MATERIALS and METHODS

5. Materials and methods

5.1. Materials and cell culture

Oleuropein was from Extrasynthese (Lyon, France). Almond β-glycosidase (EC 3.2.1.21) was from Fluka (Sigma-Aldrich, Steinheim, Germany). hIAPP (Calbiochem, La Jolla, CA, USA) was dissolved in 80% HFIP (Sigma-Aldrich) to a concentration of 512 μM and stored at−20°C until use. Caspase-3 fluorogenic substrate N-acetyl-DEVD-7-amino-4-trifluoromethyl coumarin (Ac-DEVDAFC) and inhibitor N-acetyl-DEVD-aldehyde (DEVD-CHO) were from Biomol (Exeter, UK). Anti-amylin rabbit polyclonal antibody (H-50) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Alexafluor 488-labeled chicken anti-rabbit IgG antibody was from Molecular Probes (Eugene, OR, USA); propidium iodide was from Fluka (Sigma-Aldrich). 1,2-Dioleoyl-snglycero-3-[phospho-L-serine] (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were from Avanti Polar Lipids (Alabaster, AL, USA). Formvar/carbon-coated 400 mesh copper grids were from Agar Scientific (Stansted, UK). The CytoTox-ONETM Homogeneous Membrane Integrity Assay was from Promega. ThT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), calcein and all other chemicals and supplements, unless otherwise stated, were from Sigma-Aldrich.

Rat RIN-5F insulinoma cells were from ATCC (American Type Culture Collection). This cell line was extensively used by the researchers to assess the toxicity caused by amyloid hIAPP aggregates [7,21,22]. The cells were cultured in RPMI medium supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich, Steinheim, Germany), 2.25 g/L glucose, 10 mM HEPES, 1.0 mM sodium piruvate, glutamine and antibiotics and maintained in a 5.0% CO_2 humidified atmosphere at 37°C.

5.2. Oleuropein deglycosilation

Oleuropein deglycosilation was performed according to the method developed by Konno et al. [**218**], with some minor modifications.

Briefly, a 10 mM solution of oleuropein in 310 μl of 0.1 M sodium phosphate buffer, pH 7.0, was incubated with 8.90 IU of β-glycosidase overnight at room temperature. The following day, the reaction mixture was centrifuged at 18,000 rpm for 10 min to precipitate the aglycon of oleuropein, which was then dissolved in the apolar solvent DMSO. The complete oleuropein deglycosylation was then confirmed by assaying the glucose released in the supernatant with the Glucose (HK) Assay Kit (Sigma). This test uses the following reactions:

$$\text{Glucose + ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-Phosphate + ADP}$$

$$\text{G6P + NAD} \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate + NADH}$$

Glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. Glucose-6-phosphate (G6P) is then oxidized to 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration. GC-MS (gas cromatography-mass spectrometry) analysis were also performed and they showed the absence of any glycated oleuropein in the precipitate and the substantially total recover of the aglycon in the same precipitate. The 100 mM stock solution was protected from light and used by the day it was prepared. Dilutions in aqueous buffers were made immediately before use.

5.3. Cytotoxicity assay

hIAPP aggregate cytotoxicity was assessed on RIN-5F cells by the MTT reduction assay, in which a yellow redox dye, MTT, is reduced to purple formazan [**219**] by the metabolically active cells. It is widely assumed that MTT is reduced by active mitochondria in living cells. Indeed, malate, glutamate, and succinate are able to support MTT reduction by isolated mitochondria. However, several data indicate that the role of mitochondria is not exclusive in MTT reduction by intact cells. Using a variety of approaches, MTT reduction was found to be confined to intracellular vesicles that later give rise to the needle-like MTT formazan at the external cell surface. Some of these vesicles were identified as endosomes or lysosomes. In addition, MTT was found to be membrane impermeable. MTT is probably taken up by cells through endocytosis and reduced MTT formazan accumulates in the endosomal/lysosomal compartment and is then transported to the cell surface through exocytosis [**220**]. The ability of cells to take up and reduce MTT, therefore, correlates with their viability and metabolical function, both at cytoplasm and membrane level.

The cells were seeded into 96-well plates at a density of 20,000 cells/well in fresh complete medium and grown for 7 h. hIAPP aggregates were prepared by diluting stock solution to 3.25 μM in 10 mM phosphate buffer, pH 7.4, containing 1.0% HFIP [**221**], in the presence or in the absence of 30 μM oleuropein (oleuropein molar concentration was 9X with respect to hIAPP molar concentration), and aged for different lengths of time at 25°C. Then, the aggregate mixtures were diluted 110 times in the culture medium without FCS and phenol red and given to cells (final concentrations: 30 nM hIAPP, 270 nM oleuropein). The medium without phenol red

was preferred since an inhibitory activity of phenol red on hIAPP amyloid aggregation was previously demonstrated [**215**].

In the dose dependence experiments, 3.25 μM hIAPP was also incubated with a 3X concentration of oleuropein (9.8 μM). As additional controls, the cells were also treated with oleuropein alone, or with hIAPP aggregates (obtained in the absence of oleuropein) together with 270 nM or 2.70 μM oleuropein aglycon; the secoiridoid was previously incubated at the same conditions and for the same length of time as hIAPP aggregates, before being given to cells. At the end of the exposure (24 h), the culture medium was removed and the cells were incubated for 2 h in a 0.5 mg/ml MTT solution. Finally, cell lysis solution [20 % (w/v) sodium dodecyl sulfate, 50 % (v/v) N,N-dimethylformamide] was added, and the multiwell plate was kept overnight at 37°C in a humidified incubator. Blue formazan absorbance was measured at 570 nm using an automatic plate reader (BioRad).

5.4. Caspase-3 activity assay

The cells were seeded, cultured and treated with hIAPP exactly as indicated for the MTT assay. In this case, the cells were exposed to hIAPP aggregates aged for 30 min at 25°C in the presence or in the absence of oleuropein and then diluted in the culture medium. After 3h of incubation, the cells were washed twice with phosphatebuffered saline (PBS) and then lysed for 20 min at 0-4 °C in 20 mM Tris-HCl buffer, pH 7.4, containing 250 mM NaCl, 2.0 mM EDTA, 0.1 % Triton X-100, 5.0 μg/ml aprotinin, 5.0 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 4.0 mM sodium vanadate, and 1.0 mM dithiothreitol. The lysis was completed by sonication, and total protein content was determined in the clarified lysates with the Bradford reagent. Caspase 3 activity was determined by incubating 50 μg of total proteins for 2h at 37 °C in the presence of 50 μM Ac-DEVD-AFC substrate in 50 mM HEPES-KOH buffer, pH 7.0, 10 % glycerol, 0.1 % 3-[(3-cholamidopropyl)-dimethylammonium]-1-propane sulfonate, 2.0 mM EDTA, 10 mM dithiothreitol. The fluorescent aminofluorocoumarin formation was determined at 400-nm excitation and 505-nm emission, using a 2 x 10-mm path length cuvette and a PerkinElmer LS 55 spectrofluorimeter equipped with a thermostated cell compartment. In order to determine nonspecific substrate degradation in the assay, total protein samples were preincubated for 15 min at 37 °C in the presence or in the absence of the specific caspase inhibitor (100 nM DEVD-CHO) prior to substrate addition.

5.5. Lactate dehydrogenase (LDH) release assay

LDH release was measured in the culture medium of cells exposed to hIAPP aggregates by the CytoTox-ONETM Homogeneous Membrane Integrity Assay. The latter is a fluorometric method for estimating the number of cells dying by necrosis, and thus releasing components from the cytoplasm into the surrounding culture due to membrane fragmentation. LDH released into the culture medium is measured with an enzymatic assay that results in the conversion of resazurin into the fluorescent product resorufin, as shown in Fig. 5.1.

Rin-5F cells were plated on 96-well plates at a density of 20,000 cells/well in fresh complete medium and grown for 72 h. Then the cells were treated with hIAPP exactly as indicated for the MTT assay but in this case only with hIAPP aggregates aged for 30 min at 25°C in the presence or in the absence of oleuropein and then diluted in the culture medium. Expecially for the development of this assay, the absence of serum in the culture medium resulted essential to eliminate background fluorescence resulting from the significant amounts of LDH in serum and improve assay sensitivity. Cells were exposed to aggregates for 24 h.

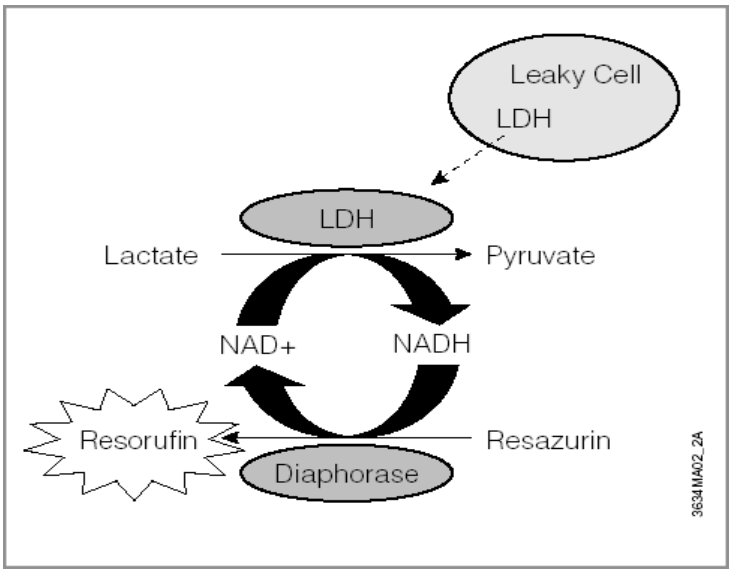

Fig. 5.1. Release of LDH from damaged cells is measured by supplying lactate, NAD+, and resazurin as substrates in the presence of diaphorase. Generation of the fluorescent resorufin product is proportional to the amount of LDH. http://www.promega.com/tbs/tb306/tb306.pdf

Controls were performed by supplementing the cell cultures with identical volumes of aggregation buffer for the same lengths of time. After treatment, 100 µl of CytoTox-ONETM Reagent containing substrates were added to each well of the plate, previously equilibrated at room temperature, and cells were then incubated at room temperature for a further 10 min. The reaction was then stopped with 50 µl of Stop Solution and the fluorescence measured using an automatic fluorescence microplate reader Fluoroskan Ascent FL (Thermo Electron), according to the manufacturer's instructions. As a positive control, cells were lysed in 9 % Triton X-100 prior to reagents addition, resulting in the maximum LDH release. The increase in the proportion of LDH release in cells treated with hIAPP aggregates is expressed as a fraction of that observed in cells treated with identical volumes of aggregation buffer.

5.6. Immunofluorescence analysis

The cells were seeded on glass cover slips and treated with hIAPP aggregates aged for 30 min at 25°C (3.25 µM in 10 mM phosphate buffer, pH 7.4, containing 1.0% HFIP, with or without 30

μM oleuropein) prior to dilution in the culture medium in the presence or in the absence of the oleuropein aglycon (final concentrations: 200 nM hIAPP, 1.8 μM oleuropein). In this case it was necessary to treat cells with a higher concentration of hIAPP (and, consequently, of oleuropein) to subsequently obtain a clear immunodetection.

After 5 h of incubation, the cells were washed with PBS, fixed with 2.0% p-formaldheyde for 20 min and incubated overnight at 4°C with rabbit anti-amylin antibodies (1:300). Then the cells were stained with Alexafluor 488-labeled anti-rabbit antibodies (1:400), and propidium iodide was used to image nuclei. Control cells, not treated with hIAPP, were incubated with primary and secondary antibodies as well. Images were obtained using a Leica SP5 laser scanning confocal microscope (Fig. 5.2).

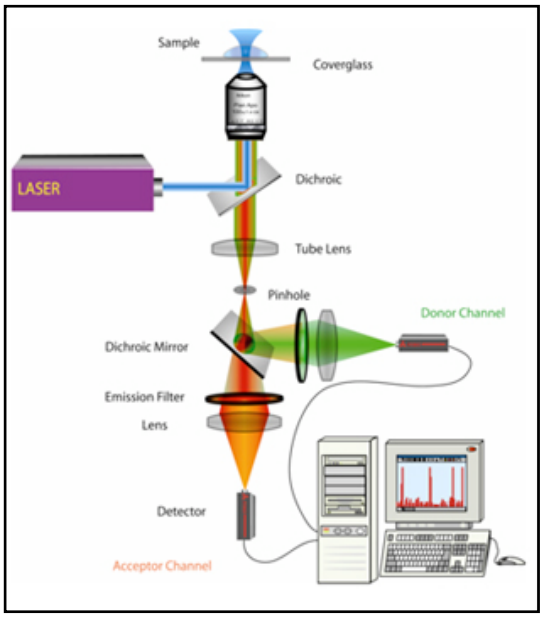

Fig. 5.2. Scheme of the operating of a confocal microscope.

5.7. Preparation of phospholipid unilamellar vesicles

DOPS and DOPC in chloroform solutions were mixed (DOPS:DOPC=3:7 [**222**]) and dried in glass tubes (8 mm in diameter) under a gentle nitrogen stream in a fume hood. For the calcein release assay, the dry lipid film was resuspended in 1.0 M Tris–HCl, pH 8.0, containing 60 mM calcein, at a final lipid concentration of 1.0 mg/ml. The resuspended samples were incubated for 1h at room temperature to allow lipid vesicles formation, vortexing occasionally. The resulting suspension was then subjected to five freeze-thaw cycles of 2.0 min each, using liquid nitrogen and a 37°C water bath, and sonicated for 20 min at 20 kHz in ice to obtain a suspension of small unilamellar vesicles (SUV) with diameters ranging between 50 and 200 nm. Sonication provides the energy input required to downsizing the lipid particles. Vesicle mean size and distribution are influenced by lipid composition and concentration, temperature, sonication time and power, volume, and sonicator tuning. Since it is nearly impossible to reproduce the

conditions of sonication, size variation between batches produced at different times is not uncommon. Also, due to the high degree of curvature of these membranes, SUV are inherently unstable and will spontaneously fuse to form larger vesicles when stored below their phase transition temperature. To remove large lipid aggregates the preparation was centrifuged for 10 min at 10,000xg. The non-encapsulated calcein was removed by gel-filtration using a Sephadex G-50 column (1.5x7.0 cm); the vesicles were eluted in 10 mM phosphate buffer, pH 7.4, and used within 48 h.

Before each experiment, the size distribution of the vesicle population was checked using a Zetasizer Nano S dynamic light scattering device from Malvern Instruments (Malvern, Worcestershire, UK) and the nonhomogeneous lipid populations were discarded. DLS provides the mean diameter and distribution of the vesicles.

5.8. Calcein release assay

The assay was performed according to Engel et al. [**222**] with some modifications. 3.25 μM hIAPP was induced to aggregate at 25°C in the presence or in the absence of 30 μM oleuropein in 10 mM phosphate buffer, pH 7.4, containing 1.0% HFIP in a multiwell microplate for increasing time periods. Control mixtures without hIAPP were similarly prepared. At the end of the aggregation periods, the sample volumes were adjusted to 200 μl and the SUV suspension (1:40) was added.

Calcein is a polyanionic fluorescein derivative carrying about five negative and two positive charges at pH 7 (Fig. 5.3).

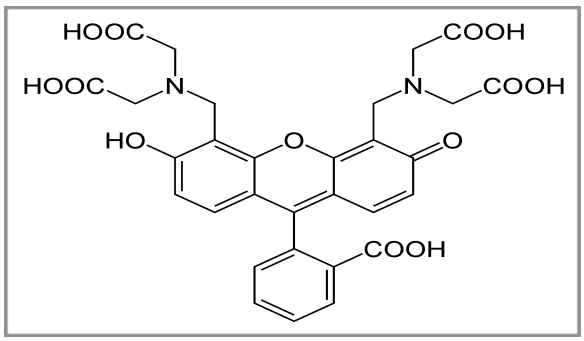

Fig. 5.3. Structure of calcein.

Calcein is self-quenched into the vesicle aqueous core; therefore, calcein leakage to the external medium upon hIAPP aggregates-vesicles interaction can be monitored as an increase in calcein fluorescence, due to its dilution and the consequent reduction of self-quenching. At regular time intervals, measurements were performed using the fluorescence microplate reader Fluoroskan Ascent FL (Thermo Electron). The excitation and emission wavelengths were 485

nm and 538 nm, respectively. The percentage of calcein release was calculated according to the following formula:

Calcein release (%)= (F- F_0) x100/ (Fmax-F_0)

where F is the fluorescence measured at the different time intervals during the experiment, F_0 is the fluorescence measured at the beginning of the experiment and Fmax is the maximum fluorescence, as determined by disrupting the vesicles at the end of each experiment by adding 0.1 % Triton X-100. Each curve was obtained after a point by-point subtraction of the data coming from its control mixture.

5.9. ThT assay

ThT is widely used as a non-covalent fluorescent probe in studies of protein aggregation and amyloid fibril formation [**9**]. It is characterized by a two ring structure arranged in an almost planar orientation (φ ~ 30°) in the minimum energy conformation (Fig. 5.4). During excitation, the conjugated benzothiazol and aminobenzol rings rotate in order to obtain the most stable excited state conformation (φ ~ 90°), which has low fluorescence efficiency. Polar solvents and viscosity can affect the reorientation of the rings and thus the ThT fluorescence. The binding of amyloid-like aggregates probably stabilizes the planar form of the molecule and leads to a 10-500-fold increase in ThT fluorescence intensity [**9**]. It is tempting to speculate that ThT may bind between the β-sheets of the fibril, however no experimental data exist to support this hypothesis.

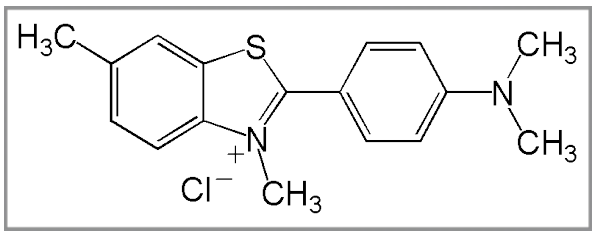

Fig. 5.4. Structure of ThT.

For these experiments, hIAPP 3.25 μM was incubated at 25°C in 10 mM phosphate buffer, pH 7.4, containing 1.0% (v/v) HFIP in the presence or in the absence of increasing concentrations of the oleuropein aglycon, for 24 h; 50 μl of this solution was withdrawn at different time intervals and mixed with 350 μl of 20 μM ThT dissolved in 0.1 M Gly/NaOH buffer, pH 8.5. Blanks were prepared similarly but without hIAPP. As a control, oleuropein was also added to every hIAPP sample aggregated in the absence of oleuropein, before or after ThT addition, and just prior to fluorescence reading. Fluorescence emission intensity was measured (440 nm

Excitation/485 nm Emission) [**223**] using a Perkin Elmer LS 55 spectrofluorimeter equipped with a thermostated cell-holder attached to a Haake F8 water-bath in order to perform the experiments at 25°C. Dose-dependence analysis was performed by assaying hIAPP samples incubated with a 1X, 3X or 9X molar concentration of oleuropein for 24 h. In experiments parallel to CD analysis 6.5 μM hIAPP was used and oleuropein concentration correspondingly adjusted.

5.10. Circular dichroism

CD spectroscopy measures differences in the absorption of left-handed polarized light *versus* right-handed polarized light which arise due to structural asymmetry. This phenomenon is exhibited in the absorption bands of an optically active molecule and it is acquired by a CD Spectrometer, an instrument that records this phenomenon as a function of wavelength.

The absence of regular structure results in zero CD intensity; instead an ordered structure leads to a spectrum which can contain both positive and negative signals. CD can be used to determine secondary structure of proteins in the "far-UV" spectral region (190-250 nm) and at these wavelengths the chromophore is the peptide bond itself.

α-helix, β-sheet and random coil structures each give rise to a characteristic shape and magnitude of CD spectrum, as showed in Fig. 5.5. For example, a β-sheet structure of an amyloid protein exhibits a spectrum with two characteristic peaks: a positive one around 193 nm and a negative one around at 218 nm.

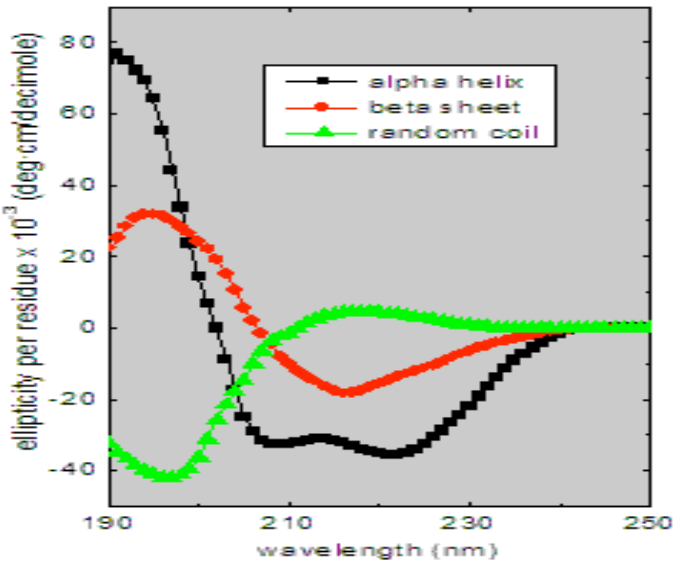

Fig. 5.5. CD spectra of different secondary protein structures.

For these kind of analysis, hIAPP 6.5 μM was diluted in 10 mM phosphate buffer, pH 7.4, containing 1.0% (v/v) HFIP, in the presence or in the absence of 50 μM oleuropein aglycon. The

spectra of the mixtures were recorded at 25°C (1.0 nm data pitch, 50 nm/min speed, and 1 s response) with subsequent accumulations, using a Jasco J-810 spectropolarimeter equipped with a thermostated cell holder attached to a Thermo Haake C25P waterbath. Background spectra were acquired for buffer with and without 50 μM oleuropein and subtracted to the sample spectra to obtain the clear spectra.

5.11. Electron microscopy

In this technique a beam of electrons is transmitted through an ultra thin specimen, interacting with it as it passes through. An image is formed from the interaction of the electrons transmitted through the specimen; then the image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film or to be detected by a sensor such as a CCD camera.

hIAPP was incubated with or without oleuropein aglycon as for CD analysis and a 3.0 μl sample was loaded onto a formvar/carbon-coated grid 400 mesh nickel grid (Fig. 5.6) which was then blotted. When the grid was dried, it was negatively stained with 1.0 % (w/v) uranyl acetate. After wicking off the excess stain, the grids were allowed to air-dry and observed under the electron microscope, using a JEM 1010 transmission electron microscope (TEM) at 80 kV excitation voltage (Fig. 5.7).

Fig. 5.6. Image of a grid used to analyze a sample at a TEM.

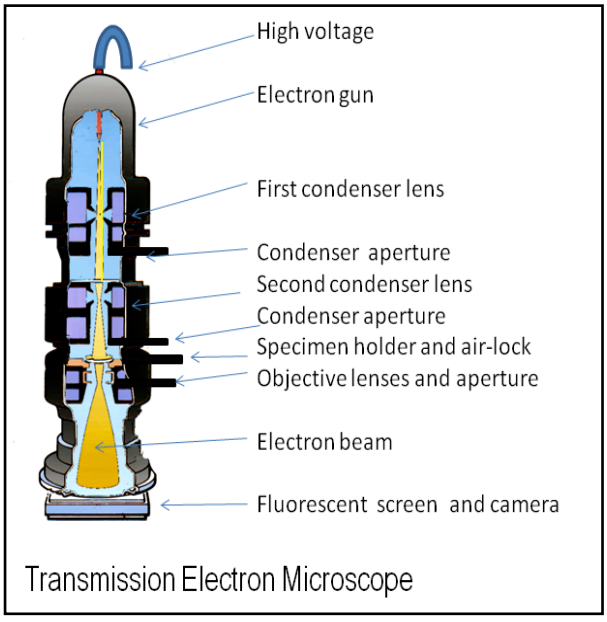

Fig. 5.7. Scheme of the operating of a TEM.