Ministry of Higher Education and Scientific Research
University of Baghdad
College of Science
Department of Biology

Practical Immunology
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المرحلة الثالثة ـ الدراستان الصباحية والمسائية
الفصل الدراسي الأول

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Lab One

Animal Marking

Lab: 1→10

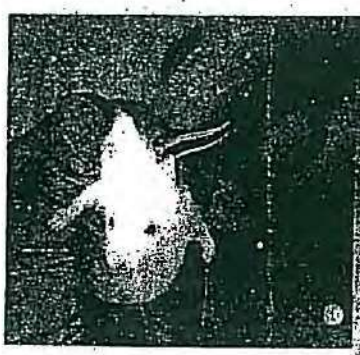
مناعـــــة على

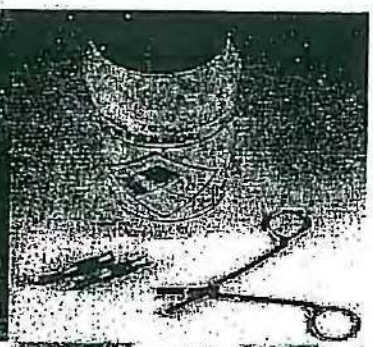
تالـــت بايـــو

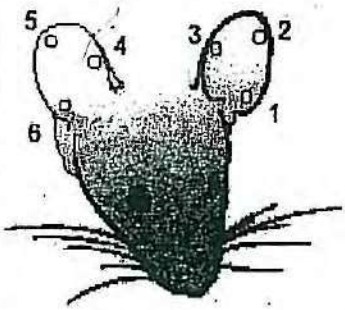
Animal Marking

1. **Temporary.**
   - Cage marking.
   - Dyes.
   - Hair clip.
   - Tagging.

2. **Permanent.**
   - Natural marking.
   - Branding.
   - Ear notching.
   - Tattooing.
   - Microchips transponders.







The marking method ultimately chosen should be simple, easy to apply, easy to read, harmless to the animal, and offer no potential interference with experimentation or interpretation of the data. When selecting marking methods, careful consideration should be given to the following factors prior to making final judgment:

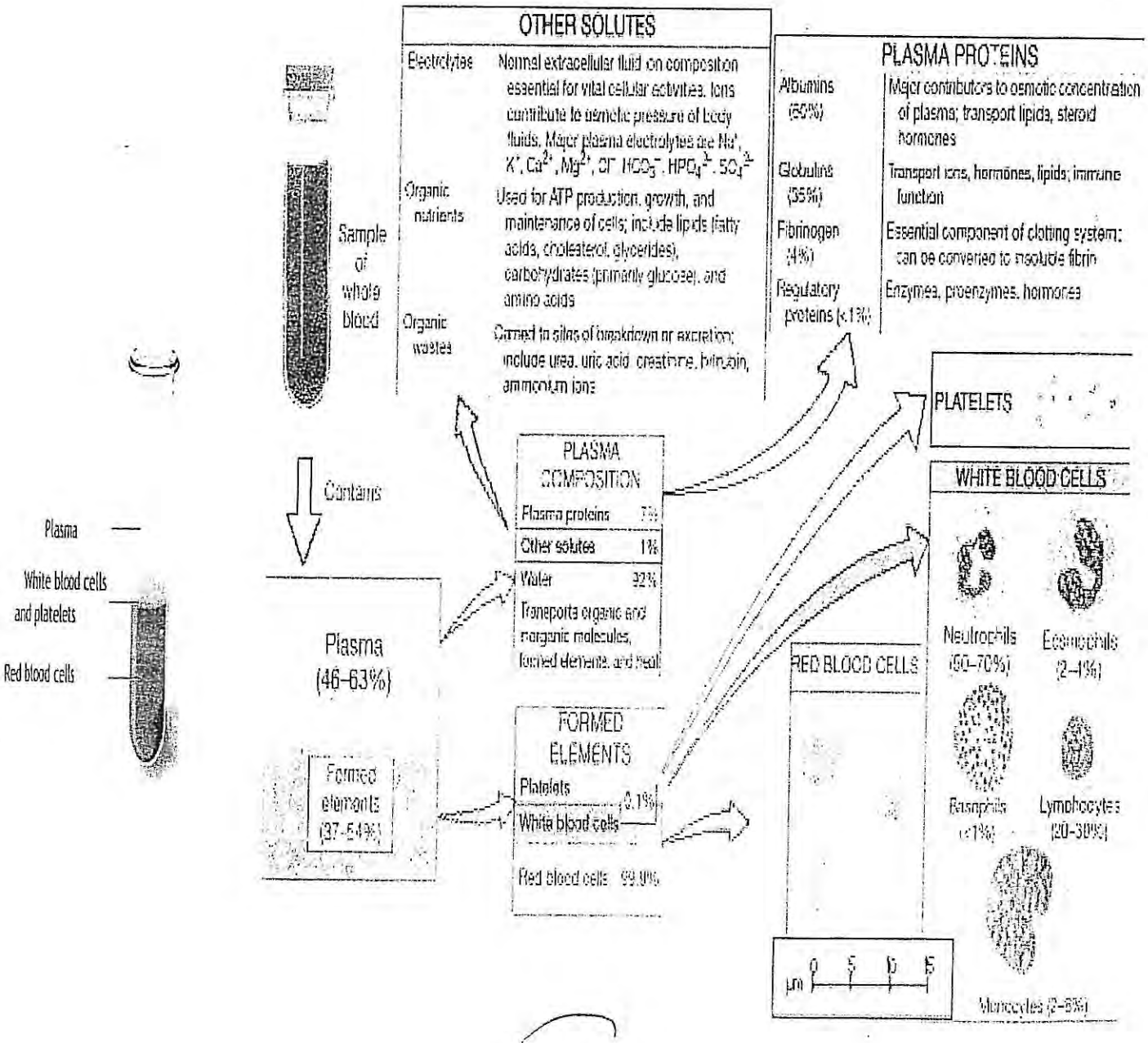
1. Species of experimental animal used. (Marking methods differ significantly and often limit usage to certain animals).
2. Number of animals in a room a group, or in a particular experiment (consideration for the limitations of a numbering system is important).
3. Size and age of the animal.
4. Duration of the experiment. (It is often very critical whether the experiment done in a short or long period, and if long, months or years?
5. Nature of the experiment.
6. Ease of maintain the species without loss of identity. (Using random source dogs or multicolored guinea pigs, for example, allows the use of body shapes and color markings to aid in identification and helps to insure identity).

Appendix lab1

Experimental animals use purposes

1. To study the immunological and pathological process of disease.
2. To study the antigenecity of vaccines.
3. For antibody production.
4. As a blood source.

Blood components



Route of injection

1. **Intravenous**

   This procedure involves direct injection into blood stream of the animal. The marginal ear vein is the most accessible and visible vein in the rabbit.

Take the rabbit then:

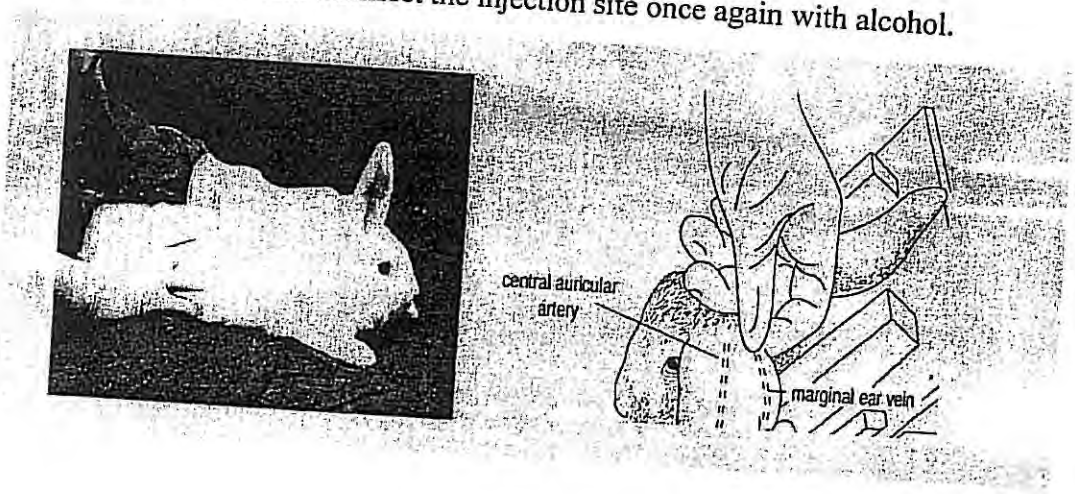
- See the vein on the dorsal edge of either ear.
- Shave the hair away from the area.
- Clean the area with disinfectant (70% ethanol).
- For the ease injection, the diameter of the vein may be enlarged before injection by heating and by applying pressure over the vein at the base of the ear.
- Hold the ear with one hand while supporting the ear from below with finger of the same hand.
- With vein clearly visible, insert the needle in the direction of blood flow (toward the head of the rabbit). Slowly inject the inoculums (Ag) while watching for signs of dilution of the blood in the vein indicating that the injected material is passing into the vein. The blunger of the syringe should move down easily and no raised area in the surrounding tissue should be visible.
- If the vein is missed, try again to inject into the same vein but a few centimeters towards the head of the animal, when injection is complete. With draw the needle and made pressure by finger to the injection site to stop bleeding and disinfectant with ethanol 70 %, the area by place apiece of cotton on site of injection.

2. **Intraperitoneal (mouse).**
   - The injection of material into the peritoneal of abdominal cavity of the animal. The animal (rabbit or mouse) should be place on its back.
   - Anesthesia may be used for this injection.
   - Clip the hair away from the injection site and disinfect the site with 70 % ethanol.
   - Grasp the skin and peritoneum with finger.
   - Insert the needle through the skin and peritoneum. The needle should be empty from bubbles.

- Inject the inoculums (Ag) carefully so as to prevent injection into abdominal organs with practice, the procedure is relatively easy.
- Remove the needle and disinfect the area once again with alcohol.

3. Subcutaneous

- This method involves the injection of material under the skin. Choose a site (usually the back).
- Cline and shave the hair and disinfect the injection site with 70% ethanol.
- Load tuberculin syringe and insert the needle almost horizontal to the animal to a depth.
- Slowly inject the inoculums and observed the formation of a slightly raised area under the skin.
- Remove needle and disinfect the injection site once again with alcohol.



properly, the plunger must be retracted to determine whether any urine, intestinal contents or blood have been aspirated.

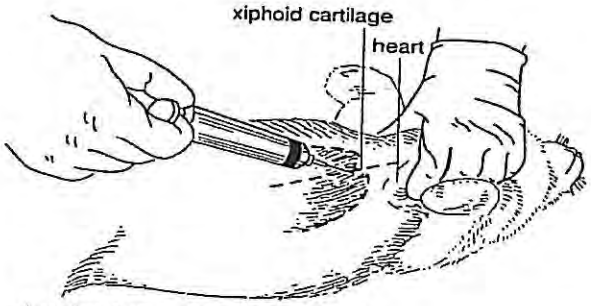
Table 1.6.1 Guidelines for Maximal Injection Volumes (in Milliliters)[a]

Species	Subcutaneous	Intramuscular	Intraperitoneal	Intravenous	Intradermal
Mouse	2-3	0.05	2-3	0.2	0.05
Rat	5-10	0.3	5-10	0.5	0.05
Hamster	3-4	0.1	3-4	0.3	0.05
Rabbit	30-50	0.5-1.0	50-100	1-5	0.1[b]

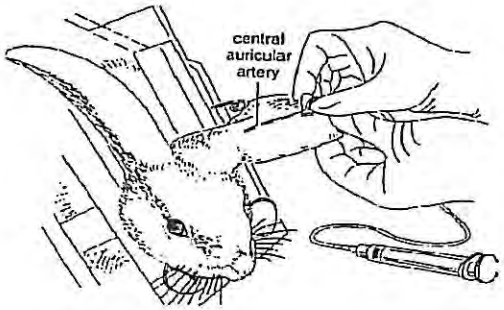
[a]Adapted from Tuffery (1987).
[b]0.05 ml, with adjuvant.

BLOOD SAMPLING TECHNIQUES

1. Cardiac puncture rabbit.



2. Auricular artery of rabbit.



3. Blood collection from tail vein of mouse.

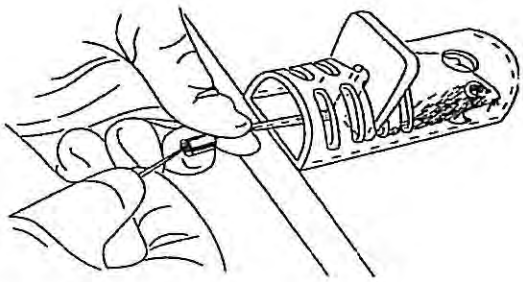


Table 1.7.1 Critical Blood Collection Parameters for Mouse, Rat, Hamster, and Rabbit[a]

Species	Hemoglobin (g/dl)	Hematocrit PCV (%)	Average adult blood volume (ml)	Maximum single sample volume (ml)	Expected volume from exsanguination (ml)
Mouse	10.2-16.6	32-54	2.5	0.3	1.2
Rat	11.1-18.0	36-52	30	2.5	12.0
Hamster	10.0-20.2	36-59	9	0.5	3.0
Rabbit	9.9-19.3	30-53	250	50.0	150

[a]Adapted from Tuffery (1987).

Blood sampling

Cardiac puncture (rabbit)

. Bleeding from the heart , commonly called cardiac puncture , is often use to obtain hyper immune serum from the rabbit after vaccination procedure is complete.

. to obtain sufficient amount of serum

.the rabbit is tied to specially designed restraining table and anesthetized . the animal must be motionless during procedure.

. clip hair from the thorax to a few centimeters below the sternum.

. sterile the area with 70 ethanol .

. use needle 18 gauge and free the syringe from bubbles . gently touch the rabbit around the area on the left side of middle with your finger , will feel a slight heart beat .

. insert the needle between ribs while pulling the blunger.

. continue to insert the needle and watch for the blood to flow into the syringe , at this point, stop advancing the needle and hold the syringe steady untile desired amount blood has been drawn .

. draw the needle slowly,and press the area by using a piece of cotton with 70 ethanol.

Lab Two

Bactericidal Effect of Serum

Practical immunologyLab. 2

Bactericidal effect of serum

Aim: Test the bactericidal effect of normal and heated serum.

Serum: the fluid portion of the blood after the blood has clotted. The proteins in the serum called globulins (albumin, alpha-1 globulin (α-1), alpha-2 globulin (α-2), beta globulin (β), and gamma globulin (γ).

The immune system consists of two parts:

1- Cellular immune response(reticuloendothelial system)
2- Humoral immune response (e.g.Antibc dy,Properdin, Beta-lysin, Lysozymes, Complement.... Etc.)

Antibodiy(Ab)=Immunoglobulin(Ig)

- Gamma (γ) globulin protein.
- Y- Shaped protein produced by b- cells.
- Used by immune system to identify and neutralize foreign objects like bacteria and viruses.

Properdin:

- Gamma (γ) globulin protein.
- Activates complement.
- Effects on G-ve and G+ve bacteria.

Beta-lysin(β-lysin):

- Amino acid produced by platelets during blood coagulation (clotting).
- Cause lysis of G+ve bacteria.

Lysozymes:

- Enzymes that damage bacterial cell wall (especially G+ve).
- Abundant in secretionssuch as tears, saliva, human milk, and mucus.

Complement:

- β globulin protein(10% of globulins).
- Present in blood and tissue fluids of human and animals (Guinea pig is a rich source of complement).
- Consist of 9 complements labeled as C1,C2,C3.....,C9.

- Thermo labile (serum heating in water bath to 56cófor 30 min leads to complement inactivation.
- Cause G-ve destruction.

Functions of complement

1- **Cell lysis**: rupturing membranes of foreign cells like bacteria, viruses, and RBCs by MAC(membrane- attack complex).
2- **Opsonization** = (opsonisation): enhancing phagocytosis of antigen (e.g.C3b).
3- **Chemotaxis**: attracting macrophages and neutrophils to the site of infection (e.g. C5a).
4- **Clearance of immune complexes**: Ag-Ab complexes are removed to the liver and spleen.

Method

1- Studied bacterial isolates cultured (*Escherichia coli&staphylococcus aureus*) in nutrient broth (N.B.) incubated at 37óC for 18 hour, and then adjusted to 104 CFU/ ml with N.B. medium as initial inoculum size.
2- Pooled normal human serum (PNHS).
3- Test tube a: 0.4ml PNHS + 0.1 ml bacterial inoculum.
4- Test tube b: 0.4ml PNHS (heated at 56óC for 30 min) +0.1 ml bacterial inoculum.
5- Control tube: 0.4 ml saline+0.1 ml bacterial inoculum.
6- All tubes are incubated at 37óC for 1 hour.
7- A standard plate counting was performed for each test tube .Spreading 0.1 ml suspension for each tube (A,B, and control) over Muller –Hinton agar plates using sterile cotton swab.
8- The percentage of bactericidal activity was calculated as following:

% Bactericidal activity=100-100[No. CFU/ml of normal serum / No. CFU/ml of heated serum]

OR No. CFU/ml of control / No. of CFU/ml control

Lab Three

Antigen Preparation

Lab 3

Antigen Preparation

Antigen (Ag): is macromolecule that induced to formation of antibody (Ab), which reacts specifically with the Ag.

♣Main characteristics of the Ag:

- High molecular weight ≥ 10000 Dalton.

-Foreignness.

-Chemical Complexity.

♣**Vaccine:** any substance when introduce artificially to human or animal body produces an immune resulting Ab.

Salmonella Ag

1- Somatic (O) antigen:

i. Found on the surface of the cell.
ii. Lipid complex (it is a polysaccharide).
iii. Stable at 100°C and in alcohol.

2- Flagellar (H) antigen:

i. Flagella.
ii. Protien.
iii. Inactivated at more than 60°C.

♣The properties of formal-saline (formaldehyde+saline):

i. Kill microoranisms.
ii. Fix the flagella on the cell wall.
iii. Keep it for along time without deterioration.
iv. Mask the body bacterial cell.

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Method:

Somatic (O) antigen preparation:

1. Streaking a few typical *Salmonella* colonies from the Salmonella-Shigella agar (S.S agar) plate onto brain heart infusion agar plates using a sterile cotton swab.
2. Plates were incubated overnight at 37°C.
3. Sterile normal saline was used to harvest the lawns and a bottle containing 250mL of bacterial suspension was immersed in a boiling water bath for 3.5 h.
4. It was left to stand at room temperature overnight and again immersed in boiling water for an additional 1.5 h on the next day.
5. The now killed bacteria were washed 3 times with normal saline.
6. The resulting product had the appearance of skim milk.
7. It was judged sterile by its failure to cause turbidity in brain heart infusion broth tubes after prolonged incubation at 37°C, and its failure to produce growth on S.S agar plates.
8. Storage was in 0.3% formal saline at 4°C.

Flagellar (H) antigen preparation:

1. Inoculated a few typical *Salmonella* colonies from the (S.S agar) plate onto a 250mL brain heart infusion broth.
2. Broth was incubated overnight at 37°C.
3. Then, 250mL 0.6% formal saline was added and the mixture was allowed to stand at room temperature for 5 d. Sterility test were performed.
4. Washing Killed bacteria and discard the supernatant.
5. Resuspend the precipitate with 0.3% formal saline and storage at 4°C.

♣ Standardization by Wellcome Opacity Tubes: set of 10 tubes containing barium sulphate, content should be dissolved.

Method:

*Take similar opacity tube NO.

*Concentration stock (Y) =tube NO. × Reciprocal dilution factor×750

Con. stock= Y× 10^6 cell/mL

*The required dilution= $\frac{X}{X+1}$ × (Y× 10^6 cell/mL)

Q: Prepare 30ml of *Salmonella* (O) antigen, when you know the concentration was 10^9 cell/mL, the No. of Wellcome Opacity tube 10 and the sample was diluted to 10 times?

♣Table show the time of injection:

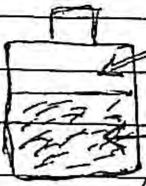
Day	O-antigen	H-antigen	Human RBCs suspension
1st	0.01ml	0.01ml	10% give daily for 8days then we leave rabbit 2weeks
3rd	0.25ml	0.25ml	
5th	0.25ml	0.25ml	
6th	0.5ml	0.5ml	
10th	0.75ml	0.75ml	
14th	1ml	1ml	

*Note: must we leave rabbit for 2weeks after the last injection.

Lab: 4 مكتبة ماسة علي

(Flagellar) HAg (H Antigen)

1-



2- Keep at room temperature for 48 hours (Why

3- check turbidity (why?)

4- check sterility (why?)

5- Transfer to centrifuge tube 

6- Centrifugation (3000 r.p.m for 10 min)

7-

8- Discard the supernatant.

9- Resuspend the precipitate with ... same

formal saline use to:-

1- Kill micro organism

2- Masking (Fixation the flagella with cell wall)

* ملاحظة في السابق نعطي البكتريا Nutrient broth مخفف الحضنة في الـ oven بدرجة ٣٧م مدة ٢٤ ساعة (لماذا؟)

O Ag (Somatic Ag) preparation

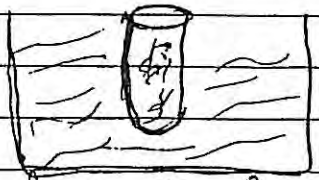
bacterial growth

Washing by saline 10ml by using pipet

→ transfer to centrifuge tube

bacterial suspension

Water bath for 2½ hr. at 100°c (Why?)



Centrifugation for 10 min at 3000 r.p.m

O Ag

resuspend the precipitate with formal saline

Lab Four

Phagocytosis *In VIVO*

Phagocytosis in vivo

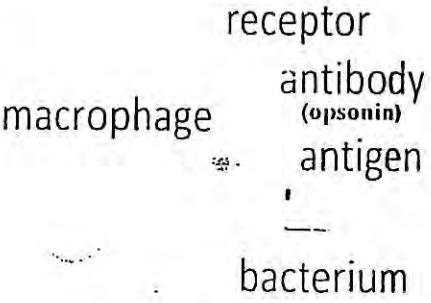
Aim: study of phagocytosis in mice by intaperitoneal injection (I.P.).

Grater omentum: Large fold of peritoneum has milky spots of macrophage collections.

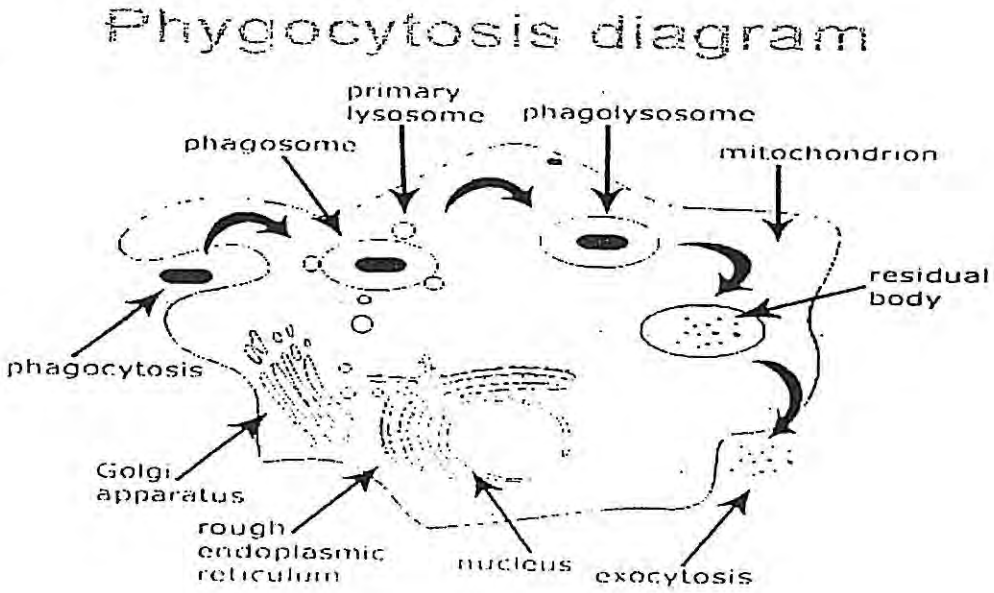
Phagocytosis: Process of cell or particulate matter engulfment by phagocytes e.g.: monocytes, neutrophils (circulating) and macrophages (resident).

Phagocytosis steps

1. **Chemotaxis:** Attraction of leucocyte or other cell by chemicals (chemotaxins) e.g. soluble bacterial factors, lysosomal enzyme, c3b and L.P.S.
2. **Opsonization and attachment:**
   - **Opsonin:** is any molecule that acts as a binding enhancer for the process of phagocytosis, for example, antibody and products of complement activation.



3. **Engulfment.**
4. **Intracellular killing and digestion:** Phagosome fuses with lysosome to form phagolysosome then the process of digestion by hydrolytic enzyme.



Method:

- Inject the mouse by 0.1 ml of *S. aureus* intraperitoneally.
- After 18-24 hrs, inject the mouse by 0.5 ml of saline, why?
- Make a slide of intraperitoneal fluid, liver, spleen, heart
- Dry the slide, stain by wright's stain, why? For 5 min.
- Wash the slide by water.
- Examine 5-10 fields and count.

$$P.I. = \frac{B}{A} \leq 1 \text{ always.}$$

A= No. of phagocytic cell.

B= No. of phagocytic cell that contain bacteria.

Peritoneal	spleen	liver	heart
A	A	A	A
B	B	B	B
P.I.	P.I.	P.I.	P.I.

- Compare P.I. for each organ then discuss the results.
- Phagocytic index depends on:
  - Route of injection.
  - No. of injected bacterial cells.
  - No. of phagocytic cells in the organ.
  - Ability of phagocytic cells to engulf bacterial cells.
  - Ability of bacterial cells to reach all organs.

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Lab Five

ABO Blood Group System

ABO Blood group system

Landsteiner's rule

An antigen on a red blood cell and its corresponding antibody do not present

Blood group	Ag (RBCs)	Ab in serum
A	A	Anti-B
B	B	Anti-A
AB	AB	—
O	—	Anti-A and Anti-B

- O: Universal donor. Why?
- AB: Universal recipient. Why?

Blood transfusion (compatibility test)

Serum of recipient (Ab) + Blood cell of donor (Ag)

↓

No agglutination

Ex: A recipient ⇒ Anti-B in serum

B donor ⇒ B-Ag on RBCs

→ Agglutination

Blood shall not be transferred

Rh factor

- Group of Ags on RBCs e.g. (D-Ag) (Rh^+).
- Natural antibodies do not present.
- Rh^- no D-Ag and no Anti-D in serum unless:
  - Blood transfusion.
  - Maternal immunization.

$$♀\ Rh^- + ♂\ Rh^+ \longrightarrow Rh^+\ Baby$$

- D^+ cells pass to mother ⟶ Anti-D will be formed during first delivery.
- During second pregnancy mother (Anti-D) pass over through placenta then to fetus breaking his RBCs ⟶ Erythroblastosis fetalis, (Immature RBCs), abortion or still born.

-How can we avoid this situation?

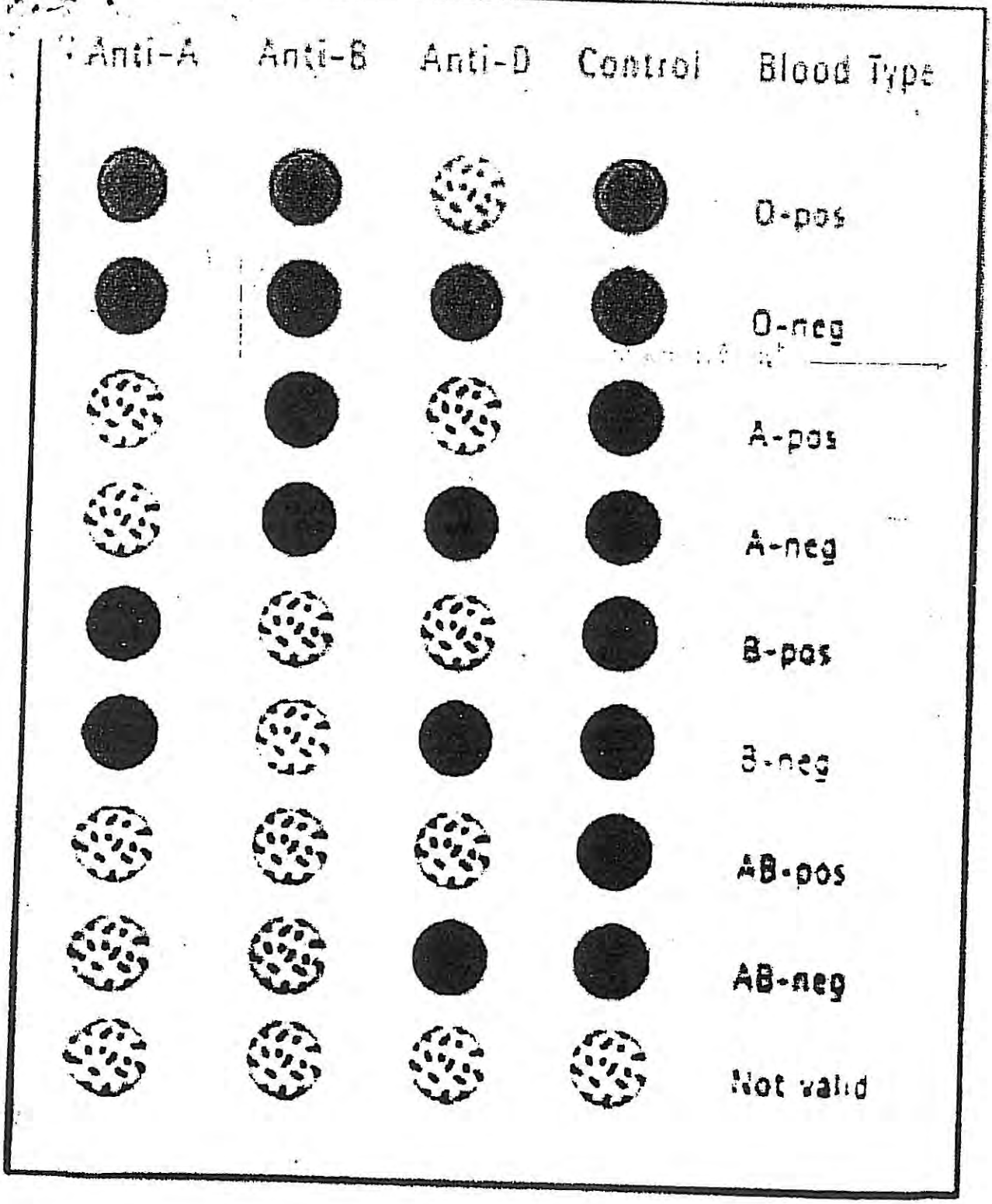
Mother receive an injection of Anti-D antibody within 72 hours of first delivery to neutralize transformed baby D-Ag and prevent the mother's immune system from producing its own Anti-D which leads to problems in the second delivery.

Methods:

- Major: 0.25 ml recipient serum + 0.1 ml donor RBCs + 0.65 ml saline.
- Minor: 0.25 ml donor serum + 0.1 ml recipient RBCs + 0.65 ml saline.
- Control: 0.1 ml donor RBCs + 0.9 ml saline.
- Control: 0.1 ml recipient RBCs + 0.9 ml Saline.

  Control to test autoagglutination

- Keep tubes in water bath at 37c° for 30 min.
- Centrifuge at 1500 r.p.m. for 5 min.
- Read results.
  - The results:
    - Circle of irregular edges at tube bottom means agglutination.
    - Circle of regular edges at tube bottom means no agglutination.


Anti-A
Anti-B
Anti-D
Control
Blood Type
O-pos
O-neg
A-pos
A-neg
B-pos
B-neg
AB-pos
AB-neg
Not valid

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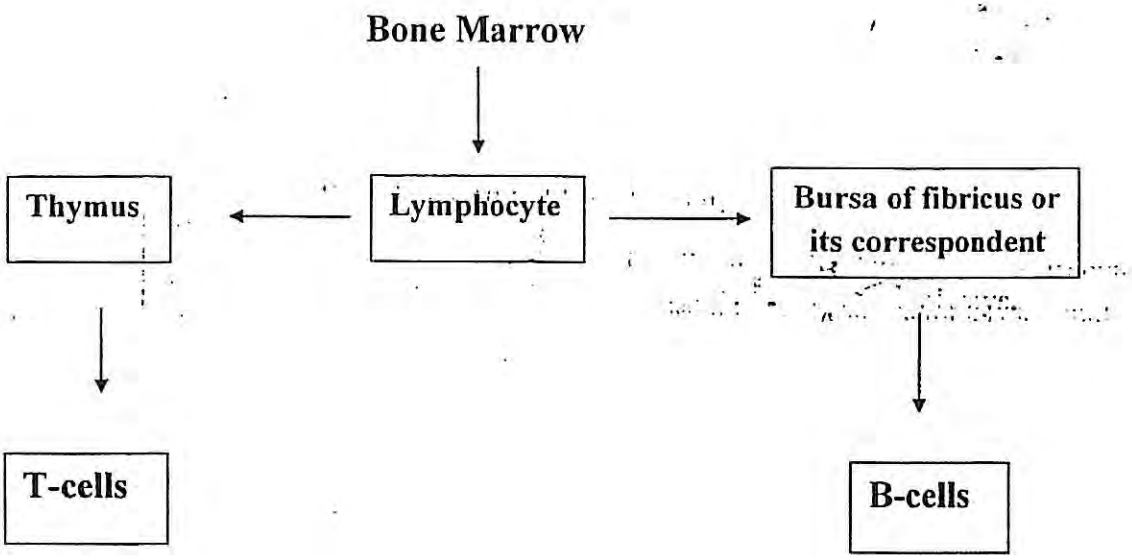
Lab Six

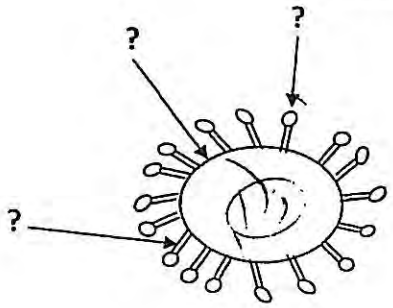
Rosette Forming Cells

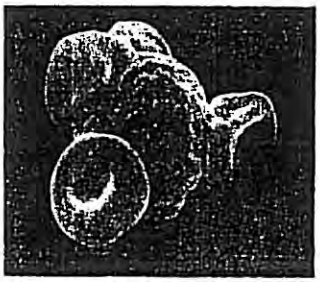
Rosette forming cells (RFCs)



Erythrocyte rosette forming cells

ERFCs



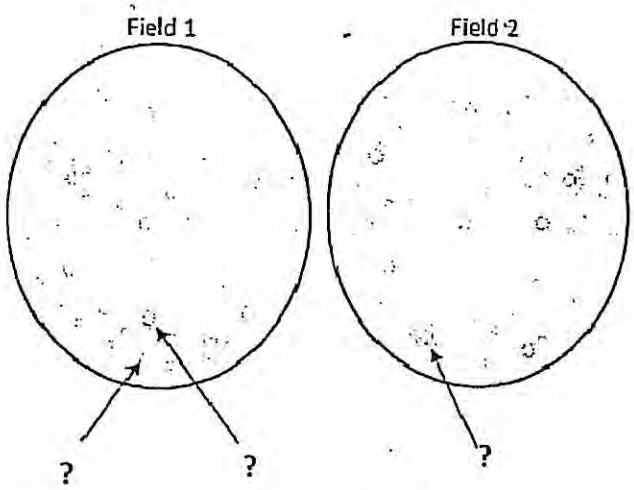


- Is this an immune reaction? Why?
- Why we don't stain the slide?

- Kill the mouse, remove the spleen.
- Put it in motor and mix with 3ml of saline.
- Homogenize the spleen by needle, why?
- Complete to 10ml then put it in centrifuge for 30 mins.
- Remove the supernatant.
- Take 1ml of the cell suspension+ 1ml (10% SRBCs), then incubate.
- Mix well then take a slide with cover (make a slide).
- Examine under microscope.
- Calculate no. of T cells in the organ

$$\frac{Avg.no.of\ RFC}{Avg.of\ total\ lymphocyte} \times 100$$

No. of Tcells = ?



- T. cell can be differentiated functionally and quantitatively by in vivo and in vitro methods:
  - In vivo:
    - Tuberculin test.
    - Contact dermatitis (Allergy).
    - Tissue rejection.
    - In vitro: Formation of rosette shape when mixed with sheep RBCs.
    - This method can be used for quantitation of T-lymphocyte in addition to differentiation from B-lymphocyte.

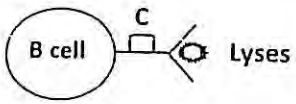
Lab Seven

Enumeration of Abs Secreting Cell

Lab. 7

Enumeration of Abs-secreting cell (B-cell)

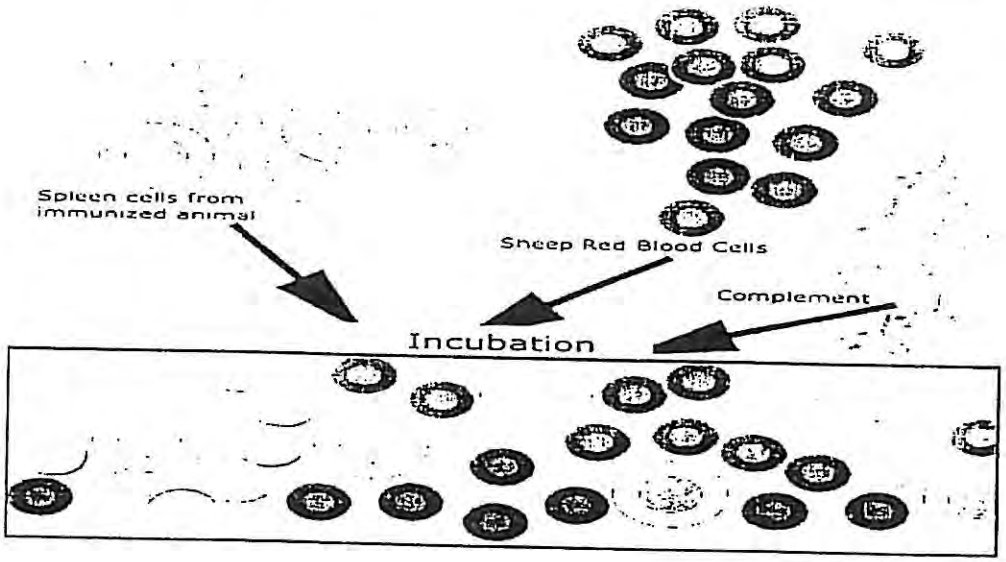
Primary antigenic challenge → Immune system stimulation → B- cell form specific Abs



Calculate no. of B cells in the organ

→ Spleen B-cells → IgM (Abs) + SRBCs (Ag)

Lyses of SRBCs as plaque ← Complement + Agarose (Why?)



Notice:

- Mice injected with SRBCs as antigens.
- Mice spleen removed and used since it is a lymphatic organ that contains large numbers of B lymphocytes.

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- Remove spleen; tear in ice bath with forceps, why?
- Add 2ml of hank's solution, why?
- Prepare 1/10 or 1/5 of hank's solution, why?
- Add 0.5ml of your suspension to a tube.
- Add 0.5ml of 20% SRBCs to tube and mix well.
- Use hot pipette (Why?) to transfer 1.5 ml of agarose to the tube (in conc. of 0.7%).
- Mix well; pour the tube into agarose containing petri, why? In conc. of 1-4%.
- Wait till hardening of agarose.
- Add complement (0.5ml), incubate for 1 hr.
- Count plaques in the plate.
- Find the number of B-cell using the next formula:

No. of B-cells= No. of plaques× dilution factor

❖ **Hank's balance salt solution (HBSS) components:**

- Nacl 8g/l.
- Cacl2 0.2g/l
- Mgso4 0.2g/l
- Kcl 0.4g/l
- KH2po4 0.1g/l
- Na2co3 1.27g/l
- Glucose 2.6 g/l
- In one liter of D.W.
- Autoclaved in 10 mins.

Lab Eight

Serological Tests

serologicaltests ملخص حاكي

تالتة - بايو
٢٠١٥ - ٢٠١٦

types of Reaction ① Agg ② precip 3-Neutralization
Ⓐ primary stage Ⓑ secondary stage ③ Ag + Ab → Reaction

Agglutination: Agglutinin + Agglutinogen → Agg. < direct / indirect
soluble insoluble haem.gg. اهم

types of method for agg.
tube method (كمية ونوعية) — slide method (نوعية + أ.)

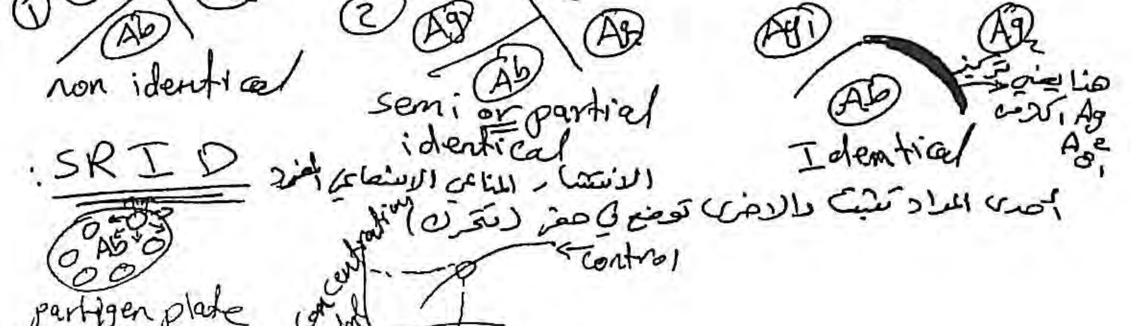
precipitation: precipitin + precipitinogen → precipitation
soluble soluble
Ab زيادة Ag زيادة
① proZon ② post Zon ③ optimum proportion
للاتساع، عدد ... أوساط.

① Soluble med. (Ring test)
② Semi-solid media (agar-agaropectin = agarose مثل) الاختبارات
Ⓐ Oüden (tube) Ⓑ Oüchterlony (Petridish) Ⓒ Single Radial Immune Diffusion (SRID) partigen plate
③ Solid media (Cellulose acetate paper) (IEF)

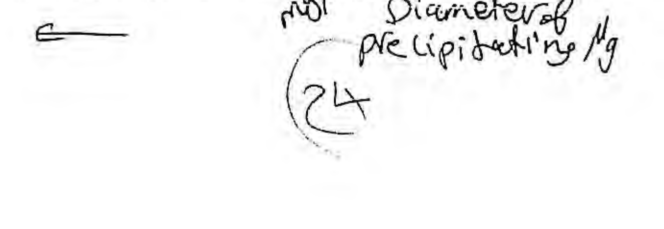
Ouden & Oüchterlony الفروق

Ouden	Oüchterlony
① in tube	① in petri dish
② Single Immune Diffusion SID	② Double (DID)
③ One of the reactant mixed with agarose other is added	③ the two reactants added and move
④ Band	④ line
⑤ number of system	⑤ Give type of reactant

Oüchterlony الخطوط في الترسيب



: SRID الانتشار، المناعي الإشعاعي الفرد
أحد المواد تثبت بالأرض توضع في حفر (تتحرك)



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Serological Tests

- Serological reactions which include Ag-Ab such as agglutination, precipitation .....etc, used in diagnosis of microbial infection, these tests can be qualitative or quantitative, highest dilution of Ag and Abs that gives positive result is "the titer" in such tests.

Agglutination

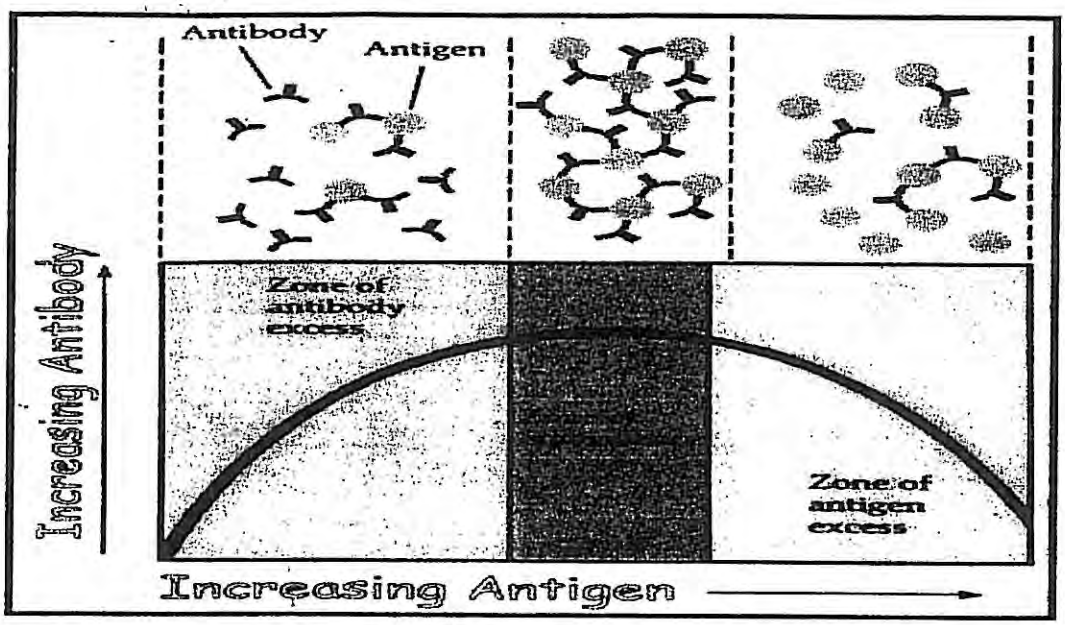
Particulate Ag with Abs.

- **Factors effect agglutination test are:**
  - Electrical charge of the medium: e.g. Bacterial cells have repulsive forces due to negative charge on their surface.
  - Antigen-Antibody ratio.
  - PH: differences cause differences in chemical structures of antigens/antibodies, affecting the "fit".
  - The number of antigen sites on the cell is important since the more antigen sites result in more antibodies being attached and forming cross-linkages. These cross-linkages result in agglutination.
  - Temperature: The optimum temperature depends on the type of antibody involved.
  - Optimum incubation time.
  - Size and structure of the antibody: IgM is a good agglutinin as compared to other types of antibodies, why?

Mechanism of agglutination

Abs (diluted) + Ag $\xrightarrow{\text{incubation}}$ **Result**

- **Prozone-antibody excess:** antibodies saturating all antigen sites; no antibodies forming cross-linkages between cells; no agglutination
- **Zone of equivalence:** antibodies and antigens present in optimum ratio, agglutination formed.
- **Zone of antigen excess (Post-zone):** too many antigens, any agglutination is hidden by masses of unagglutinated antigens.



Method:

- Dilute serum (2fold):

Tube	1	2	3	4	5	6	7
Dilution	1/10	1/20	1/40	1/80	1/160	1/320	0

- Add 0.5 ml of O.Ag.
- Incubate in a water-bath at 37°C for two hours.
- Examine each tube for agglutination.
- The titer is the highest dilution with agglutination.

❖ **Positive result:** agglutination of the suspension.
❖ **Negative result:** suspension remains turbid.
❖ **Antigen control tube:** suspension remains turbid.

Application:

1. Blood grouping.
2. Widal test for *Salmonella* (H and O antigen).
3. Diagnosis of different diseases e.g. rheumatoid arthritis.
4. Pregnancy test.

Lab Nine

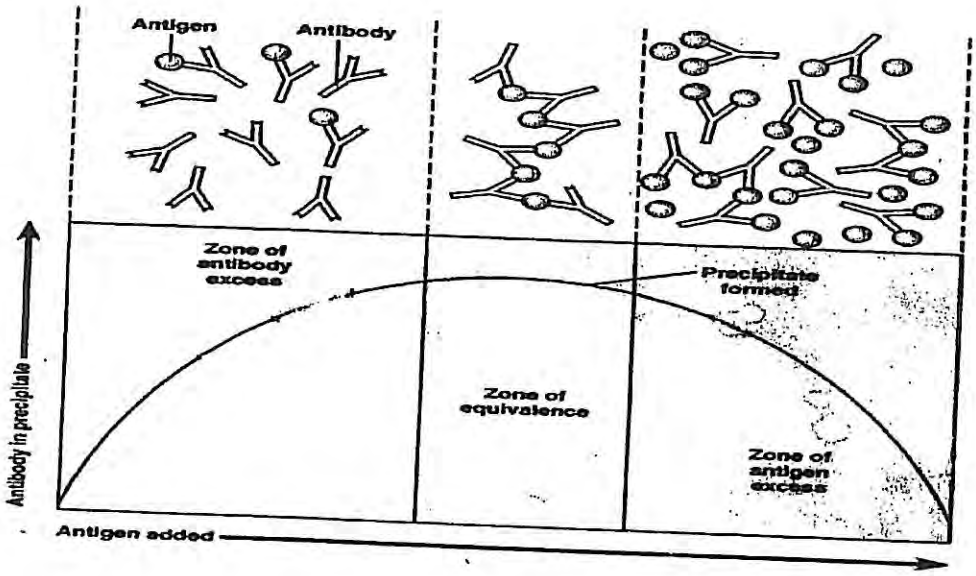
The Precipitation Test

Lab 5-

Precipitation test

Reaction in two steps:

- **Primary complex: (Ag-Ab) complex, independent of temperature.**
- **Secondary complex: Ag-Ab complex form precipitate, heat dependant.**



- Highly specific.
- Ag-Ab bond is weak, H bond, vanderval.
- High molecular weights of Ag bind to more Abs.

Method (precipitation in solution):

- Dilute serum (2fold):

Tube	1	2	3	4	5	6	7
Dilution	1/10	1/20	1/40	1/80	1/160	1/320	0

- Add 0.5 ml of soluble Ag.
- Incubate in a water-bath at 37°C for two hours.
- Examine each tube for precipitation.
- The titer is the highest dilution with precipitation.

Precipitation in gel

Immunodiffusion: the diffusion of Ags and Abs towards each forming a precipitation in gel which represents the equivalence zone of optimum antigen to antibody ratio.

Factors affect immunodiffusion:

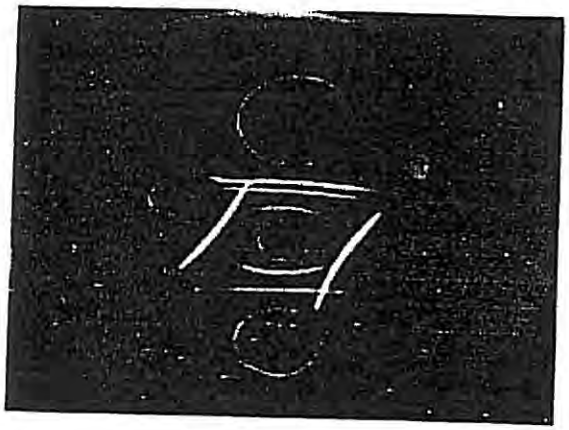
1. Chemical structure of Ag.
2. Diffusion coefficient.
3. Concentration of antigen and antibody.
4. M.wt,PH and temperature.

Ouchterlony plate test

Double diffusion, double dimension

Oudin tube test

Single diffusion, single dimension



1. **Identity reaction:** Straight line, Abs can't distinguish Ag_1 from Ag_2, Ag_1 is similar to Ag_2.

Figure 1: Reaction of identity

2. **Partial identity reaction:** Abs specific for additional Ag passes through precipitate and reaact with more complex Ag, Ag_2 is a mixture of antigens.

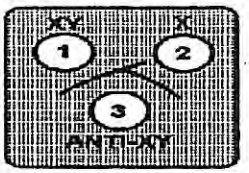


Fig. 2: Reaction of Partial Identity

3. **Non-Identity reaction:** Two antigens react with different antibodies, Ag_1 differs from Ag_2.

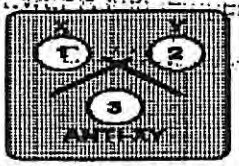


Fig. 3: Reaction of Non-Identity

Precipitation lines shape indications:

a. Ag | Ab means Ag and Ab have similar m.wt.

b. Ag (Ab means m.wt of Ag < m.wt of Ab.

c. Ag) Ab means m.wt of Ag > m.wt of Ab.

Importance of the test:

a. Identification of antigens that contained in a mixture.

b. Finding the antigenic relationship between different antigens.

c. Finding relationship between antigen-antibody systems.

Lab Ten

Complement Fixation Test

Lab 1

Complement Fixation Test

-Complement is a group of serum proteins these are:

1. Not Ig's.
2. Their concentration in serum does not increase after antigenic stimulation.
3. One IgM is required for the fixation of the first complement while two IgG molecules are needed.
4. IgA & IgE do not fix C.
5. The major activites of complement are:
   a) Lyses of G-ve bacteria.
   b) Activation of immune system (macrophage).
   c) Hemolysis of RBCs.
   d) Opsonization: complement facilities phagocytosis of bacteria.
   e) Sediment immune complex Ag-Ab.
6. Complement Fixation Test is used in diagnosis of several infections (viral).

Mechanism of CFT:

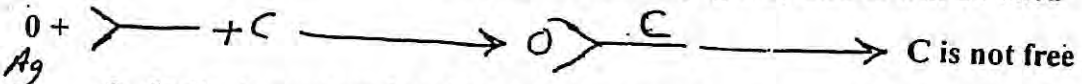
Hemolysin: is Ab with specificity for erythrocytes which in cooperation with complement will lyse RBCs.

Hemlysis: release of hemoglobin from RBCs following reaction with specific Ab in presence of complement.

CFT: with two steps

1. **Ag+Ab (serum)+ C**

If specific Abs is present the complex is formed (Ag-Ab) and C will be fixed



2. **Indicator system in which (RBCs +hemolysin) are added:**
   a) **If C is not free ⟶ no lysis to RBCs ⟶ test is +ve. (Presence of Ab's).**
   b) **If c is free (no Ab's) ⟶ lysis of RBCs ⟶ test is –ve.**

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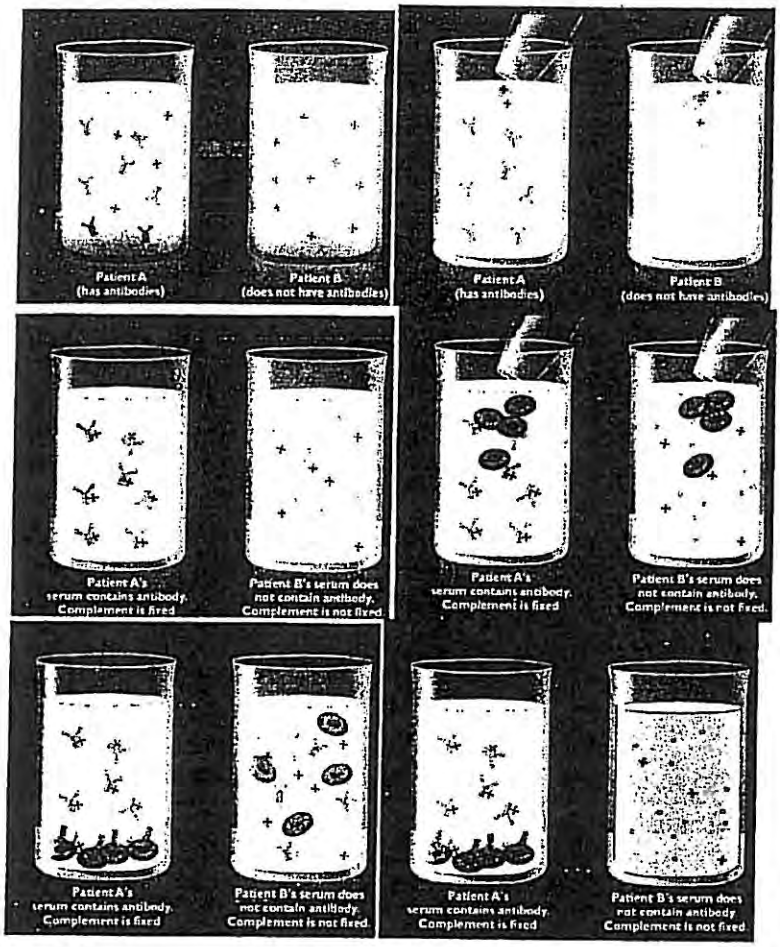

Aim of experiment:

1. Complement titration (unit).
2. Hemolysin titration (unit).
3. Optimum conc. of Ag.
4. Determine titer of unknown serum.

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Exp 1: Titration of complement

1 unit of C is the lowest dilution that results in 100% hemolysis of SRBCs in presence of specific Ab.

$$X = K\left(\frac{y}{1-y}\right)^{1/n}$$

X= concentration of complement or Hemolysin.

Y= degree of hemolysis.

K, n=constant,

$$\log X = \log K + \frac{1}{n}\log\left(\frac{y}{1-y}\right)$$

Relationshep between concentration of specific rabbit antiserum and % of a constant no. SRBCs in presence of C.

100% hemolysis: large increase in concentration of hemolysin result in only small increase in degree of hemolysis, Thus using a 100% hemolysis as atitration end point results in an easy of very low precision.

Therefore ⟹ 50% hemolysis is more sensitive.

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Method:

Tube no	1	2	3	4	5	6	7
Hemolysin (ml)	2	2	2	2	2	2	2
SRBC 1% ml	2	2	2	2	2	2	2
Mix, let Stand for 10 min.							
Buffer ml	—	0.2	0.4	0.5	0.6	0.7	1
C. (1/100) ml	1	0.8	0.6	0.5	0.4	0.3	—

incubate for 30 min. 37C

*Centrifuge 2100 rpm for 10 min

*Take supernatant of tube 7 control to blank the spectrophotometer.

*Read O.D from 1-6 tubes at 530 nm.

*Calculate:

log X =log(Con. C × volume)

$$\log\left(\frac{y}{1-y}\right) = \log\left(O.D/0.6 - O.D\right)$$

*Determine point's:

Log X					
Log $\frac{y}{1-y}$					

*Draw line:

Note:

I. Complement titration must always calculate before hemolysin titration.
II. Hemolysin should be inactivated 56C for 30 min to destroy the complement.
III. Reject tubes that give aresult of O.D < 0.09 or O.D > 0.53.
IV. SRBCs suspended in barbital buffer with 1%.

Isotonic saline

حافظ دل کریاے الدم الحمر

Factor that effect on C activity:

1- Concentration of RBCs.

2- Concentration of hemolysin.

3- Concentration of Ca^{+2} & Mg^{+2} ions.

4- Ionic force.

5- Tem., pH and time of reactions.

6- Volume of the reactants.

Exp 2: Titration of hemolysin

50% unit of hemolysin : is the amount of serum that is required to lyse 50% of RBCS in presence of 2 units of complement.

*Hemolysin is heated to in activated

***logX = log (Con.C × volume)**

***log $(\frac{y}{1-y})$ = log (O. D/0. 6 − O. D)**

Method:

Tube no	1	2	3	4	5	6
Buffer ml	—	0.2	0.4	0.6	0.8	1
Hemolysin (1/100)ml	1	0.8	0.6	0.4	0.2	—
RBC(1/100)ml	2	2	2	2	2	2
Mix, let stand for 10 min.						
C. ml	2	2	2	2	2	2

*Draw line

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RBC(1/100)ml	2	2	2	2	2	2
Mix, let stand for 10 min.						
C. ml	2	2	2	2	2	2

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