A Laboratory Manual for IMMUNOLOGY

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by

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INTRODUCTION

The purpose of this course is to acquaint students with the various fundamental principles of immunology The exercises are planned and arranged so as to fit into two-hour laboratory periods, given twice a week during one quarter. Therefore, the procedures have been made as simple and intelligible as possible without sacrificing too much accuracy. With respect to materials, the exercises have been designed so as to avoid dependence on specimens from clinical laboratories. For example, the complement fixation principle is presented on the basis of a comparatively simple procedure applicable to the diagnosis of brucellosis. If positive blood samples are not obtainable from naturally infected individuals, one can easily produce serums containing complement fixing antibodies by injecting guinea pigs, rabbits or other susceptible animals with dead or live cultures of, say, Brucella abortus.

The students must realize that the procedures, taught in these classroom exercises, may be somewhat different from those currently employed in diagnosis and research. Their task, for the present, is to acquire as thorough an understanding as possible of fundamental principles, together with a good, general working knowledge of immunological and serological technique. If these objectives are accomplished, they will have little difficulty in mastering any techniques in practical laboratory work. In addition to the laboratory work there will be 3 lectures and recitation periods per week during which the theoretical aspects of immunology will be discussed.

ACKNOWLEDGEMENT

The authors are indebted to Dr. Erma M. Hill of the Michigan Department of Health for the revision of the exercise on blood typing; and to Dr. Charles H. Cunningham, Department of Microbiology and Public Health, Michigan State University, for permission to use a slightly modified form of his exercise on virus hemagglutination.

REFERENCE BOOKS

Besredka	Immunity in Infectious Diseases
Boyd	Fundamentals of Immunology
Burnet and Fenner	The Production of Antibodies
Carpenter	Immunology and Serology
Diagnostic Procedures and Reagents -	American Public Health Association
Dubos	Bacterial and Mycotic Infections of Man
Eagle	Laboratory Diagnosis of Syphilis
Feinberg, Malkiel and Feinberg	The Antihistamines
Gay and Associates	Agents of Disease and Host Resistance
Gershenfeld	Biological Products
Huddleson	Brucellosis in Man and Animals
Kabat and Mayer	Experimental Immunochemistry
Kahn	Tissue Immunity
Karsner and Ecker	The Principles of Immunology
Kolmer	Serum Diagnosis by Complement Fixation
Kolmer and Boerner	Approved Laboratory Technique
Landsteiner	Specificity of Immunological Reactions
Raffel	Immunity - Hypersensitivity - Serology
Ratner	Allergy - Anaphylaxis and Immunotherapy
Rivers	Viral and Rickettsial Infections of Man
Schaub and Foley	Diagnostic Bacteriology
Taliaferro	The Immunology of Parasitic Infections
Topley	Outline of Immunity
Topley and Wilson	Principles of Bacteriology and Immunity
Wells	Chemical Aspects of Immunity
Zinsser	Infection and Resistance
Zinsser	Immunity--Principles & Practice as Applied in Public Health

Technics of Serodiagnostic Tests for Syphilis: Supplement No. 11 to Venereal Disease Information

Information on immunology, serology and immuno-prophylaxis and therapy will be found in textbooks dealing with pathogenic bacteriology. See reference list in A Manual for Pathogenic Bacteriology, by Stafseth - Newman - Stockton, Burgess Publ. Co., 1953.

REQUIREMENTS

1. Each student must work independently unless otherwise directed.
2. The data obtained from the various exercises must be recorded in table form whenever they lend themselves to tabulation. Brief statements, giving reasons for the results obtained must be included in the records of each exercise.
3. At the end of the term the notebook, containing the record of the laboratory work, must be handed in. It will be judged by the neatness and ingenuity displayed in making it up as well as by the correctness of the results.

VOCABULARY

AGGLUTINATION	(L. agglutinatus, to glue a thing) The serological reaction manifested by the aggregation of a cellular antigen and its specific antibody in the presence of an accessory factor.
AGGLUTINATION (Cross)	Agglutination which occurs as a result of similarity in antigenic structure.
AGGLUTINATION (Spontaneous)	Agglutination which occurs in the absence of specific antibody.
AGGLUTININ	The name designating the antibody which takes part in the agglutination reaction.
AGGLUTINOGEN	The name designating the antigen which takes part in the agglutination reaction.
AGGRESSIN	(L. aggressus, to attack + in) Substances produced by microorganisms which contribute to their virulence principally due to interference with the defensive mechanisms of the host.
ALEXIN	See complement
AMBOCEPTOR	(L. ambo, both + capere, to take) The name commonly applied to the antibody which sensitizes sheep red blood cells to the lytic action of complement.
ANAMNESTIC	(Gr. anamineskein, to recall to memory) The rapid rise in antibody titer resulting from a second injection of an antigen some time after the primary injection.
ANAPHYLACTIC SHOCK	The reaction shown by an individual when exposed to a subsequent injection of the antigen to which it has been sensitized.
ANAPHYLACTIN	The name designating the antibody concerned in anaphylaxis.
ANAPHYLACTOGEN	The name designating the antigen concerned in anaphylaxis.
ANAPHYLAXIS	(ana, without + phylaxis, protection) An artificially induced state of hypersensitiveness dependent on an antigen-antibody interaction.
ANTIBODY	Substances elaborated by animal tissue, or physico-chemical properties acquired by certain tissue constitutents, in response to the influence of an antigen. In vitro they may react with their specific antigen in the presence of the proper accessory factor producing a perceptible reaction.
ANTIBODY (Auto)	(Gr. autos, self + antibody) Antibodies produced by an individual in whose body the specific antigen is present.
ANTIBODY (Iso)	(Gr. isos, alike + antibody) Those antibodies present in the serum and other body fluids of an individual, the specific antigen for which is present in other individuals of the same species.
ANTICOMPLEMENTARY	The action of serum or antigen with complement which interferes with fixation of complement by a specific antigen-antibody complex.

ANTIGEN	(anti, against + gen, to produce) Substances which when introduced parenterally into a susceptible host will cause certain tissues of the host to produce antibodies or cause the host to become hypersensitive. In vitro they may react with their specific antibody in the presence of the proper accessory factor producing a perceptible reaction.
ANTIGEN (Iso)	(Gr. isos, alike + antigen) Those antigens present in cells and body fluids of an individual, the specific antibodies for which are present in other individuals of the same species.
ANTIGENIC FORMULA	Usually a series of numerals, numbers, or letters arbitrarily employed to designate the antigenic components of a microorganism.
ANTISERUM	See Immune Serum.
ANTITOXIN	(Gr. anti, against + toxin, poison) Serum containing antibodies which were produced in response to the influence of a toxin or toxoid.
ATOPY	Clinical forms of hypersensitiveness which are believed subject to hereditary influences.
ATTENUATION	(L. attenuatio, to make thin) The procedure for decreasing the virulence of microorganisms by physical, chemical, or biological means.
BACTERIN	A suspension of killed bacteria employed to produce an active immunity.
BACTERIN (Autogenous)	A bacterin composed only of microorganisms isolated from the individual to be immunized.
BACTERIN (Mixed)	A bacterin composed of two or more different microorganisms.
BACTERIN (Stock)	A bacterin prepared from stock cultures of microorganisms.
BACTERIOLYSIN	The antibody which sensitizes bacteria to the lytic effect of complement.
BACTERIOTROPIN	Immune opsonin specific for a given bacterium.
BLOOD TYPING	The serological procedure whereby the presence or absence of antigens A or B in human red blood cells is determined.
COMPLEMENT	A thermolabile complex substance present in blood serum of most normal animals which may produce lysis of certain cellular antigen-antibody combinations.
CROSS MATCHING	The serological procedure employed to determine the compatibility of bloods of the same type.
DESENSITIZATION	(L. dis, put down + sentire, to feel) The term used in immunology referring to the process employed to render individuals insensitive to those materials to which they are naturally or artificially sensitive.
DOSE	(Gr. dosis, a giving) An arbitrary unit of measure indicating the amount of a toxin, bacterial or viral suspension to be injected.
DOSE (L_+)	The smallest amount of toxin which when mixed with 1 unit of antitoxin and injected by a designated route into the specified animal of certain weight, produces the desired reaction in the prescribed length of time.

DOSE (L_o)	The largest amount of toxin which when mixed with 1 unit of antitoxin and injected by a designated route into the specified animal of certain weight, produces the slightest observable reaction.
DOSE	The smallest amount of antigen which when introduced parenterally into a susceptible host produces maximum antibody response. Any quantity of antigen above this amount does not produce an increase in antibody titer.
DOSE (Minimum Lethal)	The smallest amount of toxin, bacteria or virus suspension, which will produce death in the test animal within the time period designated.
DOSE (Threshold)	The amount of antigen which when introduced parenterally into a susceptible host produces the smallest detectable antibody response.
HAPTEN (Complex)	(Gr. hapten, to seize) That fraction of the antigen which determines its specificity and reacts with its specific antibody in vitro producing a perceptible reaction. In its native form, does not stimulate the production of antibodies upon parenteral injection.
HAPTEN (Simple)	That fraction of the antigen which determines its specificity and reacts with its specific antibody in vitro without producing a perceptible reaction.
HEMAGGLUTINATION	(Gr. hema, blood + agglutination) The serological reaction in which red blood cells combine with their specific antibody in the presence of an accessory factor resulting in agglutination.
HEMAGGLUTININ	The name designating the antibody which takes part in hemagglutination.
HEMAGGLUTINOGEN	The name designating the red blood cells which takes part in hemagglutination.
HEMOLYSIS	(Gr. hema, blood + lysis, dissolution) The dissolution of red blood cells resulting from their having been sensitized by their specific antibody to the lytic effect of complement.
HEMOLYSIN	The name designating the antibody which takes part in hemolysis.
HETEROPHILE	(Gr. heteros, other + philos, love or affinity for) Having affinity for other antigens or antibodies besides the one for which it is believed specific.
IMMUNITY	(L. immunitas) The condition of being immune. The property an individual possesses to resist and overcome infection.
IMMUNITY (Active)	The immunity, usually of long duration, an individual possesses as a result of the parenteral injection of an antigen, or as a result of having had an infectious disease.
IMMUNITY (Passive)	Immunity, usually of short duration, an individual possesses as a result of the parenteral injection of an immune serum.
IMMUNITY (Acquired)	Immunity an individual acquires during life.
IMMUNITY (Natural)	Immunity an individual possesses as a result of genetic inheritance.
IMMUNOLOGY	The Science or study of immunity.

INACTIVATION	The heating of serum to 56 C. for 30 minutes to destroy complement. Also used with reference to the process employed to kill microorganisms.
INFECTION	Invasion of the tissue by pathogenic microorganisms in such a way as to favor their growth with resultant injury.
INJECTION (Primary)	The initial parenteral introduction of antigen. Immediately after this, no circulating antibody can be detected, followed by a slow rise in antibody titer which after reaching a maximum slowly decreases.
INJECTION (Secondary)	The second parenteral injection of antigen into a host which some time previously received the primary injection of the same antigen. Characteristically there is a rapid rise in antibody titer to a high maximum level which is maintained for a longer period of time.
IN VITRO	(L. in, in + vitreus, glass) Within a glass; observable in a test tube.
IN VIVO	(L. in, in + vivo, living) Within the living body.
LYSIN	(Gr. lysin, to dissolve) The antibody which sensitizes its specific antigen to the lytic effect of complement.
NEGATIVE PHASE	The temporary drop of antibody titer in a sensitized individual following a subsequent injection of antigen.
OPSONIN	(Gr. opsonin, to prepare food for) The substance present in fresh normal serum which renders bacteria more susceptible to phagocytosis.
OPSONIC INDEX	The ratio of the phagocytic index of the patient's serum to the phagocytic index of normal serum.
PARENTERAL	(Gr. par, beside + enteron, intestine) Besides intestine. A method of introducing materials into the body other than by way of the alimentary canal; e.g., subcutaneously, intramuscularly, intradermally, etc.
PLASMA	(Gr. plassein, to mold) The fluid portion of blood composed of serum and fibrinogen.
PRECIPITATION	The serological reaction resulting from the union of a colloidal antigen and its specific antibody in the presence of an accessory factor producing aggregation of the antigen-antibody complexes.
PRECIPITIN	The name designating the antibody which takes part in precipitation.
PRECIPITINOGEN	The name designating the antigen, colloidal in size, which takes part in the precipitation reaction.
SERUM	(L. Whey) The clear portion of any animal liquid separated from its more solid elements; especially the clear liquid which separates in the clotting of blood.
SERUM (Homologous)	(Gr. homo, like) A serum, usually immune, employed prophylactically or therapeutically which was obtained from another individual in the same species.
SERUM (Heterologous)	(Gr. heteros, other) A serum, usually immune, employed prophylactically or therapeutically which was obtained from an individual in another species.
SERUM (Immune)	One containing antibodies.

TITRATION	The process of diluting antigen, antibody, or complement for a quantitative determination.
TITER	(Fr. titre, standard) The greatest dilution of antigen, antibody, or complement which will produce the desired reaction.
TITER (Diagnostic)	The titer determined by research believed to be of diagnostic significance.
TOXIN	(Gr. toxin, poison) Substances produced by microorganisms which may stimulate the production of antitoxic antibodies.
TOXIN (Exo)	(Gr. toxin, poison + exo, out of) Substances produced by bacteria and liberated from the viable cell.
TOXIN (Endo)	(Gr. toxin, poison + endo, within) Substances liberated from bacteria upon cellular disintegration.
TOXOID	(Gr. toxin poison + oeides, like) A detoxified toxin. One treated with formalin, or other substances, which detoxifies the toxic portion but does not appreciably alter its antigenicity.
UNIT	The term employed in designating the potency of an antitoxin, hemolysin, or complement.
UNIVERSAL DONOR	(L. universlais, including the whole + donator, donate) An individual whose blood group is O. Theoretically he can donate blood to any individual regardless of the recipient's blood type.
UNIVERSAL RECIPIENT	An individual whose blood group is A. Theoretically he can receive blood from any individual regardless of the donor's blood type.
VACCINE	Suspensions of living bacteria, living or inactivated viruses, which after parenteral injection will stimulate the production of antibodies conferring an active immunity.
VIRULENCE	(L. virulentus, poison) The relative ability of a microorganism to produce disease.

SELECTION AND CARE OF LABORATORY ANIMALS

Rabbits, Guinea pigs, and chickens will be used. They must be healthy and in good condition. In most cases it is best to employ mature animals. During the process of immunization the animals must be well fed and their body weight should be checked. If any of them show loss in weight they should be given a rest period. The cages, in which they are kept, should be so constructed as to provide comfortable and sanitary quarters.

The laboratory animals, used in this course, must be handled with care and consideration. During demonstrations and group work there must be no unnecessary commotion or noise.

PREPARATION OF MATERIALS FOR IMMUNIZATION OF ANIMALS

Definitions and Explanations

A great deal of the work in this course is done with immune serums (antiserums). Serum is the liquid part of blood after coagulation (or defibrination) has taken place. An immune serum is one containing antibodies; especially one in which the antibody content has been increased by a naturally or artificially produced infection or by parenterally introduced antigen. Antibodies are substances elaborated by animal tissues, or physico-chemical properties acquired by certain tissue constituents, in response to the influence of an antigen. Antibodies will, when brought in contact with specific or closely related antigens in vitro under proper conditions, give rise to perceptible reactions. Antibodies may also be defined as animal tissue globulins which under the influence of antigens have acquired certain physico-chemical properties by which they are able to react with specific or related antigens and thereby produce detectable immunological results in vivo and in vitro. (Whether these definitions are applicable to innate antibodies, such as normal opsonins, isoantibodies, etc., is open to question). Antigens are substances which, when introduced parenterally into susceptible animals, will cause certain tissues of these animals to produce antibodies or cause the animals to become allergic. The word antigen is also used to designate substances which, when acted upon by specific antibodies in vitro, give certain perceptible reactions such as precipitation, agglutination, cytolysis and complement fixation. Generally speaking, antigens are protein substances to which are coupled carbohydrates or lipoids or both. The protein fraction of the antigen is usually the antibody stimulating part and the carbohydrate or lipoid fractions the part that governs specificity.

Antigens may be designated as either complete or incomplete. Complete antigens stimulate the production of antibodies upon parenteral injection into susceptible animals and also give rise to perceptible reactions in vitro under specified conditions. Incomplete antigens, commonly termed haptens, will not stimulate the production of antibodies in vivo but do govern specificity of a complete antigen and will react with specific antibody in vitro. On the basis of the observability of the in vitro reaction haptens are divided as follows:

Complex Hapten: Will react with specific antibody in vitro to give a perceptible reaction. Confers specificity to an antigen but will not stimulate the production of antibody.

Simple Hapten: Will react with specific antibody in vitro but this reaction is not directly perceptible. Can be demonstrated only by the subsequent addition of a specific complete antigen or a complex hapten. Upon such addition no reaction would occur, indicating the previous union of antibody and the simple hapten. Like complex haptens, simple haptens confer specificity to a complete antigen but will not stimulate the production of antibody.

By immune animals we mean, not only animals that are resistant to disease, but animals whose tissues have been acted upon by an antigen to the extent that antibodies can be detected in the blood stream.

This statement may need clarification. The words immune and resistant are not always used synonymously. An animal that is able to ward off an infection is resistant, or immune, to the disease caused by the pathogen concerned. Such resistance may be due to the presence of antibodies or it may be due to a tissue immunity without antibodies demonstrable by presently known techniques. Resistance (immunity) may be inherited (innate) as a result of transmission of genetic characteristics from parent to offspring. It may also be acquired in several ways: (1) by transmission of antibodies from mother to fetus (congenital immunity); (2) by transmission of antibodies through colostrum or milk; (3) by having and overcoming an infection; (4) by artificial immunization, using living or nonliving microbes or certain by-products such as toxoids; (5) by administration of serums containing specific antibodies.

The presence of antibodies in the blood of an animal does not always signify immunity, even if such antibodies are specific for a certain pathogen. Furthermore, animals may possess antibodies that have no relationship to pathogenic microbes or parasites. Among such antibodies are: the iso-antibodies concerned in blood typing, hemolysins, hemagglutinins and precipitins produced by the injection of animals with red blood cells and serums or egg albumin or any other antigenic substances of nonmicrobial origin.

Broadly speaking, any animal that possesses antibodies may be said to be immune. Likewise, serums that contain antibodies, produced in response to infection or artificial immunization, are rightly or wrongly called immune serums.

Animals that produce their own antibodies are called actively immune, whereas, those that obtain their antibodies from others are called passively immune.

Animals will be immunized with red blood cells, blood serum, bacteria, bacterial toxins and a virus. The process of immunization may be expressed by an example as follows:

$$\text{Antigen} + \text{susceptible animal} \longrightarrow \text{antibodies}$$

more specifically:

$$\text{Sheep serum} + \text{rabbit} \longrightarrow \begin{cases} \text{precipitins} \\ \text{hemolysins} \\ \text{hemagglutinins} \end{cases}$$

These antibodies are specific for sheep serum and sheep cells and, in addition, are able to act to a certain degree on cells and serum from closely related species of animals such as the goat.

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EXERCISE I

1. Bleeding Sheep

Place about two dozen glass beads in a 100 or 200 cc. Erlenmeyer flask (size depends on the amount of blood wanted). Plug the flask with a cotton plug covered with one or two layers of cheese cloth and sterilize in hot-air oven for 1 hour at 170°C. or in autoclave at 121°C. for 20 to 30 minutes. Sterilize a 2.5 inch, 15 gauge hypodermic needle by autoclaving. Back the sheep into a corner so that its left side is against the wall (for a right-handed operator). Have an assistant hold the sheep's head up and slightly to the right (while leaning against and partly sitting on its right shoulder) so that the skin over the left jugular groove will be fairly tense. Clip the wool closely from an area the size of a person's hand over the jugular groove, about half-way between the head and shoulder. Wash this area and the adjacent wool carefully with 3 pieces of absorbent cotton saturated with a quaternary ammonium compound. After the last washing, remove the excess moisture to leave the area fairly dry. With the thumb of the left hand apply pressure over the lower part of the jugular groove, thus causing the jugular vein to distend so that its location can be seen or determined by palpation with the middle finger of the right hand. Now, with a quick thrust, insert the needle into the vein and allow the blood to flow into the flask. When enough blood has been obtained remove pressure from jugular vein, withdraw hypodermic needle with a jerk so as to avoid bleeding and shake flask for 15 minutes to defibrinate the blood. If the blood must be sterile a piece of rubber tubing about 18 inches long may be attached to the needle at once end and at the other end to a piece of glass tubing extending through a perforated rubber stopper into the flask. To prevent pressure build up in the flask as the blood flows into it, there should be another piece of glass tubing, plugged with cotton, extending through a second hole in the rubber stopper. Wrap a plug of cotton around the hub of the needle and insert it into a test tube. The whole apparatus may be sterilized in the autoclave.

2. Preparation of Serum and Cells for Injection

The defibrinated blood is poured into sterile centrifuge tubes (about 3/4 full). The plugs of these tubes should preferably be covered with one or two layers of gauze. When the plugs are replaced, that portion protruding from the mouth of the tube should be folded down over the rim and secured with rubber bands to prevent their being thrown into the bottom of the tubes. Rubber caps may be used in place of cotton plugs. The blood is now centrifugalized at about 1200 R.P.M. for 10 to 15 minutes. Higher speed causes too much pressure with consequent crushing of the cells, liberation of hemoglobin and discoloration of the serum. The length of time required depends on the size of the tubes and the amount of blood placed in them. If a self-balancing centrifuge is not available the tubes, containing the blood, must be weighed in order to balance opposite loads placed in the centrifuge head. When the cells have been thrown down the supernatant serum is drawn off with sterile bulb pipettes fitted with rubber suction bulbs. Suitable quantities of serum are distributed in sterile test tubes with aseptic precautions. For injections into rabbits the serum is heated in a water bath at 56°C. for half an hour. This makes the serum less toxic for rabbits. The cells are suspended in sterile saline solution (.8 to .9 per cent NaCl in distilled water) and again centrifugalized. They are washed in this way three to four times in order to remove the serum. The cells are then resuspended in sterile solution so as to make a 50 per cent suspension.

Owing to the fragility of red blood cells, it is not advisable to attempt storage of them for any appreciable time. It may not always be convenient to bleed a sheep when sheep cells are required as, for example, for one of the reagents for the complement fixation test. Red blood cells can be stored in Alsever's solution which consists of the following:

Glucose	2.05 gm
Sodium Citrate	0.80 gm
Sodium Chloride	0.42 gm
Citric Acid	0.055 gm

Distilled water q.s. ad. 100.0 ml.

This solution is sterilized by autoclaving, care being taken to avoid over heating to prevent carmelization. It is used at the rate of 1 part of the solution to 1 part of whole blood. Red blood cells drawn directly into this solution, mixed and then stored under refrigeration can be preserved for at least ten weeks for use in the complement fixation test. Stored at ordinary room temperatures blood drawn directly into Alsever's solution can be maintained satisfactorily for at least two weeks.

EXERCISE II

1. Preparation of Bacterial Suspension for Injection

In immunizing animals for classroom purposes one needs merely suspend the growth from an agar slant in sterile saline (10 cc. of saline to 1 agar slant), and heat this suspension in the water bath at 60°C. for 1 hour. It is well to filter such a suspension through a sterile cotton filter, before heating, if it is to be injected intravenously. When bacterial vaccines are made commercially for use on humans or animals more elaborate methods are required.

2. Preparation of Bacterial Vaccines

The organisms may be grown on agar slants or in broth depending on their media requirements.

A. Preparation of Bacterial suspensions from agar slants

1. With a sterile pipette place enough sterile saline solution into an agar slant culture tube, so that the surface of the saline solution is even with the top of the slant.
2. With a sterile platinum loop scrape off the growth. Stir well to disperse the bacteria evenly. Do not dig into the agar with the loop!
3. Pour this suspension into one culture tube after another, repeating the washing off process until the bacterial suspension shows the desired turbidity.
4. Pour the suspension into a bottle containing about 12 small glass beads.
5. Stopper the bottle tightly with a sterile cork or rubber stopper and shake thoroughly for at least 15 minutes to break up clumps of bacteria.
6. Filter, through a sterile cotton filter, into a sterile test tube or bottle, stopper and let stand until ready for standardization.

B. Preparation of Bacterial suspension from broth cultures

1. Fold cotton plugs of culture tubes down over the rim of the tube and secure with rubber bands, place in centrifuge and centrifugalize at about 3000 R.P.M. for at least one hour, using a Size 2 I.E.C. Centrifuge.
2. With a pipette and a rubber bulb draw off the supernatant fluid and if it is thought desirable wash the bacteria once or twice in sterile saline to remove traces of medium.
3. Finally suspend the bacteria in saline so as to obtain the desired turbidity.

3. Standardization of Bacterial Suspensions

A. Wright's Method.

Apparatus. Capillary pipette (caliber about 1/16 in.) with rubber nipple; three concave slides; three or more properly cleaned (grease free) slides; sharp needle or blood lancet; absorbent cotton; 70 per cent alcohol; citrated salt solution; diaphragm for ocular with aperture of 2 mm.; Wright's or Hasting's stain.

1. Place about 0.1 cc. of bacterial suspension and citrated salt solution on concave slides labelled correspondingly.
2. Make a wax pencil mark on the capillary pipette about one inch from its tip.
3. Carefully disinfect the tip of the middle finger with 70 per cent alcohol, let dry and prick with a blood lancet. Press out one drop of blood holding the finger in such a position that the drop will round up well.
4. Draw into the capillary pipette:

   a. one volume of citrated saline solution
   b. a small bubble of air
   c. one volume of blood
   d. a small bubble of air
   e. one volume of bacterial suspension