* value of 0 corresponds to a completely hydrophobic molecule, and the value of 20 would correspond to a molecule made up of hydrophilic components. The *HLB* value can be used to predict the surfactant properties of a molecule. The value from 3 to 6 indicates a W/O emulsifier. The value from 7 to 9 indicates a wetting agent and that from 10 to 18 indicates an O/W emulsifier.

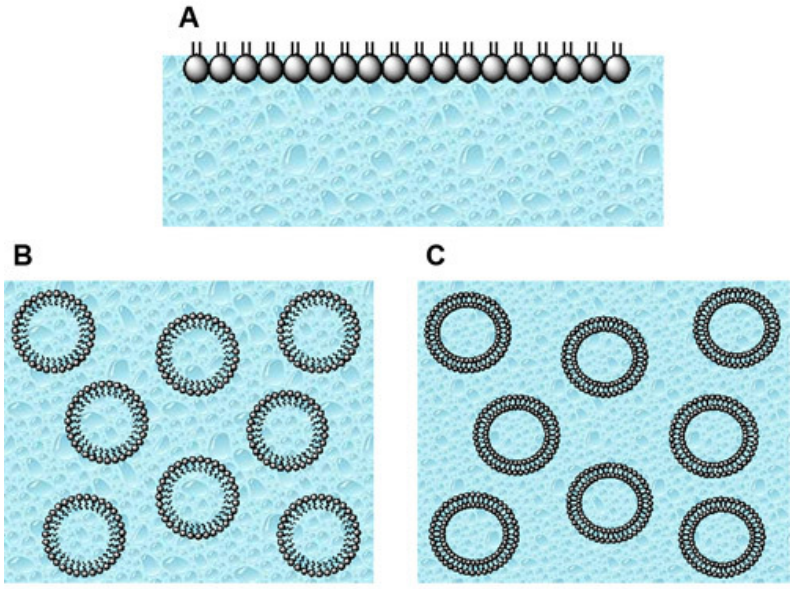


Fig. 2. Assemblies of phospholipid molecules in aqueous dispersions: (A) monolayer at the air-water interface, (B) micelles, (C) liposomes.

The phospholipids often occur with other molecules (e.g., proteins, glycolipids, cholesterol) in a bilayer such as a cell membrane and play an important role in biological systems. This membrane is partially permeable and has fluid properties, in which embedded proteins and phospholipid molecules are able to move laterally. The lipid molecules act as a solvent for all the substances and proteins. Additionally, cholesterol contributes to membrane fluidity by hindering the packing of phospholipids. The model of biological membrane is presented in Fig.3.

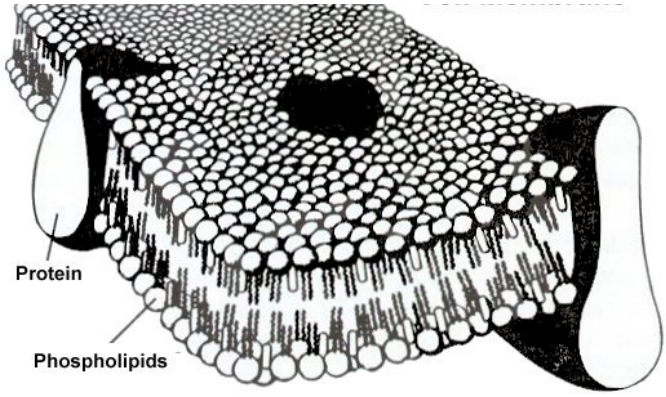


Fig. 3. Model of biological membrane.

Also, the hydrophilic and hydrophobic ends allow the phospholipids to transfer and be associated with water. They are able both to resist and associate with water at the same time. This is due to the fact that only one end of the molecule is soluble in water. Thus phospholipids act as a natural emulsifier enabling oils to dissolve freely in water.

Phospholipids called lecithin are extracted out of cooking oil and then used as food additives in many products and can be also acquired separately in diary and pharmaceutical industry.

III. 2. Classification of liposomes

The name "liposome" is derived from two Greek words: *lipos* (meaning fat) and *soma* (meaning body). A liposome can be formed in a variety of sizes as a uni-lamellar or multi-lamellar construction. A liposome is a tiny bubble (vesicle), made of the same material as a biological membrane. Liposomes were first described by Dr Alec Bungham in 1961. Liposomes can be composed of naturally-derived phospholipids with mixed lipid chains (like egg phosphatidylethanolamine) or pure surfactant components (like dioleoylphosphatidylethanolamine).

There are four types of liposomes with respect to the shape, size and number of bi-layers:

1. Small Unilamillar Vesicles, **SUV** (25–100 nm),
2. Large Unilamillar Vesicles, **LUV** (100–400 nm),
3. Multilamillar Vesicles, **MLV** (200 nm – few microns),
4. Giant Unilamellar Vesicles, **GUV** (more than 1 micron).

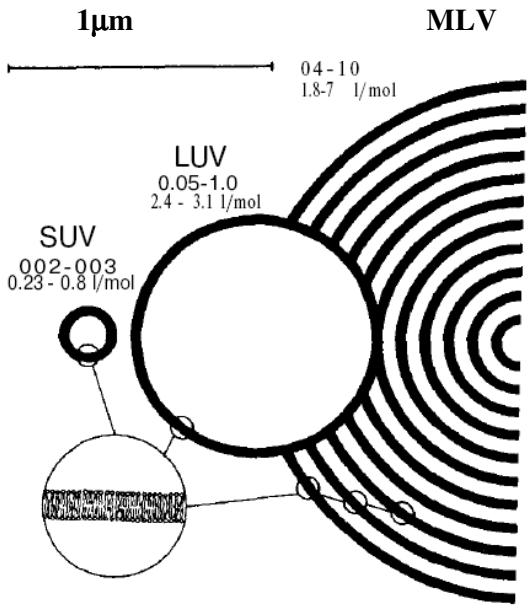


Fig. 4. Classification of liposomes and their parameters.

Low-shear sonication of phospholipids in water generally creates multilamellar liposomes **MLV** and continued high-shear sonication tends to form smaller unilamellar

liposomes **SUV**. In this technique, the liposome content is the same as that of the aqueous phase but it can damage the structure of the drug to be encapsulated. Further advances in liposome research showed that liposomes can not be detected by the body immune system. Targeted liposomes can carry nearly any cell type in the body and deliver drugs, vitamins, antibodies or specific antigens. What is important, is the fact that naturally toxic drugs can be much less toxic if delivered only to diseased tissues.

III. 3. Methods of liposomes preparation

It should be noted that formation of liposomes is not a spontaneous process. Lipid vesicles are formed when phospholipids (for example phosphatidylcholine) are placed in water and consequently form one bilayer or a series of bilayers, each separated by water molecules. The choice of liposome preparation method depends on the following parameters: 1) the nature of the medium in which the lipid vesicles are dispersed; 2) the physicochemical characteristics of the material to be delivered; 3) the concentration of the entrapped substance and its potential toxicity; 4) additional processes involved during application of the liposomes; 5) size, polydispersity and shelf-life of the vesicles for the intended application and 6) reproducibility and possibility of large-scale production of safe and efficient liposomal products.

The main methods of liposome preparation are:

- Hydration of thin lipid layer;
- Sonification by ultrasonic waves;
- Calibration;
- Extrusion by French' press;
- Injection of ethanol solution;
- Injection of ether solution;
- Detergent dialysis;
- Evaporation by reversed phases technique.

The size of liposomes is very small, in the order of a nanometer. As illustrated in Fig.5, the spheres are hollow inside and enclose some of the liquid material in which they were formed. Because of the small size of the phospholipid molecule, they can pass through the epidermis and act as a carrier for the enclosed substances. It is postulated that when they reach the outside of a living cell membrane in the dermis they may become accepted as part of the membrane, being of the same composition. Thus, they are able to carry with them any enclosed substances into the dermis and to the individual cells. Newer methods such as extrusion are employed to produce materials for the human use.

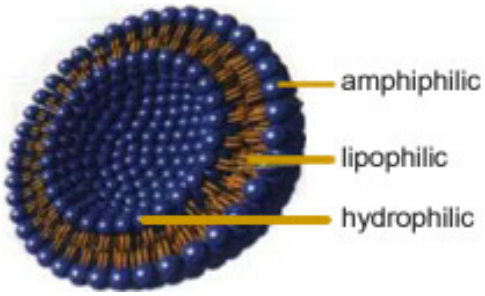


Fig. 5. Liposome structure.

The following stages in the process of liposome preparation and kind of liposomes depending on the used methods are presented in the table:

Solubilization of lipids						
Hydration of dry lipid layer			Solubilization of lipids			Emulgation
suspension MLV			colloidal solution	solution in the solvent		(micro) emulsion
bulge un-forming	calibration	fragmenta-tion	removal of de-tergent	injection		
				miscible with water	unmiscible with water	gel
LUV	LUV, SUV	SUV	LUV	SUV,ML	MLV,LUV	MLV,LUV

In the laboratory one of the simplest method of liposome preparation is slow injection of ethanol solution of lipid (ether or detergent solution) to aqueous solution and homogenization of the suspension (or sonification by ultrasonic waves). Liposomes should be prepared at temperatures higher than their phase transition temperature, but not exceed 45-50^{o}C (for DPPC the phase transition temperature is equal to 41 ^{o}C). After obtained a suspension additionally dialysis could be done to remove ethanol (ether or detergent). The obtained suspension is usually heterogeneous and calibration technology is needed to get through greatly homogeneous pores in polycarbonatic membrane filters. The scheme of this method is presented in Fig.6.

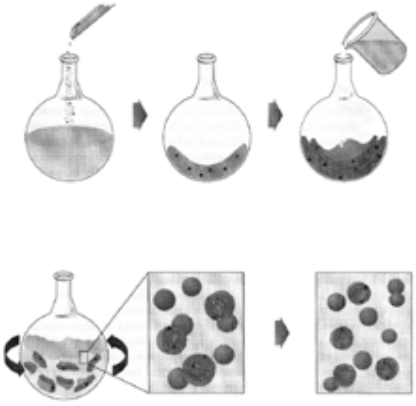


Fig. 6. Preparation of liposomes.

III. 4. Application of liposomes

The ability of phospholipids to act as the carrier mechanism has extensive implications for drug, gene and cosmetics delivery. Liposomes are composite structures made of phospholipids and may contain a small number of other molecules. Liposomes are used for delivering active ingredients directly to the cell due to their unique properties. A liposome encapsulates a region on aqueous solution inside a hydrophobic membrane; dissolved hydrophilic solutes cannot readily pass through the lipids. Hydrophobic chemicals can be dissolved into the membrane, and in this way liposome can carry both hydrophobic and hydrophilic molecules. To deliver the molecules to the sites of action, the lipid bilayer can fuse with other bilayers such as the cell membrane, thus delivering the liposome contents. By formation of liposomes in a solution of DNA or drugs they can permeate the lipid bilayer. The use of liposomes for transformation or transfection of DNA into a host cell is known as lipofection.

Though liposomes can vary in size from the low micrometer range to tens of micrometers, unilamellar liposomes are typically in the lower size range with various targeting ligands attached to their surface allowing for their surface-attachment and accumulation in pathological areas for treatment of disease. The interesting property of liposomes is their natural ability to target cancer. Anti-cancer drugs such as Doxorubicin (Doxil), Camptothecin and Daunorubicin (Daunoxome) are currently being marketed in the liposome delivery systems.

The development of liposome technology offers also the potential for many beneficial cosmetic products. The use of liposomes in nano cosmetology has many profits, including improved penetration and diffusion of active ingredients, selective transport of active ingredients, longer release time, greater stability of active, reduction of unwanted side effects, and high biocompatibility. Liposome cosmetic products are non-toxic and cause no skin irritations. They are hazardous due to their ability to carry toxic or contaminated substances into the cells, hence the careful selection of raw mate-

rials is needed. The microspheres themselves are constantly undergoing changes due to thermal activity during preparation and storage. As a result, each ingredient of the preparation can end up inside the microspheres over time. In addition, liposomes can be used as carriers for the delivery of dyes to textiles, pesticides to plants, enzymes and nutritional supplements to foods.

III. 5. Characteristics of colloidal systems. Nephelometric method

Colloidal systems are a type of chemical mixture where one substance is dispersed evenly throughout another. The particles of the dispersed substance are only suspended in the mixture, unlike a solution. This occurs because the particles in a colloid are larger than in a solution - small enough to be dispersed. A colloidal system consists of two separate phases: a dispersed phase (or internal phase) and a continuous phase (or dispersion medium). The dispersed-phase particles have a diameter of between approximately 1 nm and 500 nm and even 1000 nm. A colloidal system may be solid, liquid or gaseous.

One of the most important parameters of colloidal systems stability is the electrokinetic potential dzeta (ζ). Knowledge of this parameter gives possibility to understand most properties of colloids. Zeta potential is the electric potential in the interfacial double layer at the location of the slipping plane. In other words, the zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. It depends on the kind of molecule, dispersed medium and the present ions. As a consequence, modification of pH, temperature or compound concentration should change the zeta potential.

Electrokinetic phenomena (for example electrophoresis or microelectrophoresis) may be interpreted in terms of the zeta potential or the charge density at the surface of shear. The zeta potential depends on the electrophoretic mobility according to the Henry's equation:

$$U_e = \frac{2\varepsilon\zeta}{3\eta} f(\kappa a) \qquad \text{(III.1)}$$

where: κ – the Debay – Hückel parameter depended on the electrolyte concentration, U_e – the electrophoretic mobility, ε – the electrical permittivity of the medium, η – the viscosity of the medium, $1/\kappa$ – the effecttive thickness of the electrical double layer.

For non-conducting particles in twater suspension and low electrolyte concentrationwhere f(κa) equals 1 for small κa equation (III.1) transform to the Hückel equation, where f(κa) is equal to 1.5 for large κa equation (III.1) transform to Smoluchowski equation:

$$U_e = \frac{\varepsilon\zeta}{\eta} \qquad \text{(III.2)}$$

The **point of zero charge (pzc)** is the point when the electrical charge density on a surface is zero. The pzc value is assigned to a given substrate or colloidal particle

determined in relation to electrolyte pH. The value of pH is used to describe pzc only for the systems in which H^+/OH^- are the potential-determining ions. The **isoelectric point (iep)** is the concentration of potential-determining ions when the electrokinetic potential is equal to 0. At pzc and iep, the colloidal system exhibits minimum stability (i.e., it exhibits the maximum coagulation/flocculation rate). In literature a value of 25 mV (positive or negative) can be taken as the arbitrary value that separates low-charged surfaces from highly-charged ones and will confer stability. When the potential is low (< 25 mV), attraction exceeds repulsion and the dispersion will break and flocculate. In the colloidal systems light hits small particles and the particles scatter the light in all directions as long as the particles are small compared to the wavelength. If the light source is a laser (monochromatic and coherent) then one observes a time-dependent fluctuation in the scattering intensity. These fluctuations are due to the fact that small molecules in solutions are undergoing the Brownian motion.

The **Tyndall effect** is an effect of light scattering by colloidal particles or particles in suspension. The intensity of the scattered light depends on the fourth power of the frequency. Tyndall scattering can be used to determine the size of colloidal particles and it is also often used to describe light scattering by macroscopic particles such as dust in the air. The phenomenon is best explained by the Mie theory as the particle size is much larger than the wavelength of light. In this formulation, the incident plane wave as well as the scattering field are expanded into radiating spherical vector wave functions. The internal field is expanded into regular spherical vector wave functions. Colloidal particles are much larger than atoms or molecules. However, this phenomenon is more like reflection, as the macroscopic particles become visible in the process.

Nephelometry is a widely used method to measure size of colloidal particles (for example emulsion). It is performed by shining light on a sample, and measuring the amount of light scattered (or reflected). The amount of scattering is determined by collecting the light at an angle (usually about 90 or 45 degrees). It is based on the principle that a dilute suspension of small particles will scatter light passed through it rather than simply absorbing it.

The effect of light radius on the behaviour of different particles is described by the Mie theory. When size of particles is smaller than the wavelength of light it is possible to use the simplification of this theory, named the Rayleigh – Gans - Debye theory. All particles smaller than 1/10 of the wavelength of incidence light show the behavior consistent with the Rayleigh theory, the light is scattered isotropically (the same energy is scattered in all directions). In this way, it gives the relationship of light scattered intensity I by spherical and colourless particles as a function of light incidenced intensity I_o:

$$I = 24\pi^3 I_o \left(\frac{n_1^2 - n_2^2}{n_1^2 + 2n_2^2} \right)^2 \frac{Nv^2}{\lambda^4} \qquad \text{(III.3)}$$

where: n_1 and n_2 – the refractive indices of the dispersed phase and dispersion medium respectively, N – the total amount of dispersed particles, v – the volume of particle, λ – the wavelength of incidenced light.

The relationship of the scattered light intensities, I_1 i I_2, for two colloid systems differentiated by the size of dispersed particles is proportional to the size of both sol particles v_1 i v_2:

$$\frac{I_1}{I_2} = \frac{v_1}{v_2} \qquad \text{(III.4)}$$

When both colloid systems have the same size of particles, the relationship of the scattered light intensities is proportional to their concentrations:

$$\frac{I_1}{I_2} = \frac{c_1}{c_2} \qquad \text{(III.5)}$$

On the basis of relationship (III.5) it is possible to find the concentration of the investigated colloid of known intensity of the scattered light and the concentration of model colloidal system. Therefore, this method is widely used in the quantitative analysis and monitoring of processes (e.g. coagulation).

The colloid systems have specific qualities, one of them is kinetic quality –**diffusion**. Diffusion is the movement of particles from an area of high concentration to a lower one. The result of diffusion is a gradual mixing of material. The most characteristic mechanical quality is the **Brownian motion.** It is the seemingly random movement of particles suspended in a fluid described by the expression:

$$\bar{x}^2 = \frac{RT}{N_A} \frac{t}{3\pi\eta r} \qquad \text{(III.6)}$$

where: $\bar{x}^2$ – the second power of the average projection of colloidal particles on the selected axis, η – the viscosity index, r – the radius of colloidal particle, t – the time.

IV. Experimental

A. Apparatus and materials

1. Apparatus: Turbidimeter 2100AN IS,
   Homogenizer MPW- 120.
2. Equipment:
   - calibrated cylinder (100 cm^3) – 1 p.,
   - pipette (5 cm^3) – 1 p.,
   - beaker (50 cm^3) – 1 p.
   - siringe (2 cm^3) – 1 p.,
   - cuvettes – 4 p.,
   - silicone oil for the cuvette cleaning,
   - thermometer,
   - wash bottle,
   - tea-pot.
3. Reagents: cosmetic lecithin suspension in ethanol (1.85 g/100 cm^3 ethanol), 10^{-1} M NaCl, 10^{-1} M $CaCl_2$, 10^{-1} M $AlCl_3$.

B. Task scheme

1. Homogenization of liposome suspension in water and electrolyte solution.
2. Turbidity measurement *Z* of liposome suspension as a function of time.
3. Evaluation of the obtained results.

C. Instruments description

1. Turbidimeter 2100AN IS

The Hach Model 2100AN IS Laboratory Turbidimeter (Figure 7) is designed for turbidity and attenuation measurement in accordance with the International Turbidity Measurement Standards. FNU (Formazin Nephelometric Unit) and FAU (Formazin Attenuation Unit) measurements as large as 1000 FNU and 10,000 FAU can be measured directly. Measure the solutions with larger turbidity levels using the NTU measurement mode or by dilution with the filtered sample and a simple calculation. The 2100AN IS Laboratory Turbidimeter also provides direct measurements in the units of NTU (Nephelometric Turbidity Unit, 0-10,000), EBC (European Brewing Convention, 0-2,450), % Transmittance and Absorbance. In addition, two Application Specific Calibrations (ASC) can be specified by the analyst. The Application Specific mode of operation uses the Nephelometric optical system and the NTU measurement mode. Special method development and sample characterization can also be accomplished using the signal output from any of the four detectors. The optical system includes an 870 ± 30 nm light emitting diode (LED) assembly, a 90° detector to monitor scattered light, a forward-scatter light detector, a transmitted-light detector, and a back-scatter light detector.

Note: The model 2100AN IS Turbidimeter does not require warming up for lamp output or electronic stabilization.

The electronic and optical design of the 2100AN IS Turbidimeter provides long-term stability and minimizes the need for frequent calibration. The multi-detector rationing optical system compensates for electronic and optical system variations between calibrations. Hach recommends calibrating the instrument before it is used for the first time. The standards (<0.1-, 20-, 200-, 1000-, 4000- and 7500-NTU) are stable for one year from the date it is received. Periodically, as experience or regulating authorities indicate, verify the instrument calibration using one of the StablCal® standards supplied with the instrument. If the reading in the range of use is not within 10% of the standard assigned value, recalibrate the instrument.

Calibrating the Turbidimeter

Note: Calibration frequency is given by the demonstrator

1. Press **CAL/Zero**. The Cal mode annunciator lights, and the small green LED digits in the mode display flashes **00**. The FNU value of the dilution water used in the previous calibration is displayed.
2. Select the StablCal® Stabilized formazin vial labelled <0.1-NTU. Wipe the cell clean and apply a thin film of silicone oil to its surface. Place the capped sample cell into the cell holder (without shaking) and close the cell cover.
Note: Install all StablCal vials with the orientation mark aligned with the cell holder reference mark.
Press **ENTER**. The instrument display counts down from **60** to **0**, and then makes a measurement. This result is stored and used to calculate a correction factor. The instrument automatically increments to the next standard, the display shows 20-NTU.
Note: To exit the calibration procedure at any time without changing any stored value, press **UNITS/Exit**.
3. Select the StablCal vial labelled 20-NTU and gently shake. Place the capped sample cell into the cell holder and close the cell cover. Press **ENTER**. Wait for the instrument to count down as before from **60** to **0** and display the next standard. Remove the vial from the cell holder.
4. Repeat this procedure to record the other standards.

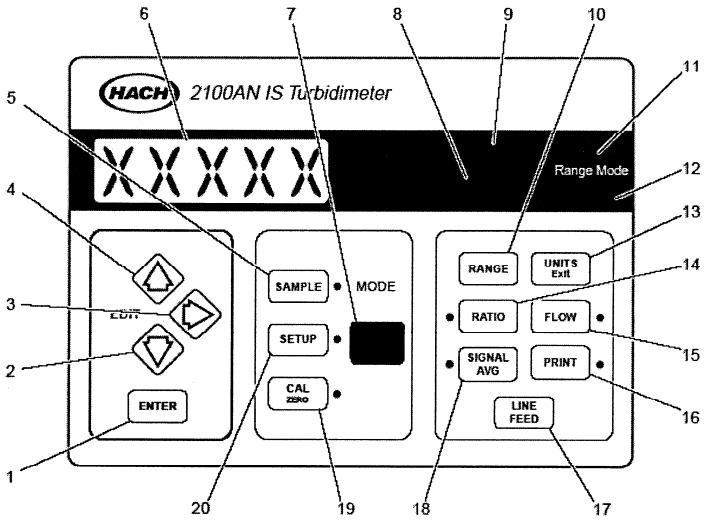


Fig. 7. Laboratory Turbidimeter 2100AN IS

Operating Features and Functional Descriptions

1 – ENTER Used in calibration to select the value of the formazin calibration standard and to initiate measurement of the standard. Selects functions during instrument setup and initiates zeroing;
2, 3, 4 – Down arrow the same as the up arrow except for direction of steps. Right arrow advances the cursor position during calibration standard editing, instrument setup and sample number selection, up arrow edits LED digits in the calibration mode and SETUP number (in the mode display) during the instrument setup procedure;
5 – SAMPLE Initiates editing of the sample (green LED);
6, 7 – Mode display Displays the calibration standard number, setup parameter number or sample number;
8 – Lamp lighted annunciator indicates when the instrument lamp is on;
9 – CAL? Lights to indicate the calibration information recorded when the calibration process is out of the acceptable range (may be an operator calibration error or an instrument malfunction). The instrument must be recalibrated if the CAL? annunciator flashes;
10 – RANGE Selects Auto Ranging or Manual Ranging;
11 – Manual Range, 12 – Auto Range;
13 – UNITS Exit Selects unit of measure. Available options are NTU, EBC, NEPH, %T, Absorbance, CU and two ASC (Application Specific) units;
14 – RATIO Turns ratioing on or off. Flashes to indicate over-range of 40 NTU in the ratio off mode;
15 – FLOW Lighted annunciator indicates that the flow mode of operation is on. A flashing annunciator indicates that the flow cycle is complete;
16 – PRINT Transmits the result of measurement to a computer or printer;
17 – LINE FEED Advances the internal printer paper one line each time the key is pressed;
18 – SIGNAL AVG Turns the signal averaging function on or off;

19 – CAL/Zero Initiates calibration in NTU, EBC, NEPH and ASC measurement modes.
Initiates analytical zeroing in %Transmittance and Absorbance modes;
20 – SETUP Initiates editing of the setup number to configure the instrument for specific operational functions.

Nephelometric Measurement Procedure

- To power up the instrument put down the measurement cell lid and press the **I/O** switch placed on the back instrument panel. Immediately after powering, dark readings of the detector are displayed. An error code **E7** may be displayed if the cell cover is left open during powering up;
- Measurements can be taken with the average signal switched on or off using key **SIGNAL AVERAGE** as well as with auto or manual range module;
- Select 'automatic range' by using the **RANGE** key. If the reading is out of the range only 9 will be displayed and under the range 0 will be displayed.
- Select the appropriate measurement unit (NTU, % or ABS) by pressing the **UNITS Exit** key;
- Transfer the sample containing the examined solution to the cell up to the mark (approximately 30 mL). Hold the cell by its top part and screw cap on. Holding the cell by cap, wipe its walls with a cloth saturated with silicone oil to remove any stains and finger prints. The cell should be nearly dry with little or no visible oil;
- Insert the sample cell aligned with the cell holder reference mark measurement chamber and close the cell cover. Press **ENTER** to update the display;
- While the lid is open, the code **DOOR** is displayed;
- Place the next sample cell into the measurement chamber, close the lid and press **ENTER**;
- Read and record the results. In the course of this procedure do not keep the sample cell all time in the turbidimeter;
- After thev measurements, clean the sample cells carefully and switch off the instrument by pressing **I/O** key on its back panel.

2. Homogenizer MPW–120

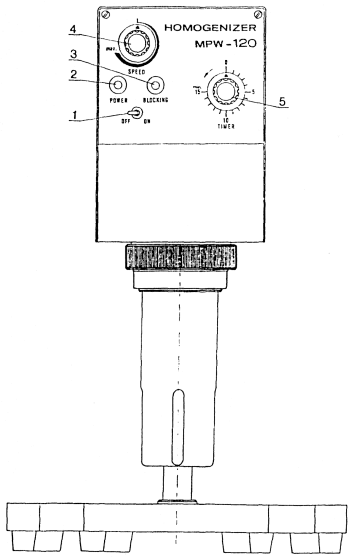


Fig. 8. Head of homogenizer:
1 – power switch,
2 – power indicating light,
3 – lock indicator light,
4 – rotation speed control,
5 – timer switch.

Homogenizer of MPW-120 type is designed for production of homogeneous mixtures and suspensions, as well as pharmacologic, medical and physicochemical samples. MPW-120 is equipped with the rotation speed control in the range 1,000–15,000 rpm, and the maximum work time of 15 minutes.

Before measurements:

- Set all handwheels in the initial position and **switch** [1] in the **OFF** position;
- Plug the instrument to the power socket. Fill the metal container with solution for homogenization and screw it on the homogenizer head;
- **Rotation speed control** handwheel [4] set in 10000 rpm position – arrow directed vertically down;
- **Timer switch** [5] set for 1 min., **Note: the direction of switch rotation is anticlockwise** ;
- Push **switch** [1] to **ON** position, (power indicator light [2] should come on, but lock indicator light [3] should remain off);
- Light [3] is on, the automatic lock is applied (i.e. head is fitted wrongly or steel container is attached incorrectly). After the time set with the timer, the homogenizer will switch off;

– After measurements all used elements of homogenizer should be striped, cleaned and dried;

– Push **switch** [1] to the **OFF** position.

D. Program of activity

1. Preparation of liposome suspensions

a) liposome suspension in water

i. Using a kettle in the fume cupboard heat distilled water up to 50°C precisely.

ii. Measure off 95 cm^3 of heated water using a measuring cylinder and transfer it to the metal container of homogenizer.

iii. Mix lecithin in the ethanol suspension vigorously and extract 5 cm^3 into the beaker.

iv. Take 1 cm^3 of this suspension with a syringe and inject it into water in the container (Note: needle should be immersed in water). Next attach the metal container to the homogenizer and set the timer for 1 min and the rotation speed control for 10,000 r.p.m (Remember the timer handwheel works anticlockwise!).

To achieve a homogeneous suspension of liposome the procedure from step (iv) has to be repeated 4 times, up to the total consumption of lecithin in the ethanol suspension in the beaker (5 cm^3). **As $t = 0$ [min] is taken, homogenization of the whole suspension ends.**
After homogenization clean the metal container - fill it up worm water, attach in the homogenizer head and switch on for 2 minutes.

b) liposome suspension in the electrolyte solution

i. Preparation of $CaCl_2$ 10^{-2} M solution: Take 10 cm^3 of 10^{-1} M $CaCl_2$ solution and transfer it to the measuring cylinder. Heat up distilled water to 50°C precisely and top up the measuring cylinder with $CaCl_2$ solution up to 95 cm^3. Transfer the solution prepared in this way to the metal container.

ii. Mix lecithin in the ethanol suspension vigorously and extract 5 cm^3 into the beaker.

iii. Take 1 cm^3 of this suspension with a syringe and inject it into the prepared solution in the container (note: needle should be immersed in solution). Attach the metal container to the homogenizer and set the timer for 1 min and the rotation speed control for 10, 000 rpm. Remember the timer handwheel works anticlockwise! Repeat the procedure as with water suspension.

iv. Procedure needs to be repeated with NaCl and $AlCl_3$ solutions (10^{-2} M) according to the instructions of the demonstrator.

2. Stability measurements

Following the **Nephelometric Measurement Procedure (in NTU** units) turbidity measurements will be taken. Zeroing of the instrument is done by turbidity measurement of a clean sample cell with distilled water. Next the cell is filled with the liposome suspension and placed in the measurement chamber and the reading of turbidity is taken to check liposome suspension stability.

Start taking turbidity measurements Z in NTU units for t = 5 min. (time from the end of homogenization), and every five minutes after up to 25 minutes after homogenization. In the meantime, you should start homogenizing the next liposome suspension in 10^{-2} M $CaCl_2$. Remember about proper washing of metal container for homogenization and rinsing it with distilled water. After the end of measurements all equipment should be properly washed.

E. Results and discussion

1. Results of turbidity Z obtained for each suspension record in the Table:

Liposome suspension in water			Liposome suspension in $CaCl_2$ solution		
t [min]	*Z [NTU]*	*v [nm]*	*t [min]*	*Z*	*v [nm]*
5		280	5		
10			10		
15			15		
20			20		
25			25		

The measurements values must be presented on one graph Z = f (t) for each measurement series. Based on the results verify the electrolyte influence on liposome suspension stability.

For each measurement estimate the size of liposome in electrolyte solution, based on the measurement of intensity light scattering according to the equation:

$$\frac{Z_1}{Z_2} = \frac{v_1}{v_2} \qquad \text{(IV.1)}$$

For the calculation purpose the average size of liposome particle in water v_1 under the measurement conditions is equal to 280 nm (value measured by dynamic light scattering).