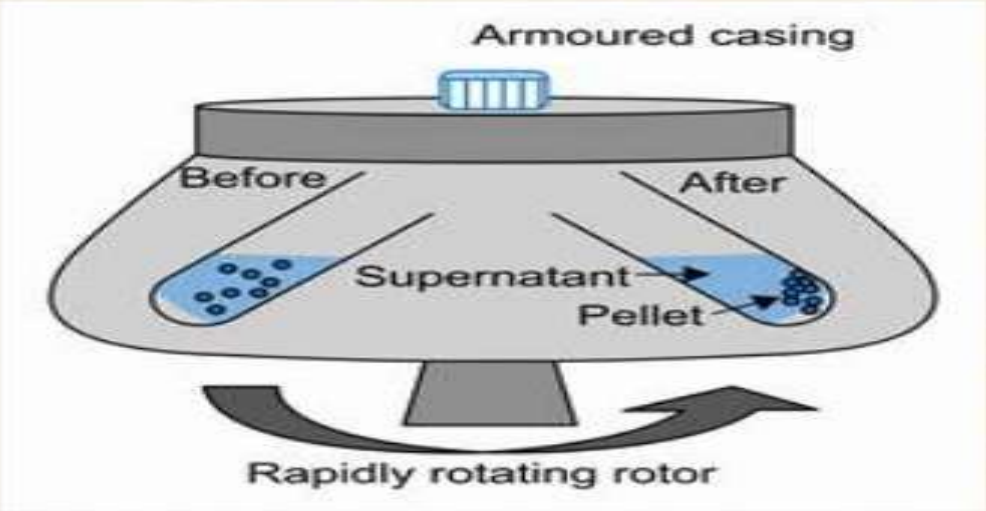
CENTRIFUGATION

For Sem IV

Continuation from last class lecture

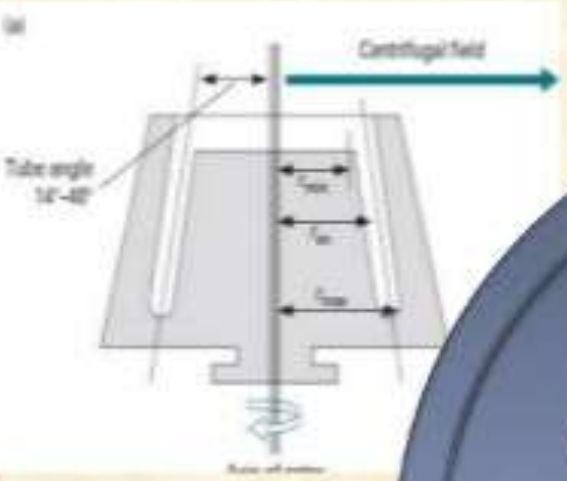


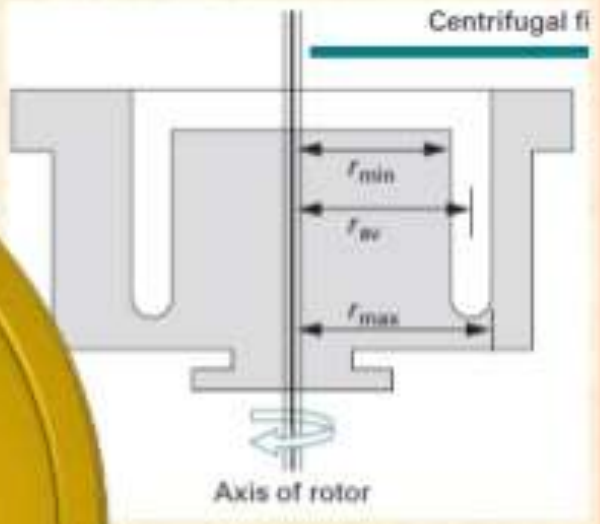
- Common feature of all centrifuges is the central motor that spins a rotor containing the samples to be separated.

CENTRIFUGE ROTOR

- A centrifuge rotor is the rotating unit of the centrifuge, which has fixed holes drilled at an angle. Test tubes are placed inside these holes and the rotor spins to aid in the separation of the materials.

Types of rotor

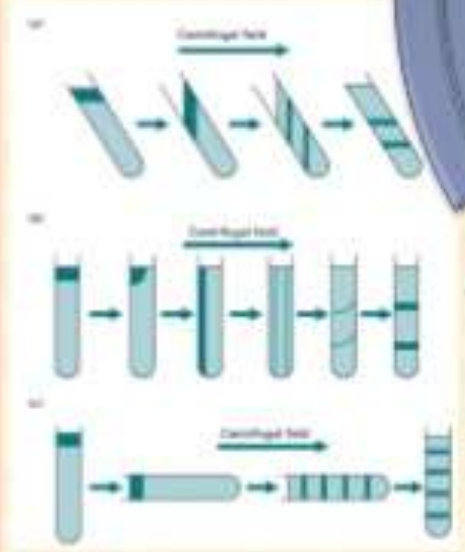


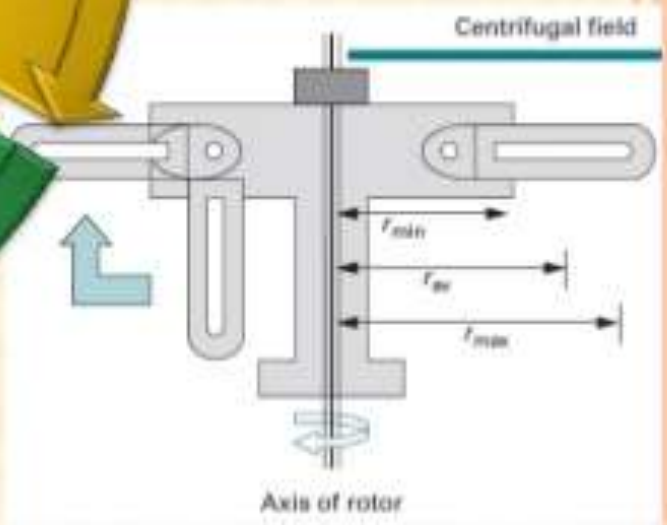


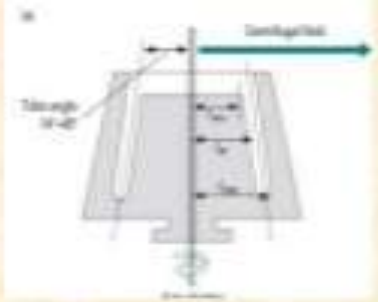
Fixed angle rotors

Vertical tube rotors

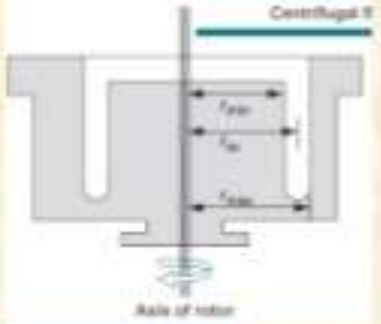
Swinging-bucket rotors







Types of rotor



Fixed angle rotors

- Tubes are held at angle of 14 to 40^0 to the vertical.
- Particles move radially outwards, travel a short distance.
- Useful for differential centrifugation
- Reorientation of the tube occurs during acceleration and deceleration of the rotor.

Vertical tube rotors

- Held vertical parallel to rotor axis.
- Particles move short distance.
- Time of separation is shorter.
- Disadvantage: pellet may fall back into solution at end of centrifugation.

Fixed-angle Rotor

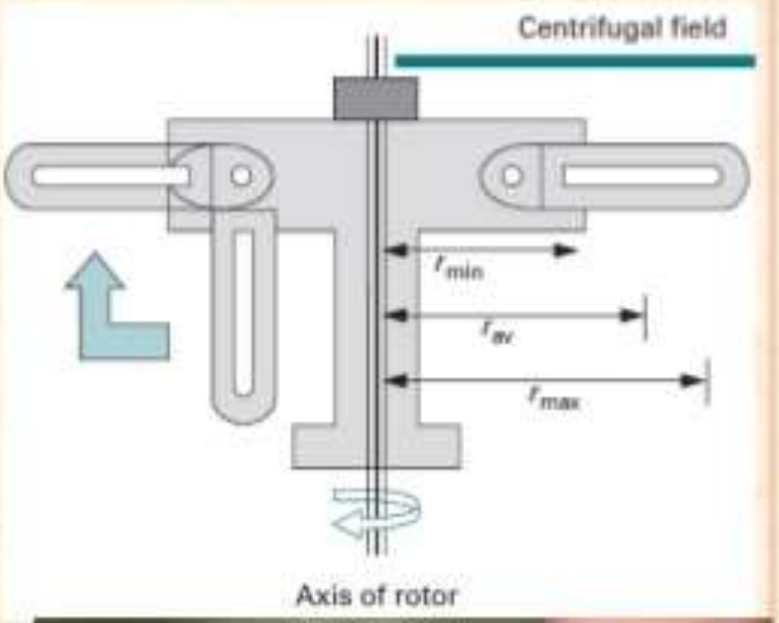
Vertical Rotor

swing-bucket Rotor

Types of rotor

Swinging-bucket rotors

- Sing out to horizontal position when rotor accelerates.
- Longer distance of travel may allow better separation, such as in density gradient centrifugation.
- Easier to withdraw supernatant without disturbing pellet.
- Normally used for density-gradient centrifugation.



Types of centrifuge

- Desk top centrifuges
- High speed centrifuges
- Ultracentrifuges
  - Analytical
  - Preparative

LOW SPEED CENTRIFUGE

- Most laboratories have a standard low-speed centrifuge used for routine sedimentation of heavy particles.
- The low speed centrifuge has a maximum speed of 4000-5000rpm.
- These instruments usually operate at room temperatures with no means of temperature control.
- Two types of rotors are used in it, fixed angle and swinging bucket.
- It is used for sedimentation of red blood cells until the particles are tightly packed into a pellet and supernatant is separated by decantation

Desk top centrifuge

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HIGH SPEED CENTRIFUGE

- High speed centrifuges are used in more sophisticated biochemical applications, higher speeds and temperature control of the rotor chamber are essential.
- The operator of this instrument can carefully control speed and temperature which is required for sensitive biological samples.
- Three types of rotors are available for high speed centrifugation-fixed angle, swinging bucket, vertical rotors

High speed centrifuges

ULTRA CENTRIFUGE

- It is the most sophisticated instrument
- Intense heat is generated due to high speed thus the spinning chambers must be refrigerated and kept at high vacuum.
- It is used for both preparative work and analytical work.

Ultracentrifuges

- Operate at speed of 75,000rpm, providing the centrifugal force of 500,000g.
- Rotor chamber is sealed and evacuated by pump to attain vacuum.
- Refrigeration system (temp 0-4^{0}C).
- Rotor chamber is always enclosed in a heavy armor plate.
- Centrifugation for isolation and purification of components is known as preparatory centrifugation, while that carried out with a desire for characterization is known as analytical centrifugation.

History:

- The first analytical ultracentrifuge was developed by Svedberg in 1920

ULTRACENTRIFUGATION

- Svedberg coined the term “ultracentrifuge". He was colloid chemist.
- He used the ultracentrifuge to determine the MW and subunit structure of hemoglobin , studies which changed the ideas concerning the structure of proteins.
- The first commercial ultracentrifuge was produced in 1940 by SPINCO.

Preparative centrifugation

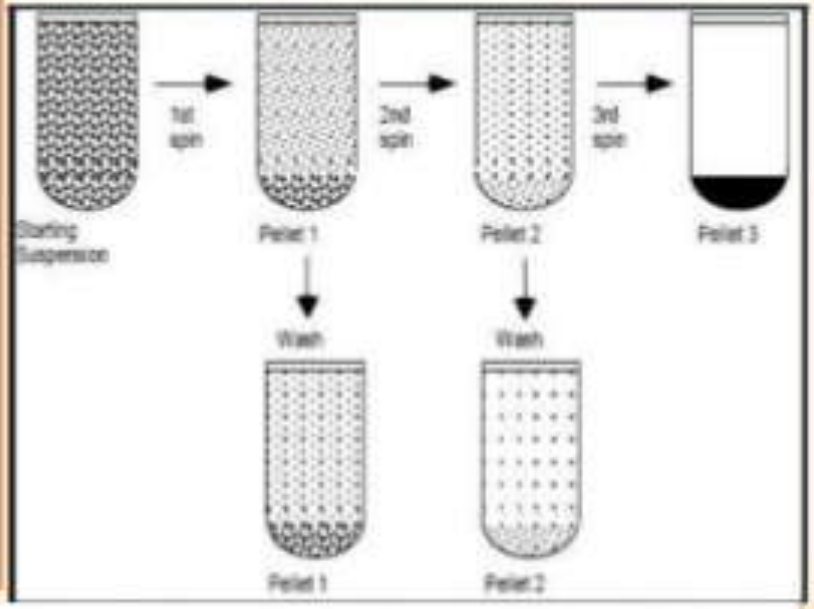
- Is concerned with the actual isolation of biological material for subsequent biochemical investigations.
- Divided into two main techniques depending on suspension medium in which separation occur.
  - Homogenous medium – differential centrifugation
  - Density gradient medium – density gradient centrifugation

1. Differential centrifugation

- Separation is achieved based in the size of particles in differential centrifugation.
- Commonly used in simple pelleting and obtaining the partially pure separation of subcellular organelles and macromolecules.
- Used for study of subcellular organelle, tissues or cells (first disrupted to study internal content)

$$t = \frac{9\eta}{2r_p^2\omega^2(\rho_p - \rho_m)} \ln \frac{r_{bottom}}{r_{upper}} \text{-----(5)}$$

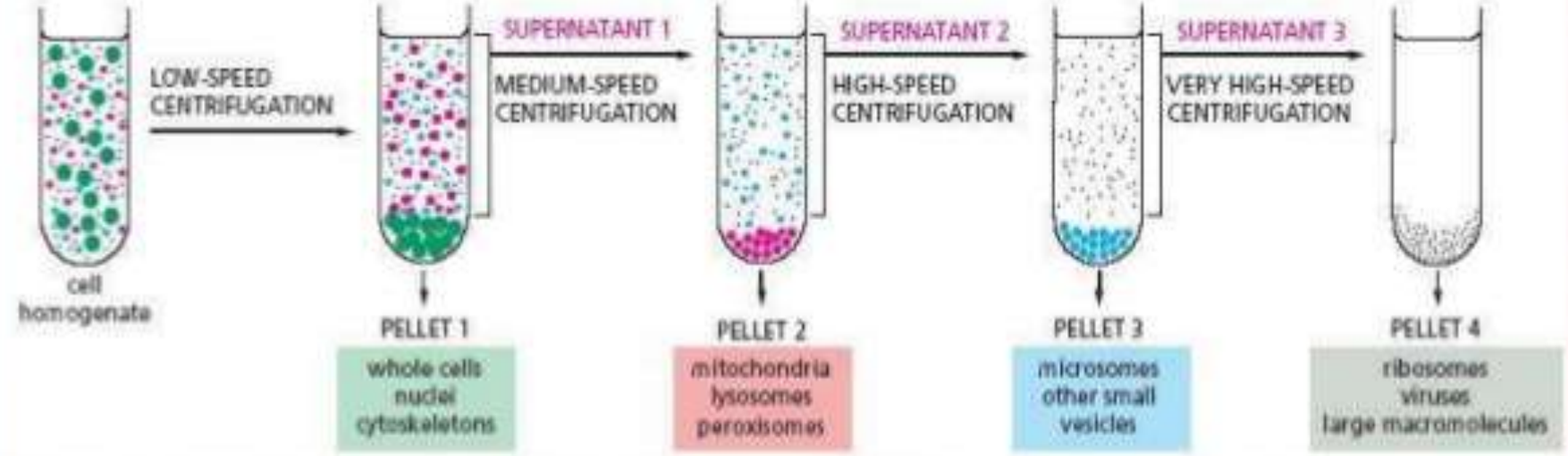
- During centrifugation, larger particles sediment faster than the smaller ones.
- At a series of progressive higher g-force generate partially purified organelles.



DIFFERENTIAL CENTRIFUGATION

Repeated centrifugation at progressively higher speeds will fractionate cell homogenates into their components.

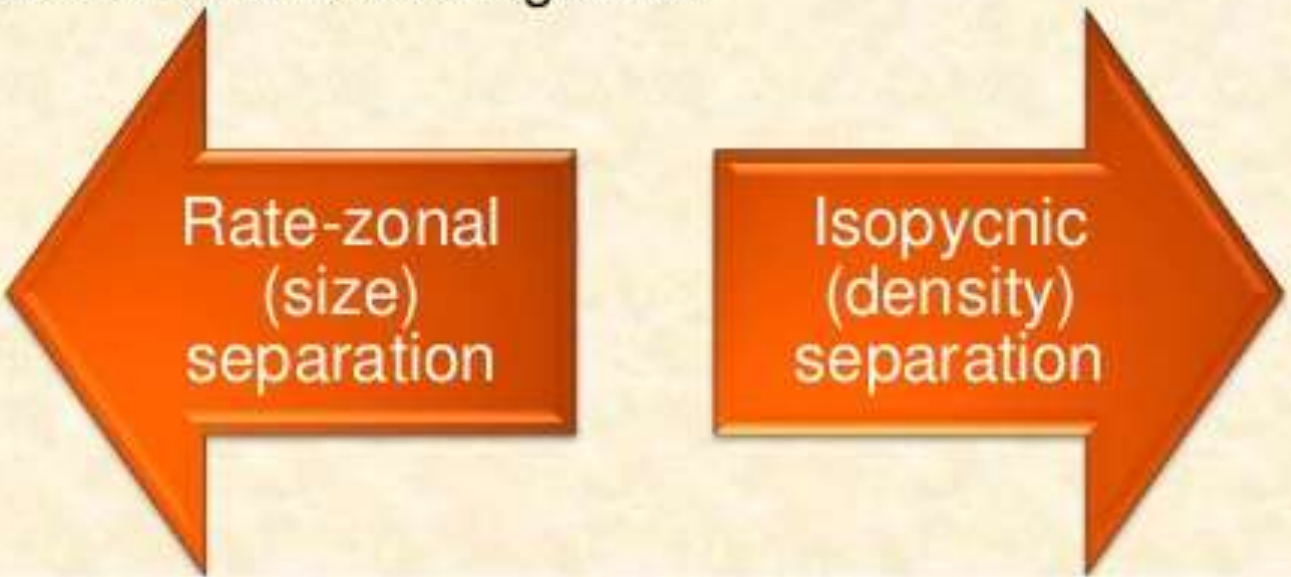
Centrifugation separates cell components on the basis of size and density. The larger and denser components experience the greatest centrifugal force and move most rapidly. They sediment to form a pellet at the bottom of the tube, while smaller, less dense components remain in suspension above, a portion called the supernatant.



- Inspite of its reduced yield differential centrifugation remains probably the most commonly used method for isolation of intracellular organelle from tissue homogenates because of its;
  - relative ease
  - Convenience
  - Time economy
- Drawback is its poor yield and fact that preparation obtained never pure.

2. Density gradient centrifugation

- It is the preferred method to purify subcellular organelles and macromolecules.
- Density gradient can be generated by placing layer after layer of gradient media such as sucrose in tube, with heaviest layer at the bottom and lightest at the top in either.
- Classified into two categories:



2.1 Rate zonal Centrifuagation

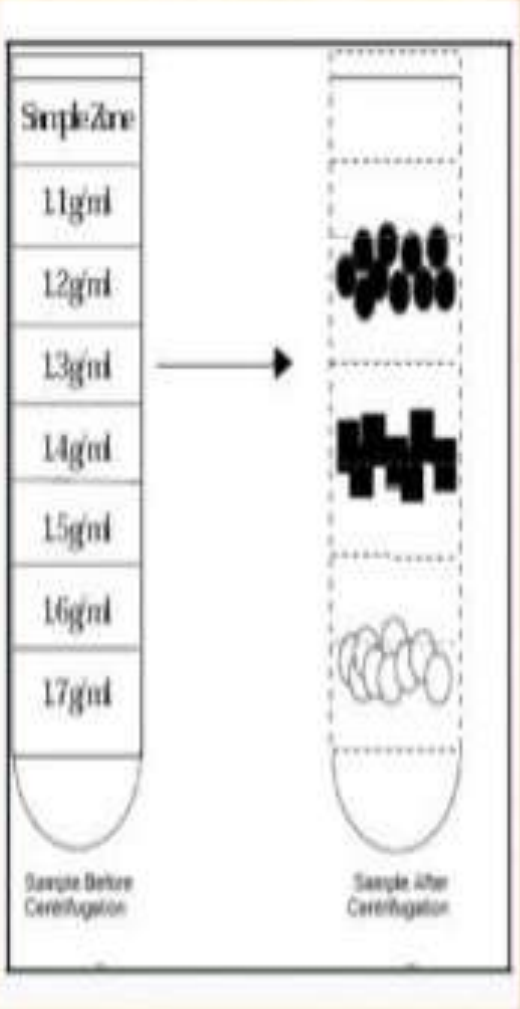
$$v = \frac{2}{9}\frac{r_p^2(\rho_p - \rho_m)\omega^2 r}{\eta}$$

$$\rho_{m(\max)} < \rho_{p(\min)}$$

- Here the gradient used has maximum density below that of least dense sedimenting particle.
- Hence the density gradient is reasonably shalow
- The sample particle travel through the steep gradient and form discrete zones depending upon their sedimenting time
- Particles which differ in size but not in density are separated

2.1 Rate zonal centrifugation

- Gradient centrifugation.
- Take advantage of particle size and mass instead of particle density for sedimentation.
- Ex: for common application include separation of cellular organelle such as endosomes or proteins (such as antibodies)



2.2 Isopycnic centrifugation

$$v = \frac{2}{9}\frac{r_p^2(\rho_p - \rho_m)\omega^2 r}{\eta}$$

$$\rho_{m(\max)} > \rho_{p(\max)}$$

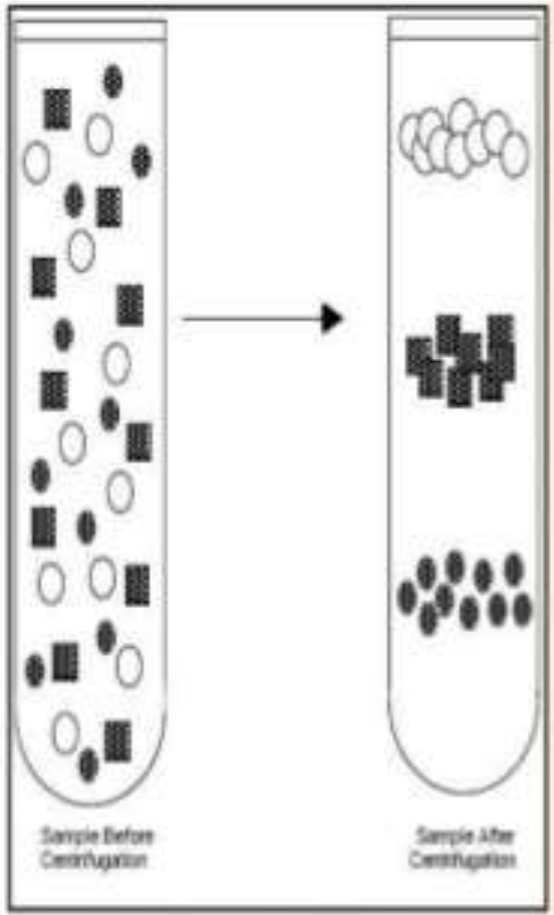
Also known as sedimentation equlibrium centrifugation

- Here the gradient used here has maximum density greater than that of most dense sedimenting particle.

2.2 Isopycnic centrifugation

- Particle of a particular density will sink during centrifugation until a position is reaches where the density of the surrounding solution is exactly the same as the density of the particle.
‘
- Once quasi-equilibrium is reached, the length of centrifugation doesnot have any influence on the migration of particle.
- Ex: separation of Nucleic acid in CsCl (Caseium chloride) gradient.

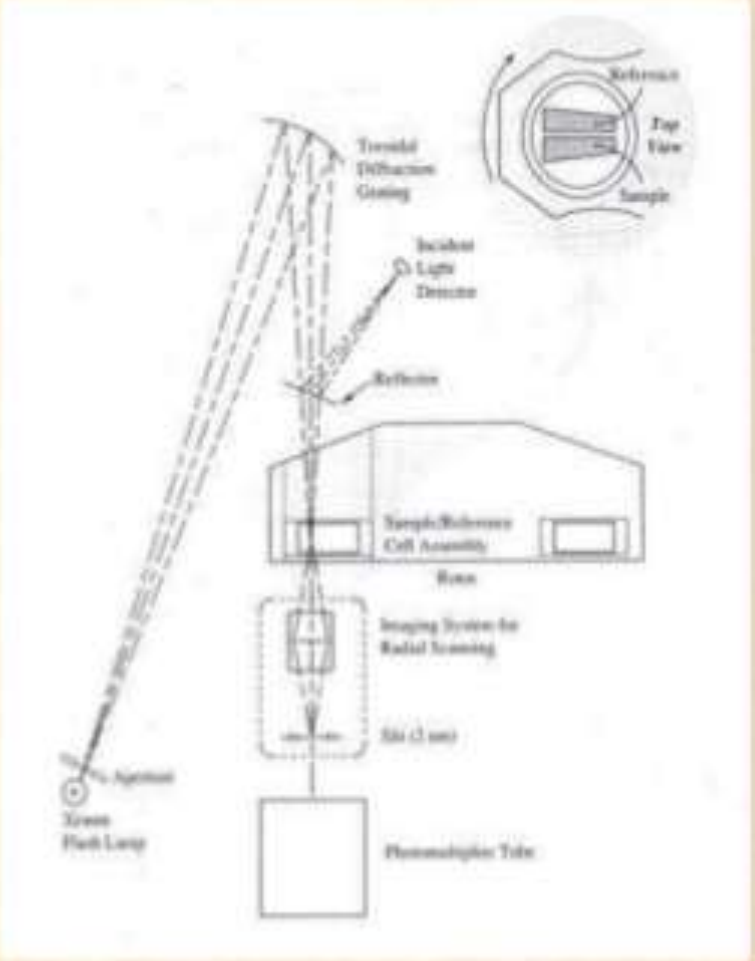
Figure 3. ISOPYCNIC (DENSITY) SEPARATION



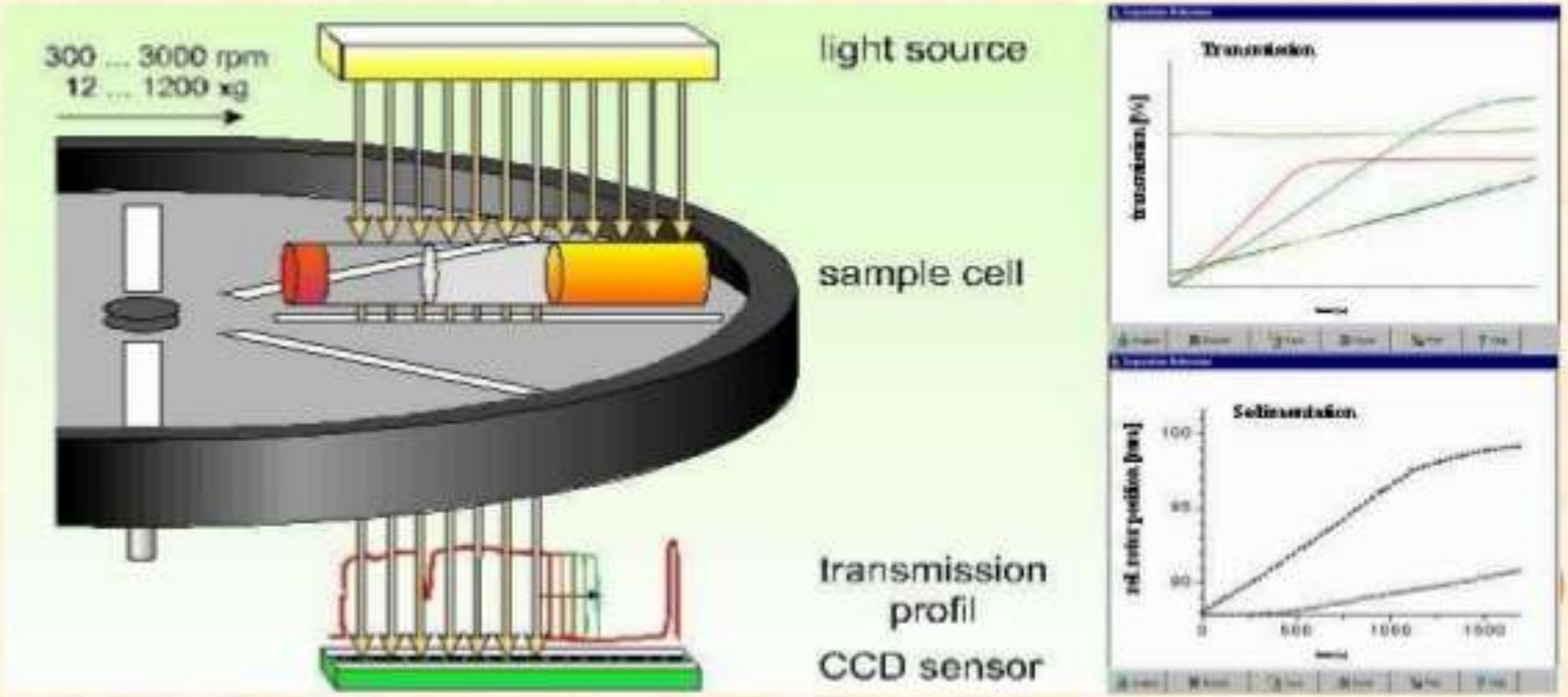
	Rate-Zonal	Isopycnic
Synonym	S-zonal, sedimentation velocity	Density equilibrium, sedimentation equilibrium
Gradient	•Shallow, •Maximum gradient density less than the least dense sedimenting specie, •Gradient continuous.	•Steep, •Maximum gradient density greater than that of the most dense sedimenting specie, •Continuous or discontinuous gradients.
Centrifuga-tion	•Incomplete sedimentation, •Low speed, •Short time	•Complete sedimentation till equilibrium is achieved, •High speed, • Long time.
Separation	RNA- DNA hybrids, ribosomal subunits, etc.,	DNA, plasma lipoproteins, lysosomes, mitochondria, peroxisomes, etc.,

Analytical centrifugation

- Speed – 70000 rpm, RCF – 5 lakh g
- Motor, rotor ,chamber that is refrigerated and evacuated and optical system
- Optical system has light absorption system ,schleiren system & Rayleigh inferometric system
- 2 cells – analytical cell and counterpoise cell



- Optics used – schlieren optics or Rayleigh interference optics
- At beginning , peak of refractive index will be at meniscus.
- With progress of sedimentation, macromolecules move down – peak shifts giving direct information about the sedimentation characteristics.



Analytical centrifugation

- Purity of macromole
- Relative molecular mass of solute (within 5% SD)
- Change in relative molecular mass of supermolecular
- complexes
- Conformational change of protein structure
- Ligand-binding study

Types of Centrifuges & applications			
	Types of centrifuge		
Characteristic	Low Speed	High Speed	Ultracentrifuge
Range of Speed (rpm)	1-6000	1000-25,000	20-80,000
Maximum RCF (g)	6000	50,000	6,00,000
Refrigeration	some	Yes	Yes
Applications			
Pelleting of cells	Yes	Yes	Yes
Pelleting of nuclei	Yes	Yes	Yes
Pelleting of organelles	No	Yes	Yes
Pelleting of ribosomes	No	No	Yes
Pelleting of Macromolecules	No	No	Yes

Operation

- Tubes recommended by their manufacturer should be used.
- Top of tube should not protrude so far above the bucket.
- Properly balanced- weight of racks, tubes, and content on opposite side of a rotor should not differ by more than 1%. (Centrifuges auto balance are available).
- Should centrifuge before unstopper the tubes.
- Cleanliness –minimizing the possible of spread of infection (hep Virus).
- Spillage and break of tube should be considered as the bloodborne pathogen hazard.
- Speed of centrifuge should be checked once 3m.
- Centrifuge timer to be checked per week.

Application

- In clinical laboratory, centrifugation is used to;
  - Remove cellular elements from blood to provide cell free plasma or serum for analysis.
  - Remove chemically precipitated protein from an analytical specimen.
  - Separate protein bound from free ligand in immunochemical and other assay.
  - Separation of the subcellular organelle, DNA, RNA.
  - Extract solutes in biological fluids from aqueous to organic solvents.
  - Separate lipid components.

Meselson and Stahl experiment

- Matt Meselson and Frank Stahl designed **the most beautiful experiment in biology.**

- The experiment tests the DNA replication models.

- They used bacteria grown in a media of a heavy isotope of nitrogen .

Meselson and Stahl experiment

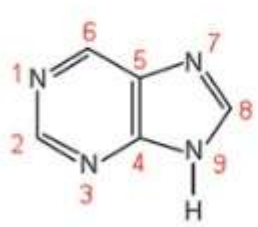
- In nature, some elements have different isotopes which they differ in the number of neutrons.
- The difference in the number of neutrons makes the isotopes' atoms heavier.

Meselson and Stahl experiment

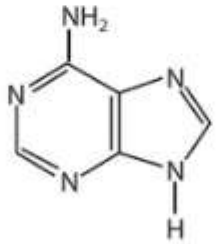
- The abundant nitrogen is N^{14} where there are 7 protons and 7 neutrons and it weights 14g/ mole.
- N^{15} is a stable isotope of nitrogen and has 7 protons and 8 neutrons and weights 15g/mole.

Why heavy nitrogen isotope N^{15}?

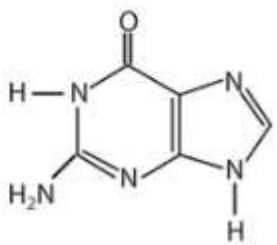
RNA only DNA only both DNA and RNA



Purine



Adenine (A)



Guanine (G)

Remember DNA bases contain **Nitrogen**

Meselson and Stahl experiment

The experiment:

1) Grow bacteria in a media containing the heavy N^{15} for many generations.

Result: all bacteria has N^{15} in their DNA.

Meselson and Stahl experiment

The experiment:

2) Transfer some N^{15} bacteria to grow in N^{14} media and allow to grow for several generations.

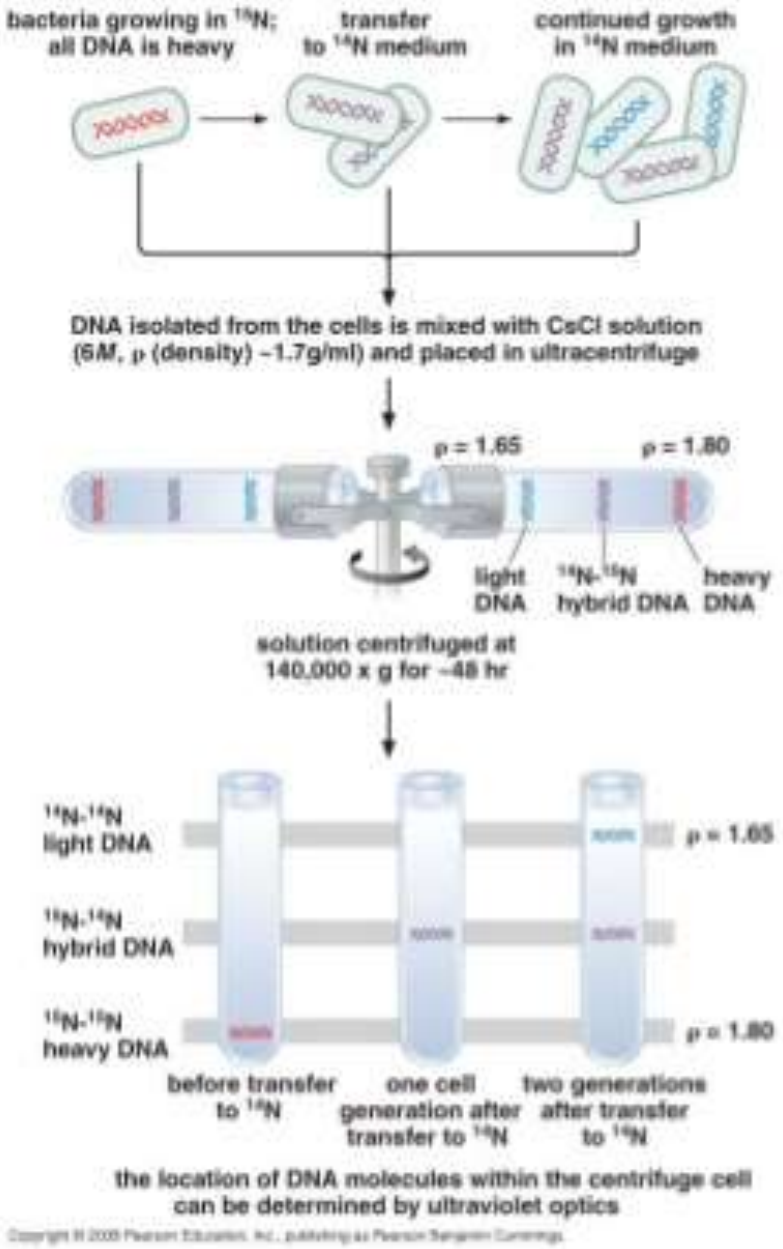
Result: DNA of newly divided bacteria will have N^{14} instead of N^{15}.

Meselson and Stahl experiment

The experiment:

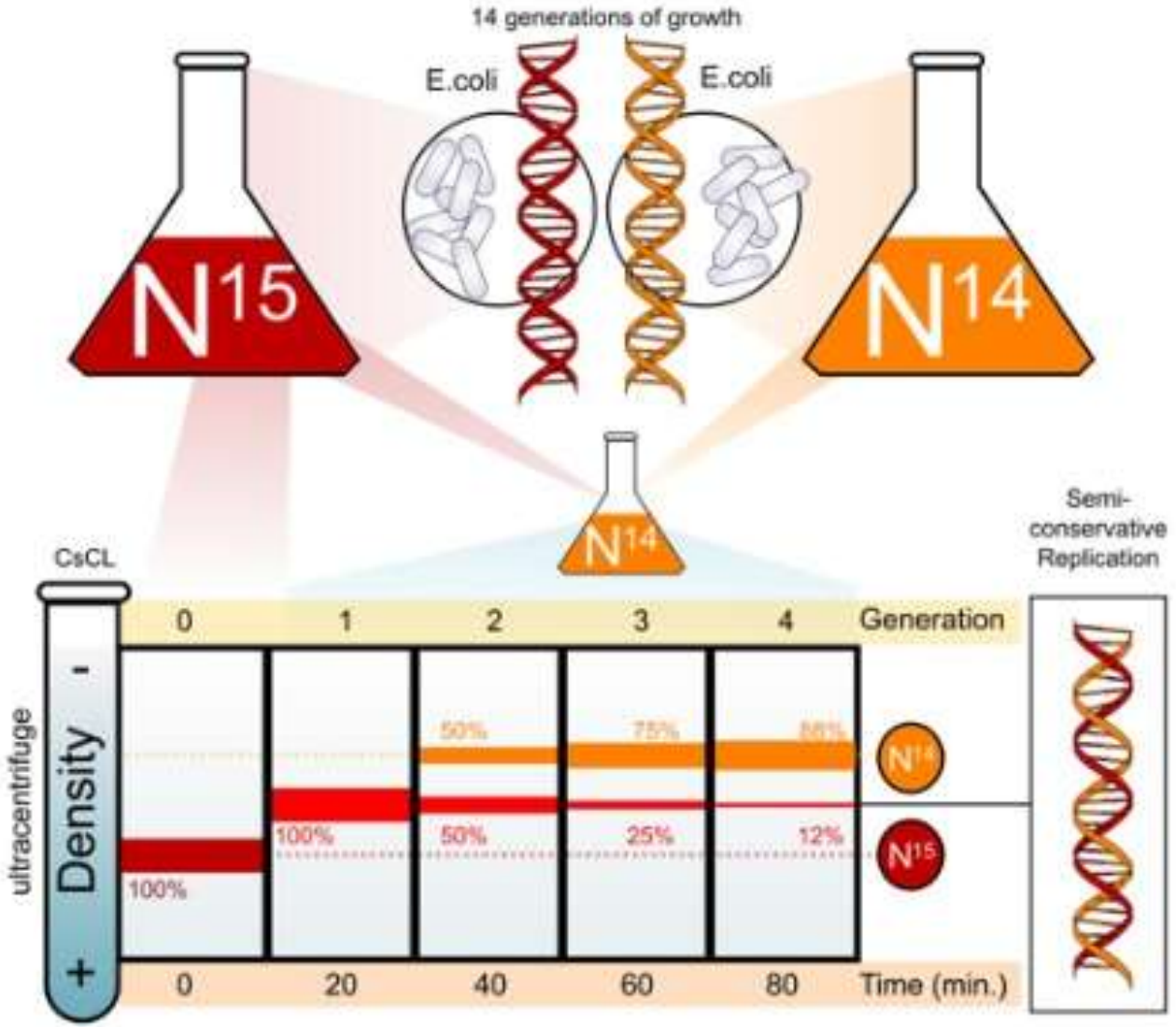
3) Take samples from the growing bacteria at different time and study the density of the DNA of the cells.

Meselson and Stahl experiment



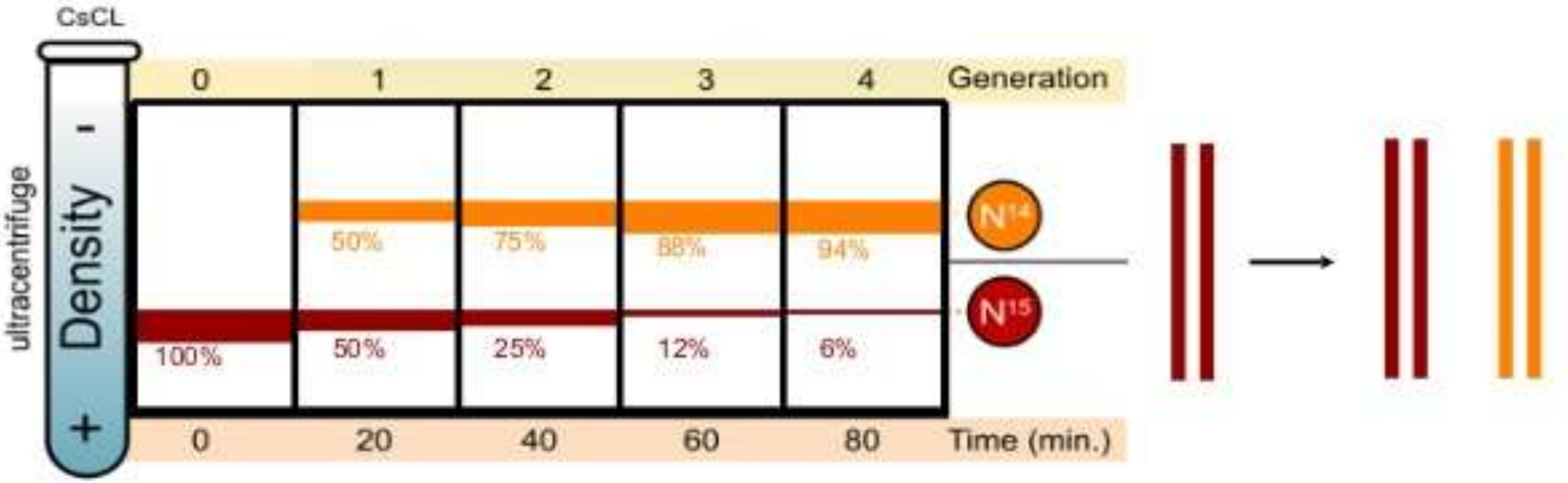
Simply the idea is to mark the old DNA and see what happens to the newly synthesized DNA.

Meselson and Stahl experiment



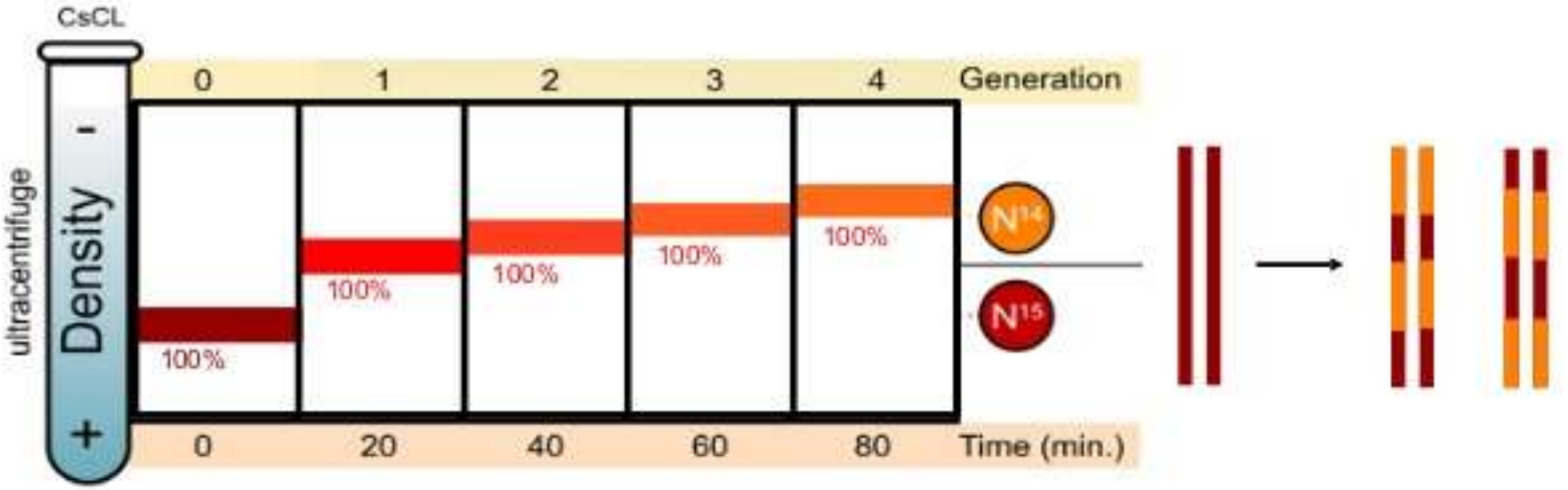
What would the results be if another model?

Conservative replication



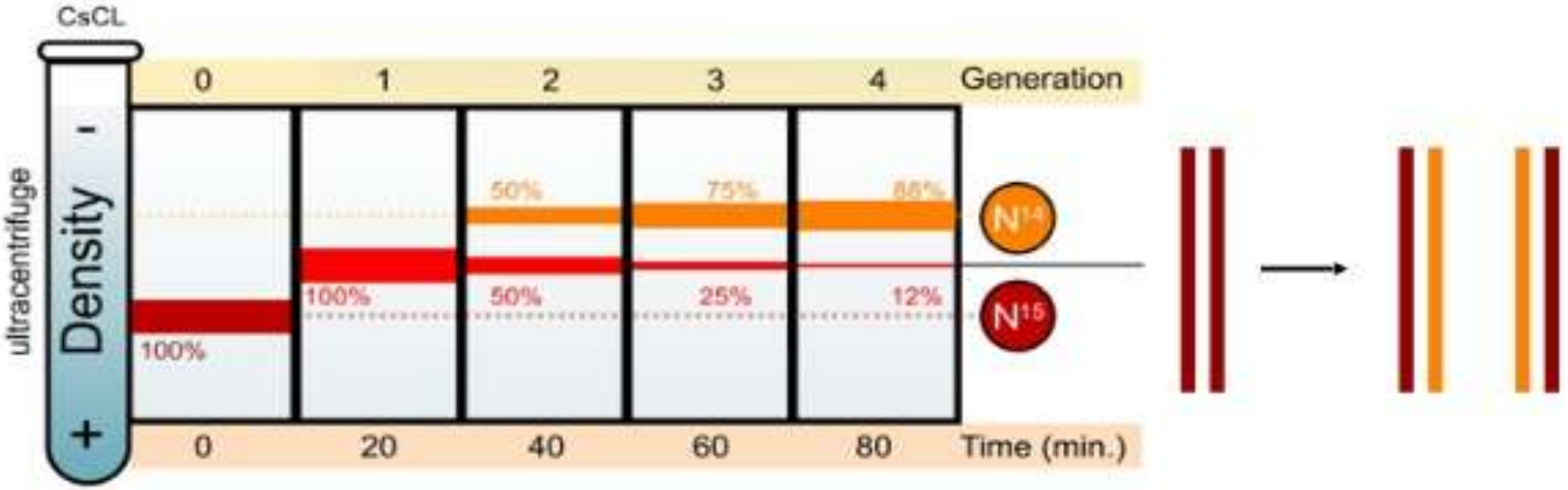
What would the results be if another model?

Dispersive replication



What would the results be if another model?

Semi-conservative replication



References :

- **Tietz** – Clinical Chemistry And Molecular Diagnostic
- **Keith Wilson and John Walker** – Principle And Technique In Biochemistry And Molecular Biology.
- **Avinash Upadhyay** – Biophysical Chemistry.
- Internet sources.

THANK YOU