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ABSTRACT

Presented are laboratory procedures of a serologic or immunologic nature for use in the diagnosis of a variety of infectious and noninfectious conditions. Included are tests for the detection of specific antibodies in the patient's serum, as well as immunologic tests for the detection of other products in the patient's serum and biological fluids. For the more frequently performed tests, the principle of the test, the reagents and the equipment needed, the preliminary steps, the complete test procedure, reporting results, and potential sources of error are presented. Additionally, the significance and interpretation of results of the tests are discussed in each chapter. (Author/CS)

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MEDICAL SERVICE

CLINICAL LABORATORY PROCEDURES—SEROLOGY

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Chapter 1

INTRODUCTION TO SEROLOGY

SECTION A—INTRODUCTION

1-1. Serology Defined. It is logical that the sooner an accurate diagnosis of a disease is made, the sooner appropriate therapy can be started. Consequently, much research, with many advances, has been directed toward more rapid and accurate diagnoses. One of the areas of clinical laboratory science that has been expanding most rapidly along these lines is clinical serology.

a. Basically, serology can be defined as the study of serum. More specifically, serology is diagnosis through the use or detection of serum globulins, known as antibodies. These antibodies may even have been produced in another animal and then used to diagnose disease in man. In effect, any in vitro diagnostic test using antibodies can be called a serologic test or serotest. However, antibodies are only half of the reactants in serotests. Antibodies must have something to react with—namely, antigens.

b. This manual is concerned with mixing these antigens with antibodies and reporting the resulting antigen-antibody reaction, if any, to the physician. General characteristics of antigens, antibodies, and their reactions are covered in the next chapter with the remainder of the manual devoted to laboratory techniques and serotests for specific diseases or conditions.

c. Due to the special techniques and time-consuming steps used in preparing them, many of the reagents used in serologic tests are available in kit form. Many kits are available through regular supply channels while others are available commercially. Attachment 3 is a partial list of sources of serologic kits and reagents. Some of these kits contain everything needed to accomplish the test. Consequently, relatively few items of glassware and laboratory equipment are used in serologic testing. In fact, more and more disposable items are being used. Glassware and equipment used in serotests are covered in the next two sections of this chapter.

SECTION B—GLASSWARE

1-2. Test Tubes. Several serotests are performed in test tubes. In addition, the patient's serum is the most frequently assayed specimen, thus requiring more test tubes. Those test tubes most commonly used are 12X75mm (Kahn), 13X100mm (Wassermann), and centrifuge tubes. Both reusable and disposable types are available through the supply system.

1-3. Slides. Many, in fact most, recently developed serotests are performed on glass slides or plastic-coated paper cards instead of in test tubes. These slide tests use very small quantities of reagents and, in addition, many use the disposable paper cards, thus making these tests very efficient for the busy laboratory. Other tests use just plain glass slides; black glass slides or tiles; slides with molded concavities; or slides with ceramic or paraffin rings. Each test kit usually comes complete with the recommended slide. Others must be purchased separately or prepared in the laboratory. Be sure to use the specific type of slide that is indicated for each given procedure.

1-4. Pipets. Serotests require the mixture of accurately measured volumes of reagents. Although many tests utilize medicine droppers to dispense reagents, even these droppers are manufactured to deliver a specific size of drop. Several serotests require larger volumes of reagents than can be delivered conveniently by droppers. For these tests, pipets are more efficient. Several reusable types are available.

a. Types of Pipets Used in Serology:

(1) Serological Pipets. Appropriately, the pipets most commonly used in serotests are serological pipets. Examples of common sizes are shown in figure 1-1. As indicated by the etched mouthpieces, these pipets are allowed to drain freely and, then, the last drop is blown out. Serological pipets are calibrated to deliver (T.D.) a given volume at a specific temperature, usually about 20°C. Therefore, for most accurate use, they should not be rinsed out, but this practice is, at times, ignored in serotesting. Serological pipets must not be confused with Mohr pipets which are also T.D. pipets. Mohr pipets are

not calibrated to their tips and must be used only with the point-to-point pipetting technique. It is best to keep Mohr pipets out of the serology area.

(2) Capillary Pipets. These pipets are also called Pasteur or transfer pipets. They are most useful in transferring reagents, such as serum, from a clot to another tube. They work most efficiently with a rubber bulb but may also be filled by mouth. Two sizes of Pasteur pipets are available. In addition, they may be easily made from glass tubing by heating and drawing out the tubing.

b. **Using Serological Pipets:**

(1) Select the smallest pipet that will deliver the volume required. Use only clean, dry pipets without chipped tips. Those with chipped tips should be discarded.

(2) Draw fluid slightly above the desired mark, using the tip of the forefinger (index finger) to control the flow.

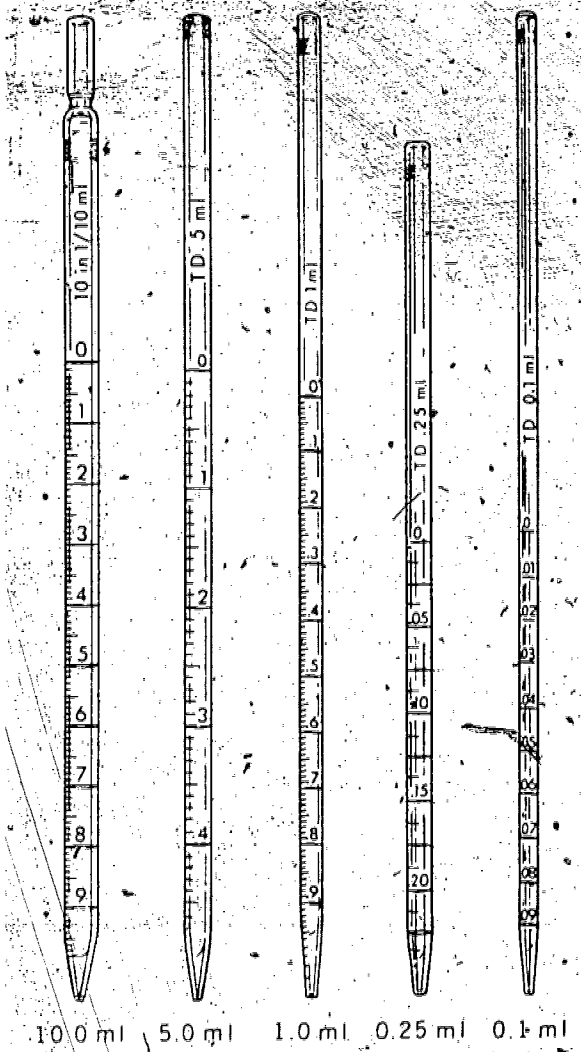


Figure 1–1. Serologic Pipets.

(3) Hold the pipet vertically and wipe the tip (wet area) with gauze or lint-free absorbent paper.

(4) Holding the pipet vertically so that the fluid level is at eye level and reading from the bottom of the meniscus, let the fluid down exactly to the desired mark. Do not put the tip of the pipet back into the fluid during this operation. Touch the tip of the pipet to the inner wall of container to dislodge any droplet that might be hanging from the tip.

(5) Recheck the fluid level. Repeat the previous steps if the fluid is not at the desired mark.

(6) Transfer the pipet to the intended container and let the fluid drain freely before blowing out the last drop. Do not immerse the tip while draining. If increased accuracy is desired, a point-to-point pipetting technique may be used but this is seldom required in serologic testing, unless Mohr pipets are used. The specialized use of serological pipets in making serial dilutions of serum is covered in chapter 4.

1–5. **Cleaning Glassware.** Most serotests are performed under closely controlled conditions of pH and ionic concentrations. Without prerinsing, even disposable glassware fresh from the manufacturer may not be clean enough for serotests. Although these tests rarely require sterile glassware, all reusable glassware that has been in contact with patient's blood or infectious organisms must be sterilized before cleaning to reduce the danger of transmitting infectious hepatitis and other infections. Methods for prerinsing and cleaning glassware are given below.

a. **Prerinsing Glassware.** For reusable glassware, the most useful way to assure cleanliness for future tests is to prerinse the glassware immediately after use. Of course, if live organisms are used, sterilize the items first. Assuming that no viable pathogenic organisms were used in the test, the simplest way to prerinse is with running tapwater followed by immersing the glassware completely in tapwater with or without a detergent. In other words, don't let the reagents dry in or on the glassware. Glassware containing relatively small amounts of reagents could be placed directly in the water without rinsing. In addition, pipets are especially inconvenient to rinse immediately after use, so these should be immersed completely in a vertical position in water containing detergent. This is best accomplished in a cylinder with a cotton-lined bottom to prevent chipping.

b. **Cleaning Test Tubes and Slides:**

(1) If desired, these items may be soaked overnight in hot tapwater containing a neutral detergent such as any of the well-known liquid household dishwashing detergents. Highly alkaline detergents should not be used for serologic glassware because of the difficulty in removing residual alkali that may affect test results. In addition, prolonged exposure to alkali will etch the glassware.

(2) Actual cleaning of the glassware can usually be accomplished with a nylon or similar brush in hot tapwater containing neutral detergent. Only excessively dirty items will normally have to be subjected to stronger cleaning solutions such as the chromic acid cleaning solution covered in paragraph 1-5f.

(3) Rinse the glassware several times in hot running tapwater and, then, several times in fresh distilled water.

(4) Dry in an inverted position in a drying oven (100°C) and store in a dust-free area. Be sure to discard chipped or cracked tubes. Using cracked centrifuge tubes could be especially wasteful of both time and reagents—not to mention the possible inconvenience to the patient.

c. **Cleaning Larger Pipets.** Pipets in sizes of one ml or larger can be cleaned efficiently in automatic pipet washers such as described in paragraph 1-6. Cleaning smaller pipets will be covered in paragraph 1-5d. Excessively dirty pipets may be cleaned in chromic acid cleaning solution.

(1) Sort the prerinsed pipets, removing those smaller than one ml.

(2) Make sure the tips are not clogged with debris.

(3) Place the pipets in the holder (basket) with tips uppermost.

(4) Place basket in washer and wash at least 2 hours. Most washers can be set to cycle at least 10 times per hour. Be sure to observe several cycles to be certain that the correct water pressure has been applied and that the washer is cycling properly. Do not simply turn on the water unless the pressure has been preregulated.

(5) Remove the pipets and recheck for clogged tips. Clogged pipets will not wash by this method.

(6) Allow the pipets to drain briefly and rinse 5-6 times in fresh distilled water, making sure to drain the pipets completely after each rinse.

(7) Dry in a hot air oven (100°C).

d. **Cleaning Smaller Pipets.** Pipets smaller than one ml will not flush adequately by gravity as employed in automatic pipet washers. Consequently, small pipets must be washed by aspiration using a vacuum such as produced by a faucet vacuum pump. Several adapters for various numbers of pipets are available. After rinsing for several minutes with tapwater, the pipets are rinsed with distilled water, drained, and dried in a hot air oven (100°C) or they may be dried by flushing with acetone followed by air.

e. **Precleaning Disposable Glassware.** Disposable test tubes are very convenient for serotests. However, most disposable glassware is made from very cheap glass. This glass may still contain contaminating alkali unless it has been precleaned at the factory at added cost. Because serotests are easily affected by chemical contamination, this alkali must be neutralized. Neutralization is accomplished by immersing the glassware overnight in 2% (v/v) hydrochloric acid. Then, the glassware is rinsed several times in hot running tapwater followed by fresh distilled water and dried.

f. **Chromic Acid Cleaning Solution.** Unusually dirty glassware may be cleaned by immersion in chromic acid cleaning solution. However, due to the difficulty of insuring adequate rinsing, serologic glassware should not be cleaned this way unless absolutely necessary.

(1) Preparing Chromic Acid:

Sodium dichromate ($Na_2Cr_2O_7 \cdot 2H_2O$) or potassium dichromate ($K_2Cr_2O_7$), technical grade	100 g
Tapwater (or distilled water)	1000 ml
Sulfuric acid (H_2SO_4), concentrated, technical grade	1000 ml

Dissolve the dichromate in the tapwater in a large sturdy container. Once the dichromate is dissolved, place the container in a sink with cold circulating water covering the level of the liquid. While wearing rubber gloves, an apron, and safety glasses, slowly add the sulfuric acid with constant stirring. (ALWAYS add acid to water slowly with stirring.) The solution will change from orange to dark brown as acid is added. This solution is usable until it turns green. Glassware containing large amounts of serum or other organic substances will rapidly reduce the useful period of the solution. Therefore, prerinsing and at least partial drying of the glassware is advisable before cleaning in chromic acid solution.

(2) Using Chromic Acid Cleaning Solution. Prerinsed and partially dried glassware is immersed CAREFULLY in the acid while wearing rubber gloves and safety glasses.

Special care must be taken to slowly immerse pipets as they may spurt acid if immersed too rapidly. Be sure test tubes and pipets are completely filled. Let stand overnight; then, using the previously mentioned safety precautions, rinse the glassware thoroughly in hot running tapwater followed by distilled water, and dry at 100°C. Larger pipets can be rinsed in a pipet water after acid cleaning.

SECTION C—LABORATORY EQUIPMENT

1–6. Pipet Washer. One of the more useful pieces of equipment used in serology is a pipet washer. Various automatic models are available—some with built-in dryers. Most can wash about 200 1-ml pipets at one time. Cleaning is by surging water as the washer repeatedly fills and empties. Pipets smaller than 1 ml should not be washed in these washers because the surging action is hampered by the small bore of the pipets. In addition, care must be exercised in setting the water pressure of these washers so that an effective surging action is achieved. A typical pipet holder and washer are shown in figure 1–2.

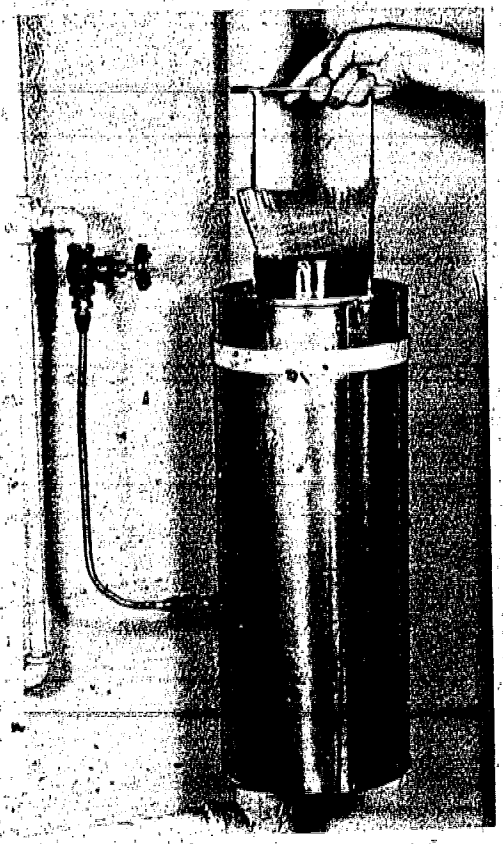


Figure 1–2. Pipet Washer.

1–7. Centrifuges. Centrifuges are used in serology mainly to separate patients' sera from blood clots. In addition, centrifugation is a step in the preparation of certain antigens, such as erythrocyte suspensions. Several types of centrifuges are applicable to serologic testing. Two examples are shown in figures 1–3 and 1–4.

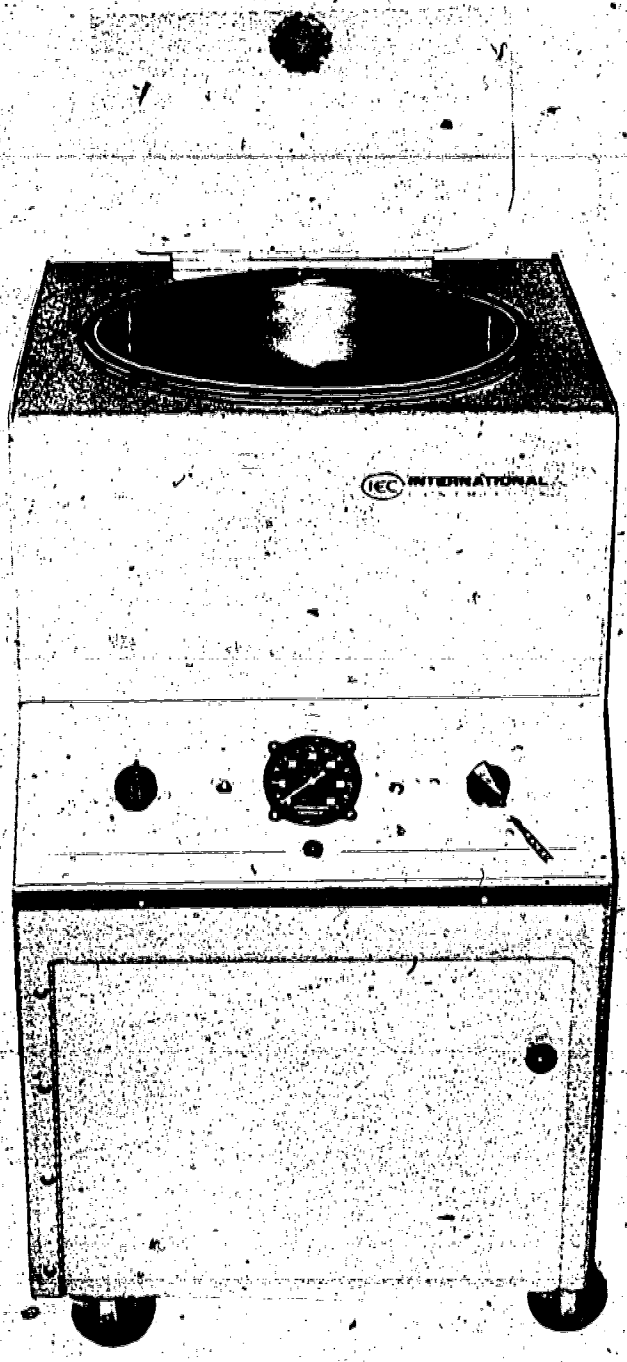


Figure 1–3. Centrifuge, Floor Model.

a. **Use of Centrifuges.** Usually each serologic procedure will specify the time and revolutions per minute (rpm) that centrifugation should be applied. These settings will be adequate for most general purpose centrifuges. However, certain procedures require very critical centrifugation. For these procedures, a specific relative centrifugal force (rcf) or gravity (g) is given. The rcf and g will be considered as synonymous for serologic purposes. Both rcf and g are based on rpm and the radius of the centrifuge. Although tachometers on centrifuges measure only rpm, most instruments will have conversion tables or nomograms to convert rpm to rcf (g). The required rpm for achieving a given rcf may also be calculated.

b. **Calculating rpm From rcf (or g):**

(1) Determine the radius (in centimeters) of the centrifuge head by measuring to the outermost TIP of the centrifuge tube. Centrifuges with swinging-type cups must have the cups extended horizontally before measuring the position of the tip of the centrifuge tube.

(2) Calculate the required rpm setting for the desired rcf using the following formula:

$$\text{Required rpm} = \frac{\text{Desired rcf (or g)} \times 105}{1.11 \times \text{radius (in cm)}}$$

c. **Centrifugation Precautions.** Valuable time and reagents can be lost if glassware shatters during centrifugation. In general, glass centrifuge tubes will not withstand relative centrifugal forces (rcf or g) of more than about 2000 X g. Therefore, know your centrifuge and when to switch to polyethylene or steel centrifuge tubes.

1-8. Rotating Machines. Slide rotators are invaluable for serotests that require prolonged rotation of the reactants on slides. Many serotests require closely controlled rotating speeds and times so machines with variable speeds and timers are especially useful. Be sure to calibrate rotators for each procedure. An example of a slide rotator is shown in figure 1-5.

1-9. Constant Temperature Devices. Several serologic tests must be performed or incubated at some constant temperature, usually body temperature. In these tests, a variation of 1°C from the prescribed temperature may not be acceptable. In other tests, a range of several degrees may still yield valid results. Four main types of constant temperature devices are useful in serology.

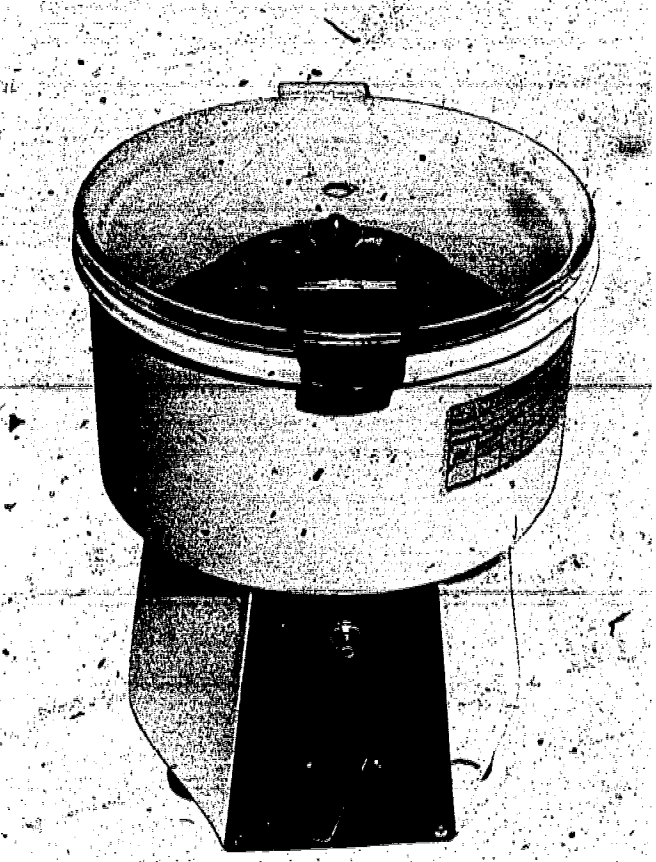


Figure 1-4. Centrifuge, Table Model.

a. **Freezers.** The best way to preserve serum, if it cannot be tested immediately, is to freeze it at minus 20°C or colder. This is because storage of serum at room temperature may allow microorganisms to denature the antibodies and, thus, render the serum useless for serotesting. Be sure to check storage instructions before freezing serologic reagents, because some reagents are destroyed by freezing.

b. **Refrigerators.** Refrigerators set at 4-6°C are used to store many serologic reagents. In addition, serum may be stored for short times at this temperature. Only two serotests, the cold hemagglutination and CRP tests, are performed in a refrigerator.

c. **Water Baths.** Variable-temperature water baths are available that can maintain temperatures within 0.5°C of any desired temperature from slightly above room temperature up to about 60-70°C. Although heat transfer to test tubes immersed in water is excellent, these tubes must be handled carefully as dripping water could ruin a test. A typical water bath is shown in figure 1-6.

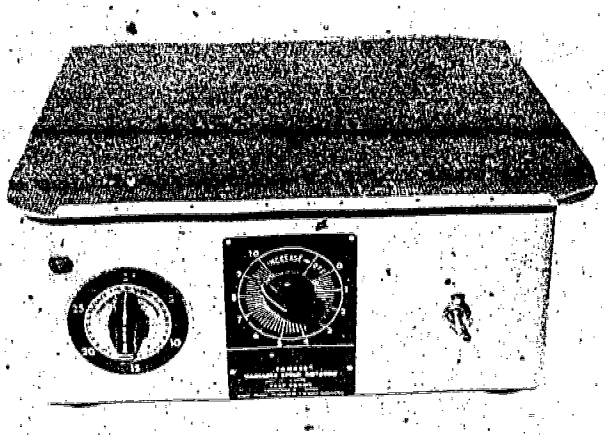


Figure 1-5. Slide Rotator.

d. **Dry-Heat Blocks.** Heating blocks can maintain temperatures as constant as a water bath and are useful for temperatures including boiling and higher. However, heat transfer is not as efficient as with a water bath and test tubes exactly the diameter of the heating block must be used. Fortunately, units with interchangeable tube holders are available. See figure 1-7 for an example of a dry-heat block.

1-10. **Microscopes.** Microscopes are required to read certain antigen-antibody reactions that do not yield macroscopically visible reactions. Two main types of microscopes are used in serology.

a. **Light Microscopes.** The use of ordinary light (compound) microscopes is declining rapidly in serology as more and more macroscopic tests are developed. However, certain tests for syphilis still rely on light microscopes. The specific use in these tests is covered in chapter 6. Information on the care and use of light microscopes may be found in AFM 160-51, Clinical Laboratory Procedures—Hematology.

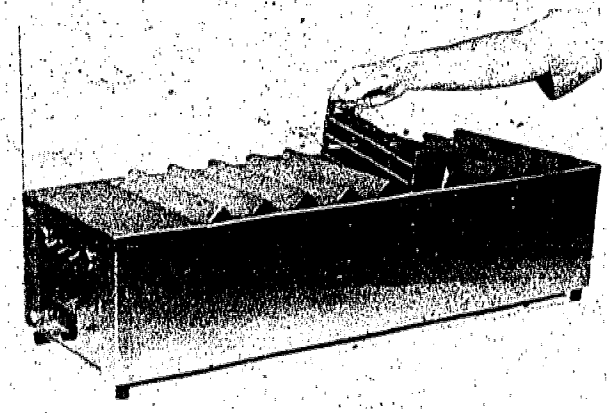


Figure 1-6. Water Bath.

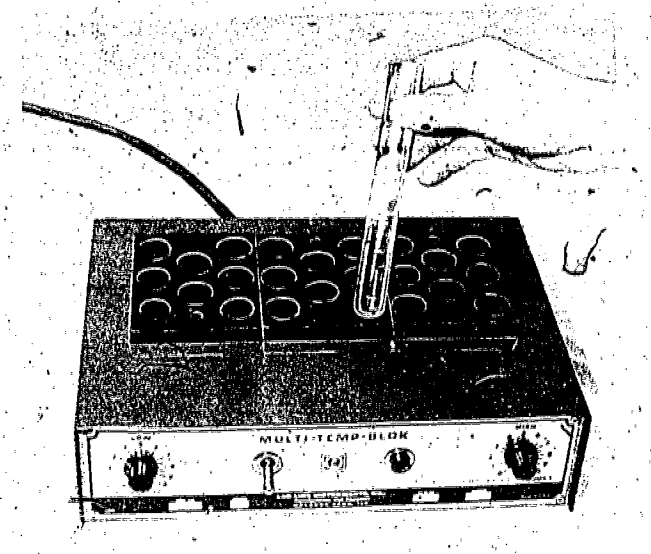


Figure 1-7. Dry-Heat Block.

b. **Fluorescent Microscopes.** Fluorescent microscopes are basically light microscopes that have a system of barrier filters built into their barrels. Most of these microscopes can still be used as light- and dark-field microscopes too. The important difference with a fluorescent microscope is the light source.

(1) The light is usually from a high-pressure mercury vapor bulb that emits light rich in wavelengths of 300-400nm—the upper UV area. A system of filters removes most other wavelengths, thus exposing the object under study to these UV wavelengths. If the object is stained with certain fluorescent dyes, the object will emit light of a color that is characteristic of the dye.

(2) After passing the object, extraneous UV light is filtered by the barrier filters in the barrel of the microscope. These filters serve two purposes. First, they protect the eyes of the observer. Second, allow the object to be seen in its characteristic colors against a dark background. Use of a dark-field condenser also enhances this contrast, but some procedures use bright-field condensers.

(3) The principle behind the use of fluorescent microscopes in immunofluorescence (fluorescent antibody) testing is covered in paragraph 2-12. Specific applications of the technique covered in this manual are in the fluorescent treponemal antibody-absorption (FTA-ABS) test in chapter 6 and the antinuclear antibody (ANA) test in chapter 12. An example of a fluorescent microscope with its power supply and light source is shown in figure 1-8.

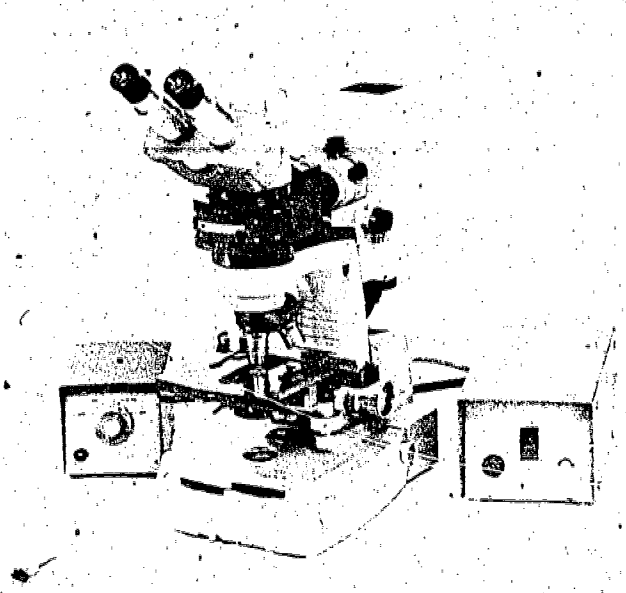


Figure 1–8. Fluorescent Microscope Apparatus.

1–11. **Other Equipment.** Several other items of equipment are used in serologic testing. Some are used only on rare occasions or only in larger or specialized laboratories. These will be discussed briefly.

a. **Timers.** Most serologic tests must be carefully timed. Several types of timers are available, some of which can be used to start or stop other equipment. In addition, many pieces of equipment, such as centrifuges, rotators, et cetera, may have built-in timers.

b. **Sterilizers.** Sterile glassware is rarely needed for serotests. Only tests that are incubated at body temperature for several hours would require sterile conditions. Of course, any equipment or glassware contaminated with viable organisms must be sterilized before being discarded or cleaned.

c. **Microtitration Equipment.** The development of microtitration equipment has proven to be a very useful substitute for test tubes and pipets in certain serotests. At times these tests use as little as one-tenth the amount of reagents as the original test method using test tubes, et cetera. In addition, microtitration is many times faster in setting up a test procedure than other methods. The basic equipment consists of calibrated microdroppers, plastic plates with wells in them, and the microdiluters. These items are available commercially. They are shown diagrammatically in figure 1–9. Many accessories, including automatic pipetters and diluters, are available to complement these items. Although microtitration equipment is required for only one procedure in this manual (the rubella titer in chapter 14), the technique has been applied to several other serotests. Examples are the antistreptolysin-O (ASO) test in chapter 9, tests for bacterial agglutination as in chapter 5, complement fixation tests, and others.

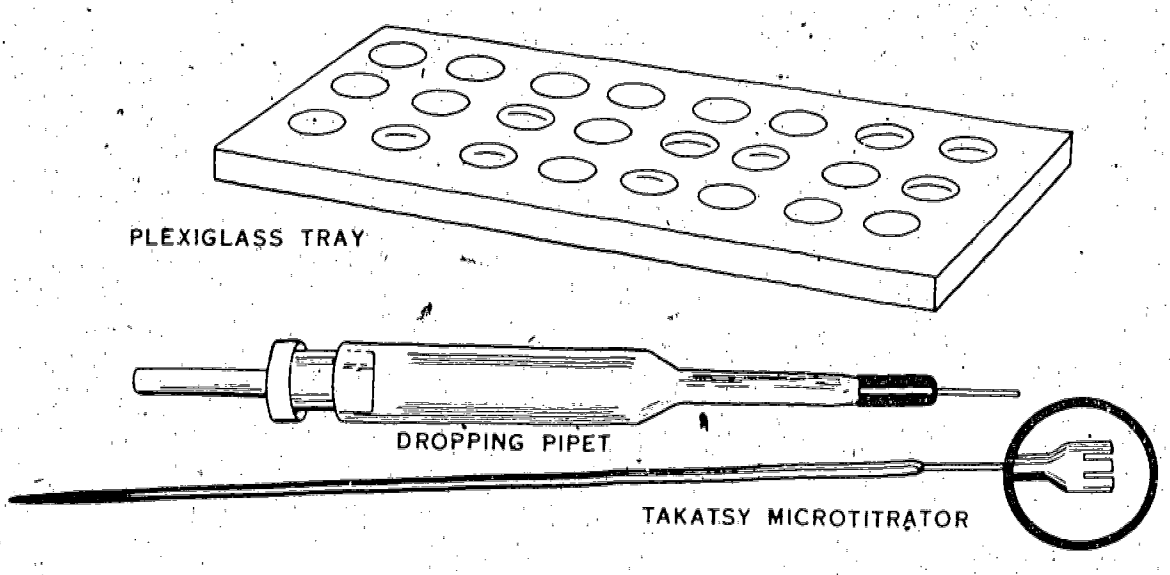


Figure 1–9. Microtitration Apparatus.

Chapter 2

IMMUNOLOGICAL BASIS FOR SEROLOGIC TESTS

SECTION A—IMMUNITY

2-1. Introduction. Resistance or immunity to disease can be classified under two broad headings—innate immunity and acquired immunity. If the disease-resisting ability is an inherited characteristic, the individual is said to possess innate immunity. Innate immunity is usually very general in action and not directed specifically at a given pathogenic organism. In contrast, acquired immunity is directed against a specific microbe or microbial product.

a. The specificity of acquired immunity involves inactivation of the pathogen or foreign substance by specific globulins, known as antibodies, that have been produced by the body in response to the pathogen or some foreign substance gaining entry into the body. These antibodies are found in body fluids—especially blood. Antibody production can also be stimulated by the injection of killed or attenuated organisms used as vaccines. Even parts of organisms or their toxins may stimulate antibody production. Immunity can also be acquired by producing antibodies in one individual or an animal and then injecting these antibodies into another individual, thus hopefully protecting the recipient.

b. Although all immunity involving antibodies may be classified as acquired immunity, not all antibodies are of a protective nature. Many antibodies arise from contact with foreign substances that are not connected with disease at all. In some diseases, the mere presence of certain antibodies, whether protective or not, is diagnostic. In other diseases, an increase in antibody level is significant. The detection and study of antibodies and their reactions provide the basis for the science of serology.

2-2. Innate Immunity. Innate immunity is the type where man is immune to canine distemper, while dogs do not contract syphilis. Each species has innate immunity to that particular disease. There are many degrees of innate immunity displayed. All species do not possess the absolute immunity described in the example. Even members of the same species vary in their ability to resist a given disease. Many defense mechanisms are active in innate immunity. Although there is much overlapping, these defense mechanisms can be divided into two categories—anatomical and chemical, excluding antibodies. A few of these will be described.

a. **Anatomical Defense Mechanisms:**

(1) Skin. The intact skin is an effective physical barrier to most disease-causing organisms. In addition, chemical mechanisms also operate here because the acid pH of the surface of the skin inhibits many microbes.

(2) Phagocytes. Throughout the body there is found a wide variety of cells which actively engulf (phagocytize) foreign particles, including microbes. White blood cells are well-known as phagocytes. Kupfer cells of the liver and microglial cells of the brain are examples of phagocytes which are localized in specific organs. As usual, chemical mechanisms cannot be excluded, for even the actual attraction of the phagocyte to the foreign particle is chemically or ionically mediated. Also, chemical degradation of the microbe takes place in the phagocyte if the phagocyte has the appropriate enzyme systems to destroy that particular microbe.

(3) Mucus. Mucus may act as a physical "trap" for disease agents. In combination with other mechanisms such as the cough reflex and cilia of the respiratory tract, mucus becomes an effective factor in preventing disease. Mucus can also contain phagocytes, enzymes, and antibodies which add effectively to disease resistance.

(4) Mouth. The simple act of swallowing moves potential pathogens to the stomach where few can survive. Coughing followed by swallowing aids in the removal and destruction of respiratory pathogens. Many enzymes and other microbial inhibitors are active in the salivary juices of the mouth too.

(5) Urinary Tract. The flushing effect of the urinary tract helps to physically remove potential pathogens.

(6) Eyes. The flushing action of tears, along with the presence of enzymes in the tears, provides one of the best examples of innate immunity. Acquired immunity is of minimal importance here. Even so, the eyes

are perhaps the most healthy organs of the body when one considers infectious disease.

(7) Lymphatic System. This system of ducts running throughout the body has nodes at intervals. These nodes can physically filter microbes from lymphatic fluid. Also, the nodes are the sites of accumulation of white blood cells, including fixed tissue macrophages. These cells are then in an ideal position to phagocytize the offending microorganisms.

b. **Chemical Defense Mechanisms, Excluding Antibodies:**

(1) Lysozyme. This enzyme is present in many body fluids such as mucus, tears and saliva. Primarily, it attacks the cell walls of gram-positive bacteria.

(2) Interferon. This protein is produced by the body in response to a viral infection or after the injection of dead viruses. It differs from antibodies in that interferon may provide protection against challenge by another completely unrelated virus while antibodies normally act only against the specific injected virus.

(3) Properdin. This high-molecular-weight protein is effective against certain viruses and also against some gram-positive bacteria and even a few gram-negative ones. Properdin might be considered to be an antibodylike substance as its action requires the presence of complement and magnesium ions, both of which are active in certain antibody reactions. In fact, properdin has been called a "natural antibody," but it lacks the specificity of an antibody and attacks many different organisms.

(4) Inflammation. Invasion by certain microbes causes inflammation which brings several defense mechanisms into action. For one, the temperature at the site of the inflammation or even throughout the body may rise, thus becoming a less favorable environment for many pathogens. The increased permeability of blood vessels at the site allows antimicrobial factors and white blood cells to readily contact the infectious agent. Alteration of the chemical composition at the site also acts as a defense mechanism. For example, the pH goes down as lactic acid and carbon dioxide accumulate. All of these changes during inflammation combine to resist further reproduction and dissemination of the pathogen.

2-3. Acquired Immunity.

a. **Active Immunity.** Active immunity is an acquired immunity because it involves specific antibodies against a microorganism or foreign substance. It is called active immunity because the individual actively produced these antibodies himself at some stage in his life. The antibodies may arise as a result of a clinical or subclinical infection by the given microbe. They may also be produced in response to vaccinations with killed or attenuated organisms. In either case, the individual produces the antibodies. Antibodies produced following actual infection are said to confer naturally-acquired active immunity. Vaccination produces artificially-acquired active immunity. The resulting antibodies may contribute to immunity in several ways or not at all. Some may cause a microbe to be more readily phagocytized, destroy it directly, neutralize some toxin produced by the organism, or combine these actions. Man may also produce antibodies that have no apparent protective value at all but their detection may be useful diagnostically.

(1) Naturally-Acquired Active Immunity. The fact that a person has recovered from an infectious disease does not guarantee resistance to another attack by the same microbe. For example, influenza and gonorrhea result in very short-lived immunity and repeated attacks are common. On the other hand, a single infection of measles or chicken pox usually confers life-long immunity to reinfection. There are other examples of these extremes of immunity and also many that fall between the extremes.

(a) Especially significant to the serologist is the rise in antibody titer during the course of a disease. In many cases, serological tests are the only practical diagnostic tools available. Perhaps isolation of the causative organism would be impossible, too expensive, or take too long to benefit the patient.

(b) Another important aspect of serological testing is the determination of antibody levels at a given time. A good example of this is the testing of serum from pregnant females for antibody titers against rubella (German measles). In this case, a certain titer usually indicates that the pregnant woman is immune to the rubella virus. Consequently, the safety of the developing fetus will most likely be assured in the event the immune woman should become exposed to rubella.

(2) Artificially-Acquired Active Immunity. For many diseases, vaccination has proven to be an effective method of providing immunity. This immunity is also not absolute and the relative efficiency of each vaccine can be guessed by the frequency that

reimmunization is required. In endemic areas, cholera and plague shots are given every 6 months; yellow fever shots, every 10 years. Obviously, the yellow fever vaccine is much more effective than cholera and plague vaccines. Although antibodies produced as a result of vaccination may benefit the patient, these same antibodies usually confuse the serologist. His tests usually will not differentiate between artificially-acquired antibodies and those produced in the presence of active disease. Consequently, a negative history with a rising antibody titer becomes very important in serodiagnosis. Of course, the problem is minimized in diseases where vaccination is not practiced.

b. **Passive Immunity.** Passive immunity is another type of acquired immunity because antibodies are involved. It differs from active immunity by the fact that the antibodies are produced in another individual or animal and then injected into the recipient. The recipient passively receives the immunity conferred by these antibodies. Although passively-transferred, antibodies are important to the recipient; they are short-lived and rarely assayed by the clinical serologist. As with active immunity, passive immunity may be acquired both naturally and artificially.

(1) Naturally-Acquired Passive Immunity. This type of immunity is significant mainly in the survival of the newborn infant. The infant passively acquires antibodies from its mother. The antibodies may pass from the immune mother to the fetus across the placental barrier. In addition, the infant may acquire these antibodies from its mother's milk which is rich in antibodies for a short time after birth. Of course, immunity is transferred only if the mother is immune to a given disease. Passive immunity is especially important to the newborn because they are incapable of producing antibodies of their own for a few months after birth. The antibodies received via natural transfer from the mother are relatively short-lived with protection seldom exceeding about 6 months. Fortunately, by this time the infant's immunological system is fully functional.

(2) Artificially-Acquired Passive Immunity. Antibodies that have been produced in another individual or animal and then administered by injection to the recipient provide this type of immunity. This method has been used extensively in the past in the treatment of diphtheria and tetanus through the injection of antibodies produced in horses. Before the advent of antibiotics, passively-administered antibodies were used as the treatment for pneumococcal pneumonia. Currently, passive immunization is mainly used for prophylaxis following exposure to such diseases as rubella and infectious hepatitis. This is usually accomplished by injecting the recipient with antibodies (gamma globulin) which have been extracted from the blood of immune persons. These antibodies provide protection for a relatively short time and then are removed from the system.

SECTION B—ANTIGENS AND ANTIBODIES

2-4. Antigens:

a. **Introduction.** Any foreign substance that stimulates the body (or any animal's body) to produce antibodies is called an antigen. To qualify as an antigen, these foreign substances must also react in some specific way with the antibodies that have been produced. An antigen might be a microbe, part of a microbe, some microbial toxin, or even some foreign product completely unrelated to microorganisms and the diseases they cause. Although antigens comprise a wide variety of substances, they have several characteristics in common. All function together to qualify a substance as an antigen.

b. **Characteristics of Antigens:**

(1) Foreign to the Body. Normally, the body does not respond by producing antibodies against itself. A notable exception to this is the production of antibodies against the lens of the eye. This may be explained by the fact that the lens is not in intimate contact with the reticuloendothelial system which produces the antibodies so the lens is, therefore, recognized as foreign. In addition, there are other examples of so-called autoimmune diseases in which the body produces antibodies against itself but these are usually associated with some malfunction of antibody production or antigen recognition. In general, however, the reticuloendothelial system responds only to antigens that are foreign, but even members of the same species have many antigens that are foreign to other members of the species. In man, the red blood cell antigens with their significance in blood transfusions and erythroblastosis fetalis provide good examples of foreignness within a species. Another example is seen in kidney transplantations, where usually only kidneys from very close relatives are accepted by the recipient. In time, kidneys from unrelated donors are usually rejected due to reaction with antibodies produced against kidney antigens. However, the mere fact that a substance is foreign to an individ-

ual does not confer antigenicity. Other characteristics must be present too.

(2) Molecular Weight. Another characteristic of antigens is that they are generally complex chemicals of very high molecular weight. A molecular weight of 10,000 is considered to be the minimum, but antigens with a molecular weight down to about 3,000 have been known. Some antigens have molecular weights in the millions. In general, the strongly antigenic substances have higher molecular weights than weakly antigenic substances.

(3) Chemical Nature. Most antigens are proteins or proteins complexed with carbohydrates or lipids and, as such, are derived from living organisms. As a general rule, carbohydrates and lipids are not antigenic unless in a protein complex. Only humans and mice appear to be able to produce antibodies against pure carbohydrates, such as certain polysaccharides, but this is a rare occurrence in nature.

(a) Each antigen has two parts—a carrier and a determinant. The carrier is usually a protein. Aside from contributing most of the molecular weight and increasing the rigidity of the antigen, the carrier functions mainly as its name implies—a carrier. The determinant is the important part of an antigen. It makes an antigen a specific, foreign substance. Although the determinant cannot stimulate antibody production by itself, it can react with the antibodies once they are produced. A determinant functioning in this fashion without its normal carrier is called a hapten. Many consider determinants and haptens as synonymous.

(b) Determinants (or haptens) comprise only a small portion of the total antigen molecule. For example, even the stereoisomers, D-tartaric acid and L-tartaric acid (M.W. 150), when conjugated with a carrier protein, form specific antigens. Each stimulates the production of antibodies which react only with the given isomer. In addition to lending specificity to the antigen and stimulating the body to produce specific antibodies against them, determinants are the reaction sites for the antibodies in antigen-antibody reactions. Each antigen molecule usually has about a dozen of these reaction sites (determinants) but some molecules may have a hundred or more. By contrast, the most common type of antibody molecule has only two reaction or combining sites while other types may have up to ten sites. Therefore, most antibodies are said to be bivalent, while antigens are multivalent. The valence of antigens and antibodies is especially important to the serologist in mixing the proper proportions of each in serologic tests.

(4) Reactivity with Antibodies. The last characteristic that an antigen must possess is that it must be able to react in some demonstrable way with the antibodies which have been produced in response to that antigen. The utilization and observation of these antigen-antibody reactions provide the basis for serological diagnosis and are covered later in this chapter and throughout this manual.

c. **Fate of Antigens.** Antigens may enter the body in a variety of ways. They may be acquired during the normal course of living, during disease, or following vaccinations. They may also be injected accidentally such as during a blood transfusion. Once they enter the body, they are very rapidly localized in the fixed macrophages of the liver, spleen, and bone marrow. The macrophages apparently process the antigen in some way, but the lymphocytes actually produce the antibodies. Some antigens are physically present in the macrophages during the time that the lymphocytes are actively producing antibodies. Whether this is necessary for continued antibody production is under investigation. Many antigens appear to be present for several months after injection; so retention may be essential for antibody production to occur.

2-5. Antibodies:

a. **Introduction.** Just as antigens are defined in terms of their reactivity with antibodies, all antibodies are intimately associated with their antigens. These antibodies must be able to react in some demonstrable way with the antigen which stimulated their production. Antibodies of significance to the serologist are found in the serum (or plasma) fraction of blood, but there are some antibodies that remain fixed to certain tissue cells. These fixed antibodies are of minimal significance to the serologist although their importance to the health of the individual should not be discounted. Whether fixed to cells or free in the serum, antibodies have several characteristics in common.

b. **Characteristics of Antibodies:**

(1) Specificity. In general, each antibody will react only with the antigen that stimulated the body to produce that antibody. In other words, each antibody possesses a high degree of specificity. As with any biological system, there are exceptions to the rule. These exceptions will be discussed later un-

der antigenic variation and as they apply to each test procedure.

(2) Chemical Nature. Antibodies are found in the globulin fraction of serum protein. More specifically, most antibody activity is in the gamma globulin fraction of serum globulin. Because these globulins are active in immunity, they are frequently called immunoglobulins as a synonym for antibodies.

(a) The immunoglobulins (antibodies) of man can be further classified based on the fact that they are antigenic themselves when injected into certain animals. The animals produce antibodies which precipitate the human immunoglobulins. Five main classes of immunoglobulins have been identified in man by this method. They are IgG, IgM, IgD, IgA, and IgE. Most antibody activity in human serum is due to IgG, IgM, or IgA with certain diseases causing the production of more of one class than another. For example, antibodies against bacterial endotoxins are primarily IgM; while those against the mumps virus are IgG. In addition, the class that is produced in greatest quantity varies with the number of times the individual has had contact with a particular antigen. Usually on the first contact, such as a vaccination, IgM is the main antibody produced; but on a second or subsequent contact, IgG usually predominates.

(b) The five classes of immunoglobulins also vary somewhat in molecular weight and structure. All are very large molecules with most classes having a molecular weight of 150,000 to 200,000; however, the molecular weight of IgM is about 900,000. Each immunoglobulin molecule consists of a combination of two types of chains of amino acids. These chains have been designated as light chains and heavy chains. The most abundant immunoglobulin molecule, IgG, is made up of two light and two heavy chains. An IgM molecule has ten light and ten heavy chains. Each chain of the molecule has a region with a constant amino acid sequence and a variable region. It is the variable region of the antibody molecule that was produced in response to a specific antigen. The variable region is also the binding site in antigen-antibody reactions. Figure 2-1 shows a schematic structure of an antibody (IgG) molecule.

2-6. Antigenic Sharing (Cross-Reactivity).

a. **Introduction.** So far, antigens, antibodies, and their reactions have been described as very specific entities. However, there are three main factors which serve to destroy the totality of the preceding discussion. First of all, a given organism, such as a bacterium, may possess several entirely different antigens, each of which stimulates the produc-

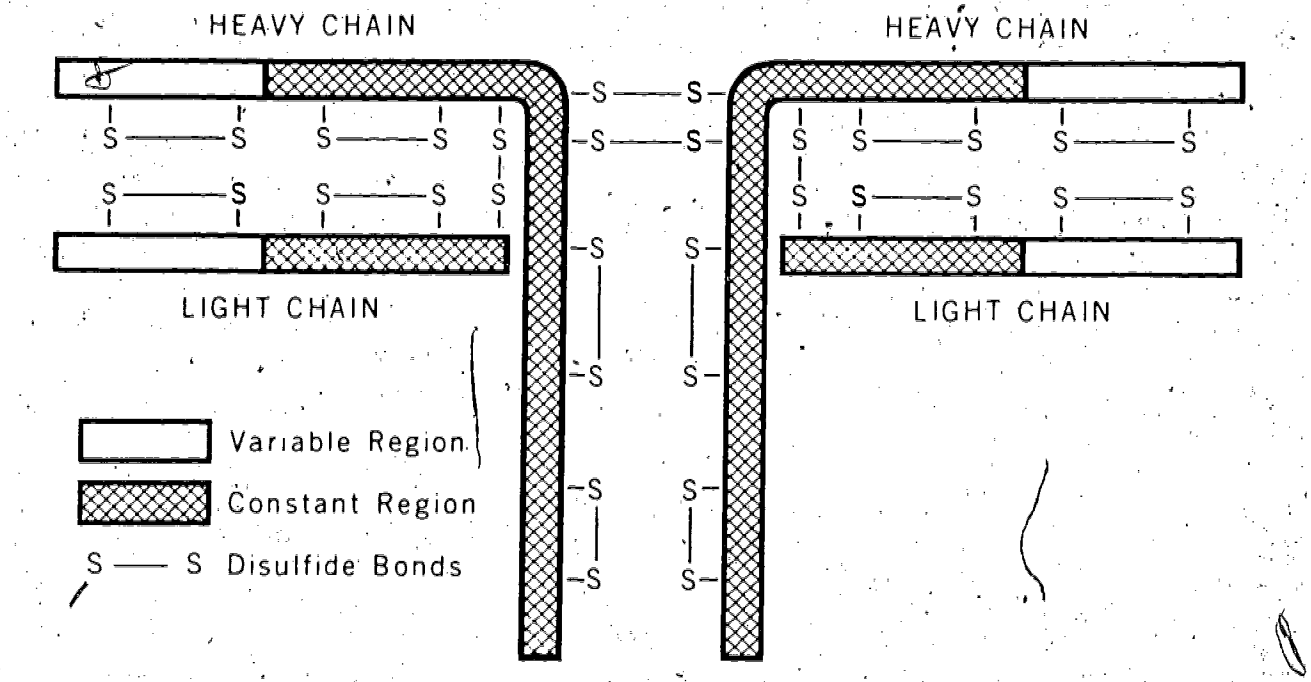


Figure 2-1. Schematic Structure of an Antibody (IgG).

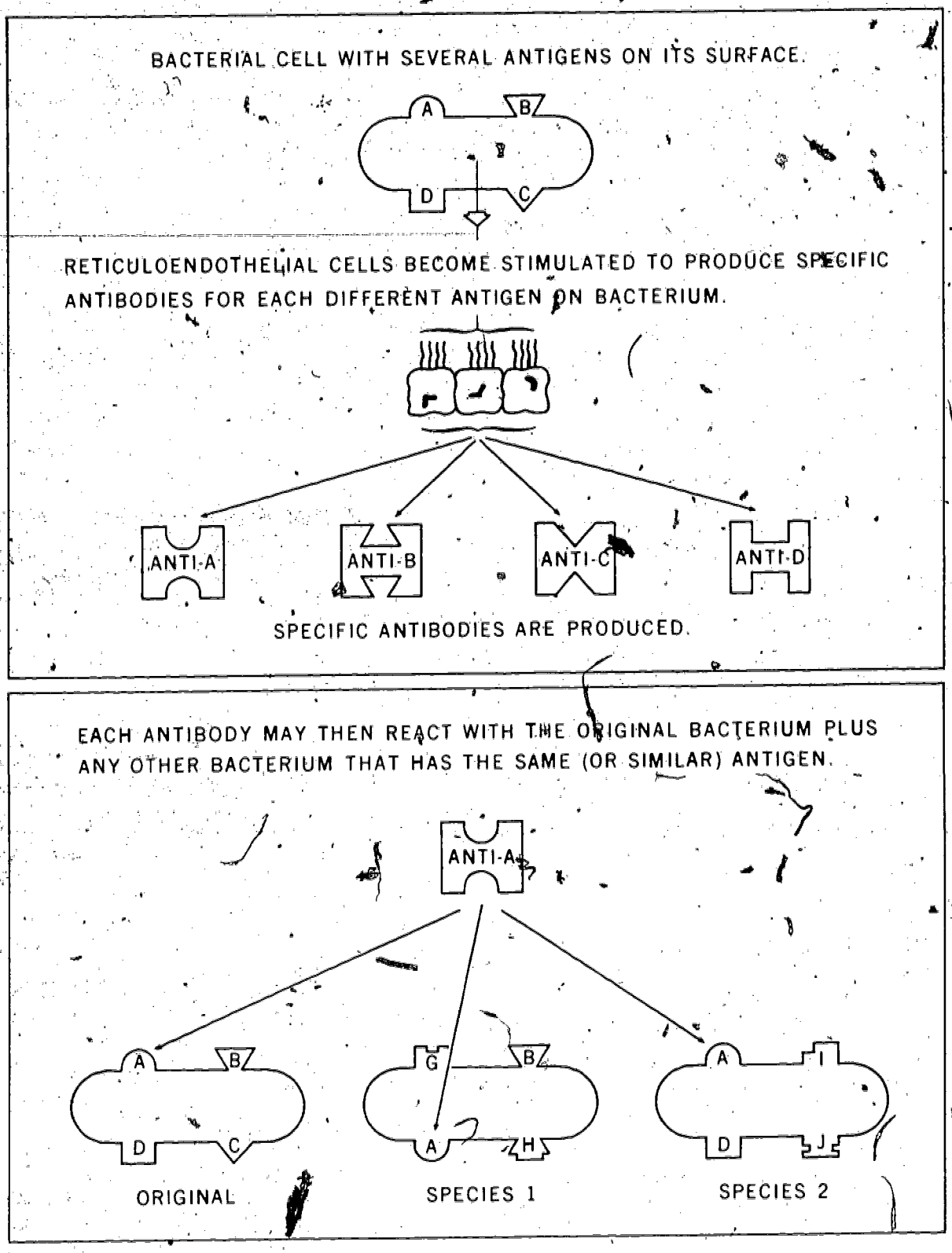


Figure 2-2. Cross-Reactivity.

tion of antibodies against that antigen. Secondly, evolution has resulted in seemingly unrelated organisms which may possess antigens in common with each other or even several organisms. Finally, the number of possible antigens (compounds) is limited by the number of elements occurring in nature so that duplications are inevitable. The end result is the production of antibodies which will react with identical or nearly identical antigens from two or more completely unrelated organisms. See figure 2-2. In serologic testing, this antigenic sharing results in what is referred to as cross-reactivity.

b. **Significance of Cross-Reactivity.** To the serologist, cross-reactivity is both a useful and, at times, a very confusing occurrence. The serologist in performing his tests must be constantly aware of the possibility that some completely unrelated antigen, such as a nonpathogen, may have stimulated the production of the antibodies under study. He must learn to rule out these so-called "false positive" reactions. At times this poses an insurmountable obstacle. On the other hand, the principle of cross-reactivity provides the basis for many useful serological tests. For example, patients with the supposedly viral disease, infectious mononucleosis, produce antibodies against sheep and horse red blood cells. This fact is then used in the laboratory diagnosis of infectious mononucleosis because sheep or horse red blood cells are more readily available and easier to use then the causative virus. In fact, no practical test is available for the detection of the infectious mononucleosis virus. Another good example is seen with rickettsial diseases, such as typhus and Rocky Mountain spotted fever. These rickettsiae have some antigens in common with certain bacteria of the genus *Proteus*. Since bacteria are more readily cultivated than rickettsia, the more economic route is selected by using bacterial antigens (*Proteus sp.*) in serologic testing for the unrelated rickettsial diseases. These are but two examples of the beneficial use of cross-reactivity by the serologist. Many more uses of cross-reactivity are seen in serologic testing including the most common of serologic tests—the ones for syphilis. Additional uses of cross-reactivity will be described as they apply to the procedures presented in this manual.

SECTION C—ANTIGEN-ANTIBODY REACTIONS

2-7. Introduction. Although in vivo antigen-antibody reactions are important to the health of a patient, the clinical serologist uses only in vitro reactions. After mixing antigens and antibodies in test tubes or on slides, the serologist must evaluate the reaction he observes, if any, and report his findings to the physician. Many times the mere presence or absence of a reaction is adequate for the physician, but at times an estimation of the antibody level is required. This is especially important in following the course of a disease where a change in antibody levels may be significant. Actually, no practical method is available for directly measuring antibody levels. However, analyzing the reacting capacity or titer of antibodies in a given serum provides a practical measure of antibody levels. This is usually accomplished by making serial dilutions of the patient's serum and noting the greatest dilution that still gives a positive reaction. The titer is then expressed as the reciprocal of the highest serum dilution to give the positive reaction. For example, if a serum dilution of 1/1280 was the highest to react, the titer would be reported as 1280 or 1:1280 against that antigen. This titer, while not an absolute measure of antibodies, provides a mathematical base for future tests on the same patient.

a. **Sources of Antigens and Antibodies.** As mentioned above, in many serologic tests the serologist will be trying to detect, and perhaps titer, antibodies in a patient's serum. He will accomplish this by reacting the patient's serum with some known antigen. However, in a few tests, he will be testing for the presence of a given antigen from man by using known antibodies (antisera) that have been produced in some other animal, such as a rabbit. Except for these antigens of human origin, antigens used in serologic tests come from a variety of sources. As mentioned in the discussion of antigenic (cross-reactivity), very useful serologic tests have been devised using antigens from sources which are apparently completely unrelated to the disease process under study. In addition, many antigens have been modified by the serologist in order to make the reactions more readily visible, increase the sensitivity of a test, or enhance the test in some practical way.

b. **Stages of Antigen-Antibody Reactions.** In vitro antigen-antibody reactions usually occur in two stages. The first is a nearly instantaneous physical combination of the reactants without a visible reaction. Some reactions stop here. There are tests available to detect these first-stage reactions; but, fortunately, many antigen-antibody reactions go to a second stage—a visible reaction. Al-

though the manifestations of a visible reaction may take minutes or even hours to become evident, the serologic tests presently in use make wide use of second-stage reactions. In fact, many test reactions, that normally would not be visible, have been modified to provide visible reactions for the serologist.

c. **Types of in vitro Antigen-Antibody Reactions.** There are several types of antigen-antibody reactions used by the serologist in his profession. Although there are many modifications of the basic reactions, they can be classified into the following five main categories: precipitation, agglutination, neutralization, complement fixation, and immunofluorescence reactions or tests. Since a given antibody may react in many different ways, there may be tests available from several of these categories for a given disease. For example, a disease may stimulate the production of IgM antibodies which are most efficient in agglutination reactions, but these antibodies will react in other types of reactions too. Consequently, test selection must be based on the antibody present as well as on the equipment and personnel capabilities of a given laboratory.

2-8. Precipitation Reactions. Precipitation is the second-stage reaction in which the antibody (precipitin) reacts with a soluble antigen. The visible precipitate results from the sequential combination of antibodies and antigens until antigen-antibody aggregates large enough to be seen are formed. This formation of visible aggregates will occur only when optimum proportions of both antigen and antibody are present. Most precipitation reactions take several hours for a visible precipitate to appear. The tests are usually performed by one of two basic methods—in capillary tubes or by immunodiffusion in agar.

a. **Capillary-Tube Precipitin Tests.** In this technique, the soluble antigen and the antibodies (antisera) are introduced successively by capillary attraction into the tube so that the layers contact each other. After incubation in an upright position, the precipitate usually forms at the antigen-antibody interface or throughout the mixture if the proper antigen and antibody are present. This test is relatively insensitive as it detects only large amounts of antibodies. Another more obvious limitation of this method is that both antigen and antibody solutions must be clear, as cloudiness may interfere with reading the final results. This technique is used for the detection of C-reactive protein in the serum. Precipitin tests also have applications in bacteriology in the classification of streptococci.

b. **Immunodiffusion Tests.** There are many tests being developed which depend on the diffusion of either the antigen or antibody (or both) through a buffered agar before they react. Some of these methods require sophisticated laboratory equipment, such as for the detection of radioisotopes, and are beyond the scope of this manual, but the increased sensitivity and specificity of these methods may soon make them practical. However, there are a few immunodiffusion tests which rely on the formation of a visible precipitate. Some of these are rather sophisticated tests too, but their importance warrants at least brief mention. In general, these tests are based on the migration of the antigen and/or antibody through the agar until optimum proportions of each are reacted and a precipitate appears. As usual, the appearance of the precipitate takes several hours, but a modification by combining diffusion with electrophoresis has speeded the reaction and has great promise for the future in serologic diagnosis.

(1) Double Diffusion Tests. In double diffusion tests, the antigen and antisera are placed in separate wells cut into the agar and each diffuses toward the other. A precipitate line appears where optimum proportions of the reactants are present. See figure 2-3. Although the technique yields only qualitative results, it is simple and can be used with mixed antigen systems or in many combinations of known-unknown situations. The test is referred to as the Ouchterlony test. It has been applied to the detection of antibodies in coccidioidomycosis.

(2) Single Radial Diffusion Tests. In this method, one reactant, usually the antibody, is incorporated into the agar. The antigen is placed in a well and diffuses radially into the agar. A ring of precipitate forms whose diameter is proportional to the antigen concentration (see figure 2-4). Standard antigens of known concentrations may be tested at the same time. The diameter of the reactions of the standards can be plotted against their concentration; and, then, the concentration of unknown samples can be read directly from the standardization curve. This method is presently used for estimating the concentration of immunoglobulins (IgG, IgM, etc) and complement (C'3). The specific details of these tests are described in chapter 15. Several other antigenic substances can also be assayed by this method.

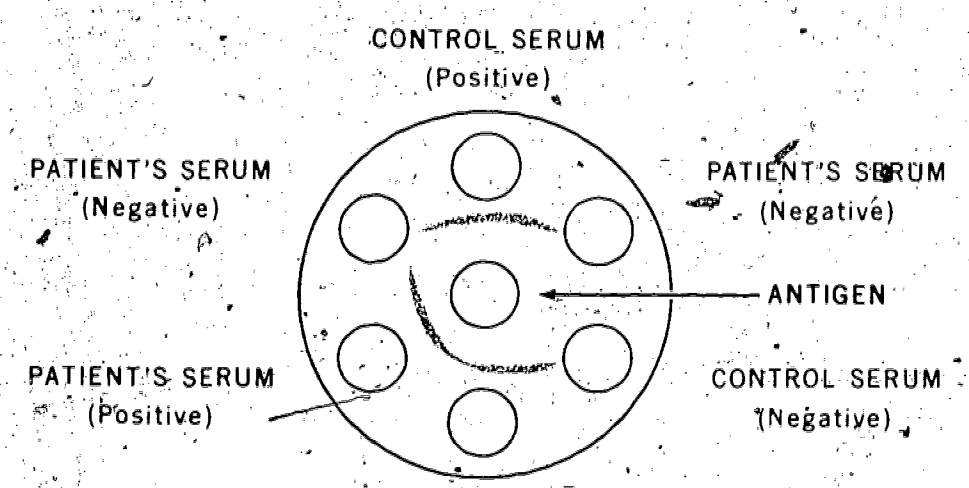


Figure 2-3. Double Diffusion (Ouchterlony)

(3) Immunoelectroosmophoresis (IEOP). This technique is also called counterelectrophoresis, counter-current electrophoresis, counterimmunoelectrophoresis and others. Basically the principle is a combination of simultaneous diffusion and electrophoresis followed by precipitation at the point of optimal proportions of reactants. In the test, antigen and antibody are placed in separate wells in the agar and an electrical current applied. The pH and ionic strength of the agar as well as the nature of the antigen and antibody determine the migration characteristics of the system. As applied to the detection of the hepatitis associated antigen (HAA) in the blood of potential blood donors, the antigen (HAA) is electrophoresed toward the known antibodies which are diffusing through the agar toward the antigen. A precipitate line appears when optimum proportions of each have reacted (see figure 2-5). The main advantages of IEOP over double diffusion tests are increased speed and the ability to detect very small quantities of antigen—both important considerations for the clinical serologist.

2-9. **Agglutination Reactions.** These reactions are similar to precipitation reactions in that antigen-antibody aggregates are formed. The main difference is that agglutination is used to describe the aggregation of particulate antigens. These antigens might be red blood cells, bacteria, or even inert particles such as latex particles that have been coated with a given antigen. Flocculation is at times used erroneously as a synonym for agglutination. Some authors use agglutination when describing reactions involving cellular antigens (red blood cells, etc) and flocculation for inert antigens (latex, etc). Many varieties of agglutination (or flocculation) reactions have been applied to clinical serol-

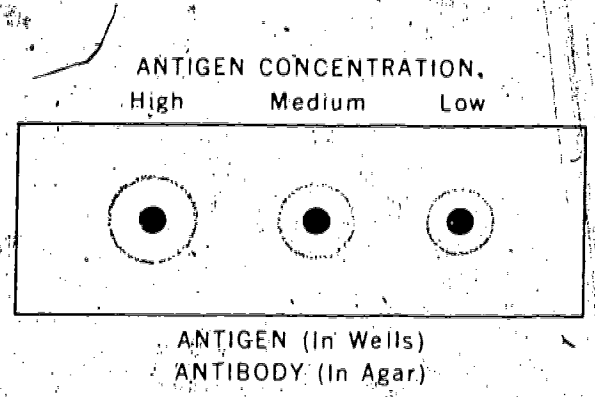


Figure 2-4. Single Radial Diffusion.

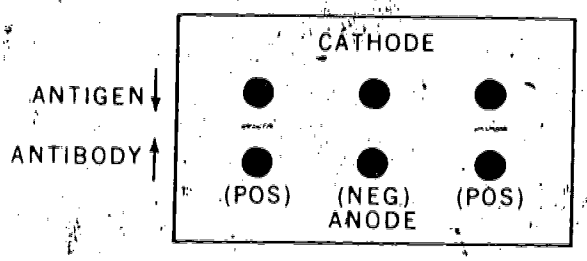


Figure 2-5. Immunoelectroosmophoresis (IEOP), Example Using Australia Antigen.

ogy. They are faster than precipitin reactions and many can be observed macroscopically.

a. **Bacterial Agglutination.** The first practical application of serologic test in medicine was for the detection of agglutinating antibodies (agglutinins) against *Salmonella typhi* in the serum of patients with typhoid fever. Although this test was devised before the turn of the century, it is still in use and the principle has been applied to the serodiagnosis of certain other bacterial and rickettsial diseases such as tularemia, brucellosis, and typhus. In these tests, known bacterial antigens are used to detect unknown antibodies in the patient. The bacteria may be live or killed and preserved in some fashion such as with heat and/or formalin. In the case of typhus and related rickettsial diseases, a good example of the application of cross-reactivity is seen. Agglutination tests for rickettsial diseases use bacteria of the genus *Proteus* as antigens instead of the rickettsiae that actually cause the disease. The febrile agglutination tests in chapter 5 are bacterial agglutination tests. Another important application of bacterial agglutination is in bacteriology, where known antisera (antibodies) are used to identify unknown bacteria.

b. **Hemagglutination.** The agglutination of erythrocytes by antibodies or viruses provides the basis for many serotests. In addition, hemagglutination reactions are the main type used in immunohematology or blood banking but this field is covered in another manual. There are several modifications of the basic test. Each will be discussed separately.

(1) Direct Hemagglutination. In these tests the antigens on the erythrocytes are present normally. Although the cells may be preserved to increase their useful lifespan, they are essentially unaltered. Direct hemagglutination serotests are applied mainly to two diseases, primary atypical pneumonia and infectious mononucleosis. Both are excellent examples of the application of cross-reactivity to serotests. The antibodies in primary atypical pneumonia agglutinate human group "O" or the patient's own erythrocytes although the disease is caused primarily by a pleuropneumonia-like organism. In infectious mononucleosis, erythrocytes from sheep, horses, and oxen have been used to detect antibodies. Tests for infectious mononucleosis and primary atypical penumonia are found in chapters 7 and 8, respectively.

(2) Passive (Indirect) Hemagglutination. The antigens in this type of hemagglutination test have been adsorbed onto erythrocytes. The erythrocytes are simply physically carrying the antigens. Sheep erythrocytes are frequently used for this purpose. A wide

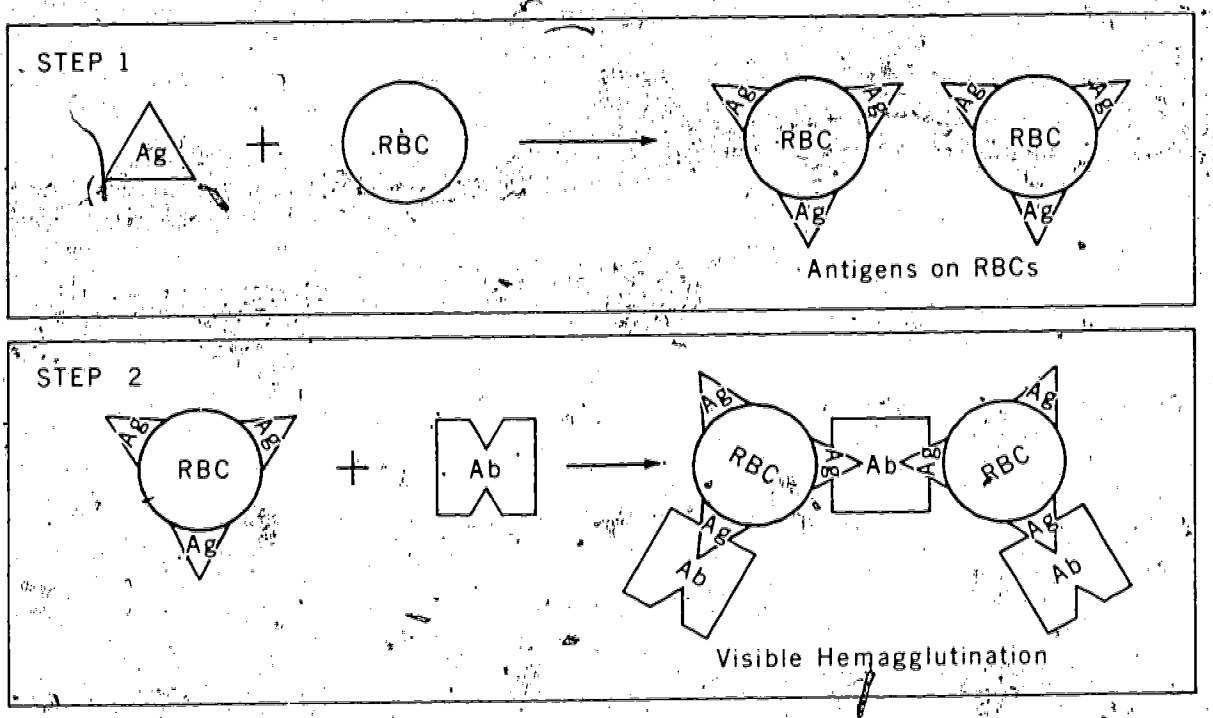


Figure 2–6. Passive (Indirect) Hemagglutination.

variety of antigens which do not normally give a second-stage reaction of any kind can be detected if the antigens are adsorbed on erythrocytes. Agglutination of the erythrocytes is indicative of the antigen-antibody reaction. Many polysaccharide antigens will adsorb directly onto erythrocytes but protein antigens usually require pretreatment of the erythrocytes with tannic acid before the antigens will adhere. Antigens prepared in this way provide a very sensitive method of testing for antibodies. The technique has been used in the diagnosis of amebiasis, plague, Hashimoto's thyroiditis, and others. The reaction is presented graphically in figure 2-6.

(3) Passive Hemagglutination Inhibition. Another useful application of antigens artificially adsorbed onto erythrocytes has been in tests to inhibit hemagglutination that would take place in the presence of the proper antibody. The test is used mainly to detect the presence of free antigens in solutions such as urine and serum. It is carried out in two steps. First, a known antibody against the antigenic substances under study is mixed with the solution that is suspected of

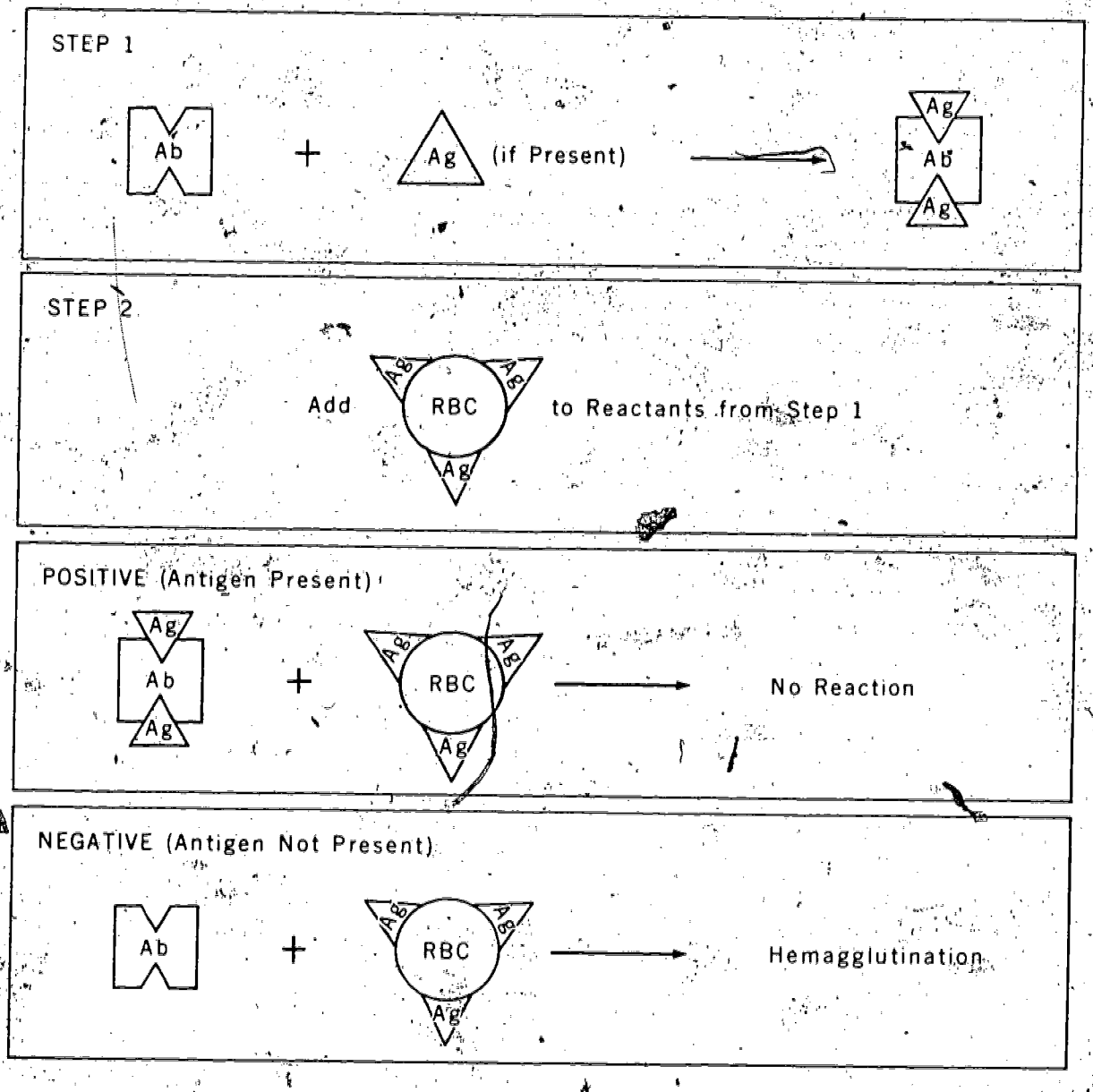


Figure 2-7. Passive Hemagglutination Inhibition for Detecting an Antigen.

containing the antigen. If the antigen is, in fact, present, the antibody will be bound or inhibited. In the second step of the test, antigen-coated erythrocytes are added to the test. Agglutination now indicates that the antibody has not been inhibited and is free to react with the antigen-coated erythrocytes—a negative test. It means that the antigen was not present in the solution under study. No agglutination of the erythrocytes indicates a positive test. These reactions are presented graphically in figure 2-7. This technique has been applied successfully in pregnancy tests (see chapter 13) for the detection of human chorionic gonadotropin (HCG) in the urine.

(4) Viral Hemagglutination Inhibition. Certain viruses have the ability to agglutinate certain erythrocytes. For example, the rubella (German measles) virus agglutinates erythrocytes from day-old chicks. Antibodies against the virus inhibit this agglutination. The reaction is presented graphically in figure 2—8. Determining the titer of rubella

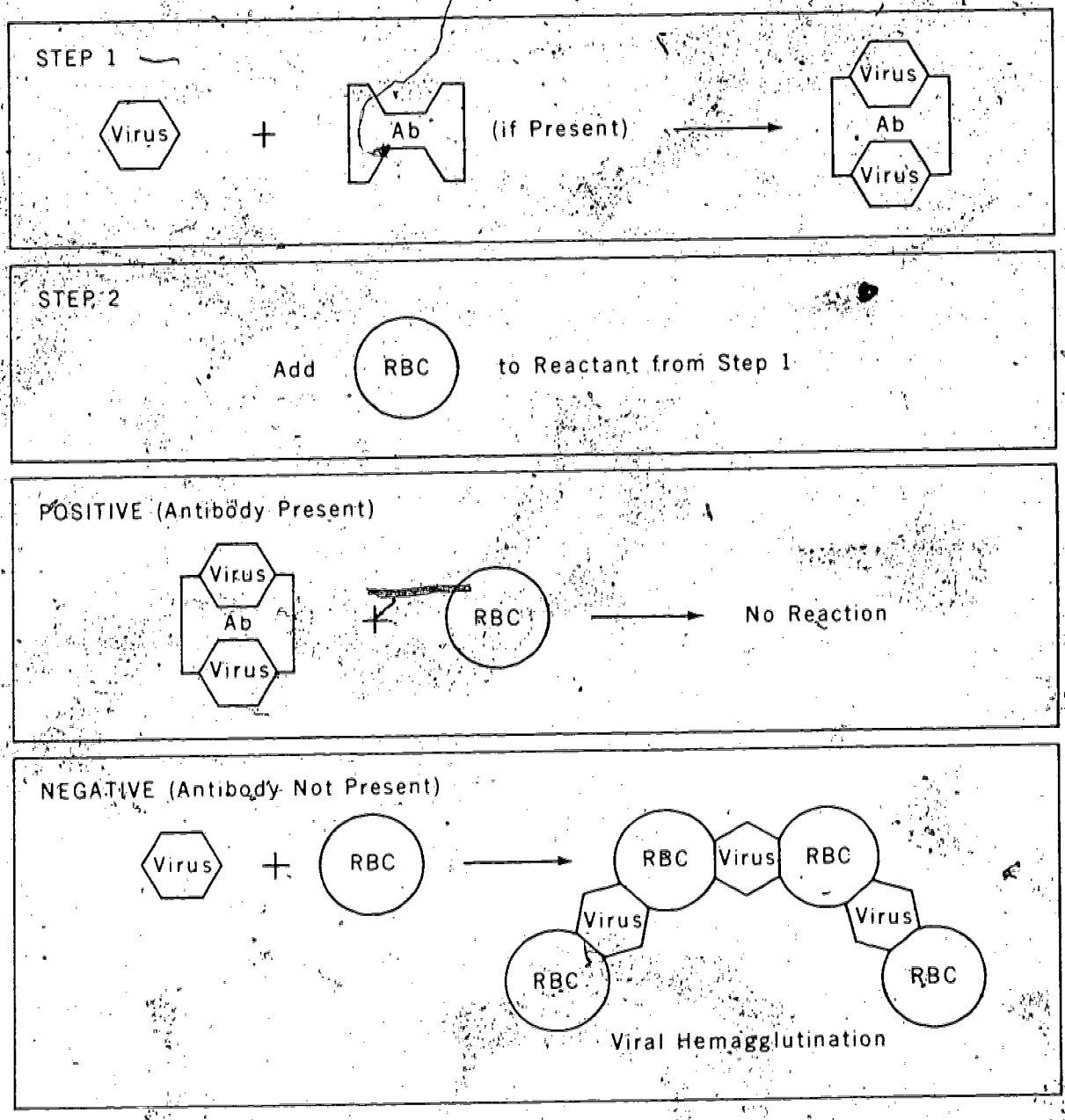


Figure 2-8. Viral Hemagglutination Inhibition for Detecting Antibodies.

hemagglutination-inhibiting antibodies in a patient's serum is a useful measure of the patient's ability to resist future infection by the rubella virus. This test for inhibiting antibodies has been used extensively on pregnant women where a rubella infection could result in severe damage to the developing fetus. Tests for rubella antibodies are in chapter 14. The same principle has been applied to the serodiagnosis of influenza and certain arbovirus diseases which also result in the production of hemagglutination-inhibiting antibodies.

(5) Agglutination Involving Inert Particles. Several inert, acellular particles have been coated with either antigen or antibody and used in serotests. Some of these are latex, bentonite, charcoal, and others. The reactions involved may be either direct agglutination of the coated particles or agglutination inhibition. Too many diverse combinations of antigens and inert particles exist to cover adequately at this point so each will be covered in this manual as they apply to each test, but tests for syphilis and rheumatoid arthritis are examples.

2-10. Neutralization Reactions. Usually neutralization is used only to describe antigen-antibody reactions which require a laboratory animal to detect whether or not a reaction has taken place. The antibodies and antigens, such as toxins or viruses, are mixed in vitro. Then the mixture is injected into the test animal. Subsequent observation of the animal for characteristic symptoms indicates whether the toxin or virus has been neutralized. As can be imagined, the maintenance of an animal colony is beyond the scope of the average clinical laboratory. However, if one uses the term, neutralization, in a broader sense, there are several serologic tests that could be categorized as neutralization reactions. For example, the hemagglutination inhibition tests previously discussed might apply, but the best example is the antistreptolysin-O (ASO) test. The antigen in this test is a hemolysin, streptolysin-O, that is produced by certain streptococci. Antibodies neutralize the hemolysin in vitro so that when erythrocytes are eventually added to the test, the hemolysin can no longer hemolyze them. This test is presented in chapter 9.

2-11. Complement Fixation Reactions. Complement is a set of 11 serum constituents which become inactivated (fixed) in many antigen-antibody reactions. In fact, complement is required for the completion of many of these reactions. However, since many antigen-antibody reactions do not result in a visible manifestation, the detection of complement fixation provides a useful serologic test. To detect whether complement fixation has occurred in the first reaction, a second antigen-antibody system is added to the test. Sheep erythrocytes and antisheep erythrocyte antibodies (hemolysins) are usually used in this second step. The sheep RBC hemolysins require the presence of complement to lyse the erythrocytes. Therefore, lysis of the erythrocytes indicates that free complement is still present in the system or that no complement fixation (no antigen-antibody reaction) occurred in the first step of the test. Conversely, no hemolysis indicates a positive complement fixation test. This test has many applications in the serodiagnosis of viral diseases and has also been used extensively in syphilis serology. However, since the test is performed only in larger laboratories, procedures will not be included in this manual and reference should be made to procedures in the medical literature when needed.

2-12. Immunofluorescence Reactions. Also called fluorescent antibody (FA) reactions, these tests are based on the fact that certain chemicals emit visible light when exposed to ultraviolet (UV) light. Some of these chemicals (for example, fluorescein isothiocyanate) can be conjugated with antibodies without destroying antibody reactivity. Although there are several modifications, two main types of reactions are used in the fluorescent antibody tests in use in clinical serology.

a. **Direct Fluorescent Antibody Technique.** In this method, a specific antibody is conjugated with fluorescein. The labeled antibody is reacted on a slide with its specific antigen, such as bacteria, spirochetes, viruses, or similar particulate antigens. The specific antibodies adhere to the cells. After washing away excess antibody (and any other antibodies not specific for the antigen), the preparation is exposed to UV light and viewed with a microscope fitted with special filters. In the case of fluorescein isothiocyanate-labeled antibodies, the organisms will now emit a blue-green light. This technique is used mainly to detect an antigen in tissue or from culture by using known, labeled antibodies. It has been applied to the detection of the gonococcus, meningococcus, streptococci, plague bacilli, and several others in clinical specimens.

b. **Indirect Fluorescent Antibody Technique.** This modification of the fluorescent antibody

technique is useful because it permits the detection of antibody in a patient's serum without the time-consuming process of having to label the antibodies of each patient with the fluorescein dye. As usually applied, the unlabeled patient's serum is reacted with a known antigen (bacteria, spirochetes, etc.) on a slide and the excess antibody washed away as in the direct technique. Next, fluorescein-labeled antihuman globulin, that has been produced in an animal following the injection of human globulin is added. The labeled antihuman globulin reacts with the patient's antibodies (globulins) that have coated the antigens. When examined by UV microscopy, the same fluorescence as in the direct test is seen. The steps in the reaction are diagrammed in figure 2-9. In addition, this test can be performed on serial dilutions of the patient's serum in order to determine the antibody titer. The Fluorescent Treponemal Antibody Absorption (FTA-ABS) test for syphilis in chapter 6 and the Antinuclear Antibody Test for lupus erythematosus in chapter 12 are based on this indirect FA method.

2-13. Other Antigen-Antibody Reactions. Several other types of reactions occur which require at least brief mention. Some of these could have been included in the five categories described in the preceding paragraphs but are considered to be of lesser importance than the examples given. Others are tests of the future. A few of these will be described briefly.

a. **Opsonification.** This process is the alteration of foreign particles by antibodies (opsonins) which enhance phagocytosis of the particles. In vitro tests are available to detect these opsonins.

b. **Quellung Reaction.** Antibodies against certain encapsulated bacteria, such as pneumococci, cause the capsules to swell. The reactions are very specific. In the past these reactions were used extensively in selecting the proper antiserum to administer to pneumonia patients, but their use has nearly disappeared since the advent of antibiotics. This capsular swelling by specific antibody has also been used in the identification of *Haemophilus influenzae* and *Bacillus anthracis*.

c. **Immunoelectrophoresis (IEP).** Electrophoresis of serum antibodies in agar may be followed by the addition of known precipitating antiglobulins, such as anti-IgM, anti-IgG, etc. When the amount of precipitate is compared with known normal standards, a semiquantitation of each class of immunoglobulin is possible. Through the use of radioisotopes, many modifications of the basic technique are possible.

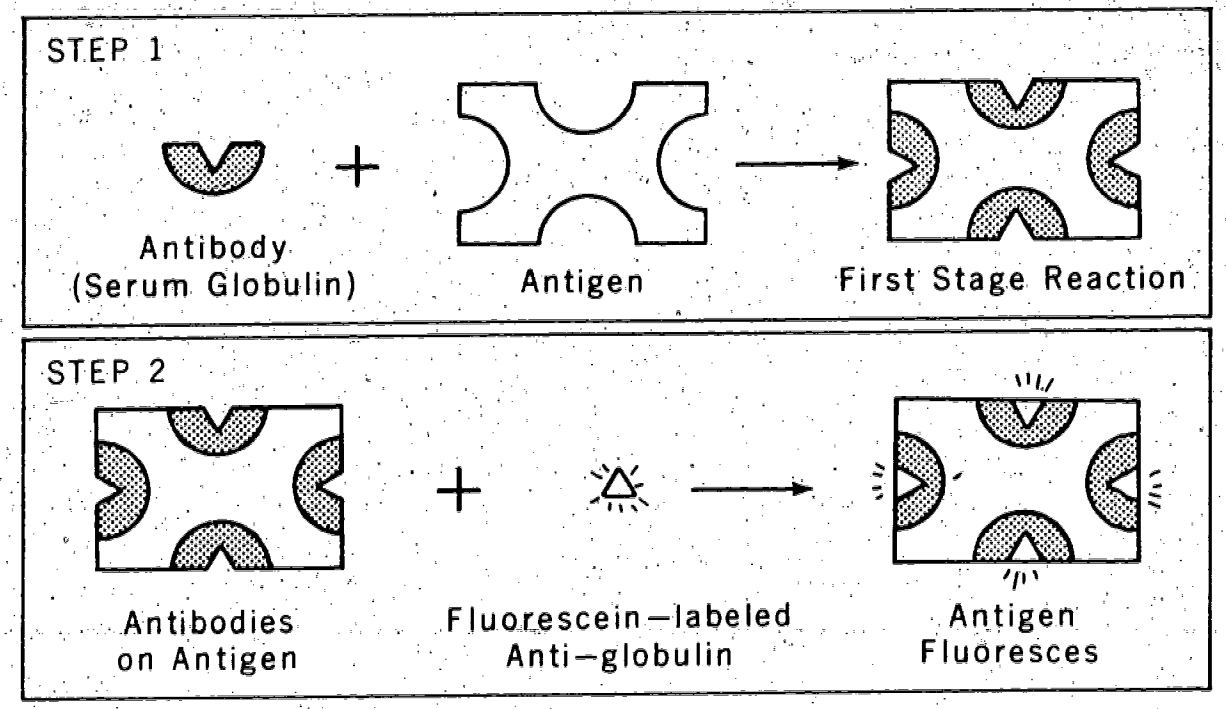


Figure 2-9. Indirect Fluorescent Antibody (Immunofluorescence).

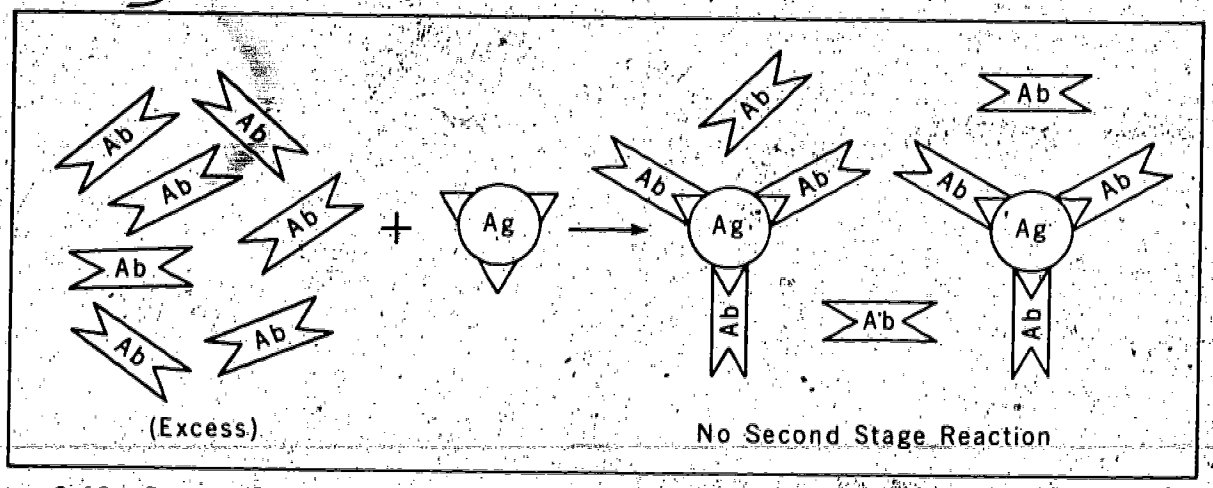


Figure 2-10. Prozone (Excess Antibody) Reaction.

d. **Radioimmunoassay (RIA).** This term applies to serologic tests which employ radioisotopes in them. Although limited at present to larger laboratories, these tests are likely to become more widely used due to their increased sensitivity and greater accuracy than other methods. Presently under development is a test for the hepatitis associated antigen, that is several times more sensitive than older methods.

2-14. ZONAL REACTIONS. There are several factors, such as ionic concentration, temperature, incubation time, and others, that affect antigen-antibody reactions. However, the serologist rarely has to worry about these factors because most reagents used in serology are commercially manufactured. All the serologist has to do is follow the manufacturer's directions with adequate controls, and these factors will be kept under control. However, there are two situations of which the serologist should be aware. Both may result in a weak reaction or lack of visible manifestation of the expected reaction due to alterations in the optimal proportions of antigen to antibody.

a. **Prozone Reactions.** These are weak or negative reactions due to an antibody excess. For example, in an agglutination reaction, each antigenic determinant might be combined with a single antibody molecule so that agglutination would be impossible. A prozone reaction is depicted graphically in figure 2-10. Suspected prozone reactions can be detected by making dilutions of the antibodies (serum) before adding the antigen.

b. **Postzone Reactions.** These are weak or negative reactions due to an excess of antigen in the test. Although rarely encountered by the

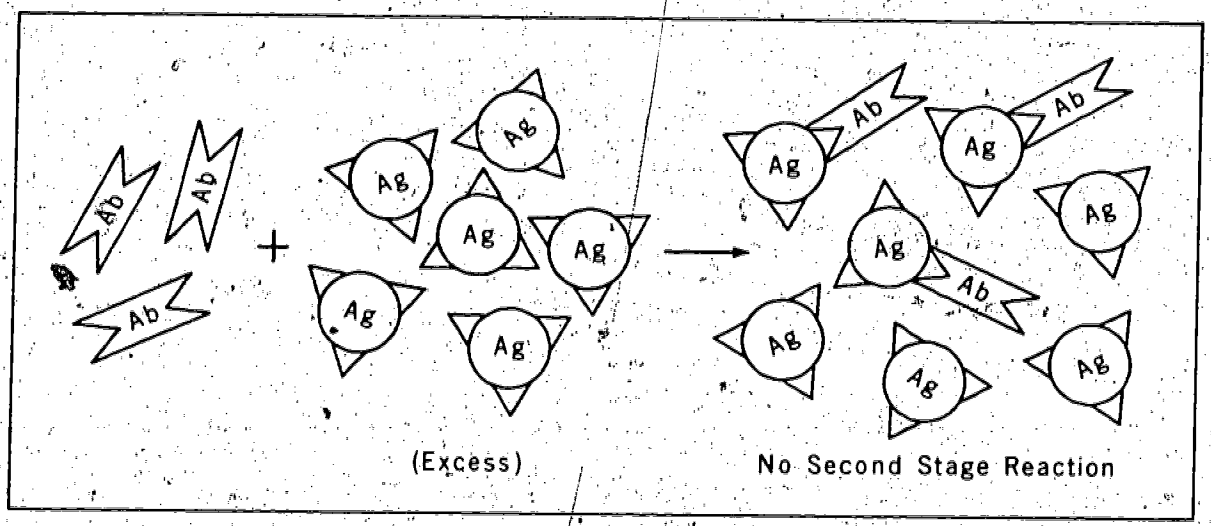


Figure 2-11. Postzone (Excess Antigen) Reaction.

serologist unless he is devising or modifying a test, postzone reactions are, in fact, what are seen early in a disease before an optimal antibody level is achieved. Some undetectable antibody may be present. A postzone reaction is depicted graphically in figure 2-11.

Chapter 3

SPECIMEN COLLECTION AND PREPARATION

3-1. Types of Specimens. Valid serologic tests must be performed on properly collected and prepared specimens. Since most serotests are performed on the patient's serum, proper collection and preparation usually refers to blood collection and separation of serum. This chapter is devoted primarily to the processing of serum. However, certain serotests are performed on other body fluids. In addition to serum, some serotests are performed on plasma, cerebrospinal fluid (CSF), and urine. The characteristics of each type of specimen will be described briefly.

a. **Serum.** Serum is the supernatant portion of whole blood that has been allowed to clot. Serum is the most frequently assayed specimen in serology because antibodies are found in the serum. Certain abnormal components, such as C-reactive protein (CRP) and rheumatoid factors, are also found in serum by using serotests.

b. **Plasma.** Plasma is the fluid portion of blood that has been prevented from clotting by the addition of anticoagulants, such as oxalates, heparin, EDTA, and others. The only significant difference between plasma and serum is that (in addition to anticoagulants) plasma contains the fibrinogen that is removed during the clotting process. Plasma is rarely used in serotests because fibrinogen precipitates when plasma is heated, and anticoagulants could alter the sensitive chemical balance of a serotest. However, certain tests for syphilis may use unheated plasma and unheated serum interchangeably. Therefore, be sure to use plasma only if specified in a given test procedure and then only if the correct anticoagulant is used.

c. **Cerebrospinal Fluid (CSF).** A physician collects this specimen and usually presents the laboratory with three tubes of fluid numbered in the order collected. The third tube is usually reserved for serotests, but any blood-free sample could be used. Serotests on CSF are usually limited to tests for syphilis, but tests are available for detecting albumin and immunoglobulins in CSF.

(1) Cerebrospinal fluid is also frequently tested for its effect on various colloidal suspensions, such as colloidal gold, gum mastic, and benzoin. Although colloidal tests are not really serologic tests, some serologic techniques are employed in performing them so the serologist is usually responsible.

(2) One very important precaution to keep in mind when handling CSF is that, like any other biological fluid, CSF, must be considered to contain pathogenic microorganisms until proven to the contrary. The specimen may have been collected from a patient with meningococcal meningitis, tuberculosis, or some other infectious disease transmittable to the unwary technician.

d. **Urine.** Pregnancy tests are about the only serotests performed on urine in the average clinical laboratory. These are tests for the presence of a hormone, human chorionic gonadotropin (HCG), in the urine during pregnancy. Usually a freshly collected morning specimen will be used in these tests. In addition to pregnancy, HCG, may be detected in the urine in certain diseases. Urine is also occasionally tested for the presence of certain immunoglobulins that may be passed in abnormal conditions, but these tests are usually beyond the scope of the average clinical laboratory.

3-2. Blood Collection, Venipuncture. There are several reasons why serum from blood collected by venipuncture is the specimen of choice for many serologic tests. Primarily, if the antecubital region of the forearm is used as a collection site, the patient's discomfort is minimal and of short duration. In addition, the venipuncture is easily performed by a technician, and enough blood can be collected at one time for several tests or repeat tests. The techniques to be described here are applicable to all serotests requiring serum or plasma except for the tests for cold agglutinins. The special techniques for collecting serum for cold agglutinins are covered in chapter 8.

a. **Venipuncture Site.** Venous blood can be collected from several body sites. A physician should make all collections from the scalp, femoral, or jugular veins. However, even minimally trained technicians should be able to collect blood from the antecubital region of the arm of most patients. If necessary, the technician may also collect blood from the

back of the hand or top of the foot. However, the back of the hand and foot are more sensitive to pain, and the veins tend to roll easily so that the arm is usually selected as the site of choice. The venous system of the antecubital region of the arm is shown diagrammatically in figure 3–1. The actual collection site is usually the medium cubital vein or another vein in that vicinity.

b. **Equipment.** In general, sterile equipment or glassware should be used in performing venipunctures for serotests. The main items used in venipunctures are as follows:

(1) Syringes or vacuum blood-collecting tubes (usually without anticoagulant).

(2) Needles, sterile, disposable, about 20 gauge, 1½ inches long (or double-pointed needles).

(3) Tourniquet.

(4) Isopropyl alcohol, 70%. If made from 99% isopropyl alcohol, q.s. 70 ml of alcohol to 99 ml with distilled water.

(5) Alcohol-soaked sponges. Use 70% alcohol.

(6) Gauze pads, sterile, 2 × 2 inches.

(7) Labeled collecting tubes.

c. **Preparatory Steps:**

(1) Label the collecting tubes with the patient's name and other identifying information.

(2) Wash your hands thoroughly.

(3) Assemble the needle and syringe or vacuum tube system. Align the bevel with the markings on the syringe. Examine the needle and discard damaged ones. Keep the plastic shield on the needle until ready to use. Make sure that the plunger of the syringe is free and pushed completely into the barrel to prevent injecting air during the venipuncture.

(4) If ambulatory, have the patient seated with the selected arm extended palm upward and resting on a table or similar device. Bed patients may rest their arms on the bed.

d. **Syringe Technique:**

(1) Place the tourniquet about 3 inches above the patient's elbow just tight enough to check venous flow but not arterial flow. The patient should still have a pulse at the wrist.

(2) Have the patient open and close the fist several times.

(3) Select the desired vein by inspection and palpation (see a, figure 3–2).

(4) Clean the skin over the selected puncture site with an alcohol-soaked sponge (see b, figure 3–2). Wipe off excess alcohol with a dry, sterile gauze pad. Maintain aseptic technique from this point on.

(5) Have the patient clench his fist.

(6) Grasp the syringe in the right (or left) hand and remove the plastic shield from the needle. The forefinger may be placed on the hub of the needle as a guide. Do not touch the needle. With the other hand, hold the skin taut about 2 inches below the puncture site (see c, figure 3–2).

(7) With the needle held bevel up, insert the needle alongside and parallel to the vein for a short distance and then into the vein, all in one motion (see d, figure 3–2).

(8) Gently aspirate the desired quantity of blood. Make sure to firmly anchor the barrel of the syringe while aspirating (see e, figure 3–2).

(9) Remove the tourniquet prior to removing the needle (see f, figure 3–2) and have the patient relax his fist.

(10) Place a dry, sterile gauze pad over the puncture site and apply light pressure. Withdraw the needle in one smooth motion (see g, figure 3–2).

(11) Have the patient keep constant pressure on the site for 3–5 minutes while keeping his arm extended. Remove the needle from the syringe and gently deliver the blood specimen down the side of the appropriately labeled tube (see h, figure 3–2).

e. **Vacuum Blood Collecting Tube Technique:**

(1) Label the vacuum tube with the pa-

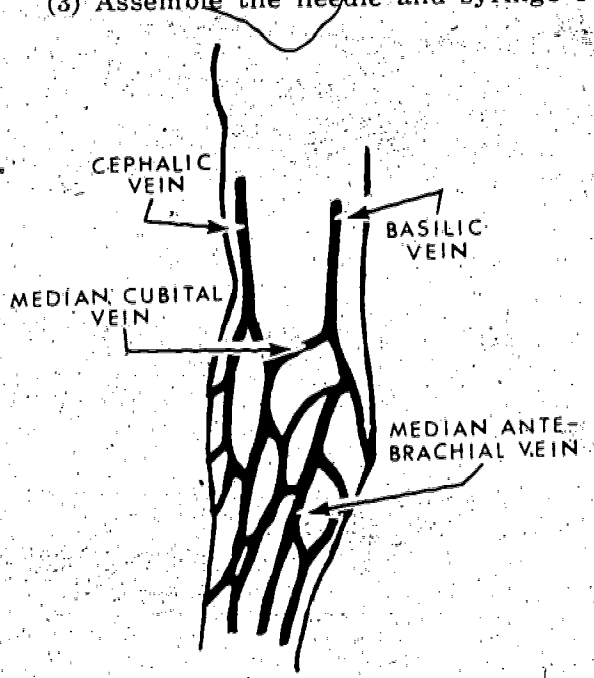


Figure 3–1. Site of Venipuncture.

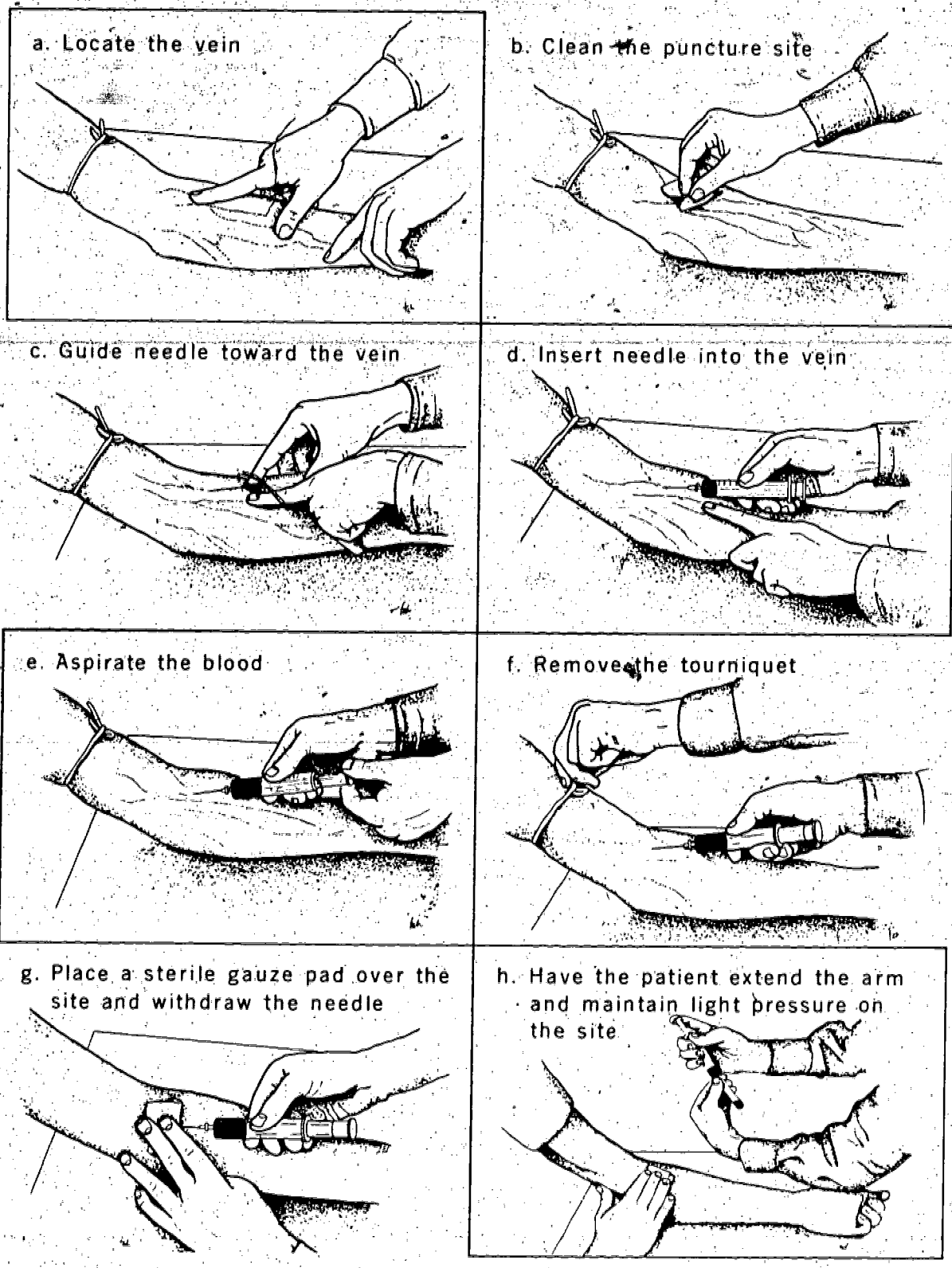


Figure 3-2. Venipuncture Procedure.

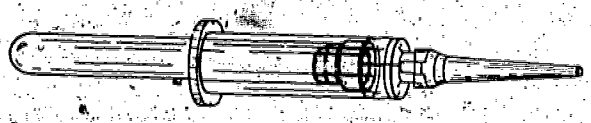


Figure 3–3. Vacuum Blood Collection System.

tient's name and other identifying information.

(2) Screw the double-pointed needle into the holder, leaving the plastic shield over the venipuncture point.

(3) Embed the short needle in the stopper until the stopper reaches the guideline on the holder. Do not break the vacuum (see figure 3–3).

(4) Prepare the patient and insert the needle as indicated in paragraph d.

(5) Once in the vein, push the tube all the way into the holder. Constant pressure on the end of the tube may be required if the needle is equipped with the rubber shut-off sleeve for collecting multiple specimens. The sleeve tends to push the tube from the short needle.

(6) Change tubes if multiple tubes are to be collected.

(7) Remove the tourniquet, apply a sterile gauze pad and remove the needle as described in paragraph d. Be sure that the patient maintains constant pressure on the site for 3–5 minutes following the venipuncture.

f. **Discussion:**

(1) Aseptic technique is required for the patient's and technician's safety.

(2) If possible, collect blood at least an hour after the patient has eaten, since chylous serum could interfere with certain serologic tests.

(3) To minimize the development of a hematoma, be sure to remove the tourniquet before withdrawing the needle or if difficulty is encountered in inserting the needle.

(4) Blood collected in syringes must be transferred quickly and gently to the specimen container. Hemolyzed blood is unacceptable for serologic tests.

(5) Always allow vacuum tubes to fill completely because hemolysis may occur due to rushing air as the needle is withdrawn.

(6) The blood-collecting techniques described in this chapter are applicable to all serotests requiring serum except for cold hemagglutinin tests. Special collection techniques for cold agglutinins are given in chapter 8.

3–3. Blood Collection, Capillary Puncture. Blood collection from capillary punctures is seldom required for serologic tests because venipuncture is a relatively simple and efficient technique. However, capillary puncture may prove to be the most practical method with certain patients, such as infants.

a. **Puncture Site:**

(1) Adults. Usually the lateral surface of the tip of the ring or middle finger is used when the patient is an adult. Heavily caloused areas should be avoided. As an alternate site the edge of the ear lobe is acceptable.

(2) Infants. Either the heel or the big toe can be used for collecting blood by capillary puncture from infants.

b. **Reagents and Equipment:**

(1) Isopropyl alcohol, 70%. If made from 99% isopropyl alcohol, q.s. 70 ml of alcohol to 99 ml with distilled water.

(2) Alcohol-soaked sponges. Use 70% alcohol.

(3) Gauze pads, sterile, 2 × 2 inches.

(4) Lancets, finger-bleeding, sterile.

(5) Capillary tubes, with or without heparin as determined by the test procedure.

c. **Technique:**

(1) Be sure the selected puncture site is warm and has adequate circulation. The site may be warmed with warm (38°C–40°C) water for a few minutes. Dry the site before the next step.

(2) Clean the site with an alcohol-soaked sponge. Wipe the site dry with a dry, sterile gauze pad. Be sure the site is completely dry to prevent hemolysis of the blood by alcohol.

(3) Make the puncture and wipe away the first drop of blood. NOTE: An "X" incision is advised for small infants to assure a freely flowing sample.

(4) Fill the capillary tubes about three-fourths full by capillary attraction.

(5) Apply pressure to the wound with a sterile gauze pad (or have the patient apply pressure) until bleeding stops.

3–4. Separation of Serum:

a. **Clotting.** Blood for all serotests, except cold agglutinins (see chapter 8), can be allowed to clot at room temperature. A few minutes after the blood is collected, free the developing clot from the tube by ringing the clot with an applicator stick. Usually enough serum for most tests will be expressed after about 30 minutes to an hour. If greater quantities of serum are required and the delay is not significant, the clot may be allowed to retract more completely overnight in the refrigerator (4°C–10°C). When adequate serum has been expressed from the clot, the clot is sedimented by centrifugation.

b. **Centrifugation.** Depending on the amount of serum required for testing, centrifugation for as little as 5 minutes at 1500 rpm may be adequate. For more complete packing of the clot, increase the time to about 15 minutes. Actually neither the time nor the speed is critical as long as the glassware can withstand the speed and adequate sedimentation of the clot is achieved. Blood collected in capillary tubes is best separated using a microhematocrit centrifuge after first freeing the clot from the glass with a bacteriological needle and sealing one end.

c. **Separation.** If serotests are to be performed immediately after centrifugation and no further treatment of the serum is required before testing, the serum may be left on the clot. Just be sure not to disturb the clot when taking a sample of serum. However, never store serum with the clot. If prolonged storage or shipment of the serum is anticipated, transfer the serum to another appropriate container. Be sure that the new container is tightly stoppered to prevent contamination and desiccation. After centrifugation, serum may be collected from capillary tubes by first scoring the tube with a file and then breaking the tube above the cells.

3–5. Preservation of Serum. Ideally, serotests should be performed as soon as possible after the serum is collected. This is necessary because antibodies in the serum are readily denatured by many microbial contaminants. However, since immediate processing is not always practical (or even possible, if the specimen must be shipped to another laboratory), some method of preserving the serum must be used. Specifically, the antibodies must be preserved. Both physical and chemical preservation methods are available.

a. **Physical Preservation:**

(1) Refrigeration. The best short-term preservation method for serum (and the least likely to interfere with the test) is simple refrigeration at 4°C–10°C. If separated from the clot, serum in tightly stoppered containers may be stored at this temperature for 2–3 days or longer. Serum should not be stored on the clot longer than overnight. Since most serologic tests are temperature-sensitive, be sure to allow the serum to return to the specified temperature before testing. For example, serum for most tests for syphilis must be at room temperature when tested.

(2) Freezing. Serum can be kept nearly indefinitely if frozen at minus 20°C–70°C in tightly stoppered containers. The serum must be free of cells before it is frozen because they will lyse on thawing. In addition, serum for serotests to be shipped to other laboratories is preferably shipped frozen in dry ice. Care must be taken to thoroughly (but gently) mix the serum for testing once it has thawed because it tends to separate on thawing. Of course, the thawed serum must also be allowed to attain the appropriate temperature before testing.

(3) Lyophilization. Removing the water from serum by freeze-drying the serum under high vacuum is an effective way to preserve serum. Lyophilized serum can be stored in tightly stoppered containers for long periods of time, especially if refrigerated. In addition, lyophilized serum can be shipped without refrigeration. The serum is reconstituted by simply replacing the appropriate volume of water and gently mixing. Unfortunately, lyophilization requires powerful vacuum pumps and other specialized equipment, thus usually limiting the technique to larger laboratories. The technique is usually not practical on a small scale anyway.

b. **Chemical Preservation.** Normally, chemical preservatives should not be added to serum that is to be used for serotests. However, serum and cerebrospinal fluid for syphilis testing can be effectively preserved with sodium ethylmercurithio-salicylate (Merthiolate, Eli Lilly and Co.). Be sure that Merthiolate will not interfere with test results before this preservative is used for other tests. Preservation is only by bacteriostasis and does not prevent denaturation of antibodies by heat or other means. Dry, powered Merthiolate must be used. Do not use Tincture of Merthiolate. Tubes are prepared by adding 0.1 ml of 1% (w/v) aqueous Merthiolate. The tubes are desiccated over calcium chloride in a vacuum desiccator in the dark. The tubes are tightly stoppered and stored in the dark, preferably in the refrigerator where they may be kept for several months. This amount of Merthiolate inhibits most bacterial growth in 2–8 ml of serum or spinal fluid.

3–6. Inactivation of Serum Complement. As described in chapter 2, the fixation of complement in certain antigen-antibody reactions is a useful serologic test. However, the native complement in the patient's serum also interferes with several other serotests and must be inactivated before the test is performed. In fact, even in complement fixation tests, the patient's native complement must be inactivated and a known amount of comple-

ment added to the test. On the other hand, there are certain serologic tests that require the presence of native complement, so follow each test procedure carefully in this regard. If inactivation of the native complement is required for a given procedure, two basic inactivation methods are available. Complement may be inactivated by carefully applied heat before the test or by certain chemicals in the reagents used in the test.

a. **Heat Inactivation of Complement.** Serum complement may be inactivated by heating at 56°C for 30 minutes in a water bath or dry-heat block. The serum must be at room temperature before heating; it must also be well mixed if it was previously frozen. Be sure that the serum is cell-free because the cells will hemolyze at inactivation temperatures. After heat inactivation, the serum should normally be allowed to cool to room temperature before testing. However, since some complement activity is regained on standing, any serum that has stood at room or refrigerator temperatures for longer than 4 hours after inactivation should be reheated at 56°C for 10 minutes and then allowed to recool before testing. Heat inactivation of plasma complement is impractical because the fibrinogen precipitates on heating. This precipitate must be removed before the plasma can be tested.

b. **Chemical Inactivation of Complement.** Both serum and plasma complement can be inactivated efficiently by incorporating choline chloride into the test reagents. Since heating is not required, the fibrinogen will not precipitate from plasma nor is the extra time (30 minutes) for heat inactivation required. With choline chloride, the inactivation apparently occurs instantaneously. At the present time, choline chloride is used primarily in certain tests for syphilis, which use unheated serum or plasma.

Chapter 4

FUNDAMENTAL SEROLOGIC PROCEDURES

4-1. Preparation of Saline. Saline is one of the most frequently used reagents in serology. Basically, saline is an aqueous solution of sodium chloride (NaCl), but there are many different concentrations and modifications in use so the technician must be sure to use the saline specified for each given procedure. Some of the variations in saline are as minimal as one procedure requiring saline containing 0.85% NaCl while another procedure may specify 0.90%. Other variations are more pronounced. For example, certain tests require saline that contains NaCl plus buffers such as glycine or phosphates. Many of these buffered salines are prepared to very critical specifications and pH ranges. Fortunately, most test kits provide the appropriate buffered saline as part of the kit. The buffered saline in these kits may be in aqueous form and ready to use or the constituents may be in powder form and need to be reconstituted by the serologist. Although most types of saline are available through supply channels, two varieties of saline can be prepared economically in the laboratory because large quantities of these are used regularly.

a. **Normal Saline, 0.90%.** Saline containing 0.90% (w/v) NaCl is called normal or physiological saline. Normal and physiological are also used to describe 0.85% saline. Except for the small difference in the amount of NaCl in each, 0.90% and 0.85% are essentially identifical and for practical purposes can be used interchangeably. Saline (0.9%) is available through normal supply channels but may be easily prepared in the laboratory.

(1) Drying the Sodium Chloride. Dry the NaCl (reagent grade) in a flat, open container in a dry-air oven at 170%C ± 10°C for about ½ hour. Do not overheat the NaCl. Drying at 110°C for about 3 hours is also acceptable. Weigh the desired amount as soon as the container can be safely handled.

(2) Preparing the Saline. Weigh accurately 0.90 g of NaCl for each 100 ml of saline desired. Dilute the NaCl to the appropriate volume with distilled or deionized (demineralized) water.

b. **Phosphate Buffered Saline (PBS), pH 7.2 ± 0.1.** This buffered saline is used in large quantities in the Fluorescent Treponemal Antibody-Absorption (FTA-ABS) test. The premixed chemicals for this PBS are available commercially in powdered form. The powdered mixture requires only a single weighing before reconstitution. However, this PBS can also be prepared economically in the laboratory from the basic ingredients.

(1) Drying the Salts. Dry NaCl, Na_2HPO_4 and KH_2PO_4 for about 2 hours at 100°C–110°C in a dry-air oven. Do not dry phosphates containing water of hydration. Dry only anhydrous phosphates and do not exceed 100°C with these.

(2) Weighing the Reagents. Weigh accurately the following reagents for each liter of PBS required.

NaCl	7.65g
Na_2HPO_4	0.724 g
KH_2PO_4	0.21 g

(3) Preparing the PBS. Dissolve the salts and dilute to the appropriate volume with fresh or recently boiled distilled or deionized (demineralized) water. Determine the pH of the PBS on a pH meter. Lots that do not fall within ± 0.1 of pH 7.2 should be discarded.

(4) Storage of PBS. Store the PBS in tightly stoppered polyethylene or high-quality glass container preferably in the refrigerator. Be sure to allow an aliquot to warm to room temperature before pipetting.

4-2. Preparing Red Blood Cell Suspensions. Several serotests use suspension of erythrocytes (RBCs) as antigens. Depending on the particular serotest, these cells may be from man or from animals, usually rabbits or sheep. In addition to a specific animal source for the erythrocytes, the cells for each serotest must be of a specific blood group and concentration—usually from 2% to 5% by volume. Too many cells (antigens) in the test could result in a weak reaction due to the postzone effect (antigen excess). A weak reaction could also be produced by too few cells being used—the prozone effect (antibody excess). Another important factor is the age of the RBCs. Therefore, the serologist must be able to prepare accurate concentrations of RBC suspensions from cells of the proper age and blood group as well as from the proper animal source.

a. **Collecting the Blood.** Depending on the animal source, either venous or cardiac

puncture blood may be used for RBC suspensions for serotests. The blood may be either defibrinated or mixed with an anticoagulant but anticoagulants are easier to use. If the cells are to be stored for significant periods of time, a preservative anticoagulant, such as ACD or Alsever's should be used.

(1) Acid-Citrate-Dextrose (ACD). Dissolve 1 g sodium citrate (monohydrated) and 1.5 g dextrose in 60 ml of water. Mix 1 part of ACD with 4 parts of blood. Blood collected in ACD will keep for several weeks in the refrigerator. Vacuum blood-collecting tubes containing ACD are available commercially. Blood collected in ACD for transfusion may be used for certain serotests, but be sure to use the proper blood group and age of cells as specified in the test procedure.

(2) Alsever's Solution, Modified pH 6.1:

Sodium citrate (dihydrate)	0.80 g
Dextrose	2.05 g
Sodium chloride (NaCl) (Reagent Grade)	0.42 g
Distilled water, q.s. to	100 ml

Adjust the pH to 6.1 with 10% (w/v) citric acid. Mix equal parts of Alsever's and blood. Blood collected in Alserver's solution will keep several weeks in the refrigerator.

b. Washing the Cells:

(1) Determine the total volume of RBC suspension required for the number of tests to be performed.

(2) Calculate the minimum volume of packed RBCs required to prepare this total volume of the required concentration of cells by using the following formula:

$$\text{Packed cell volume} = \frac{\text{Total volume required} \times \% \text{ desired}}{100\%}$$

EXAMPLE: If you need 75 ml of a 2% RBC suspension:

$$\text{Packed cell volume} = \frac{75 \text{ ml} \times 2\%}{100\%}$$

$$= \frac{150 \text{ ml}}{100}$$

= 1.5 ml of packed cells needed.

(3) Put a quantity of blood at least three times the required packed cell volume in a graduated centrifuge tube. The actual volume should take into consideration the amount of anticoagulant in the blood.

(4) Add about 10 volumes of normal saline and mix gently. Centrifuge at 1500–2000 rpm for 5 minutes.

(5) Using a faucet filter pump (Richard's pump) or similar device (see Figure 4–1), siphon off the supernatant fluid and the buffy coat (white blood cells).

Figure 4–1. Aspiration of Supernate, With a Faucet Filter Pump.

(6) Resuspend the RBCs in about 10 volumes of saline and repeat steps 4 and 5 at least two more times. The washing is repeated until supernatant is clear (not blood-tinged). Usually about three washings is adequate. Cells that must be washed more than five times to produce a clear supernatant should be discarded and fresh blood collected and washed.

(7) At the last washing, centrifuge the cells for 15 minutes to pack them firmly. Before removing the supernatant, record the volume of packed cells and then carefully remove the supernatant without disturbing the cells. Resuspend the cells to the desired concentration using the diluent specified in the procedure.

NOTE: Although normal saline is used to wash the cells, the final suspension must be made with the diluent specified in the procedure.

c. Resuspending the Washed, Packed Cells:

(1) Calculate the total volume of RBC suspension that the acquired packed cell volume will prepare by using the following formula:

Total volume =

$$\frac{\text{Observed packed cell volume} \times 100\%}{\text{Desired \% cell concentration}}$$

EXAMPLE: If 2.5 ml of packed cells have been prepared and at least 75 ml of a 2% RBC suspension is required, the 2.5 ml of packed cells will make the following total volume:

$$\text{Total volume} = \frac{2.5 \text{ ml} \times 100\%}{2\%}$$

$$= \frac{250 \text{ ml}}{2}$$

$$= 125 \text{ ml}$$

(2) Calculate the amount of diluent to add to the packed cells as follows:

ml diluent to add =

Total volume that packed cells will prepare

− (minus) packed cell volume.

EXAMPLE: In the example started above,

ml diluent to add = 125 ml − 2.5 ml
= 122.5 ml.

Therefore, the requirements of the hypothetical serotest that needs 75 ml of a 2% RBC suspension will be met with some RBC suspension to spare.

d. **Outline for Preparing RBC Suspension:**

(1) Collect the blood (see paragraph 4–2a).

(2) Wash the RBCs at least 3 times with normal saline after first performing the following:

(a) Determine the total volume needed.

(b) Calculate the minumum volume of packed cells that will yield the desired concentration and total volume (see paragraph 4–2b).

(3) At the last washing, firmly pack the cells, record the packed cell volume; and carefully removed the supernatant.

(4) Calculate the total volume of RBC suspension that can be prepared from the actual packed cell volume of washed cells (see paragraph 4–2c).

(5) Subtract the packed cell volume from the total volume to be prepared (see paragraph 4–2c).

(6) Resuspend the packed cells in the appropriate diluent to bring the suspension to the desired concentration (see paragraph 4–2c).

4–3. Serial Dilutions.

a. **Significance of Serial Dilutions.** As described in chapter 2, paragraph 2–7, the only practical method that the serologist has available for measuring serum antibody levels is to determine the titer (reacting capacity) of the serum against a given antigen. However, to determine this antibody titer, the serologist must first make a planned, progressive or serial dilution of the serum in some diluent, such as saline. These serial dilutions can be performed using pipets or with the microtitration equipment described in chapter 1. However, the serial dilutions described here will be for using pipets and test tubes. Microtitration techniques will be described in chapter 14.

(1) To prepare a serial dilution using pipets and test tubes, diluent is added to a series of tubes and then a measured sample of serum is transferred successively from tube to tube so that each tube contains progressively smaller amounts of serum. After the serum has been diluted, the next step is usually to add some of an antigen (such as an RBC suspension) to each tube and record the highest dilution of serum that still gives a specified antigen-antibody reaction. The serum dilution usually is figured to include the added antigen too. The reciprocal of this highest serum dilution is the TITER of that particular serum against that particular antigen. For example, if the highest serum dilution to react to the extent specified in the test procedure is 1/640, the titer would be reported as Positive—1:640 against that antigen.

(2) Although the antibody titer of a serum is not an absolute measure of the antibody content of the serum, the titer does provide a useful mathematical base for certain serotests. Even the titer of a single serum sample could be diagnostic if the titer is higher than usually expected. Or a disease in question might be ruled out if the serum had no titer or a very low one. However, the best use of antibody titers is when a serotest is performed on two serum samples from the same patient. With two samples collected several days or weeks apart, a rise in titer in the second would be most significant. Assuming that the patient had not been immunized recently with the antigen under study, a rise in titer would most likely indicate the presence of active disease or at least recent disease.

(3) Serial dilutions are also very useful for detecting prozone (antibody excess) reactions in serotests. Sometimes undiluted

serum gives only a very weak reaction, but when the test is performed on serially diluted serum, an optimum proportion of antigen to antibody may be achieved at some higher serum dilution with a stronger reaction noted than with undiluted serum.

(4) Not all serial dilutions are limited to serum. Cerebrospinal fluid is used in some of the same tests as serum. An example of making a serial dilution of an antigen is the assay of human chorionic gonadotropin (HCG) in urine. In two conditions, hydatidiform mole and choriocarcinoma, the amount of HCG in the urine is much greater than during pregnancy. So the serologist determines the titer of an antigen (HCG) in these diseases using antibodies (anti-HCG) that have been produced in rabbits.

b. **Types of Serial Dilutions.** Most serologic tests are designed so that the serial dilution of serum, if required, follows a relatively simple pattern. One of the more common dilution patterns has the serum diluted in half in each succeeding tube of the series. This pattern is called a twofold dilution. Other tests call for fourfold dilutions wherein each succeeding tube contains one-fourth as much serum as the preceding tube. A tenfold dilution is used in still other tests.

(1) Nearly all serologic tests in this manual use twofold serial dilutions. However, the serologist may modify a twofold dilution to obtain just about any dilution sequence he wants. He does this by making a preliminary dilution of the serum before he starts the serial dilution. For example, if he made a preliminary dilution of the serum of one-to-ten (1:10) and then started a twofold dilution of this, the second tube would have a dilution of 1:20; the third 1:40 et cetera. By varying the starting point of a simple twofold serial dilution a nearly unlimited variety of dilutions can be achieved.

(2) Unfortunately, not all serologic tests use simple serial dilutions. The antistreptolysin-O (ASO) titer (see chapter 9) is a good example of a very complex serial dilution technique. In one modification of the ASO titer, three different preliminary dilutions are made. In a method for determining the titer of human chorionic gonadotropin (HCG) (see chapter 13), the serial dilution of urine starts out as tenfold and then switches in midseries to a fivefold dilution. Consequently, the serologist must be sure to follow all serial dilution procedures carefully but especially these complex ones.

c. **Performing a Typical Serial Dilution.** In this manual, most procedures that require serial dilutions will be presented in written form with the major steps also outlined diagrammatically in a table. The examples given in this chapter will be similar to those used throughout the manual.

(1) Preliminary Steps. Before starting the serial dilution, examine the test procedure and assemble the equipment and reagents required. Special attention should be paid to the following:

(a) Water baths or heating blocks; operating at the proper temperature.

(b) Test tubes; clean and of specified size.

(c) Pipets; generally the smallest usable size.

(d) Diluent; saline or modification, as specified.

(e) Antigen suspension; prepared as specified and at the specified temperature before use.

(f) Serum or other specimens; fresh or inactivated as specified. For frozen serum, be sure it is thawed, mixed gently and allowed to warm to room temperature before inactivation. Also be sure all specimens are at room temperature before pipetting.

(2) Procedure, Example:

(a) Place seven test tubes (12 × 75 mm) in a rack. Label the first tube with the patient's name, et cetera, and number each tube consecutively. Some test tube racks have numbered slots so if the test tubes do not have to be removed from the rack during the test, only the first tube needs to be labeled. However, a good habit is to label all tubes, if practical.

(b) With a 5 ml serological pipet used with the point-to-point technique or with an accurately calibrated, syringe-type automatic pipetter, put 0.5 ml of normal saline in each tube.

(c) In a separate test tube, make a preliminary 1:5 dilution of fresh serum (not inactivated) by adding 0.2 ml of serum to 0.8 ml of normal saline. Use a separate pipet for each reagent and the smallest, usable size.

(d) With a 1.0 ml serological pipet, mix the contents of the preliminary 1:5 dilution of serum by carefully sucking the mixture into the pipet and then gently blowing it back into the test tube. Repeat the mixing at least 3 times, but do not create bubbles in the mixture. After mixing, suck slightly more than 0.5 ml of mixture into the pipet and lower the meniscus exactly to the 0.5 ml mark. Remove droplets on the tip of the pipet by rubbing the pipet around the inside of the test tube as the pipet is withdrawn. The

pipet is not wiped dry as it would be in normal pipeting technique.

(e) Transfer the 0.5 ml of 1:5 dilution to the first tube and blow out the last drop. NOTE: For greatest accuracy, this pipet should now be discarded and a new pipet used to mix and dilute each tube. However, for practical purposes the same pipet is used throughout the serial dilution. The important considerations are to be consistent and at the same time try to remove as much external fluid as possible from the pipet as it is removed from each tube.

(f) Using the same pipet, mix and transfer 0.5 ml from tube #1 to tube #2 and repeat through tube #6. After mixing, discard 0.5 ml from tube #6. Tube #7 is the Antigen Control and does not receive any serum.

(g) Examine the fluid level of each tube. Each should have 0.5 ml if the serial dilution has been performed properly.

(h) Add 0.5 ml of antigen, such as a bacterial or RBC suspension to each tube including #7.

(i) Incubate the tubes and read the reactions as specified for the particular procedure. The preceding procedure is presented diagrammatically in table 4-1.

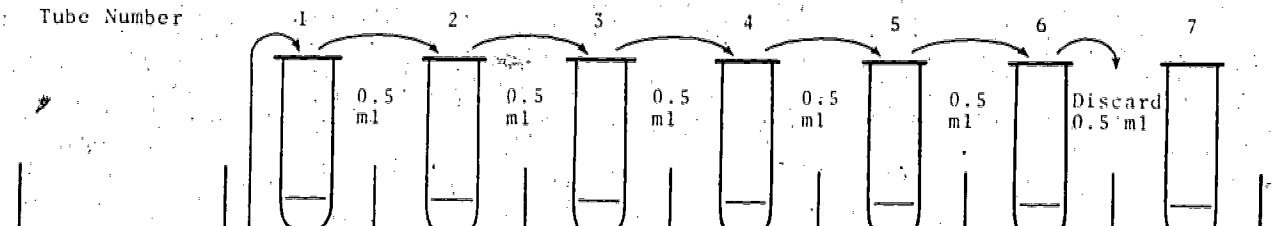
d. **Calculating the Titer.** As defined, the antibody titer is the reciprocal of the highest serum dilution that still gives a positive antigen-antibody reaction. Unfortunately, this definition must be qualified because some tests have been designed that included the addition of the antigen before figuring the serum dilution while other tests use the dilutions before the antigen is added. Therefore, be sure to follow the system used for each given serotest.

(1) The serum dilution of a given tube is most easily determined by considering the ratio of serum to the total volume in the tube as a fraction. This most easily accomplished if the numerator is converted to one. For example, in the preliminary dilution made in the sample serial dilution given in paragraph 4-3c(2), 0.2 ml of serum is mixed with 0.8 ml of saline for a total volume of 1.0 ml. The dilution of serum is 0.2 ml serum/1.0 ml total volume which is proportional to 1 ml serum/5 ml total volume or a 1:5 dilution.

(2) Once the dilution of the preliminary tube is known, calculating the dilution of

Table 4-1. Example, Typical Serial Dilution Procedure.

Table 4-1. Example, Typical Serial Dilution Procedure.



Saline, ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	0.5 milliliters of:						
Serum, ml	1:5 (From separate tube)	1:10	1:20	1:40	1:80	1:160	None (Control)
Serum Dilution After Transfer	1:10	1:20	1:40	1:80	1:160	1:320	---
Appropriate Antigen Suspension, ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total Volume, ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Final Dilution of Serum	1:20	1:40	1:80	1:160	1:320	1:640	---

other tubes in the series is simply a matter of multiplying fractions and can usually be performed mentally. For example, from the example in paragraph 4–3c, a twofold dilution is made starting with the preliminary 1:5 dilution. Therefore, before the antigen is added, the serum dilution in the first series would be:

$$1/5 \times 1/2 = 1/10 \text{ or } 1:10$$

After an equal volume of antigen is added, the final dilution of serum in the first tube becomes:

$$1/10 \times 1/2 = 1/20 \text{ or } 1:20$$

Each succeeding dilution can be calculated in this fashion but be sure to check the test procedure for whether the antigen is considered as part of the serum dilution or not. Another important step to remember is to always represent the serum to total volume ratio as one part of serum to whatever total volume exists; then the reciprocal of this fraction will be expressed in the proper term for reporting titers as one part of serum in some total volume, such as 1:640, 1:1280, et cetera.

4–4. Reporting Results of Serologic Tests. As soon as practical after the completion of a serotest, the results should be recorded on the patient's laboratory slip and dispatched to the appropriate office. The proper way to report the results is provided with each serotest in this manual. However, there are certain general principles that should be described.

a. **Tests Without Serial Dilution.** Several serotests do not require a serial dilution as part of the test procedure. These tests are usually reported simply as either POSITIVE or NEGATIVE. However, a variation of the positive-negative report is used with tests for syphilis. Syphilis tests are reported as either REACTIVE or NONREACTIVE.

b. **Tests with Serial Dilutions:**

(1) Positive (Reactive) Tests. Positive results from tests involving serial dilutions are usually reported as positive (or reactive) followed by the titer. For example, a test result might be reported as follows: Positive—1:20, Pos-titer 1:20; or some similar format. CAUTION: Before reporting any titer, be sure that the test has been read as specified in the test procedure. This precaution must be emphasized because many serotests specify some degree (strength) of antigen-antibody reaction on which to base the titer. Usually the reactions are rated from 1 to 4+ with 1+ being a very weak reaction and 4+ the strongest possible. Using this system, a given test procedure may specify that the titer is the highest dilution of serum to give a 2+ or greater reaction. A 1+ reaction might be reported as BORDERLINE so be sure to follow the procedure in determining the end point of a reaction.

(2) Negative (Nonreactive) Tests. Do NOT report a serotest as NEGATIVE if none of the tubes in a serial dilution show a reaction. The best way to indicate these results is to name the antigen and indicate that the reaction may have been at some point below the lowest serum dilution used in the test. For example, a report might read: *Salmonella typhi* H—less than 1:20. This report would indicate to the physician that a 1:20 dilution was the lowest serum dilution tested against that particular antigen but does not rule out the possibility that a 1:5, 1:15, or even 1:19 dilution might have reacted. The fact is that these lower dilutions have not been tested so it would be unfair to suggest they had been tested by calling the results negative. Each test has been designed so that titers less than the first tube of the series are usually insignificant but unless they are tested they should not be reported.

Chapter 5

AGGLUTINATION TESTS FOR FEBRILE DISEASES

5-1. Significance of Febrile Agglutinins. Although direct diagnosis of infectious disease by isolating and identifying the causative organism is usually the preferred method, this is not always practical—or even possible. For example, isolation of the organism may be difficult, especially if the clinical specimen is collected late in the course of the disease or after antibiotic therapy has been initiated. In certain other diseases, such as the rickettsioses, the organisms normally could not be isolated and identified in time to benefit the patient. Fortunately, however, several febrile diseases, such as certain bacterial and rickettsial diseases, stimulate the body to produce agglutinating antibodies (agglutinins) against the causative bacteria or, in the case of rickettsial diseases, against antigenically related bacteria. In some of these febrile diseases, bacterial agglutinins appear in the blood early enough in the disease to be of practical diagnostic value.

a. To detect bacterial agglutinins, suspensions of known bacteria (usually acquired commercially) are added to the serially diluted serum. After mixing and incubating as prescribed in the test procedure, a macroscopically visible agglutination (clumping) of the bacteria occurs in mixtures containing an optimum proportion of bacteria (antigen) and agglutinin (antibody). Usually each serum is tested against several different bacterial antigens as a battery because so many of these so-called febrile diseases have similar symptoms. Several combinations of febrile antigens are available commercially in kit form. The agglutinin titer against each antigen used in the test battery is reported to the physician.

b. The febrile agglutinin titer of a single serum sample rarely provides the physician with conclusive evidence of infection by a given organism, but the information might help him to initiate therapy. However, in the absence of recent immunizations, a rise in titer on a subsequent serum sample provides the physicain with at least indirect evidence of a clinical or subclinical disease caused by the test organism or some antigenically related organism.

c. The fact that antigenically related organisms and immunizations can stimulate the production of febrile agglutinins identical to those detected during actual disease constitutes one of the main limitations in using data from these tests. Therefore, the patient's complete history (immunizations, past infections, and so forth) must be considered before drawing conclusions from test results. Another limitation on the usefulness of these tests is that the time of appearance of detectable levels of agglutinins in the serum varies with the causative organism, the patient's immunologic status, and other variables. At other times, no agglutinins are ever produced. This variability is another good reason for using a battery of antigens on serum collected every few days, usually from 3–7 days apart. Eventually a significant rise in titer against one or more of the antigens may be detected. Even with these limitations, there are several febrile diseases for which bacterial agglutination tests are the most practical tests available to the average clinical laboratory

d. Bacterial agglutination tests have been used in the diagnosis of such diverse diseases as plague, brucellosis, tularemia, typhus, Rocky Mountain spotted fever, typhoid fever, and others. For some of these diseases, newer tests (for example, fluorescent antibody tests) have nearly replaced febrile agglutination tests. In addition, many of these diseases are rare in the United States today. However, because of the possibility of encountering these diseases with increased frequency in other parts of the world, the more common bacterial agglutination tests warrant inclusion in this manual.

(1) Widal Reaction. The first serologic test used to diagnose human disease was for the detection of agglutinins in the serum of typhoid fever cases. These sera agglutinated suspensions of the typhoid bacillus, *Salmonella typhi*. This reaction is the so-called Widal reaction—named for one of the early discoverers of this phenomenon. As currently used, the Widal reaction is a general term for agglutination tests for all salmonelloses, including typhoid and paratyphoid fevers.

(a) Most salmonellae possess two types

of antigens—O (somatic) and H (flagellar). The O antigens are usually prepared by extracting bacterial cultures with either phenol or alcohol. This extraction preserves the O antigens and destroys the H (flagellar) antigens at the same time. H antigens may be prepared by extracting cultures of motile salmonellae with formalin, which destroys the O antigens. Both types of antigens are available commercially for several groups of salmonellae.

(b) The O antigens are especially important because the titer of O agglutinins rises earlier in the disease and then drops faster than H agglutinins. Testing for H agglutinins is usually unimportant because titers are slow to rise in a disease and then add to the confusion by remaining elevated for several years. Immunization also results in elevated H agglutinins while O agglutinins are not affected as much. Unfortunately, however, many manufacturers still customarily include H antigens in their febrile antigen kits so some discretion must be used by the serologist in selecting the antigens for his test battery.

(c) Currently only about 20,000 cases of salmonellosis are reported in the United States each year. Most of these cases are due to salmonellae of Groups B, C_1, C_2, and D with Group B being the most frequent. Only about 400 cases of the most severe salmonellosis, typhoid fever, occur each year in the US. Other groups of salmonellae are more prevalent in other countries. Therefore, the serologist must also consider the prevalent strains for each given locality in selecting his test antigens.

(2) Weil-Felix Reaction. Some of the rickettsial diseases stimulate the production of bacterial agglutinins against certain nonmotile *Proteus* OX strains. This is referred to as the Weil-Felix reaction. This practical application of cross-reactivity has proven useful because rickettsiae require special techniques, such as animal inoculations, to isolate the organisms. Although fluorescent antibody and other serologic techniques can be used for the serodiagnosis of certain rickettsial diseases, the average clinical laboratory still makes use of *Proteus* agglutination tests for these diseases. Three strains of *Proteus* are commonly used as antigens. They have been designated *Proteus*, OX-19, OX-2, and OX-K. The production of agglutinins against these three strains varies from disease to disease and even from patient to patient. However, at times the agglutinins may be detected in the serum just a few days after the onset of disease, so these tests have proven very useful.

(a) Fortunately, very few cases of rickettsioses occur in the US today. Only about 300-400 cases of Rocky Mountain spotted fever and 30 cases of endemic flea-borne (murine) typhus occur in the US each year. No epidemic louse-borne typhus, the disease that has decimated armies, has been seen in the US for several years. However, rickettsial diseases are more prevalent in other parts of the world, expecially murine typhus and scrub typhus (tsutsugamushi fever) in Southeast Asia. With the declining use of chlorinated hydrocarbons (DDT and others) as insecticides, rickettsioses might be expected to increase in frequency as their arthropod vectors increase in numbers.

(b) The variable production of Proteus agglutinins in rickettsial diseases makes interpreting the results of the Weil-Felix reaction a difficult task. First of all, the reaction is not seen with all rickettsioses. In addition, certain diseases besides rickettsioses may stimulate the production of *Proteus* agglutinins. These false positives must also be considered in differential diagnosis. The usual Weil-Felix reactions against the three *Proteus* strains in each rickettsial disease are shown in table 5-1.

(2) Brucellosis-Tularemia Agglutinins. Two other bacterial antigens that may be included in febrile agglutinin batteries are somatic (O) antigens of *Brucella abortus* and *Francisella (Pasteurella) tularensis*. Both brucellosis and tularemia are relatively rare diseases in the US with only about 200 cases of each reported per year. However, these diseases must be considered when undiagnosed febrile diseases occur.

(a) Both of these diseases are difficult to diagnose by bacteriological methods. Even if an organism is isolated, definitive identification is still a time-consuming project. Part of this problem arises from the relative rarity with which the bacteriologist encounters these organisms. Therefore, most cases of brucellosis and tularemia are detected serologically.

(b) The strain of *Brucella abortus* used as the febrile antigen will detect agglutinins produced against all three species infecting man, namely, *B. suis* and *B. melitensis* as well as *B. abortus*. In addition to this across-reactivity, significant cross-reactivity is seen between *Brucella* and *Francisella* and vice versa. Consequently, titers using both antigens will have to be performed in order to obtain more definitive information on the

Table 5-1. Weil-Felix Reactions in Rickettsial Diseases

Disease	Organism	Reaction of Serum with *Proteus* OX-19	OX-2	OX-K
Epidemic Typhus, Brill's Disease	*Rickettsia prowazekii*	+	-(or+)	-
Endemic Typhus	*R. typhi*	+	+(or-)	-
Scrub Typhus	*R. tsutsugamushi*	-	-	+
Rocky Mountain Spotted Fever and Related Tick-borne Rickettsioses	*R. rickettsii* and others	+(or-)	+(or-)	-
Rickettsialpox	*R. akari*	-	-	-
Trench Fever	*R. quintana*	-	-	-
Q Fever	*Coxiella (R.) burnetii*	-	-	-

Table 5-1. Weil-Felix Reactions in Rickettsial Diseases.

actual disease agent causing the antibody production.

e. The actual selection of the antigens to be used in the febrile agglutinin battery must be made by the serologist. This selection must take into consideration the disease prevalence of the geographic locality of the patient's history and symptoms as well as the availability of the antigens. Table 5-2 lists some suggested antigens for use in the US. If the patient has recently traveled to the Far East, *Salmonella* O Group A and *Proteus* OX-K should be considered as possible additions. Travel to other parts of the world may suggest additional modifications of the battery.

Table 5-2. Suggested Febrile Antigens for Use in the Continental U.S.

Suggested Antigen	Most Frequent Causative Organisms
Salmonella Group D somatic	*Salmonella typhi* *Salmonella paratyphi*
Salmonella typhi flagellar d	*S. typhi*
Salmonella Group B somatic	*Salmonella schottmuelleri* *Salmonella paratyphi*
Salmonella Group C somatic	*Salmonella choleraesuis* *Salmonella typhimurium*
Proteus OX-19, OX-2	*Rickettsia typhi* *R. prowazekii*
Brucella abortus	*Brucella abortus* *Brucella melitensis* *Brucella suis*

Table 5-2. Suggested Febrile Antigens for Use in the Continental US.

5-2. Slide Test for Febrile Agglutinins:

a. **Principle.** This test should be used for screening purposes only. In the test, a heavy suspension of known bacteria is added to a series of measured small quantities of serum on a slide. Macroscopic agglutination of the bacteria indicates the presence of agglutinins in the serum. Although the quantities of serum placed on the slide have been correlated with the serum dilutions used in the tube test, for most accurate results, the tube test should be performed on all positive—and especially weakly positive—slide tests. Only completely negative (that is, less than 1:20) results should be reported solely on the basis of the slide test.

b. **Reagents and Equipment:**

(1) Febrile antigens, slide test type (available in kits or separately). See table 5-2 for suggested antigens to use.

(2) Positive control sera (available commercially).

(2) Clear, fresh (unheated) patient's serum.

(4) Glass plates, 3" × 5" or larger.

(5) Pipets, serological, 0.2 ml.

(6) Wooden applicator sticks or toothpicks.

c. **Preliminary Steps:**

(1) Allow the antigens and sera to warm to room temperature before testing—about 30 minutes is adequate. Frozen sera must be thawed and mixed gently prior to testing.

(2) With a marking pencil, rule off the glass plates into 1" × 1½" blocks. Five spots

(blocks) will be needed for each antigen with each serum or control serum.

d. **Procedure:**

(1) With a 0.2 ml pipet, deliver 0.08, 0.04, 0.02, 0.01, and 0.005 ml of serum to a row of blocks on the slide. Touch the tip of the pipet to the slide to assure complete delivery of each aliquot. For greater accuracy a 0.1 ml pipet may be used because of 0.005 ml aliquot must be estimated when using a 0.2 pipet. Set up one row of blocks for each antigen-serum combination to be tested.

(2) Repeat (1) above for each control serum.

(3) Resuspend the antigen by gently shaking the container until an even suspension is obtained.

(4) Add 0.03 ml (or 1 drop, if a dropper is provided) of the appropriate antigen to each quantity of serum in a row and to the corresponding control serum row.

(5) Starting at the RIGHT (0.005 ml spot) and proceeding to the LEFT, mix each antigen-serum mixture thoroughly with an applicator stick or toothpick. Use a new stock for each antigen row. ALWAYS mix each row from lowest (right) to highest (left) serum quantity to minimize carryover of serum to the next spot. As an alternative, a new stick may be used to mix each spot.

(6) Rotate and tilt the plate by hand for 3 minutes (or as indicated by the antigen manufacturer) over a light such as an Rh typing view box. Do not heat the mixtures with the light. Read the agglutination immediately at the end of the 3 minutes. Read the reactions of the control sera first to check the sensitivity of the antigens. The slide test procedures is shown graphically in table 5–3.

e. **Reporting Results.** If the control sera produce the expected results, report the patients' sera as follows:

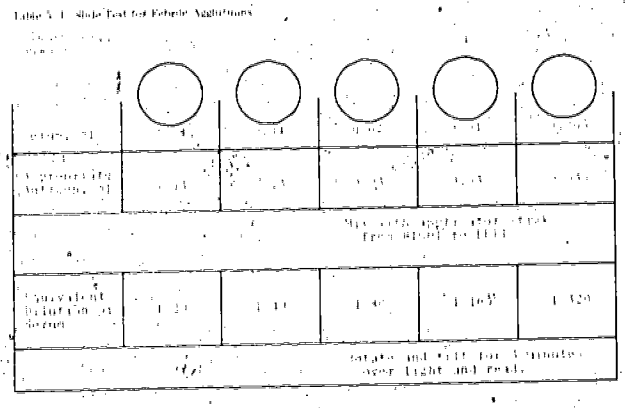


Table 5–3. Slide Test for Febrile Agglutinins.

Table 5–4. Readings of Slide Test for Febrile Agglutinins.

Amount of Agglutination	Read as
100 percent agglutination (complete)	4+
75 percent agglutination	3+
50 percent agglutination	2+
25 percent agglutination	1+
Trace to 25 percent agglutination	±
No agglutination	–

Table 5–4. Readings of Slide Test for Febrile Agglutinins.

(1) No agglutination—Less than 1:20. This is the lowest equivalent dilution of serum actually tested.

(2) Agglutination—Ideally, these sera should be titered by the tube method; however, if this is impractical, each antigen-serum mixture is read as indicated in table 5–4. The endpoint is the least amount of serum to produce a 2+ (50% agglutination) or greater. The equivalent dilution of serum as determined from table 5–3 is then reported as the titer. For example, if 0.02 ml of serum produced a 2+ reaction with a given antigen while 0.01 and 0.05 ml reacted 1+ or less, the titer would be reported as 1:80 against that antigen.

f. **Sources of Error:**

(1) Antigens must be specified for slide test use.

(2) Improperly resuspended antigen may simulate agglutination.

(3) Mixing the antigens with the serum on the slide from HIGH or LOWER dilutions with the same applicator stick may result in falsely elevated titers due to serum carryover.

(4) Cold sera and antigens may result in weak reactions.

(5) Failure to check weak or doubtful reactions by the tube method may result in a significant antibody level being missed due to the prozone effect. *Brucella* agglutinins frequently cause these prozone reactions. In addition, when all spots are positive, the tube test must be applied to find the true titer of the serum.

(6) Readings must be made immediately at the end of 3 minutes (or the time indicated by the manufacturer), because false-positive reactions may develop if reading is delayed.

(7) Antigens may lose sensitivity or become hypersensitive unless they are stored as directed by the manufacturer. The use of positive control sera serves as a check on the antigens.

5-3. Tube Test for Febrile Agglutinins:

a. **Principle.** The tube test uses a light suspension of bacteria as the antigen—usually prepared by diluting the slide test antigen. The dilute antigen is added to serially diluted serum. After incubation as specified by the antigen manufacturer, agglutination of the bacteria indicates the presence of agglutinins in the serum. The agglutinin titer is the highest dilution of serum to give a 2+ (50% agglutination) or greater reaction.

b. **Reagents and Equipment:**

(1) Febrile antigens, diluted according to manufacturer's directions. See table 5-2 for suggested antigens to use.

(2) Positive control sera (available commercially).

(3) Clear, fresh (unheated) patient's serum.

(4) Saline, 0.9%.

(5) Water baths, 37°C and 48-50°C.

(6) Test tube racks, to fit water bath and tubes.

(7) Test tubes, 12 × 75 mm.

(8) Pipets, serological, 1.0 ml, 5.0 ml.

c. **Preliminary Steps:**

(1) Allow the antigens and sera to warm to room temperature before testing—about 30 minutes is adequate. Frozen sera must be thawed and gently mixed prior to testing.

(2) Determine the volume of dilute antigen required and dilute the slide test antigen as directed by the manufacturer (usually 1:20 or 1:100). Be sure to use the diluent (usually saline or formalized saline) as specified by the antigen manufacturer.

(3) Be sure that water baths are at the temperatures specified by the antigen manufacturer.

(4) Place seven test tubes (12 × 75 mm) in a rack for each antigen to be tested. Label each row with the patient's identification and the antigen used.

d. **Procedure:**

(1) Pipet 0.9 ml of saline to the first tube and 0.5 ml to the remaining six tubes of each row.

(2) With a 1.0 ml pipet, add 0.1 ml of serum to tube #1 mix at least 3 times by aspiration, and transfer 0.5 ml of saline-serum mixture to tube #2. NOTE: For greater accuracy, this pipet may be discarded and a new one used for each tube.

(3) Mix and transfer 0.5 ml to tube #3, etc. Discard 0.5 ml from tube #6. Tube #7 is the antigen control and receives no serum. This makes a twofold serial dilution starting with 1:10 in tube #1.

(4) Examine the tubes. Each should contain 0.5 of fluid.

(5) Repeat the above steps for each serum-antigen combination to be tested. As a timesaving alternative, a larger aliquot of serum may be serially diluted with 0.5 ml of each dilution transferred to the appropriate antigen row.

(6) Add 0.5 ml of the appropriate diluted antigen to each of the seven tubes. This makes the final serum dilution in the first tube—1:20, second—1:40, and so forth. The tube test procedure is shown graphically in table 5-5.

(7) Shake the rack vigorously to mix the contents of the tubes.

(8) Incubate the tubes according to the antigen manufacturer's directions. Usually the times and temperatures are similar to those in table 5-6. Some manufacturers may require a refrigeration step after incubating.

(9) At the end of the incubation period, examine the antigen controls (tube #7). All must be negative. The tubes may be examined over a concave microscope mirror as shown in figure 5-1.

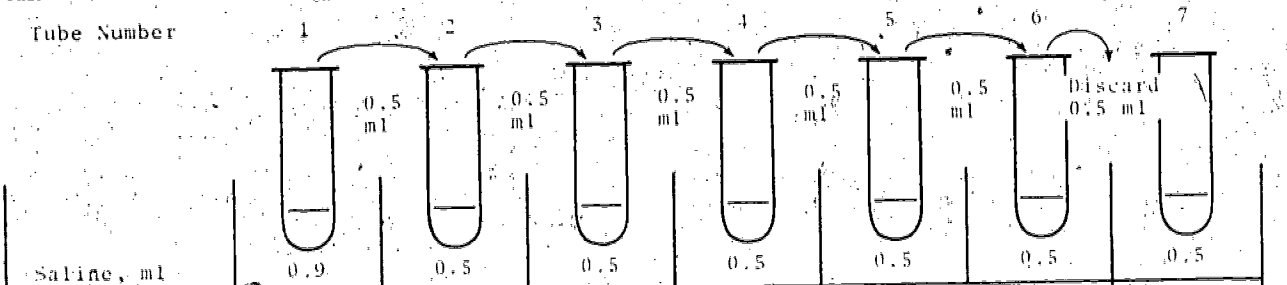
(10) Next, read the tests on the positive control sera. Each must render the expected results.

(11) Finally, read the tests on the patient's serum. GENTLY shake the tubes to examine the agglutination. Floccules of H antigens are relatively large and easily broken up, while O antigen floccules are smaller and more difficult to disrupt. Read the agglutination of each tube as indicated in table 5-7. Report the highest dilution of serum to produce a 2+ or greater agglutination as the titer.

e. **Reporting Results.** If the control sera and

Table 5–5. Tube Test for Febrile Agglutinins.

Table 5–5. Tube Test for Febrile Agglutinins.



Saline, ml	0.9	0.5	0.5	0.5	0.5	0.5	0.5
Serum, ml	0.1	0.5 milliliters of: 1:10	1:20	1:40	1:80	1:160	None (Control)
Serum Dilution After Transfer	1:10	1:20	1:40	1:80	1:160	1:320	---
Appropriate Antigen Suspension, ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total Volume, ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Final Dilution of Serum	1:20	1:40	1:80	1:160	1:320	1:640	---

the antigen controls produce the expected results, report the patients' sera as follows:

(1) No agglutination—Less than 1:20. This is the lowest serum dilution actually tested.

(2) Agglutination—The highest serum dilution to give a 2+ or greater reaction is reported as the titer. NOTE: The serum dilution INCLUDES the dilution due to the antigen.

f. **Sources of Error:**

(1) Although properly stored antigens are usually stable for years, positive control sera should be tested with each run to detect changes in antigen sensitivity. This is important because the antigens tend to become more sensitive with age and must be discarded.

(2) Antigens must be diluted as recommended by the manufacturer to prevent zonal reactions.

(3) The proper diluent (usually saline) must be used to dilute the antigen and prepare the serial dilution of the serum. Visible agglutination may not occur without the presence of electrolytes such as in saline.

(4) Use fresh serum, because heated (inactivated) serum may yield lower titers.

(5) Shaking the tubes too vigorously while reading may disrupt the agglutination especially of flagellar antigens.

(6) Tests with all six tubes showing agglutination, while the control sera show valid results, must be repeated using more tubes to determine the actual endpoint.

Table 5–6. Incubation of Febrile Agglutinin Tube Tests.

Table 5–6. Incubation of Febrile Agglutinin Tube Tests.

Febrile Antigen*	Temperature (°C)*	Time (hours)*
[illegible somatic (O)]	48–50	18–24
[illegible flagellar (H)]	48–50	*
[illegible OX-19, OX-2, OX-K]	48–50	18–24
[illegible]	56	48

* Be sure to use temperature and time indicated by antigen manufacturers.

5–4. Interpretation of Results. The results of febrile agglutination tests can only be interpreted after careful consideration of the patient's history and other factors. Special attention must be paid to past infections and immunizations as well as the patient's present and past residences and travels. Even after the patient's history has been examined, positive test results still only provide indirect evidence of infection. A twofold or

Figure 5-1. Reading Agglutination Tests With a Concave Mirror.

greater rise in titer on a second serum sample collected later in the course of the disease provides the most valid serologic evidence of infection. Unfortunately, due to cross-reactivity, even a twofold rise in titer does not definitively identify the causative organism unless an extensive battery of antigens has been tested. In addition, not all patients respond to a disease by producing agglutinins or the production may be delayed. All of these variables make interpreting the results of febrile agglutinin tests a subjective enterprise at best. However, there are certain generalizations that can be made about the reactions discussed in this chapter.

a. **Salmonelloses (Widal Reaction):**

(1) Typhoid Fever. Salmonella typhi O (Group D) agglutinins start to appear in the serum about a week after the onset of the disease. At the end of a month or so, over 90% of the cases usually have significant titers. Although a rise in titer during the disease is most significant, a titer of 1:80 or greater on a single specimen is suggestive of active typhoid fever, especially on a specimen collected early in the disease but even negative results do not exclude the disease. These titers of O agglutinins tend to drop sharply in convalescence. On the other hand, the H agglutinins in typhoid fever rise more slowly and rarely to very high levels but they remain elevated much longer than O agglutinins. In addition, high titers of H agglutinins are seen following typhoid immunizations. These titers may remain elevated for several years while O agglutinins are essentially unaffected by immunizations, thus giving the detection of O agglutinins greater diagnostic value.

(2) Other Salmonelloses. Salmonelloses such as paratyphoid and other enteric fevers are diseases that are usually much less severe than typhoid fever. Most do not have the blood phase that is seen in typhoid fever. Consequently, antibody titers may not be detected as early in the disease or to the high levels attained in typhoid fever. Since Salmonella Group A and Group B antigens are included with typhoid antigens in the classical TAB immunization, elevated H agglutinins are usually seen to these antigens, too. In addition, considerable cross-reactivity is seen between the groups of salmonellae. Therefore, do not make hasty conclusions regarding the causative organisms without first testing a positive serum against the entire battery of *Salmonella* antigens. Only the antigen showing a significantly higher titer than other salmonellae should be considered as the most probable cause of the disease.

Table 5-7. Readings of Tube Test for Febrile Agglutinins.

Agglutination and Sedimentation	Clearing of Supernate	Read as
Complete	100%	4+
Marked	about 75%	3+
Moderate	about 50%	2+
Slight	about 25%	1+
None	less than 25%	-

Table 5-7. Readings of Tube Test for Febrile Agglutinins.

b. **Rickettsial Diseases (Weil-Felix Reaction).** The Weil-Felix reaction has been most useful for the diagnosis of classical epidemic typhus but less effective for other rickettsioses. As indicated in table 5-1, rickettsialpox, Q fever, and trench fever do not stimulate the production of *Proteus* OX agglutinins while other diseases show variable cross-reactivity between the three OX strains. *Proteus* OX agglutinins are also provided in certain nonrickettsial diseases such as leptospirosis, relapsing fever due to *Borrelia*, and urinary tract infections due to *Proteus*. All of these factors must be considered when interpreting the results of Weil-Felix tests.

(1) Epidemic Typhus. Also called louseborne typhus, epidemic typhus may stimulate the production of detectable levels of

Proteus OX-19 agglutinins in as few as 3-5 days after onset. The titers peak in a month or less and then decline rapidly over the next few months. Titers in the thousands have been noted. A titer of 1:80 on a single sample should be considered as significant with no history of recent immunization or recent residence in an area endemic for typhus. In addition to *Proteus* OX-19 agglutinins, OX-2 agglutinins may also be found in epidemic typhus, but usually in lower titers. No *Proteus* agglutinins are produced in the recrudescent-type of typhus, Brill's disease.

(2) Endemic Typhus. *Proteus* OX-19 and OX-2 agglutinins are also found in endemic typhus, which is also called murine or flea-borne typhus. However, the titers are usually lower than those due to epidemic typhus. In addition, occasionally agglutinins against *Proteus* OX-K are produced.

(3) Scrub Typhus. This disease is also called tsutsugamushi disease or mite-borne typhus. Although frequently no *Proteus* agglutinins are produced in this disease, *Proteus* OX-K agglutinins may be produced. If OX-K agglutinins are produced, they usually appear in a few days, peak in about 2 weeks, and decline shortly thereafter.

(4) Tick-Borne Rickettsioses. Rocky Mountain spotted fever and other tick-borne rickettsial diseases produce a variety of *Proteus* OX reactions. None of these diseases produce OX-K agglutinins but any possible combination may be seen with OX-19 and OX-2. For example, some cases of Rocky Mountain spotted fever produced OX-19 agglutinins; some OX-2; some both types; and some cases produce no OX agglutinins at all. Consequently, along with the patient's history, a positive test might be helpful, but a negative test still does not rule out the presence of disease. If *Proteus* OX agglutinins are produced, they first appear in about a week, peak in less than 1 month and fall rapidly. Only rarely do titers exceed 1:160 or so.

c. **Brucellosis-Tularemia.** Agglutinins in both of these diseases appear 2-3 weeks after onset and peak at about 2 months. Titers of 1:80 are considered significant for both diseases. Although the titers fall rapidly in brucellosis, they may persist for years after a tu-

Table 5-8. Summary of Febrile Agglutinins Reactions.

Table 5-8. Summary of Febrile Agglutinins Reactions.

Disease	Febrile Antigen	Agglutinins First Appear	Peak Titer Reached	Minimum Significant Titer
Typhoid Fever	*Salmonella* Group D somatic	1 week	3-5 weeks	1:80
	Salmonella typhi flagellar d	later	later	1:40-1:80 (with no immunization)
Other Salmonelloses	*Salmonella* Group A, B, C, D, and E somatic	1 week	3-5 weeks	1:80
Epidemic Typhus	*Proteus* OX-19	3-5 days	2 weeks	1:160
Endemic Typhus	*Proteus* OX-19	3-5 days	2 weeks	Lower than Epidemic typhus
Scrub Typhus	*Proteus* OX-K	4-5 days	2 weeks	Frequently negative
Rocky Mountain Spotted Fever	*Proteus* OX-19, OX-2	4-5 days	Later than 2 weeks	Rarely exceeds 1:160
Brucellosis	*Brucella abortus*	2-3 weeks	3-5 weeks	1:80
Tularemia	*Pasteurella tularensis*	2-3 weeks	4-8 weeks	1:80

laremia case. Very high titers with prozone reactions may be seen in brucellosis; thus any weak reaction with the *Brucella* antigen should be studied with additional serial dilution of the serum. In spite of these differences between the agglutinins of brucellosis and tularemia, cross-reaction between them is often striking. Occasional cases of tularemia will have titers as high against *Brucella abortus* as against the causative organisms itself. This must also be considered when interpreting the results of these tests. A summary of the interpretation of results of all febrile agglutination tests is presented in table 5-8.

Chapter 6

TESTS FOR SYPHILIS

SECTION A—THE DISEASE

6-1. Introduction:

a. **Venereal Route of Infection.** Syphilis is a contagious venereal disease caused by the spirochete, *Trepanema pallidum*. The usual site of invasion of the treponeme is the male's glans penis and female's cervix, but other parts of the genitalia may be the invasion site. Although the disease is contracted primarily during direct sexual contact, invasion may be through other mucous membranes, such as of the mouth or anal region. Even abrasions or cuts in the skin of other parts of the body may serve as sites of entry of the treponeme—a fact that makes syphilis an occupational hazard for members of the medical profession.

b. **Congenital Syphilis.** Another infection route—one that justifiably generates a considerable amount of laboratory work—is the transmission of treponemes from a syphilitic pregnant woman across the placental barrier to her developing fetus. The resulting disease is referred to as congenital syphilis. This condition may (and should) be treated in utero by treating the mother as soon as her infection is detected. Many of these cases result in abortions and stillbirths. However, some infants are born alive with signs of syphilis already present. Some syphilitic infants are born without overt signs of the disease, but disfigurement, blindness, deafness, and other complications may develop unless treatment is started as soon as possible.

c. **Treatment.** Fortunately, the treatment of all types of syphilis, including congenital syphilis, is adequately performed with penicillin. Only rarely have cases failed to be completely cured by one or two doses of the drug. Therefore, the persistent (and increasing) incidence of syphilis is not due to inadequate therapy, but the disease persists due to changing sociological standards and—of prime interest to the serologist—frequent difficulties in diagnosing the disease. These diagnostic difficulties are compounded by the fact that untreated venereal syphilis may go through four distinct stages, but exact stages and their manifestations are not predictable from patient to patient.

6-2. Stages of Untreated Venereal Syphilis:

a. **Primary Syphilis.** After an incubation period of about 3 weeks (range: about a week up to 3 months), a primary chancre appears at the site of invasion of the treponemes. Usually only one chancre is found. The only other findings may be enlarged lymph glands in the region of the chancre. This highly infectious chancre disappears in about a month even in untreated cases—more quickly, if treated—but the treponemes are still in the bodies of untreated cases.

(1) Diagnosis of primary syphilis is accomplished mainly by darkfield microscopic examination of material from the chancre for treponemes. Darkfield microscopy and the morphology of the treponemes will not be described in this manual. Although a small percentage of cases have detectable levels of antibodies during the primary stage of the disease, a nonreactive serologic test for syphilis at this time does not rule out the disease.

(2) Usually, after the apparent spontaneous resolution of the primary chancre in untreated syphilis, there is a period when no visible signs of the disease are present. The serologic tests are nearly always reactive during this period, which may last up to several months. Occasionally, however, the secondary stage of syphilis may start before the primary lesion has disappeared.

b. **Secondary Syphilis.** This stage of syphilis usually starts before or within a few months after the disappearance of the primary lesion. It is characterized by fever, malaise, mucous patches in the mouth, condylomata lata of the ano-genital region, and a localized or generalized rash. All of these lesions are highly infectious. In some patients, these lesions last only a few days, but they may last up to 1 year. Periodic recurrences of these signs and symptoms may occur for a couple of years.

(1) Diagnosis of secondary syphilis may be made by darkfield examination of the lesions. In addition, nearly 100 percent of

Material in Chapter 6 was adapted from *Manual of Tests for Syphilis*, 1969. PHS Publication No. 411.

patients in this stage will have reactive serologic tests on serum.

(2) With treatment, the symptoms of secondary syphilis disappear rapidly. Even without treatment, symptoms eventually disappear, and the disease usually enters a latent period unless the disease progresses directly into the tertiary stage of syphilis.

c. **Latent Syphilis.** This stage of the disease is characterized by reactive serologic test for syphilis on serum, but the cerebrospinal fluid (CSF) must be nonreactive. If the CSF is reactive, the disease has already entered the tertiary stage. No other manifestations of syphilis are present during the latent stage, which may last several years or for the life of the patient. An additional feature of latent syphilis is that the disease is no longer communicable to another individual, EXCEPT transplacentally to a fetus. A given patient with latent syphilis may have his disease develop in one of three ways.

(1) In about one-fourth of the untreated patients, the latent period (that is, a reactive serologic test) may last for life without any other manifestations of syphilis.

(2) Another fourth of the patients experience what might be considered a spontaneous cure. In other words, their serologic tests become nonreactive and no additional manifestations of syphilis ever develop. Unfortunately, some of these apparently spontaneous cures may have developed into the most serious stage of syphilis—tertiary (or late) syphilis—even though the sera of these patients may be nonreactive in serologic tests. Usually these cases can be detected because they develop reactive serologic tests on their spinal fluid.

(3) In addition to the apparently spontaneous cures that really have developed tertiary syphilis, about half of all cases of latent syphilis progress to tertiary syphilis unless they are treated at some earlier stage.

d. **Tertiary (Late) Syphilis.** The manifestations of tertiary syphilis are varied and generally do not appear for several years after the primary infection. Cases may resemble an apparent latent stage for up to 40 years before symptoms appear. Tertiary syphilis may involve the central nervous system with the possibility of insanity, blindness, and other neurological problems. The cardiovascular system is also frequently affected with the most serious complication being the development of an aortic aneurysm which may rupture and result in nearly instantaneous death. In addition, lesions may be produced in the skin, bone, and internal organs. These lesions are not infectious, EXCEPT that a developing fetus may become infected even during this late stage of the disease. All of these serious complications of syphilis make the early diagnosis and treatment of syphilis a professional challenge. Fortunately, most cases of tertiary syphilis, as well as secondary and latent syphilis, will have reactive serologic tests for syphilis. In addition, the spinal fluid of cases with central nervous system involvement will usually have reactive tests. These facts dictate a major role for the serologist in the diagnosis of syphilis. In fact, serologic tests for syphilis are the most frequently performed serologic tests.

6-3. Types of Serologic Tests for Syphilis. The serodiagnosis of syphilis is based on the detection of two broad categories of antibodies. Most of the tests (especially the screening tests) are based on the detection of antibodies against antigens that have no logical relationship with the causative treponemes. These tests are referred to as nontreponemal or reagin tests. In addition, several tests have been developed that use antigens that are treponemal in origin. Examples of both types will be presented in this chapter.

a. **Nontreponemal (Reagin) Tests.** The antigen used in most of these tests is an extract of beef heart, known as cardiolipin. The cardiolipin may be mixed with lecithin and cholesterol for use in microscopic flocculation tests such as the VDRL and USR tests. Cardiolipin has also been combined with carbon particles (charcoal) to produce macroscopic tests, such as the RPD tests. In addition, various nontreponemal antigens have been used in complement fixation as well as several other varieties of flocculation tests. These nontreponemal tests are usually referred to collectively as a "blood test," "serology," or, more specifically "STS," which translates to "serologic test for syphilis." These tests are also frequently called a "VDRL," but "VDRL" actually refers to one particular reagin test.

(1) The syphilitic antibodies against nontreponemal antigens are considered to be antibody-like, because the antigens in use cannot stimulate their production, so they are not true antibodies. They are also called reagins or reaginic antibodies to differentiate them from treponemal antibodies.

(2) Because nontreponemal (reaginic) antibody production is not specifically stimulated by the treponemes, several other conditions can cause these antibodies to be produced. Malaria, leprosy, hepatitis, and cer-

tain immunizations are just a few of the conditions that can cause reactive nontreponemal tests at varying rates. These results are called biological false positives—BFP, for short. These BFPs, along with the social implications of syphilis, are the reason that serologic tests for syphilis are reported as "Reactive, Weakly Reactive or Nonreactive" and NOT "Positive" or "Negative." These conditions causing BFPs must be differentiated from actual treponemal infections. Reactive tests due to related treponemal infections, such as bejel, pinta, and yaws, should not be classified as BFPs. Fortunately, tests on spinal fluid rarely produce BFPs; a reactive usually indicates syphilis—in fact, tertiary syphilis.

(3) In addition to their usefulness as screening tests for syphilis, nontreponemal (reagin) tests are helpful in following the effectiveness of therapy. The antibody titer in these tests drops rapidly following successful treatment of primary syphilis. In secondary syphilis, the titer drops more slowly but usually will eventually become nonreactive. Unfortunately, titers in treated latent and tertiary syphilis may remain fairly constant for years or life. By contrast, titers of the tests using treponemal antigens do not normally drop; therefore, these reagin tests are at least useful in following treatment of the early stages of syphilis.

b. *Treponemal Tests.* Serologic tests for syphilis that utilize the treponeme as the source of antigen generally require more sophisticated equipment and techniques than reagin tests. However, the main advantage of treponemal tests, such as the Fluorescent Treponemal Antibody-Absorption Test (FTA-ABS), is that biological false positives are essentially eliminated. In fact, the FTA-ABS test is frequently used to confirm or reject the findings of one of the nontreponemal tests when these results are questionable. Although the FTA-ABS test is currently the most widely used treponemal-type test, two other tests are still in use, but declining. These are the Treponema Pallidum Immobilization Test (TPI) and the Reiter-Protein Complement Fixation Test (RPCF). Only the FTA-ABS will be presented in detail in this chapter.

SECTION B—TESTS ON SERUM

6-4. Rapid Plasma Reagin (RPR) "18 MM Circle" Card Test:

a. **Principle.** Unheated serum (0.05 ml) is spread within an 18 mm circle on a plastic-coated card. A drop (1/60 ml) of carbon-containing RPR antigen is added to the serum without mixing. The card is rotated on a slide rotator circumscribing a 3/4-inch circle for 8 minutes at 100 rpm. Macroscopic flocculation of the carbon particles indicates the presence of reaginic antibodies in the serum.

b. **Reagents and Equipment:**

(1) Patient's serum, unheated.

(2) Control sera of known reactivity, available commercially or collected locally.

(3) RPR card test kit, 18 mm circle, available through federal supply channels complete with cards, capillaries for dispensing serum, and antigen suspension with a dispensing needle. NOTE: Several other types of RPR kits are available from Hynson, Westcott, and Dunning, Inc. These kits, including one for hand rotation, may more adequately fulfill the objectives of a given laboratory. Be sure to follow all instructions for the test selected.

(4) Slide rotator, circumscribing a 3/4-inch circle and set at 100 rpm.

(5) Humidifying cover with moist blotter to cover cards during rotation.

c. **Preliminary Steps:**

(1) Preparation of Sera and Antigen.

(a) Collect patient's serum. The serum may be left on the clot after sedimentation.

(b) Allow all sera, including controls, and the RPR antigen to warm to room temperature for about 30 minutes prior to testing.

(2) Testing of Delivery Needles. When the antigen is at room temperature, test the accuracy of the dispensing needle (20-gage without bevel). This needle must deliver 60 ±2 drops of antigen suspension/milliliter. Crimp or ream the bore of the needle, as appropriate, and retest until an accurate drop is dispensed.

(3) Pretesting of RPR Antigen. Check the suitability of the antigen with unheated control sera of known reactivity by using the procedure in paragraph d below. If the expected results are obtained, proceed with testing the patients' sera. Discard antigen lots that fail to react properly.

d. **Procedure:**

(1) Place 0.05 ml of unheated serum on a circle of the test card using a calibrated capillary, serological pipet or Dispenstir (Hynson, Westcott and Dunning, Inc.).

(2) Spread the serum over the entire surface of the 18 mm circle with the wooden sticks or Dispenstirs. Avoid scratching the card.

(3) Gently shake the antigen suspension and dispense one drop (1/60 ml) of antigen to

each serum with the needle held vertically. Do not stir the antigen-serum mixture.

(4) Rotate the card for 8 minutes at 100 rpm. Be sure to use a humidifying cover to prevent drying of the mixture.

(5) Immediately following rotation, examine the cards under a light for macroscopic flocculation of the carbon particles. This reading must be made while the mixture is wet. Brief tilting of the card may aid interpretation of questionable readings.

e. **Reporting Results.** If the control sera produce the expected results, report the patients' sera as follows:

(1) Sera producing no flocculation of the carbon particles or only a slight roughness—Nonreactive.

(2) Sera producing flocculation (clumping) of the carbon particles—Reactive, preferably along with a titer. Procedures for a quantitative card test are included with the manufacturer's instructions.

f. **Sources of Error:**

(1) Failure to follow manufacturer's instructions, especially storage instructions and expiration dates of the antigen, may produce invalid results.

(2) Scratched cards may resemble flocculation.

(3) All reagents must be at room temperature for testing. Cold reagents may decrease test sensitivity; if they are too warm, the test may be hypersensitive.

(4) Dispensing needles must be accurate. Each serologic test may use a different size of antigen drop so don't interchange needles without testing them. Before storing the needle, be sure to blow all fluid from it, because dried antigen may alter the drop size, but do not wipe the needle as this removes the silicone coating.

(5) Do not confuse the 18 mm circle test with the 14 mm card test which uses different volumes of antigen and plasma. Still other tests for syphilis use 14 mm ceramic or paraffin rings on glass slides.

g. **Discussion:**

(1) Biological false positives are possible with this test.

(2) Plasma collected with a variety of anticoagulants may be substituted for serum. Check the manufacturer's instructions for acceptable anticoagulants.

6–5. Qualitative VDRL (Cardiolipin Microflocculation) Slide Test:

a. **Principle:** Cardiolipin antigen (1/60 ml) containing lecithin and cholesterol is added to 0.05 ml of inactivated serum on a slide with 14 mm paraffin or ceramic rings. The slide is rotated for 4 minutes at 180 rpm. After rotation, the mixture is examined microscopically at 100X magnification for flocculation of the antigen particles. Flocculation indicates the presence of reaginic antibodies in the serum.

b. **Reagents and Equipment:**

(1) Patient's serum, inactivated.

(2) Control sera of known reactivity, inactivated, available commercially or collected locally.

(3) VDRL (Cardiolipin Microflocculation) slide test antigen.

(4) VDRL buffered saline, pH 6.0 ±0.1.

(5) Pipets, capillaries, or Dispenstirs.

(6) Slides, with 14 mm paraffin or ceramic rings. NOTE: Slides, such as boerner slides, are not recommended for this test.

(7) Bottle, 30 ml, round, glass-stoppered, with flat innerbottom. NOTE: Be sure the innerbottom is flat.

(8) Water bath, 56°C.

(9) Slide rotator, set at 180 rpm.

(10 Ringmaker, if paraffin-ringed slides are to be used.

(11) Syringe, 1 ml.

(12) Needles, hypodermic, 18-gage without bevel or 23-gage with bevel, pretested to deliver 1/60 ml/drop.

c. **Preliminary Steps:**

(1) Preparation of Serum:

(a) Inactivate clear sera at 56°C for 30 minutes. Sera, such as control sera, that have been inactivated previously or that were inactivated more than 4 hours before being tested must be reheated for 10 minutes at 56°C.

(b) Cool all sera to room temperature before testing.

(2) Preparation of VDRL Antigen Emulsion:

(a) Pipet 0.4 ml of buffered saline to the bottom of the 30-ml bottle.

(b) Load the bottom half of a 1-ml serological pipet with 0.5 ml of VDRL antigen and introduce the tip of the pipet into the upper third of the 30-ml bottle.

(c) Start rotating the bottle at about three times/sec on a flat surface so that the center of the bottle circumscribes a circle about 2 inches in diameter. Do not make the buffer splash.

(d) Add the antigen dropwise while rotating the bottle, blowing out the last drop. This addition should take about 6 seconds.

(e) Continue rotating the bottle for 10 seconds more.

(f) Add 4.1 ml of VDRL buffered saline to the emulsion in the bottle.

(g) Place the glass stopper in the bottle and shake the bottle vigorously for 10 seconds. The fluid should be thrown from the bottom to the top of the bottle.

(h) Use the antigen only on the day of preparation.

(i) This quantity of antigen is adequate for about 250 tests. If more is needed, multiply the quantities of antigen and buffer and prepare a larger batch. Do NOT make smaller batches than the quantities indicated above.

(3) Testing of Delivery Needles:

(a) Gently mix the antigen emulsion and fill a 1-ml syringe fitted with a 23-gage needle with bevel or 18-gage without bevel (bevel removed).

(b) Holding the bevel of the 23-gage needle HORIZONTALLY (or the 18-gage needle VERTICALLY), determine the number of drops of antigen emulsion/milliliter. The needle must deliver 60 drops ± 2 drops/milliliter.

(c) Adjust the bore of the needle until the 60 drops/ml are delivered.

(4) Pretesting of VDRL Antigen Emulsion:

(a) Test known control sera (Reactive, Weakly Reactive, and Nonreactive) using the procedure in paragraph d below. Be sure the sera have been properly inactivated before testing.

(b) Discard antigen emulsions that do not produce the expected results.

d. **Procedure:**

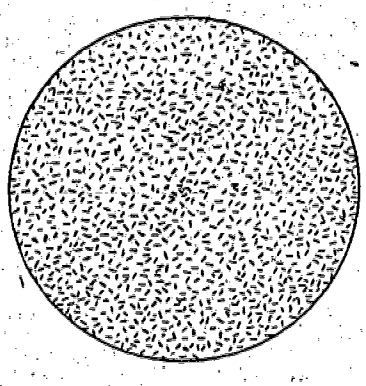
(1) Pipet 0.05 ml of inactivated serum to a 14 mm paraffin or ceramic ring on a slide. A calibrated capillary pipet or Dispenstir may also be used.

(2) Add one drop (1/60 ml) of antigen to each serum with a pretested 18- or 23-gage needle.

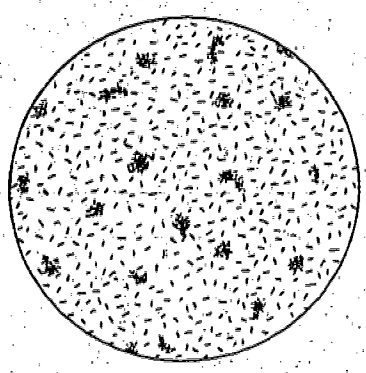
(3) Rotate the slide for 4 minutes at 180 rpm. Use a humidifying cover in a dry climate.

(4) Read the tests at 100× magnification (10× ocular and 10× objective) for flocculation of the antigen particles. See figure 6-1 for artist's conceptions of the reactions.

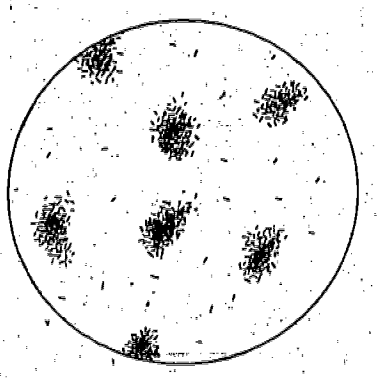
e. **Reporting Results.** Before any results can be reported, the control sera must produce the expected results. If the controls indicate valid results, report the patient's sera according to table 6-1, preferably along with the



Nonreactive



Weakly Reactive



Reactive

Figure 6-1. VDRL Slide Test Reactions.

Table 6-1. Reporting Results of VDRL Slide Tests.

Table 6-1. Reporting Results of VDRL Slide Tests.

Reading	Report
No clumping or very slight roughness.	Nonreactive (N).
Small clumps.	Weakly Reactive (WR).
Medium and Large Clumps.	Reactive (R).

titer of all Reactive, Weakly Reactive, or "rough" sera. See paragraph 6-6 for the VDRL quantitative test.

f. **Sources of Errors:**

(1) All reagents must be at room temperature for testing. Cold reagents may decrease test sensitivity; if they are too warm, the test may be hypersensitive.

(2) This test must be performed only on clear serum. Cloudy serum or heated plasmas may contain confusing precipitates.

(3) Biological false positives are possible.

(4) Prozonal reactions may occur; therefore, all Weakly Reactive, as well as "rough" reactions, should be titered to detect potentially high titered sera. See paragraph 6-6 for methods.

(5) Mix the antigen emulsions frequently to insure an even suspension of antigen particles.

(6) The pH of the buffered saline is critical. Alterations may affect test sensitivity.

(7) The antigen emulsion should only be used on the day it is prepared. For a test using a more stable antigen see the USR test in paragraph 6-7.

6-6. Quantitative VDRL (Cardiolipin Microflocculation) Slide Test:

a. **Principle.** Reactive, Weakly Reactive, and "rough" sera are serially diluted in 0.9% saline. Each serial dilution is tested as if it were an individual serum by the VDRL slide test. The highest serum dilution to produce a Reactive (not Weakly Reactive) Result is reported as the titer.

b. **Reagents and Equipment.** All items listed for the qualitative VDRL slide test in paragraph 6-5b are needed, plus the following:

(1) Saline, 0.9%.

(2) Test tubes, 12 × 75 mm.

c. **Preliminary Steps:**

(1) Prepare sera and the antigen emulsion, test the delivery needle, and pretest the antigen emulsion according to instructions in paragraph 6-5c.

(2) Prepare a twofold serial dilution of all Reactive, Weakly Reactive, and "rough" patients' sera as follows:

(a) Place five test tubes (12 × 75 mm) in a rack.

(b) Put 0.5 ml of 0.9 percent saline in each tube.

(c) Pipet 0.5 ml of inactivated serum to the first tube. NOTE: For greatest accuracy, this pipet may be discarded and a new one used to mix and transfer the saline-serum mixture to each succeeding tube.

(d) Mix by aspiration and transfer 0.5 ml of saline-serum mixture to the second tube. Repeat this through the fifth tube. Leave the pipet in the fifth tube in case more dilutions are needed. The resulting dilution is twofold starting with 1:2 in the first tube. This dilution scheme is shown graphically in table 6-2.

d. **Procedure.** Test each dilution like an individual serum by the VDRL slide test described in paragraph 6-5d. Continue the serial dilution if all five dilutions should be reactive.

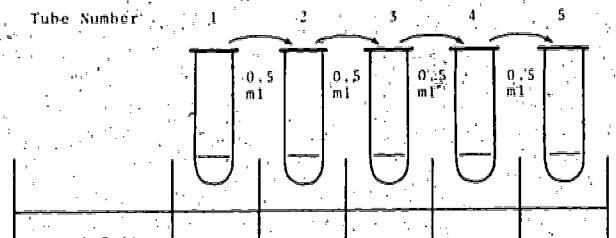
e. **Reporting Results.** The highest dilution of serum producing a Reactive (not Weakly Reactive) result is reported as the titer. Examples of quantitative VDRL test results are shown in table 6-3.

6-7. Unheated Serum Reagin (USR) Test:

a. **Principle.** Unheated serum (0.05 ml) is placed on a 14 mm paraffin- or ceramic-ringed slide. A drop (1/45 ml) of a cardiolipin antigen containing choline chloride to inacti-

Table 6-2. Quantitative VDRL Serum Dilutions.

Table 6-2. Quantitative VDRL Serum Dilutions.



Normal Saline, ml	0.5	0.5	0.5	0.5	0.5
		0.5 milliliters of:			
Serum, ml	0.5	1:2	1:4	1:8	1:16
Final Serum Dilution	1:2	1:4	1:8	1:16	1:32

Table 6-3. Example, Reports of Quantitative VDRL Serum Dilutions.

Table 6-3. Example, Reports of Quantitative VDRL Serum Dilutions.

Specimen	Reaction with serum diluted								Report
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
Serum A	R	R	R	R	R	R	WR	N	Reactive, titer 1:64
Serum B	R	R	R	WR	N	N	N	N	Reactive, titer 1:8
Serum C	WR	N	N	N	N	N	N	N	Reactive, titer undiluted only.
Serum D	WR	R	R	R	R	R	R	N	Reactive, titer 1:128

vate serum complement and EDTA to preserve the antigen emulsion is added to each serum without mixing. The slide is rotated on a slide rotator circumscribing a 3/4-inch circle for 4 minutes at 180 rpm. After rotation, the mixture is examined microscopically at 100× magnification for flocculation of the antigen particles. Flocculation indicates the presence of reaginic antibodies in the serum.

b. **Reagents and Equipment.** All items listed for the qualitative VDRL slide test in paragraph 6-5b are needed, except that the needle must deliver 1/45 ml/drop and the water bath is not needed.

(1) Centrifuge, angle-head with tachometer, capable of achieving an rcf of 2000 g.

(2) Tubes, stainless steel, for centrifuge.

(3) Phosphate (0.02 M) Merthiolate (0.2%) solution. Dissolve 1.42 g Na_2HPO_4, 1.36 g KH_2PO_4, and 1.00 g Merthiolate in distilled water in a 500-ml volumetric flask. When dissolved, q.s. to 500 ml with water, the pH should be 6.9. Store in the dark at room temperature. Stable up to 3 months.

(4) Choline chloride solution, 40%. Dissolve the entire contents of a 250 g bottle of choline chloride in 625 ml of distilled water. Filter and store at room temperature. Stable 1 year. Refilter if needed. NOTE: Use the entire bottle due to the hygroscopic nature of choline chloride.

(5) EDTA, 0.1 M. Q.s. 3.72 g EDTA (disodium salt) to 100 ml with distilled water. Stable 1 year.

(6) USR antigen emulsion (see paragraph c(3) below).

c. **Preliminary Steps:**

(1) Preparation of Serum. Clear, unheated serum at room temperature is needed.

(2) Preparation of USR Antigen Resuspension Solution. Prepare this solution each time antigen emulsion is to be prepared. Be sure the reagents are not outdated. Prepare a volume equal to the volume of antigen to be prepared. To prepare 10 ml of resuspending solution, combine the following:

EDTA (0.1 M)	1.25 ml
Choline chloride (40%)	2.5 ml
Phosphate (0.02 M), Merthiolate (0.2%)	5.0 ml
Distilled water	1.25 ml

(3) Preparation of USR Antigen Emulsion:

(a) Prepare a large batch of VDRL slide test antigen according to instructions in paragraph 6-5c(2). For example, 100 ml of antigen emulsion may be prepared by dropping 10 ml of antigen into 8 ml of buffered saline in a 250 ml glass-stoppered bottle. After an additional 10 sec of rotation, add 82 ml of buffered saline, stopper the bottle and shake according to instructions for the VDRL antigen.

(b) Centrifuge measured amounts of the VDRL antigen emulsion at an rcf of 2000 g for 15 minutes (time only when the desired speed is reached). See paragraph 1-7 for calculation of the desired rpm.

(c) Decant the supernate OPPOSITE the side containing the sediment. Wipe excess fluid from the inside of the tube with gauze without disturbing the sediment.

(d) Using freshly prepared resuspension solution, resuspend the sediment in a volume equal to that of the VDRL antigen originally centrifuged.

(e) Pool all tubes of antigen in a tightly stoppered bottle and gently shake the bottle to produce an even suspension. NOTE: The antigen is now ready for pretesting with

known control and for future use. The antigen is usually stable for at least 6 months if kept at 3°C–10°C. Remove only an aliquot for each test run, and include control sera with each run. The antigen aliquot must be at room temperature for testing.

(4) Testing of Delivery Needle:

(a) Gently mix the antigen emulsion and fill a 1-ml syringe fitted with an 18-gage needle with the bevel removed.

(b) Holding the needle vertically, determine the number of drops/milliliter. This test requires 45 drops ± 1 drop/milliliter.

(c) Adjust the bore of the needle until 45 drops/ml are delivered.

(5) Pretesting of USR Antigen Emulsion:

(a) Test known control sera (Reactive, Weakly Reactive, and Nonreactive) using the procedure in paragraph d below. All sera and the antigen must be at room temperature for testing.

(d) Discard antigen emulsions that do not produce the expected results.

d. **Procedure:**

(1) Pipet 0.05 ml of clear, unheated serum at room temperature to a 14 mm paraffin or ceramic ring on a slide. A calibrated capillary or Dispenstir may be used.

(2) Add one drop (1/45 ml) of USR antigen emulsion at room temperature to each serum with a pretested 18-gage needle.

(3) Rotate the slide for 4 minutes at 180 rpm. Use a humidifying cover in a dry climate.

(4) Read the tests at 100× magnification (10× ocular and 10× objectives) for flocculation of the antigen particles. See figure 6–1 for artist's conceptions of the reactions.

e. **Reporting Results.** Results are reported in the same manner as for the qualitative VDRL slide test given in paragraph 6–5e and table 6–1.

f. **Sources of Error.** Sources of error are the same as for the qualitative VDRL slide test with the following additions:

(1) Keep the USR antigen emulsion in the refrigerator at all times. Remove only as much as is needed for a given batch of tests. Allow this aliquot (as well as refrigerated sera) to warm to room temperature for about 30 minutes before testing.

(2) Check the antigen during each test run with known controls. Discard antigen lots when they fail to produce the expected results.

(3) The serum is not heated for the USR test. The choline chloride in the antigen emulsion inactivates the complement.

6–8. Fluorescent Treponemal Antibody-Absorption (FTA-ABS) TEST:

a. **Principle:** First, the patient's serum is absorbed with an extract of Reiter treponemes to adsorb antibodies that are common to both saprophytic and pathogenic treponemes. The adsorbed serum is then added to the Nichols strain of *Treponema pallidum* that has been dried on the slide and fixed with acetone. After incubation at 37°C, excess serum is rinsed from the slide with phosphate buffered saline (PBS). Then, fluorescein-labeled anti-human globulin (conjugate) is added to the *T. pallidum* smear, and the slides are incubated again at 37°C. At the end of this incubation period, excess conjugate is rinsed from the slide with PBS. The slides are coverslipped with a glycerine mounting medium and examined by fluorescent microscopy using the appropriate exciter and barrier filters. The presence of treponemal antibodies is indicated by a greenish fluorescence of the treponemes. The observations are compared with the fluorescence due to known control sera.

b. **Reagents and Equipment:**

(1) The following items are available from several commercial sources and are usually purchased rather than prepared in the laboratory. Store and reconstitute these according to the manufacturer's instructions.

(a) *Treponema pallidum* (Nichols Strain) (FTA-ABS) antigen, lyophilized.

(b) Fluorescein-labeled antihuman globulin (conjugate).

(c) Sorbent, FTA-ABS.

(d) Reactive (4+) control serum.

(e) Minimally reactive (1+) control serum (prepared from 4+).

(f) FTA nonspecific serum control.

(2) Patient's serum, inactivated.

(3) Phosphate buffered saline (PBS), pH 7.2 ± 0.1.

NaCl	7.650 g
Na_2HPO_4	0.724 g
KH_2PO_4	0.210 g
Distilled water	q.s. to 1 liter

Check the pH of the PBS frequently and discard lots that vary by more than ± 0.1 from pH 7.2. NOTE: The mixed chemicals for PBS of the proper pH are also available commercially in powder form.

(4) Tween 80 (Atlas Chemical Industries, Inc.).

(5) PBS containing 2 percent Tween 80. Heat PBS and Tween 80°C to 56°C. Pipet 2 ml of Tween 80 to 98 ml of PBS with rinsing. Store in the refrigerator. Discard if a precipitate forms or the pH changes.

(6) Mounting medium, 1 part PBS plus 9 parts glycerine.

(7) Acetone, reagent grade, dry.

(8) Microscope slides, frosted end, about 1 mm thick.

(9) Cover-slips, No. 1, 22 × 40 mm.

(10) Staining dish, with removable slide carrier.

(11) Pipets, serological, 0.2 ml.

(12) Pipets, capillary.

(13) Test tubes, 12 × 75 mm.

(14) Incubator, bacteriological, at 35°C–37°C.

(15) Fluorescent microscope with darkfield condenser and UV light source.

(16) Moist chamber. A plastic, 25-capacity, slide box with a tight cover may be used. Set the box on end with wet gauze at the bottom end to keep the reactants on the slide uppermost.

(17) Water bath, 56°C.

(18) Diamond-point pencil.

(19) Bibulous paper.

(20) Loop, bacteriological, 2-mm from 26-gage platinum.

(21) Immersion oil, low-fluorescence.

c. **Preliminary Steps:**

(1) Preparation of Sera. Control sera should be reconstituted and stored according to the manufactuer's instructions. All sera, including control sera, must be heated at 56°C for 30 minutes before testing. Sera that have been heated previously should be reheated for 10 minutes at 56°C. Do NOT heat the conjugate.

(2) Preparation of Minimally Reactive (1+) Control Serum. Starting with 1:2, prepare a twofold serial dilution of the Reactive (4+) serum control in PBS. Test each dilution using the procedure in paragraph d below to detect the greatest dilution that still produces minimal, but readily detectable, fluorescence. This dilution is used as a reading standard for the test.

(3) Reconstitution of Fluorescein-labeled Anti-human Globulin (Conjugate). The lyophilized conjugate is initially reconstituted (usually with water) according to the manufacturer's instructions. The reconstituted conjugate may be dispensed in small (never than 0.3 ml) aliquots and frozen at −20°C or lower. Before use, the conjugate is diluted with PBS containing 2 percent Tween 80 to the "working dilution" indicated by the manufacturer. NOTE: This "working dilution" should be checked by the using laboratory if any results are in doubt. This is done by testing a twofold serial dilution of conjugate that includes the "working dilution" indicated by the manufacturer against a Reactive (4+) control serum. The "working dilution" is defined as one tube (one twofold dilution) less than the greatest conjugate dilution to give a 4+ reaction. For example, if a 1:200 dilution of conjugate is the greatest dilution to give a 4+ reaction, a "working dilution" of 1:100 should be used.

(4) Preparation of Antigen Smears:

(a) Make two circles (1 cm inside diameter) on a clean slide with the diamond-point pencil. Circles should be separated as far as possible, but close enough together so that both may be covered completely by the 22 × 40 mm cover glass. Remove loose glass particles.

(b) Resuspend FTA-ABS antigen according to the manufacturer's instructions. Mix by aspiration with a capillary pipet at least 10 times. Check an aliquot by darkfield microscopy for even dispersion of the treponemes. Remix, if needed.

(c) Using the bacteriological loop, spread a loop of antigen in each circle on the slide.

(d) Air dry the slides at least 15 minutes.

(e) Fix smears in acetone for 10 minutes. Do not fix more than 60 slides/200 ml of acetone.

(f) Air dry the slides thoroughly.

(g) Store the acetone-fixed smears at −20°C or lower. These smears should not be thawed and refrozen, but they may be thawed and used as long as the expected reactions with control sera are produced.

(h) Allow frozen smears to warm to room temperature before testing.

d. **Procedure:**

(1) Set up each control and patient's serum (previously heated) in properly labeled test tubes according to the scheme provided in table 6–4. Add each control or patient's serum to 0.2 ml of the appropriate diluent (PBS or sorbent) using the bottom 0.05 ml of a 0.2 ml serological pipet. Mix the serum-diluent mixture by aspiration at least eight times and leave the pipet in the mixture. NOTE: The acceptable reaction for each control is also indicated in table 6–3. ALL controls must give these acceptable reactions before the results on patients' sera can be reported.

(2) Label antigen smears on the frosted end with a lead pencil. Preferably, each control and patient's serum should be run in duplicate (that is, use one slide for each fluid to be tested), if economically feasible.

(3) Carefully cover an entire antigen

Table 6-4. FTA-ABS Control Schema and Acceptable Reactions.

Table 6-4. FTA-ABS Control Schema and Acceptable Reactions.

	Serum	Diluent		Acceptable Reaction
		PBS	Sorbent	
Reactive Serum Controls				
1:5 in PBS	0.05 ml	0.2 ml	----	R(4+)
1:5 in Sorbent	0.05 ml	----	0.2 ml	R(4+ or 3+)
Minimally Reactive Reading Standard	0.05 ml	----	0.2 ml	R(1+)
Nonspecific Serum Controls				
1:5 in PBS	0.05 ml	0.2 ml	----	R(2+ to 4+)
1:5 in Sorbent	0.05 ml	----	0.2 ml	N
Nonspecific Staining Controls				
Due to PBS	----	0.2 ml	----	N
Due to Sorbent	----	----	0.2 ml	N
Patient's Serum	0.05 ml	----	0.2 ml	*

* Reported only if ALL controls are acceptable.

smear circle with approximately 0.03 ml of each dilution or control listed in table 6-3. The fluid may be spread by carefully rotating the tip of the pipet WITHOUT touching the antigen smear. Do not scratch the smear.

(4) Place the slide in a moist chamber with the reactants uppermost.

(5) Incubate the slides (in the moist chamber) at 35°C-37°C for 30 minutes.

(6) Following incubation, rinse the reactants from the slide with running PBS.

(7) Place all slides in slide carriers and soak in two changes of PBS for a total of 10 minutes (5 minutes each). Agitate the slides by dipping about 10 times during each change of PBS.

(8) Rinse the slides briefly in distilled water.

(9) GENTLY blot the slides dry with bibulous paper. Do not scratch the smears.

(10) Prepare the "working dilution" of conjugate with PBS containing 2 percent Tween 80. Spread 0.03 ml of "working dilution" of conjugate over each antigen circle.

(11) Replace the slides in the moist chamber and incubate them at 35°C-37°C for 30 minutes.

(12) After incubation, repeat the rinsing and GENTLE blotting as in steps 6-9 above.

(13) Add a SMALL drop of the PBS-glycerine mounting medium to each antigen circle and cover slip the slides.

(14) Assemble and align the fluorescent microscope and UV light source according to the manufacturer's instructions. Start the UV light and allow a warmup period as indicated by the manufacturer.

(15) Examine the slides by fluorescent microscopy using a darkfield condenser and 450× magnification (high-dry objective). Slides may be stored in the dark for about 4 hours before reading.

(a) Exciter Filters. Either a BG 12 or AO 702 filter may be used.

(b) Barrier Filters. Several types of barrier filters have been used. Some of these are OG 1, AO 724, AO 1124, B and L Y-8, and Zeiss 50/- (II/0).

Table 6-5. Reporting Results of FTA-ABS Tests.

Table 6-5. Reporting Results of FTA-ABS Tests.

Reading	Report
2+ to 4+	Reactive (R)
1+	Reactive (R)*
< 1+	Borderline (B)*
-	Nonreactive (N)

* Repeat all 1+ and < 1+ readings before reporting the results. A second serum should be tested on all Borderline results.

(16) Examine all Nonreactive smears by normal darkfield microscopy to confirm the presence of treponemes. Each smear should have about 30 treponemes/high-dry field.

(17) Compare all readings with the Minimally Reactive (1+) serum control.

e. **Reporting Results.** Before any results can be reported, all controls listed in table 6-3 must give acceptable reactions. If all are acceptable, report the results according to table 6-5 with the following exceptions:

(1) All 1+ and Borderline reactions should be retested before reporting results.

(2) A second serum should be requested on all cases identified as Borderline. Additional testing is probably not indicated if this second is also Borderline. Each patient's historical and physical findings will have to be carefully analyzed in these cases.

f. **Sources of Error:**

(1) The interpretation of fluorescence of the treponemes is subjective and only experience with the microscope will build confidence. Acceptable reactions on ALL controls must be achieved for valid results.

(2) Scratches in the antigen smear may produce confusing fluorescence.

(3) The "working dilution" of the conjugate indicated by the manufacturer should be checked to compensate for different equipment and procedures.

(4) Bacterial contamination of sera and conjugate may destroy the antibodies.

(5) Conjugate should not be frozen and thawed more than once. Freeze small aliquots and thaw only as much as required for a test run.

(6) Antigen smears should not be thawed and refrozen.

(7) Check all nonreactive smears by normal darkfield microscopy to verify the presence of treponemes.

g. **Discussion:**

(1) False-positive reactions have been reported in cases of lupus erythematosus (L.E.) in this test. Many of these demonstrate a peculiar "beaded" fluorescence of the treponemes. Other false positives may be detected as the test is studied more.

(2) Other treponemal diseases (pinta, bejel, and yaws) should be expected to give reactive results.

(3) FTA-ABS antibodies usually appear in the serum during the primary stage of syphilis and persist for life.

(4) Even after treatment the test usually remains reactive for life while most other serologic tests for syphilis become nonreactive, especially if treatment occurs before the tertiary stage of the disease.

(5) The FTA-ABS test is especially useful in the diagnosis of tertiary syphilis because the reagin tests may be nonreactive in about one-fourth of these cases.

SECTION C—TESTS ON SPINAL FLUID

6-9. VDRL Slide Test on Spinal Fluid:

a. **Principle.** VDRL slide test antigen is made more sensitive by adding an equal volume of 10-percent saline. A drop (1/100 ml) of this sensitized antigen is added 0.05 ml of unheated spinal fluid in a 16 mm diameter by 1.75 mm deep concavity of an agglutination slide. The slide is rotated for 8 minutes at 180 rpm. After rotation, the mixture is examined microscopically at 100× magnification for flocculation of the antigen particles. Flocculation indicates the presence of reaginic antibodies in the spinal fluid.

b. **Reagents and Equipment:**

(1) Patient's spinal fluid, unheated.

(2) Reactive, minimally Reactive and Nonreactive control sera, diluted at least 1:80 before testing.

(3) VDRL (Cardiolipin Microflocculation) slide test antigen.

(4) VDRL buffered saline, pH 6.0 ± 0.1.

(5) Saline, 0.9%.

(6) Saline, 10%. Q.s. 10 g of NaCl to 100 ml with distilled water.

(7) Pipets, serological, 0.2 ml.

(8) Slide, agglutination, with 16 mm diameter by 1.75 mm deep concavities.

(9) Bottle, 30 ml, round, glass-stoppered, with flat innerbottom, the same as used for VDRL tests on serum.

(10) Slide rotator, set at 180 rpm.

(11) Syringe, 1 ml.

(12) Needle, hypodermic, 22-gage with bevel removed, pretested to deliver 1/100 ml/drop.

c. **Preliminary Steps:**

(1) Preparation of Spinal Fluid. Usually the third tube of spinal fluid collected is reserved for serologic tests. The fluid should be free of hemolysis and cells. Centrifuge the specimen and test the clear supernate.

(2) Preparation of Sensitized VDRL Antigen Emulsion:

(a) Prepare a batch of VDRL slide test antigen emulsion according to instructions in paragraph 6–5b (2).

(b) Within 2 hours of testing time, mix 1 part of freshly prepared VDRL antigen emulsion with 1 part of 10-percent saline. Mix well and let stand at least 5 minutes, but use the sensitized antigen in less than 2 hours.

(3) Testing of Delivery Needle:

(a) Gently mix the sensitized antigen emulsion and fill a 1-ml syringe fitted with a 22-gage needle with the bevel removed.

(b) Holding the needle vertically, determine the number of drops/milliliter. This test requires 100 drops ± 2 drops/milliliter.

(c) Adjust the bore of the needle until 100 drops/ml are delivered.

(4) Preparation of Control Sera. Controls for this test are prepared from unheated serum, NOT spinal fluid. The Reactive serum used as a control must have a titer of 1:80 or greater when tested by the VDRL test for spinal fluid. Test the Reactive serum after it has been frozen for several days.

(3) Starting at 1:80, prepare a twofold serial dilution of unheated Reactive serum in 0.9-percent saline.

(b) Test each dilution by the VDRL test for spinal fluid and record dilutions that give Reactive, minimally Reactive, and Nonreactive results. Use these three dilutions as controls each time the test is performed.

d. **Procedure:**

(1) Allow all reagents to warm to room temperature.

(2) Pipet 0.05 ml of clear, unheated spinal fluid to a concavity in the agglutination slide.

(3) Pipet 0.05 ml of each diluted control serum to the slide.

(4) Add one drop (1/100 ml) of sensitized VDRL antigen emulsion to each control or spinal fluid with a pretested 22-gage needle.

(5) Rotate the slide for 8 minutes at 180 rpm. Use a humidifying cover in a dry climate.

(6) Read the tests at 100× magnification (10 × ocular and 10 × objective) for flocculation of the antigen particles.

e. **Reporting Results:** Before any results can be reported, the control sera must produce the expected reactions. If the controls indicate valid results, report the patient's spinal fluid as either Reactive or Nonreactive, as appropriate. NOTE: The Weakly Reactive report is not used for this test. Any reactivity is significant.

f. **Sources of Error:**

(1) Be sure that the VDRL antigen emulsion is sensitized by adding equal parts of 10-percent saline.

(2) The antigen drop (1/100 ml/drop) must be accurate within ± 2 drops/milliliter.

(3) Reactive control sera must have a titer of at least 1:80 when tested by the spinal fluid test.

(4) Spinal fluids containing gross blood or microbial contamination are unsatisfactory.

(5) All reagents must be at room temperature for testing.

g. **Discussion:**

(1) False positives rarely occur in tests for syphilis performed on spinal fluid. A Reactive test usually indicates present or past neurosyphilis, especially if the cell count and protein content of the spinal fluid are elevated.

(2) The spinal fluid must be Nonreactive in latent syphilis. A Reactive spinal fluid indicates that the disease has progressed to the tertiary stage.

(3) The spinal fluid is Reactive in about one fourth of cases in the secondary stage of syphilis, thus indicating that central nervous system involvement has begun.

6–10. Colloidal Gold Test on Spinal Fluid. The colloidal gold test on spinal fluid was widely used in the past for the differentiation of the various types of neurosyphilis and other central nervous system conditions. In the test, a colloidal suspension of gold is added to serially diluted spinal fluid. Abnormal spinal fluid proteins cause partial to complete precipitation of the gold with a variety of color changes. More recently, this test was mainly used in the study of multiple sclerosis, but the test is being rapidly replaced by the simpler, and more reproducible, gum mastic and benzoin tests.

SECTION D—OTHER TESTS FOR SYPHILIS

6–11. Reiter Protein Complement Fixation (RPCF) Test. This test is the Kolmer complement fixation method using an extract of the Reiter strain of treponeme as the antigen. In the test, complement is "fixed" during the reaction of the Reiter protein and treponemal antibodies in the syphilitic patient's

serum. Complement is, therefore, no longer available when the second antigen-antibody system, sheep erythrocytes and hemolysin, is added—hemolysis cannot occur. The complexity and relative insensitivity of the test originally led to the test being essentially replaced by the TPI test described in the next paragraph. More recently, the FTA-ABS test has replaced nearly all other treponemal tests for syphilis.

6–12. Treponema Pallidum Immobilization (TPI) Test. The TPI test detects treponemal antibodies that immobilize living Nichols strain of *T. pallidum* in the presence of complement. The requirement for live treponemes from rabbits' testes severely limits the practicality of this test for the average laboratory. Although the test is highly specific for treponemal diseases, it has been essentially replaced by the FTA-ABS test. Unlike the FTA-ABS test, the TPI test frequently becomes negative following treatment of the disease.

6–13. Automated Reagin Tests. Attempts to automate tests for syphilis have mainly involved the use of the RPR carbon-containing antigens and various AutoAnalyzer components (Technicon Instruments Corp.). Most recent applications have involved continuous filtering of the reactants on a moving strip of paper with visual interpretation of the flocculated carbon particles. Test speeds of up to 100/hr have been reported with results equivalent to manually performed tests.

Chapter 7

TESTS FOR INFECTIOUS MONONUCLEOSIS

7-1. Introduction. Infectious mononucleosis is an acute disease, characterized by fever, headache, sore throat, enlarged lymph nodes, splenomegaly, malaise, and mild hepatitis. It is believed to be caused by the Epstein-Barr virus (or some similar herpeslike virus) with transmission most likely by intimate oral contact. Young adults (16–25 years old) are most frequently affected; consequently, infectious mononucleosis is a significant problem for the military services.

a. No known treatment is available for infectious mononucleosis, but bed rest usually resolves overt illness in 2 to 4 weeks with only rare fatalities or permanent disabilities. Aside from lost time from the job, the disease is mainly important because the symptoms are shared by certain severe, but treatable, diseases. The disease has been most frequently misdiagnosed initially as diphtheria or bacterial tonsillitis. In addition, the disease must be differentiated from viral hepatitis and several other diseases. An accurate differential diagnosis of infectious mononucleosis is based primarily on laboratory studies along with the typical clinical observations.

b. One of the first laboratory findings in infectious mononucleosis concerns the white blood cells. The white blood cell count is usually over 10,000 per cmm with a differential count of over 50 percent lymphocytes. In addition, atypical lymphocytes are present. The characteristics of these lymphocytes and atypical lymphocytes can be found in the hematology manual. However, because lymphocytosis and atypical lymphocytes can also be found in other diseases, serologic tests are also necessary for the diagnosis of infectious mononucleosis.

c. The serodiagnosis of infectious mononucleosis is another good example of the use of cross-reactivity in serology. The majority of patients with the disease eventually produce antibodies (agglutinins) that agglutinate sheep and horse erythrocytes. Since these erythrocytes have no logical relationship to the causative organism of the disease, this is another example of cross-reactivity—shared antigens among different species. Unfortunately, the diagnosis of infectious mononucleosis is complicated by the fact that several microorganisms, as well as plants and animals, possess antigens that also can stimulate the production of sheep (and horse) erythrocyte agglutinins. Collectively, these antigens are called heterophile antigens and the antibodies—heterophile antibodies. The serodiagnosis of infectious monucleosis entails the detection of these heterophile antibodies along with the differentiation of the antibodies due to infectious mononucleosis from other heterophile antibodies that might be present in the serum. Since these heterophile tests are relatively simple, specific serologic tests using the virus probably never will become practical.

d. Serotests for heterophile antibodies can be divided into two categories: presumptive and differential tests.

(1) Presumptive heterophile tests. These are screening tests that utilize sheep erythrocytes (more recently, horse erythrocytes) as the antigen to detect the presence of heterophile antibodies. Several commercially available slide test kits have been developed. Many of these slide tests also include reagents to identify (differentiate) the particular heterophile antibody present.

(2) Differential heterophile tests. Most differential tests are based on the differential absorption of the various types of heterophile antibodies from the serum by suspensions of guinea pig kidneys (GPK) and beef (ox) erythrocytes (BE). One type of heterophile antibodies is adsorbed by GPK only, another by BE only, and the third by both GPK and BE. Other differential heterophile tests are based on the use of enzyme-treated sheep erythrocytes; while still another test detects ox erythrocyte hemolysins that are produced in response to infectious mononucleosis but not in other conditions that produce heterophile antibodies. One of these differential tests must usually be performed each time heterophile antibodies are detected.

7-2. Significance of Heterophile Antibodies. As with any antigenic stimulation, heterophile antigens must first gain entry into the body

before the RE system can respond by producing antibodies. For example, heterophile antigens, such as certain gram-negative bacilli of the intestinal tract, may enter through minor lesions of the intestinal wall. Or a heterophile antigen may enter the body by setting up an infection; such is the case with infectious monucleosis. In addition, heterophile antigens can be introduced by parenteral infections of horse serum. This is a rare occurrence today, but in the recent past, several diseases, such as pneumonia and diphtheria, were treated with antisera produced in horses. Regardless of the route of entry of the heterophile antigens, all stimulate the production of sheep erythrocyte agglutinins (heterophile antibodies). Fortunately, despite the multitude of heterophile antigens that man may be exposed to, heterophile antibodies can be conveniently differentiated by absorption studies and other methods into only three main types—Forssman, serum sickness, and infectious mononucleosis types. This detection and differentiation of the three types of heterophile antibodies is the responsibility of the clinical serologist.

a. **Forssman (Native) Antibodies.** The heterophile antibodies that are produced in response to incidental contact with heterophile antigens, such as certain *Salmonella*, *Shigella*, and other bacterial species as well as several other antigens of plant or animal origin, are called Forssman or native antibodies. These antibodies are found in low titers, in nearly all normal adults. The antibodies have no clinical significance, except that they must be differentiated from other types of heterophile antibodies. The differentiation is accomplished by absorbing the patient's serum (antibodies) with suspensions of guinea pig kidney (GPK) and beef (ox) erythrocytes (BE). Forssman antibodies are absorbed from the serum by the GPK, but not by the BE. In other words, after absorption with GPK, the serum no longer agglutinates sheep (or horse) erythrocytes; while serum absorbed with BE still agglutinates sheep erythrocytes. The other two types of heterophile antibodies have different absorption patterns following treatment of serum with GPK and BE.

b. **Serum Sickness Antibodies.** These sheep erythrocyte agglutinins are stimulated in man in response to the injection of horse serum or products from horse serum. This type of heterophile antibody will rarely be encountered today due to the declining use of horse serum injections. If present, they may be differentiated from other heterophile antibodies by the fact that they are absorbed from the serum by BOTH guinea pig kidney (GPK) and beef erythrocytes (BE). Of course, a history of horse serum injections is significant too.

c. **Infectious Mononucleosis Antibodies.** Heterophile antibodies due to infectious mononucleosis first appear in the serum of a low percentage of patients during the first week of illness. The precentage increases until the majority of cases have detectable titers by the fourth week, but the titers do not parallel the severity of the disease. These titers start to drop rapidly after about the fourth week and rarely persist for more than a few months. The peak titers of these antibodies are usually much higher than seen with Forssman and serum sickness, but usually the differential tests with GPK and BE still must be performed to specifically identify the source of the antibodies. Infectious mononucleosis antibodies are absorbed by BE, but not by GPK—just the opposite pattern of Forssman antibodies.

7-3. Slide Screening Test for Heterophile Antibodies:

a. **Principle.** Patient's serum is mixed with a suspension of sheep erythrocytes on a slide. Agglutination of the erythrocytes indicates the presence in the serum of unspecified heterophile antibodies.

b. **Reagents and Equipment:**

(1) Patient's serum, fresh or inactivated.

(2) Positive and negative control sera (collected from previous patients or available commercially).

(3) Sheep erythrocytes, washed, 5 percent suspension in saline. NOTE: These cells should be at least 24 hours, but less than 1 week, old. Defibrinated sheep blood for bacteriologic media may be used if the cells are of the proper age.

(4) Saline, 0.9%.

(5) Blood collecting equipment (see paragraph 3-2).

(6) Slide rotator, set at 120 rpm.

(7) Microscope slides, glass.

(8) Applicator sticks or toothpicks.

c. **Preliminary Steps:**

(1) Collect the patient's serum.

(2) Wash sheep erythrocytes with saline and prepare a 5 percent suspension of cells in saline as described in chapter 4, paragraph 4-2.

(3) Thaw aliquots of positive and negative control sera. Mix the sera gently and allow them to warm to room temperature.

(4) Label slides for positive control, negative control, and patient's sera.

d. **Procedure:**

(1) Place one drop of each serum to be tested (including controls) on appropriately labeled slides.

(2) Add one drop of a 5 percent suspension of sheep erythrocytes to each serum drop, and mix each throughly.

(3) Rotate the slide for 2 minutes at 120 rpm.

(4) IMMEDIATELY examine the mixtures for macroscopic agglutination of the erythrocytes.

e. **Reporting Results.** If the controls produce the expected results, report the patients' sera as follows:

(1) No agglutination—Sheep Erythrocyte Screening Test: Negative.

(2) Positive results (agglutination) should be confirmed by the Paul-Bunnell presumptive test (see paragraph 7-4) or one of the commercial kits. Then, if indicated, the Davidsohn differential heterophile test described in paragraph 7-5 or one of the commercially available differential tests should be performed.

f. **Sources of Error:**

(1) Drying of the mixture may be confused with agglutination.

(2) Certain lots of sheep cells may be either hypersensitive or insensitive to heterophile antibodies. Use of positive and negative control sera will detect these problems. Cell suspensions should be used only on the day they are prepared.

(3) A delay in reading the test after the rotation period may result in false positives due to delayed agglutination or drying.

(4) All reagents and sera must be used at room temperature. Cold reagents may result in delayed reactions. In addition, sera containing cold agglutinins may cause agglutination.

(5) If indicated, all negative patients should be retested at weekly intervals to detect delayed antibody production.

g. **Discussion:**

(1) This test detects only the presence of heterophile antibodies. A differential test is needed to identify the antibody present.

(2) Several slide tests using horse erythrocytes have been developed. Several concentrations of cells are used. These tests are reportedly more sensitive than sheep cells and more specific for infectious mononucleosis. Several modifications are available commercially. Some of these are described briefly in paragraph 7-6.

7-4. Paul-Bunnell Presumptive Heterophile Test:

a. **Principle.** A suspension of sheep erythrocytes is added to serially diluted serum. Agglutination of the erythrocytes indicates the presence of unspecified heterophile antibodies.

b. **Reagents and Equipment:**

(1) Patient's serum, inactivated.

(2) Positive and negative control sera (collected from previous patients or available commercially).

(3) Sheep erythrocytes, washed, 2 percent suspension in saline. NOTE: These cells should be at least 24 hours, but less than 1 week, old. Defibrinated blood may be used.

(4) Saline, 0.9%.

(5) Blood collecting equipment (see paragraph 3-2).

(6) Test tubes, 12 × 75 mm.

(7) Pipets, serological, 0.25 and 1.0 ml.

(8) Water bath, 56°C.

c. **Preliminary Steps:**

(1) Thaw aliquots of positive and negative control sera. NOTE: Only 0.1 ml of each serum is needed for the test.

(2) Inactivate the patient's serum and both control sera at 56°C for 30 minutes.

(3) Wash sheep erythrocytes with saline and prepare a 2 percent suspension of cells in saline as described in chapter 4, paragraph 4-2.

d. **Procedure:**

(1) Place 10 test tubes in racks for each serum to be tested, including the control sera.

(2) With a 1.0 ml pipet, place 0.4 ml of saline in the first tube for each serum and 0.25 ml in the remaining tubes.

(3) With a 0.25 ml pipet, place 0.1 ml of INACTIVATED serum in the first tube. (NOTE: For greater accuracy, this pipet may be discarded at this point and a new pipet used for each tube.) Mix the contents of the tube by aspiration and transfer 0.25 ml of saline-serum mixture to the second tube. Repeat this through the ninth tube from which 0.25 ml of saline-serum mixture is discarded.

(a) The tenth tube is an antigen control and receives no serum. Only one of these needs to be prepared for each batch of tests.

(b) The serial dilution prepared in this manner is a twofold dilution starting with 1:5 and ending with 1:1280 in the ninth tube. These dilutions will be increased when the antigen suspension is added.

(4) Examine the tubes. Each should contain 0.25 ml of fluid if the dilution has been performed properly.

(5) Add 0.1 ml of a 2 percent suspension

Table 7–1. Paul-Bunnell Presumptive Heterophile Test.

Table 7–1. Paul-Bunnell Presumptive Heterophile Test.

Tube Number	1	2	3	4	5	6	7	8	9	10
		0.25 ml	0.25 ml	0.25 ml	0.25 ml	0.25 ml	0.25 ml	0.25 ml	0.25 ml	Discard 0.25 ml
Saline, ml	0.4	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
		0.25 milliliters of:								
Serum, ml	0.1	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	None (Control)
Dilution After Serum Transfer:	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	
Sheep Erythrocytes, ml	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Total Volume, ml	0.35	0.35	0.35	0.35	0.35	0.85	0.35	0.35	0.35	0.35
*Final Dilution of Serum	1:7	1:14	1:28	1:56	1:112	1:224	1:448	1:896	1:1792	

of sheep erythrocyte to each tube. This makes the final serum dilution in the first tube 1:7, the second 1:14, etc.

(6) Shake the rack to mix the contents of the tubes and let them stand undisturbed at ROOM TEMPERATURE for 2 hours.

(7) At the end of 2 hours, examine the tubes for agglutination by gently shaking the tube and comparing each with the cell control (tube #10). The tubes may be examined with the aid of a hand lens or microscope mirror as shown in chapter 5, figure 5–1. Record the highest serum dilution showing definite agglutination. The Paul-Bunnelll presumptive test is shown graphically in table 7–1.

e. **Reporting Results.** If the controls produce the expected results, report the patients' sera as follows:

(1) No agglutination—Less than 1:7. This is the lowest serum dilution actually tested.

(2) In positive tests, the highest serum dilution to produce agglutination is reported as the titer. NOTE: The serum dilution INCLUDES the dilution due to the antigen. Usually positive results will have to be tested by one of the differential heterophile tests. See paragraph 7–4g for a discussion of the signficance of test titers.

f. **Sources of Error:**

(1) Certain lots of sheep cells may be hypersensitive or insensitive to heterophile antibodies. Use of positive and negative control sera will detect these problems. Cell suspensions should be used only on the day they are prepared.

(2) Unless the serum complement is inactivated, hemolysis may destroy the cells and obscure agglutination. Not all sera will contain sheep erythrocyte hemolysins, but the complement must be inactivated in all sera to avoid confusion.

(3) All sera and reagents must be used at room temperature. Cold reagents may result in delayed reactions. In addition, sera containing cold agglutinins may cause agglutination.

(4) Prozone reactions may occur with this test. For a discussion of prozone reactions see chapter 2, paragraph 2–14.

(5) If indicated, all negative patients should be retested at weekly intervals to detect delayed antibody production, because not all patients will have detectable antibody levels when first tested.

g. **Discussion:**

(1) Although titers of infectious mononucleosis antibodies are usually higher than titers of other heterophile antibodies, titers have no relation to the severity of the disease. Liver function tests are generally more reliable for following the course of the disease so that the presumptive heterophile titer is used mainly as a basis for the performance of the differential heterophile test.

(2) Titers of 1:56 or less are considered negative for infectious mononucleosis in the absence of clinical and hematologic findings.

(3) Titers of 1:224 or greater are considered to be presumptive evidence of infectious mononucleosis if clinical and hematologic signs are present. In addition, the patient must not have had recent injections of horse

serum. If these conditions are met, no further testing is required.

(4) A differential heterophile test must be performed in the following situations:

(a) A presumptive titer of LESS than 1:224, but the patient has signs of infectious mononucleosis.

(b) A presumptive titer of GREATER than 1:224, but the patient has NO signs of infectious mononucleosis.

(c) A presumptive titer of 1:224 or greater, and the patient has a history of horse serum injections.

7-5. Davidsohn and Differential Heterophile Test:

a. **Principle.** When indicated by the findings of the presumptive test (see paragraph 7-4g), separate aliquots of the patient's serum are absorbed with suspensions of guinea pig kidneys (GPK) and beef erythrocytes (BE). After centrifugation to remove the GPK and BE, the supernate from each antigen is retested by the presumptive test. A drop in titer of more than three tubes indicates absorption of the antibody by the given antigen. The absorption patterns for the three types of heterophile antibodies are summarized in table 7-2.

b. **Reagents and Equipment:**

(1) Guinea pig kidney antigen, available commercially.

(2) Beef erythrocyte antigen, available commercially.

(3) Control sera, various types are available commercially.

(4) Centrifuge.

(5) For other items, see paragraph 7-4b.

c. **Preliminary Steps:**

(1) Determine from the presumptive titer and clinical data if the differential test is indicated. Record the presumptive titer for future reference.

(2) Inactivate all sera at 56°C for 30 minutes. NOTE: Only 0.4 ml of each serum will be needed for the test.

(3) Reconstitute the GPK and BE antigens according to the manufacturer's instructions.

(4) Unless the GPK and BE manufacturer's instructions use different times and volumes, absorb the patient's serum (and each control serum) as follows:

(a) GENTLY, but thoroughly, mix the GPK and BE suspensions.

(b) Pipet 1.0 ml of GPK and 1.0 ml of BE to tubes labeled "GPK" and "BE", respectively.

(c) Add 0.2 ml of INACTIVATED serum to each tube and mix GENTLY by tapping the tube.

(b) Let the mixtures stand at ROOM TEMPERATURE for at least 3 minutes, with gentle shaking at intervals.

(e) At the end of the incubation period, centrifuge both tubes at 1500 rpm for 10 minutes.

(f) Carefully, collect at least 0.5 ml of supernate from each tube and perform the presumptive test on each supernate. NOTE: The supernate already represents a serum dilution of 1:5.

d. **Procedure:**

(1) Place two rows of 10 tubes each in racks for each serum to be tested. Label one row "GPK" and the other "BE".

(2) Starting with the SECOND tube of each row, pipet 0.25 ml of saline into each tube. The first tube receives no saline.

(3) With a 0.25 ml pipet, place 0.25 ml of GPK supernate in the first and second tubes of the row labeled "GPK". Pipet 0.25 ml of BE supernate into the first and second tubes of the row labeled "BE".

(a) The serum in the first tube needs no additional dilution, because the dilution in the supernate is already 1:5.

(b) For greater accuracy, the pipets may be discarded at this point and a new pipet used for each tube.

(4) Mix the contents of the SECOND tube by aspiration and transfer 0.25 ml of supernate-saline mixture to the third tube. Repeat this through the ninth tube from which 0.25 ml of supernate-saline mixture is discarded. The tenth tube is the antigen (sheep cell) control and receives no supernate.

(5) Repeat step #4 until all supernates are diluted.

(6) Examine the tubes. Each should contain 0.25 ml of fluid if the dilution has been performed properly.

(7) Add 0.1 ml of a 2 percent suspension of sheep erythrocytes to each tube. This makes the final serum dilution in the first

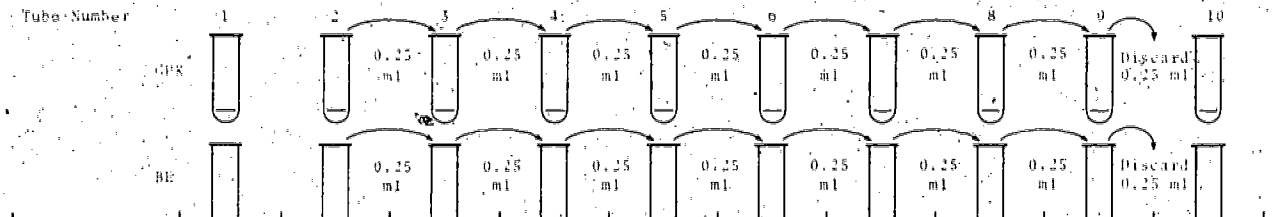
Table 7-2 Absorption Patterns of Heterophile Antibodies.

Types of heterophile antibody	Adsorbed by guinea pig kidney antigen	Adsorbed by beef erythrocyte antigen
Forssman (native)		
Serum sickness		
Infectious mononucleosis		

Table 7-2. Absorption Patterns of Heterophile Antibodies.

Table 7–3. Davidsohn Differential Heterophile Test.

Table 7–3. Davidsohn Differential Heterophile Test.



Saline, ml	None	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Supernatant Fluid (1:5 Dilution of serum)	0.25	0.25	----	----	----	----	----	----	----	----
Serum Transfer, ml	----	----	0.25 milliliters of:							
			1:10	1:20	1:40	1:80	1:160	1:320	1:640	None (Control)
Dilution After Serum Transfer	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	----
Sheep Erythrocytes, ml	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Total Volume, ml	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Final Dilution of Serum	1:7	1:14	1:28	1:56	1:112	1:224	1:448	1:896	1:1792	----

tube 1:7, the second 1:14, etc.—the same as in the presumptive test.

(8) Shake the racks to mix the contents and let them stand undisturbed at ROOM TEMPERATURE for 2 hours.

(9) At the end of 2 hours, record the titer of each supernate. The performance of the titration part of the Davidsohn differential heterophile test is shown graphically in table 7–3.

e. **Reporting Results.** Before any results can be reported, the control sera must yield the expected results. If they do, proceed as follows:

Table 7–4. Example, Results of Differential Heterophile Tests

Presumptive test titer	Differential test after absorpt. on with: guinea pig kidney	Differential test after absorpt. on with: beef red cells	Interpretation with regard to infectious mononucleosis
1:224	1:112	1	POSITIVE
1:224	1:56	0	POSITIVE
1:224	1:28	0	POSITIVE
1:224	1:14	1:112	NEGATIVE
1:224	1:7	1:112	NEGATIVE
1:56	1:56	1	POSITIVE
1:56	1:28	0	POSITIVE
1:56	1:14	0	POSITIVE
1:56	1:7	0	POSITIVE
1:28	1:28	0	POSITIVE
1:28	1:14	0	POSITIVE

Table 7–4. Example, Results of Differential Heterophile Tests.

(1) Record the presumptive heterophile titer, as well as the titers of the supernates following absorption with GPK and BE, on the patient's laboratory slip.

(2) Compare the titers of the supernates from the GPK and BE absorption with the presumptive titer. Significant absorption by the GPK and/or BE is indicated by a drop in titer of MORE than three tubes when compared with the presumptive titer. Use table 7–2 to determine the heterophile antibody present. Examples of differential test findings are provided in table 7–4.

(3) Add a statement to the patient's laboratory slip, such as "positive for infectious mononucleosis antibodies" or some appropriate comment.

f. **Sources of Error:**

(1) The sources of error listed for the presumptive heterophile test (see paragraph 7–4f) also apply to this test.

(2) The manufacturer's instructions must be followed scrupulously for the reconstitution and use of the GPK and BE antigens.

g. **Discussion:**

(1) Occasionally, GPK and BE absorption patterns will not fit the three categories listed in table 7–2.

(2) Several commercially available slide tests have been developed that incorporate presumptive and differential features into the same test. Some of these tests are based on GPK and BE absorption but offer the advantage of speed—a critical commodity in

the clinical laboratory. These tests are rapidly making the Paul-Bunnell and Davidsohn tests impractical.

(3) Due to the declining use of horse serum infections, the differential heterophile test could actually be adequately performed using the GPK antigen only. However, the use of the BE antigen does serve as a double check on the GPK results.

7-6. Miscellaneous Heterophile Tests. Several other tests for infectious mononucleosis are in use. Most of these are modifications of the basic Paul-Bunnel presumptive and the Davidsohn differential tests; a few are based on different principles. The principle of several of these tests will be described briefly and one or two examples given when commercial products are available. In all cases, be sure to adhere to the manufacturer's instructions in performing these tests.

a. **Ox Hemolysin Test.** In this test ox (beef) erythrocytes and complement are added to serially diluted serum. Patients with infectious mononucleosis produce higher titers of ox hemolysins than normal individuals. Various modifications of this test are in use, but titers of more than 1:40 to 1:48 are usually considered indicative of infectious mononucleosis. This test has been found to be quite specific and sensitive, but time-consuming.

b. **Tests with Papain-treated Sheep Erythrocytes.** The proteolytic enzyme, papain, has been found to destroy the receptor sites for infectious mononucleosis antibodies on sheep erythrocytes. Serum is tested against both papainized and untreated sheep cells. In infectious mononucleosis, the untreated cells agglutinate, but the papainized cells do not, or else their agglutination is delayed when compared to the untreated cells. Examples of commercial procedures using this principle are the Bacto-Hetrol Slide Test (Difco) and MonoStat (COLAB).

c **Modified Presumptive Tests Using Horse Erythrocytes.** Horse erythrocytes have been found to be more sensitive with fewer false positives than sheep erythrocytes for the detection of infectious mononucleosis antibodies. Both formalinized cells and cells preserved in 3.8% citrate give acceptable results. Several concentrations of cells have been tested. In addition, when properly stored, these cells are stable for up to 1 year. Examples of slide tests using formalinized horse erythrocytes are the I.M. Kit (Microbiological Research Corporation) and Mono-Test (Wampole).

d. **Modified Davidsohn Differential Tests.** A number of slide modifications of the basic GPK and BE absorption test are available commercially. Since absorption tests seem to be the most widely accepted method for the differential identification of heterophile antibodies, these slide tests offer a distinct advantage—speed—over the original tube test. In addition, these tests delete the need for a presumptive test. An example of a slide differential heterophile test that uses the traditional sheep cells as the indicator antigen is the Monosticon (Organon, Inc.) test. Another test kit from Organon, the Monosticon Dri-Dot, consists of separate spots of dried GPK and horse erythrocytes on a paper card. The serum is added to the GPK. Then, this mixture is stirred into the horse erythrocytes. Another test kit that uses horse erythrocytes, but in suspension form, along with both GPK and BE suspensions is the Monospot Slide Test (Ortho). An important consideration in using any of these tests is to be sure to follow the manufacturer's instructions at all times. Although some of these manufacturers include procedures for titrating the antibodies, titrations would not appear to be economically feasible in view of the fact that the titer does not parallel the severity of the disease. Liver function tests are considered to be better than titrations for following the course of the disease.

7-7. Interpretation of Results. In making a diagnosis of infectious mononucleosis, the physician must consider the hematologic, clinical, and serologic findings, as well as the history of the patient. Of these, the serologic findings provide the most specific evidence of disease—expecially if one of the differential heterophile tests is used. These tests are needed to identify the antibody present.

a. **Forssman (Native) Antibodies.** These are usually present in low titer in most normal adults. They are absorbed from the serum by GPK and not by BE suspensions. Forssman antibodies have no clinical significance except that they must be differentiated from other heterophile antibodies.

b. **Serum Sickness Antibodies.** These antibodies may reach relatively high titers. As the use of injections or horse serum products has declined, these antibodies have become more rare. If present, they are absorbed by both GPK and BE suspensions.

c. **Infectious Mononucleosis Antibodies.** These antibodies may reach very high titers during the disease, but not all patients produce detectable levels at the same point in the disease. A presumptive titer of 1:224 or greater with sheep erythrocytes is considered evidence of disease when hematologic, clinical, and historical findings are consistent with

infectious mononucleosis. At other times, a differential heterophile test may be required. These antibodies are absorbed by BE but not GPK suspensions. The presence of ox hemolysins and the inability of these antibodies to agglutinate papainized sheep erythrocytes can also be used to identify the disease as infectious mononucleosis.

Chapter 8

TESTS FOR PRIMARY ATYPICAL PNEUMONIA

8-1. Introduction. Primary atypical pneumonia (PAP) is a term used to describe a syndrome that is different from the "typical" lobar pneumonia caused by *Streptococcus (Diplococcus) pneumoniae*. For many years, PAP was used as a synonym for viral pneumonia, because the syndrome was considered to be caused by various viruses. In fact, some cases are due to viruses; however, since the characterization of the Eaton agent (long thought to be a virus) as a pleuropneumonia-like organism, *Mycoplasma pneumoniae*, PAP has been used primarily for Eaton agent pneumonia. A high percent of the cases of PAP were found to be caused by the Eaton agent. More specifically, the disease is called cold agglutinin-positive PAP, as well as Eaton agent pneumonia, to differentiate it from other atypical pneumonias. A correct differential diagnosis of PAP is important because the disease responds to antibiotic therapy, but viral pneumonias do not. This chapter is limited to the serodiagnosis of cold agglutinin-positive primary atypical (Eaton agent) pneumonia.

a. Cold agglutinin-positive PAP is an acute, self-limited disease. Early symptoms of chills, fever, cough, headache, and malaise are also seen in several other acute respiratory tract infections. Some of the diseases that must be considered in the differential diagnosis are influenza, adenovirus infections, Q fever, psittacosis, and others. In addition, all of these diseases may show chest x-ray changes, but these changes may be present in PAP even in the absence of overt symptoms.

b. There are several other differences between these related diseases and PAP. For example, PAP has a longer incubation period, and the course of the disease is generally longer than with the other diseases mentioned above. In addition, PAP does not occur in epidemic proportions, and there is not as much seasonal variation in the incidence of cases as seen with certain of the similar diseases. These characteristics of PAP are generally considered to be due to the fact that the disease is not highly contagious; although, under crowded conditions, such as in institutions or the military, a high percentage of the members eventually contract at least subclinical cases. In many of these cases, *Mycoplasma pneumoniae* may be cultured, and the patient may show a rise in antibody titer even in the absence of symptoms.

8-2. Types of Antibodies. The isolation and identification of *Mycoplasma pneumoniae* is a rather slow process; therefore, most cases of primary atypical (Eaton agent) pneumonia will be diagnosed serologically. There are several types of antibodies produced during the disease. Consequently, several serologic tests have been developed to detect these antibodies. Some of the tests are beyond the capability of the average clinical laboratory, but three of these newer tests will be described briefly as they may become more prevalent in the future. Tests for two other antibodies (cold and *Streptococcus* MG agglutinins) will be presented in greater detail. As with most serologic tests, detecting a rise (fourfold) in antibody titer is most significant.

a. **Immunofluorescent Antibodies.** These antibodies are detected using the indirect fluorescent antibody technique with sections of lungs from chick embryos infected with *Mycoplasma pneumoniae* serving as the antigen. This test has proven to be highly specific and sensitive for diagnosing this disease, but the limitations for the average clinical laboratory are obvious. To date, the use of *M. pneumoniae* grown in culture as the antigen for this test has been less successful than using infected chick embryos.

b. **Growth-Inhibiting Antibodies.** These antibodies may be detected by their ability to inhibit the growth of *M. pneumoniae* in culture. The organism is added to the patient's serially diluted serum in a medium containing tetrazolium. If these antibodies are present, the organism does not grow, and the tetrazolium is not reduced to a red form. The main limitations of this procedure are the need for live *M. pneumoniae* and the organism's slow growth—the test takes about 1 week to complete. Despite these limitations,

this test has proven to be highly sensitive and specific for the serodiagnosis of PAP.

c. **Indirect Hemagglutination Antibodies (IHA).** These antibodies are detected by mixing the patient's serially diluted serum with tanned sheep RBCs that have been coated with sonicated *M. pneumoniae*. Agglutination of the RBCs indicates the presence of antibodies. This test is usually considered to be too sensitive because the antibody titer rises to high levels so early in the disease that a subsequent rise in titer may be undetectable. This test has been adapted to the Microtiter system and may soon acquire a prominent role in the serodiagnosis of PAP; however, the procedure will not be included in this manual.

d. **Streptococcus MG Agglutinins.** About half or less of the patients with PAP produce bacterial agglutinating antibodies (agglutinins) against *Streptococcus* MG. Generally the presence of these agglutinins parallels the severity of the disease but their absence does not rule out the disease due to frequent false negatives. On the other hand, only rarely are false positives encountered against the *Streptococcus* MG antigen, but the true relationship of *Streptococcus* MG with *M. pneumoniae* is unknown. Both organisms may be simultaneously isolated from cases of PAP, but there appears to be no direct relationship of *Streptococcus* MG with the disease. Even though the significance of these *Streptococcus* MG agglutinins is unknown, and they are not always produced, their detection still provides a simple aid in the diagnosis of PAP. The test procedure will be covered later in this chapter.

e. **Cold Hemagglutinins.** These are antibodies that agglutinate RBCs (including the patient's RBCs) in the cold but the RBCs disperse when the mixture is heated to 37°C. The usual test temperature is about 4°C, but the temperature at which the antibodies react varies with each patient. Some patients may produce antibodies that react at temperatures as high as 35°C. This variability of reaction temperatures is especially important in specimen collection, because the patient's RBCs may absorb the antibodies from the serum before the serum is separated from the cells if the blood has cooled too much. Proper blood collection for this test will be covered later in this chapter.

(1) Unfortunately, not all patients produce detectable cold agglutinins during the course of Eaton agent pneumonia. Although cold agglutinin production seems to parallel the severity of the disease, various studies have shown that as few as 30 percent to as many as over 90 percent of patients actually produce these antibodies. In addition, several other disease conditions result in the production of cold agglutinins, but only rarely are these antibodies produced in a pneumonia other than PAP.

(2) Even with the false negatives and false positives the test for cold agglutinins still provides a simple laboratory test for PAP. The test is more valuable when performed in conjunction with another test for PAP. The most simple combination is to perform this test along with the *Streptococcus* MG agglutination test.

8-3. Screening Test for Cold Hemagglutinins (Garrow, Modified):

a. **Principle.** Blood from venipuncture or capillary puncture is added directly to an equal volume of 3.8 percent sodium citrate, and the mixture is chilled. Agglutination of the RBCs, that disappears when the mixture is rewarmed to body temperature, indicates the presence of a significant titer of cold agglutinins.

b. **Reagents and Equipment:**

(1) Blood collecting equipment (see chapter 3).

(2) 12 × 75 mm test tube with rubber stopper.

(3) Frosted ice-cube tray.

(4) 3.8% sodium citrate. NOTE: This concentration is used for many coagulation studies and is available commercially in vacuum tubes.

c. **Procedure:**

(1) Add 0.2 ml of freshly drawn blood to 0.2 ml of 3.8 percent sodium citrate in a 12 × 75 mm test tube, stopper the tube, and mix the contents.

(2) Holding only by the stopper, rub the entire test tube in the ice crystals on the frosted ice-cube tray. When the tube is covered with ice crystals, lay the tube on its side on the cold tray for 15 seconds.

(3) At the end of 15 seconds, grasp the stopper and examine the tube for agglutination of the erythrocytes.

(a) If NO agglutination is seen, report the screening test as "negative" for cold agglutinins.

(b) If agglutination is present, warm the test tube to body temperature by holding the entire tube tightly in the hand for a few seconds. If the agglutination disappears on warming, the screening test may be reported as "positive" for cold agglutinins, and the titer of these agglutinins should be determined by the method given in paragraph 8-5.

d. **Discussion:**

(1) Cold agglutinins appear in the blood about 1 week after the onset of disease. Peak titers are reached in about 2 to 3 weeks, and the titers start to fall at about the fourth week.

(2) When compared with the titration test for cold agglutinins, this screening test was found to be negative at a titer of 1:16 or less. The test was occasionally positive at 1:32, usually positive at 1:64, and always positive at titers of 1:256 or greater.

(3) A cold agglutinin titer of 1:64 (and occasionally 1:32) on a single specimen is considered to be significant. A fourfold rise in titer on a subsequent specimen would have greater significance. Even a screening test that changed from negative to positive at a later date would most likely be a significant finding.

(4) The agglutination that disappears when the tube is warmed may be reinstated by chilling the mixture again.

(5) As an alternative to chilling the tube on an ice-cube tray, the mixture could be refrigerated at 2°C to 4°C overnight, but this apparently will not increase the sensitivity of the screening test.

(6) A positive screening test should be followed by titration of the cold agglutinins in the serum. This will make it possible to identify a rise in titer in a subsequent specimen.

8-4. Collecting Blood for Cold Hemagglutinin Titration:

a. **Principle.** Although no special precautions are needed for collecting serum for most serologic tests, collecting serum for cold agglutinins is an exception. This is because the cold agglutinins can be absorbed from the serum by the patient's RBCs as the blood specimen cools. To prevent this, the blood must be maintained at 37°C until the serum can be separated.

b. **Reagents and Equipment:**

(1) Blood collecting equipment (see paragraph 3-2).

(2) Water bath, 37°C.

(3) Centrifuge.

c. **Procedure:**

(1) Prewarm the syringe and/or vacuum tube (without anticoagulant) to body temperature by holding them tightly in the hand for 2 to 3 minutes. Also prewarm the tube that will receive the blood if a syringe is to be used.

(2) Perform a venipuncture as outlined in paragraph 3-2. NOTE: This test requires 0.3 ml of serum.

(3) Keep the blood at body temperature (for example, held tightly in the hand) and put the tube in a 37°C water bath or incubator.

(4) Allow the blood to clot at 37°C for about 30 minutes, ring the clot, and centrifuge the contests IMMEDIATELY for about 3 to 5 minutes.

(5) IMMEDIATELY separate the serum, making sure that it is cell-free.

(6) Ideally the serum should be tested as soon as possible after collection, but cell-free serum may be refrigerated or frozen until it is tested.

8-5. Titration of Cold Hemagglutinins:

a. **Principle.** Serum collected and separated at body temperature is serially diluted, and a suspension of human group O erythrocytes is added to each tube. After incubation in the refrigerator at 2°C to 4°C, the tubes are examined for hemagglutination. If agglutination is present, the tubes are incubated at 37°C and reexamined. The agglutination, if caused by cold agglutinins, should disappear at 37°C. The titer is reported as the reciprocal of the highest dilution of serum causing definite hemagglutination that disappears on warming.

b. **Reagents and Equipment:**

(1) Fresh (not inactivated) serum, collected at 37°C (see paragraph 8-4).

(2) Positive control serum (collected from a previous patient and stored frozen).

(3) Saline, 0.9%.

(4) Human group O erythrocytes, 1% suspension in saline. (The patient's own cells may also be used.) Reasonably fresh blood should be used.

(5) Pipets, serological, 1 ml.

(6) Test tubes, 12 × 75 mm.

(7) Refrigerator, 2°C to 4°C.

(8) Water bath, 37°C.

c. **Preliminary Steps:**

(1) Collect serum at 37°C as described in paragraph 8-4.

(2) Wash human group O erythrocytes (or patient's erythrocytes) in saline and prepare a 1 percent suspension of cells in saline as described in chapter 4, paragraph 4-2. NOTE: If the patient's own cells are used, be sure to remove as much plasma as possible at 37°C before processing the cells. This will minimize adsorption of antibodies by the erythrocytes.

(3) Thaw and mix an aliquot of the positive control serum.

d. **Procedure:**

(1) Place ten 12 × 75 mm test tubes in a

rack. Repeat this for each serum to be tested, including the control serum.

(2) Add 0.3 ml of saline to each tube.

(3) Using a 1.0 ml pipet, add 0.3 ml of fresh (not inactivated) serum to the first tube. (NOTE: For greater accuracy, this pipet may be discarded at this point and a new pipet used for each tube.) Mix the contents of the tube by aspiration and transfer 0.3 ml of saline-serum mixture to the second tube. Repeat this through the ninth tube, from which 0.3 ml of saline-serum mixture is discarded.

(a) The tenth tube is a cell control and receives no serum.

(b) The serial dilution prepared in this manner is twofold starting with 1:2 and ending with 1:512 in the ninth tube. These dilutions will be doubled when the cell suspension is added.

(4) Examine the tubes. Each should contain 0.3 ml of fluid if the dilution has been performed properly.

(5) Add 0.3 ml of a 1 percent suspension of washed human O cells (or the patient's own cells) to each tube. This makes the final serum dilution in the first tube—1:4, the second—1:8, and so forth.

(6) Shake the rack to mix the contents of the tubes and refrigerate all tubes at 2°C to 4°C overnight. NOTE: A preliminary reading may be made after 1 hour in the refrigerator. High titered sera will usually show significant reactions in 1 hour.

(7) Examine the tubes IMMEDIATELY after removal from the refrigerator. Check the cell control (tube #10) first; this tube should have no agglutination. Hold each tube in good light, flick the bottom, and examine for agglutination. A hand lens or concave mirror may be used to aid reading. Record the highest dilution of serum that still gives visible agglutination.

(8) Incubate all tubes showing agglutination in a 37°C water bath for 30 minutes and reexamine for agglutination. All agglutination, if due to cold agglutinins, should disappear at 37°C. The cold hemagglutination procedure is shown graphically in table 8–1.

e. **Reporting Results.** If the controls produce the expected results, report the patients' sera as follows:

(1) No agglutination—Less than 1:4. This is the lowest serum actually tested.

(2) In positive tests, the highest serum dilution to produce visible agglutination that disappears on warming is the titer. NOTE: The serum dilution INCLUDES the dilution due to the antigen.

f. **Sources of Error:**

(1) Allowing the patient's blood to cool before separating the serum may result in agglutinins being adsorbed by the patient's erythrocytes.

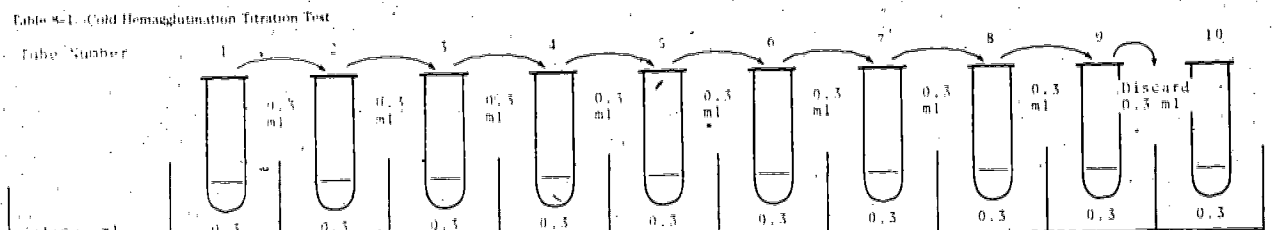
(2) The test must be read immediately after removal from the refrigerator because cold hemagglutination will disappear rapidly as the tubes warm.

(3) The presence of cold agglutinins must be confirmed by warming the tubes to 37°C. Agglutination, if due to cold agglutinins, should disappear at this temperature.

(4) A positive control serum of known titer should be included with each test series

Table 8–1. Cold Hemagglutination Titration Test.

Table 8-1. Cold Hemagglutination Titration Test



		0.3 milliliters of:								
Saline, ml	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Serum, as (not inactivated)	0.3	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	None
Dilution after serum transfer	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	----
Cell suspension, ml	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Total volume, ml	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Final dilution of serum	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	----

as a check on the suitability of the erythrocytes selected as the antigen.

g. **Discussion:**

(1) Cold agglutinins appear in the blood about 1 week after the onset of disease. Peak titers are reached in about 2 to 3 weeks, and the titers start to fall at about the fourth week. Only about 50 percent of patients with primary atypical pneumonia produce cold agglutinins, but the percentage increases with the severity and duration of the disease.

(2) Cold agglutinins are frequently produced in several other conditions, such as certain liver diseases, blackwater fever, acquired hemolytic anemias, pregnancy, trypanosomiasis and others. The antibodies in some of these diseases are present in the blood permanently; in others, they are transient. Fortunately, all of these diseases are readily differentiated from primary atypical (Eaton agent) pneumonia.

(3) Although a fourfold or greater rise in cold agglutinin titer is most significant, a titer of 1:64 (occasionally 1:32) on a single specimen in the presence of pneumonia is usually considered to be a significant finding. Of course, a negative test does not rule out the possibility of active disease due to the high percent of false-negatives.

(4) Testing at weekly intervals is most efficient for detecting rising cold agglutinin titers.

8-6. Streptococcus MG Agglutination Test:

a. **Principle.** The patient's serum is serially diluted in saline, and a suspension of the bacterium, *Streptococcus* MG, is added to each tube. After incubation at 37°C, then overnight in the refrigerator followed by reincubation at 37°C, the tubes are examined for bacterial agglutination. The titer is reported as the highest dilution of serum that produces definite agglutination.

b. **Reagents and Equipment:**

(1) Fresh (not inactivated) serum.

(2) Positive control serum (collected from a previous patient and stored frozen or commercially available *Streptococcus* MG antiserum may be used).

(3) *Streptococcus* MG suspension (available commercially), diluted according to the manufacturer's instructions.

(4) Saline, 0.9%.

(5) Test tubes, 12 × 75 mm.

(6) Pipets, serological, 1 ml.

(7) Water bath, 37°C.

(8) Refrigerator, 2°C to 4°C.

c. **Preliminary Steps:**

(1) Collect blood and separate the serum according to chapter 3. Fresh serum (0.3 ml) is needed, but no special collection techniques are required.

(2) Determine the quantity of *Streptococcus* MG suspension required and dilute stock suspension to the required volume with saline according to the manufacturer's instructions.

(3) Thaw and mix an aliquot of the positive control serum.

d. **Procedure:**

(1) Place ten 12 × 75 mm test tubes in a rack. Repeat this for each patient's serum and control serum to be tested.

(2) Add 0.3 ml of saline to each tube.

(3) Using a 1.0 ml pipet, add 0.3 ml of fresh (not inactivated) serum to the first tube. (NOTE: For greater accuracy, this pipet may be discarded at this point and a new pipet used for each tube.) Mix the contents of the tube by aspiration and transfer 0.3 ml of saline-serum mixture to the second tube. Repeat this through the ninth tube, from which 0.3 ml of saline-serum mixture is discarded.

(a) The tenth tube is an antigen control and receives no serum.

(b) The serial dilution prepared in this manner is twofold starting with 1:2 and ending with 1:512 in the ninth tube. These dilutions will be doubled when the antigen suspension is added.

(4) Examine the tubes. Each should contain 0.3 ml of fluid if the dilution has been performed properly.

(5) Add 0.3 ml of properly diluted *Streptococcus* MG suspension to each tube. This makes the final serum dilution in the first tube—1:4, the second—1:8, and so forth.

(6) Shake the rack to mix the contents of the tubes and incubate the tubes in a 37°C water bath for 2 hours.

(7) At the end of 2 hours at 37°C, incubate all tubes in the refrigerator (2°C to 4°C) overnight.

(8) The next day, reincubate the tubes at 37°C for 2 hours and read for agglutination of the bacteria by flicking the bottom of the tube and examining the contents with the NAKED eye. Record the highest serum dilution showing definite agglutination. The *Streptococcus* MG test is shown graphically in table 8-2.

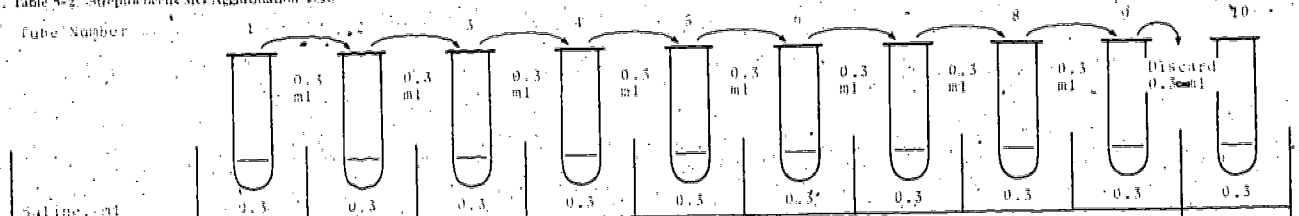
e. **Reporting Results.** If the controls produce the expected results, report the patients' sera as follows:

(1) No agglutination—Less than 1:4. This is the lowest serum dilution actually tested.

(2) In positive tests, the highest serum dilution to produce agglutination that is visi-

Table 8–2. Streptococcus MG Agglutination Test.

Table 8-2. Streptococcus MG Agglutination Test



Saline, ml	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Serum, ml (not inactivated)	0.3	0.3 milliliters of:								None
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
Dilution after serum transfer	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	----
Bacterial Antigen suspension, ml	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Total Volume, ml	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Final Dilution of Serum	1:3	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	----

ble to the naked eye is the titer. NOTE: The serum dilution INCLUDES the dilution due to the antigen.

f. **Sources of Error:**

(1) The serum must not be inactivated.

(2) The positive control serum of known titer serves as a control of the sensitivity of the antigen and should be included with each test series.

g. **Discussion:**

(1) *Streptococcus* MG agglutinins appear in the blood about 1 week after onset of disease. Peak titers are reached in 2 to 3 weeks. As with the cold agglutinins, only about 50 percent of the patients with primary atypical pneumonia produce detectable *Streptococcus* MG agglutinins, but this percentage also increases with the severity and duration of the disease. Therefore, like a negative cold agglutinin test, a negative *Streptococcus* MG agglutination test does not rule out the presence of primary atypical (Eaton agent) pneumonia.

(2) The *Streptococcus* MG agglutination test has one main advantage over the cold agglutinin test—very few false positives. Although low titers of *Streptococcus* MG agglutinins may be present in normal patients, a high titer or a fourfold or greater rise in titer usually suggests primary atypical pneumonia.

8–7. Interpretation of Results. Until the tests for immunofluorescent, indirect hemagglutination, and growth-inhibiting antibodies become more practical for the average clinical laboratory, the simultaneous performance of the tests for cold agglutinins and *Streptococcus* MG agglutinins provides the most useful information for the serodiagnosis of primary atypical (Eaton agent) pneumonia. Several possible combinations of results can be seen with these two tests.

a. For a given patient with PAP, broth tests may be positive, one or the other negative or both may be negative. Both tests are more likely to be positive if the disease is severe or of long duration.

b. Although several conditions can cause false-positive cold agglutinin tests (see paragraph 8–5g), a positive *Streptococcus* MG test usually is indicative of primary atypical pneumonia. However, if either or both tests demonstrate a fourfold or greater rise in titer in conjunction with symptoms of pneumonia, *Mycoplasma pneumoniae* is the most likely cause of the condition.

c. False-negative test results with these two tests also pose a significant interpretation problem due to the high percentage of these false negatives. Even if both tests are negative, the patient still may have PAP. In connection with these false negatives, the time of collection of the serum sample must be considered, because antibodies for both of these tests do not normally appear in the blood during the first week of the disease if they appear at all. Ideally, performing both tests on serum samples collected at weekly intervals during the disease and on into convalescence should provide the best chance for serodiagnosis of this disease.

Chapter 9

ANTISTREPTOLYSIN-O TITER

9-1. Significance of Antistreptolysin-O Antibodies. Streptolysin-O is an oxygen-labile hemolysin that is produced by most strains of group A streptococci. The hemolysin is highly antigenic, and even a simple "strep throat" may stimulate the production of antibodies that can neutralize the hemolytic effect of streptolysin-O. These neutralizing antibodies are referred to as ASO or ASTO antibodies—short for antistreptolysin-O. These same group A streptococci may also produce several other antigenic exoenzymes. Detecting ASO antibodies and antibodies against these other exoenzymes is helpful in the diagnosis of present or recent streptococcal infection, especially if cultures are not productive. Some of the suppurative streptococcal diseases, in addition to streptococcal pharyngitis or tonsillitis, that may cause the production of ASO (and other) antibodies are scarlet fever, erysipelas, impetigo, puerperal fever and others. However, in these diseases, titers generally fall to low, but persistent, levels soon after resolution of the illness, and as a rule, these diseases can be diagnosed readily by cultures. On the other hand, the ASO titer has proven especially useful for the diagnosis of two nonsuppurative diseases that are preceded by group A streptococcal infections. These diseases are rheumatic fever and acute poststreptococcal glomerulonephritis.

a. **Rheumatic Fever.** A small percentage of untreated cases of streptococcal pharyngitis with or without scarlet fever may develop into rheumatic fever. The rheumatic fever attack usually occurs several days to a month after the resolution of the original streptococcal infection. By this time, streptococci may be impossible to culture from the throat; therefore, serologic and other methods of diagnosis must be used. The disease is characterized by various combinations of the following: carditis (especially murmurs), migratory polyarthritis, chorea, and erythema marginatum. Although the disease may affect several organs of the body, the heart is the only organ to be permanently damaged; consequently, a prompt, accurate diagnosis must be made before this happens. The ASO titer generally rises to high levels in rheumatic fever, and a fall in titer suggests a favorable prognosis.

b. **Acute Poststreptococcal Glomerulonephritis.** This is another disease that may follow streptococcal pharyngitis and other streptococcal infections, but fortunately, only a few strains of group A streptococci seem to be able to cause glomerulonephritis. Like rheumatic fever, the development of glomerulonephritis follows a streptococcal infection by up to a month. The disease is immunologic in nature with complement fixation occurring in the glomeruli with an accompanying drop in serum complement levels as described in chapter 15. Proteinuria with or without hematuria along with casts may be noted on urinalysis. The disease may resolve completely after one attack, become chronic, or progress in a matter of weeks or months to complete renal destruction. Although other organisms can cause glomerulonephritis, the disease mostly frequently follows a streptococcal infection; therefore, a rising ASO titer is a useful diagnostic tool. In terminal stages of glomerulonephritis, the ASO titer may drop to very low levels.

9-2. ASO Latex Screening Test:

a. **Principle.** Patient's serum is mixed with enough streptolysin-O to neutralize 200 International Units (IU) of ASO/ml of serum. Then a drop of the serum-streptolysin-O mixture is mixed with a drop of suspension of latex particles coated with streptolysin-O. Agglutination of the latex particles indicates that the ASO titer of the patient's serum is greater than 200 IU/ml.

b. **Reagents and Equipment.** Reagents are available commercially in kit form.

(1) Patient's serum, fresh.
(2) Streptolysin-O, dehydrated.
(3) Latex particle suspension coated with streptolysin-O.
(4) Saline, 0.9%.
(5) Test tubes, 12 × 75 mm.
(6) Glass slides, black-backed.
(7) Applicator sticks.

c. **Preliminary Steps:**

(1) Reconstitute the streptolysin-O with saline according to the manufacturer's in-

structions. NOTE: After reconstitution, this reagent may be stored at 4 to 6°C for only 1 week. Do not shake this reagent violently.

(2) Mix 0.3 ml of streptolysin-O with 0.1 ml of patient's serum and incubate for 15 min at room temperature.

d. **Procedure:**

(1) After the 15-minute incubation, mix one drop (0.05 ml) of the serum-streptolysin-O mixture with one drop of ASO latex reagent on the slide. Mix thoroughly with an applicator stick and spread the mixture over an area of about 2 × 2.5 cm.

(2) Rotate the slide back and forth for 4-6 min at room temperature and examine for agglutination of the latex particles.

e. **Reporting Results:**

(1) No agglutination of the latex particles, indicating that the ASO titer of the serum is less than 200 IU/ml—Negative.

(2) Agglutination of the latex particles, indicating that the ASO titer of the serum is greater than 200 IU/ml—Positive.

f. **Sources of Error:**

(1) Streptolysin-O is oxygen-labile and does not retain its activity for long after it is reconstituted. Reconstitute and store this and other reagents according to manufacturer's instructions. Do not shake the reagent violently during reconstitution.

(2) Prozone reactions may occur with sera of unusually high ASO titers, thus causing false-negative results. Diluting these sera 1:4 with saline before testing may preclude this problem.

(3) Drying of the mixture on the slide may simulate a positive test.

g. **Discussion:**

(1) The International Unit (IU) of ASO activity is nearly identical to the Todd unit, which is described in paragraph 9–4.

(2) The ASO latex test has been found to be an effective screening test because it reduces the necessity of performing the more complicated hemolytic ASO test in about one-fourth of the cases.

(3) This test can also be performed on serially diluted serum with each dilution tested as an individual serum would be; however, the additional cost of the latex test probably makes the hemolytic ASO test more practical for titration of ASO antibodies.

9–3. Screening Test for Streptococcal Exoenzymes:

a. **Principle.** Patient's serum is mixed on a slide with treated sheep erythrocytes that have been coated with the following five streptococcal exoenzymes: streptolysin-O, streptokinase, hyaluronidase, deoxyribonuclease (DNase), and nicotinamide adenine dinucleotidase (NADase), also called diphorphopyridine nucleotidase (DPNase). Agglutination of the erythrocytes indicates the presence of antibodies in the serum against one or more of the streptococcal exoenzymes.

b. **Reagents and Equipment.** Reagents and equipment are available commercially in kit form.

(1) Patient's serum, fresh.

(2) Suspension of erythrocytes coated with exoenzymes. Store in refrigerator. Do not freeze.

(3) Positive and negative control sera.

(4) Saline, 0.9%.

(5) Capillaries, calibrated, with bulb.

(6) Test tubes, 16 × 150 mm.

(7) Pipets, serological, 0.1, 1.0 and 10 ml.

(8) Glass slide.

(9) Applicator sticks or similar stirrers.

c. **Preliminary Steps:**

(1) Allow all sera and reagents to warm to room temperature and perform a reagent check according to the manufacturer's instructions whenever new reagents are opened.

(2) Make a 1:100 dilution of serum in saline by adding 0.1 ml of serum to 9.9 ml of saline. Do NOT dilute control sera provided with the kit.

d. **Procedure:**

(1) Fill a calibrated capillary to the mark with the 1:100 serum and expel it on a slide.

(2) Resuspend the erythrocytes by shaking, add a drop of erythrocytes to the slide, and stir the two reactants together.

(3) Rotate the slide and observe for agglutination at the end of 2 min from stirring.

(4) Also test undiluted control sera by this procedure.

e. **Reporting Results.** If control sera give the expected results, report the results as follows:

(1) No agglutination within 2 min after stirring the reactants together, indicating that the streptococcal exoenzyme titer is 1:100 or less—Negative.

(2) Agglutination within 2 min, indicating that the streptococcal exoenzyme titer is 1:100 or greater—Postive.

f. **Sources of Error:**

(1) All reactants must be at room temperature before testing.

(2) Contaminated sera may produce erroneous results. Sera may be stored frozen, but they must be warmed to room temperature and mixed before testing.

(3) All slides, tubes, and other glassware must be scrupulously cleaned.

(4) Follow manufacturer's instructions at all times.

g. **Discussion:**

(1) Positive tests may be titered by preparing further dilutions of serum in saline and testing each dilution as an individual serum would be; however, the additional cost involved probably makes the hemolytic ASO test more practical for titration of ASO antibodies. There are also tests available for the titration of antibodies against the individual streptococcal exoenzymes, other than ASO, but these tests are generally impractical for the average laboratory.

(2) Whole blood may be used instead of serum if the dilution due to the erythrocytes is taken into consideration in making the 1:100 dilution.

(3) This test may be positive due to a cumulative effect of the five possible antibodies that may be reacting with the coated erythrocytes. Titers of each of the individual antibodies might be below significant levels.

(4) Although a true correlation cannot be made between the results of this test for five exoenzyme antibodies and the hemolytic ASO test, it has been found that the 1:100 dilution of serum is equivalent to a 1:166 dilution of serum (166 Todd units) in the hemolytic ASO test.

(5) This test may be repeated at weekly or biweekly intervals to detect changes in titers.

9–4. Antistreptolysin-O (ASO) Titer:

a. **Principle.** Diluted serum is incubated at 37°C with a standardized amount of streptolysin-O reagent. After incubation, a suspension of human or rabbit erythrocytes is added to each tube, and the tubes are incubated again at 37°C. The tubes are examined for hemolysis. The antistreptolysin-O (ASO) titer of the serum is the reciprocal of the highest dilution of serum to COMPLETELY inhibit hemolysis by the streptolysin-O.

b. **Reagents and Equipment:**

(1) Patient's serum, fresh or inactivated.

(2) Streptolysin-O buffer. The ingredients of the buffer are available commercially in deyhdrated and concentrated forms. The buffer may also be prepared as follows:

Sodium chloride (NaCl)	7.40 g
Potassium phosphate, monobasic (KH_2PO_4)	3.17 g
Sodium phosphate, dibasic (Na_2HPO_4)	1.81 g
Distilled water, q.s. to	1000 ml

Adjust the pH of the buffer to 6.5 to 6.7, if needed. Store the buffer in the refrigerator. Discard turbid or contaminated solutions.

(3) Streptolysin-O reagent, dehydrated. Available commercially in various sizes from several sources.

(4) ASO standard control serum. Available commercially.

(5) Red cell suspension, washed, 5% human group O or rabbit cells in streptolysin-O buffer.

(6) Saline, 0.9%.

(7) Water bath or heating block, 37°C.

(8) Pipets, serological, 1, 5, and 10 ml.

(9) Test tubes, 13 × 100 and 16 × 150 mm.

c. **Preliminary Steps:**

(1) Red Cell Suspension:

(a) Determine the quantity of 5% cell suspension needed for the test (each tube receives 0.5 ml).

(b) Using methods outlined in chapter 4, paragraph 4–2, wash human group O or rabbit's blood with saline until the supernate is free of hemolysis. Usually two or three washings should be adequate. (NOTE: Cells requiring more than five washings are too fragile and should be discarded.) At the last washing, pack the cells firmly and carefully remove the supernate.

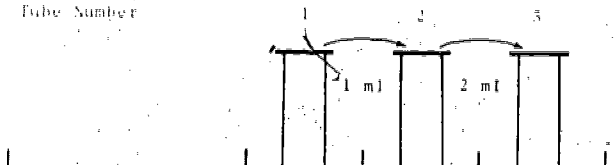
(c) Prepare the 5% cell suspension in streptolysin-O buffer.

(2) Preliminary Serum Dilutions. In 16 × 150 mm test tubes, prepare serum dilutions in streptolysin-O buffer according to table 9–1. Use a new pipet for each dilution. This dilution scheme results in dilutions of 1:10, 1:100, and 1:500.

(3) Reconstitution of ASO Control Serum. Rehydrate the control serum according to the manufacturer's instruction. Usually this control serum is rehydrated with distilled water and used as the 1:100 dilution without additional dilution. The titer is indicated by the manufacturer. This serum should be tested with each batch of tests.

Table 9–1. Preliminary Serum Dilutions for ASO Test.

Table 9-1. Preliminary Serum Dilutions for ASO Test.



ASO Buffer, ml	4.5	9.0	8.0
Patient's Serum, ml	0.5	1.0 ml of 1:10	2.0 ml of 1:100
Final Serum Dilution	1:10	1:100	1:500

(4) Reconstitution of Streptolysin-O Reagent. Reconstitute the reagent according to the manufacturer's instructions IMMEDIATELY prior to addition of the reagent to the test. If refrigerated, the reconstituted reagent is stable for about 2 hours.

d. **Procedure:**

(1) For the complete test, place 14 labeled 13 × 100 mm test tubes in a rack for the first patient's serum and 12 tubes for each additional patient's serum. Tubes #13 and #14 are the Red Cell and Streptolysin Controls, respectively.

(a) As a screening procedure only, the first seven tubes may be used, along with two tubes for controls. ASO titers that exceed the seven tubes (333 Todd units) would have to be retested using the complete test.

(b) For the ASO control serum, set up tubes #3 through #7. This serum is used as the 1:100 dilution.

(2) Accurately dispense the 1:10, 1:100, and 1:500 preliminary serum dilutions according to table 9–2.

(3) Dispense streptolysin-O buffer according to table 9–2.

(4) Gently shake the tubes to mix.

(5) At this time, prepare the Streptolysin Reagent according to paragraph 9–2c(4) and IMMEDIATELY add 0.5 ml to each tube, EXCEPT #13.

(6) Gently shake the tubes to mix.

(7) Incubate the tubes in a 37°C water bath or heating block for 15 min.

(8) Add 0.5 ml of the 5% (human group O or rabbit) red cell suspension to each tube.

(9) Gently shake the tubes to mix.

(10) Incubate the tubes at 37°C for 45 min. Shake the tubes after the first 15 min.

(11) After incubation, centrifuge the tubes to sediment the red cells.

(12) Examine the Red Cell Control (tube #13); the supernate should show NO hemolysis.

(13) Examine the Streptolysin Control (tube #14); hemolysis should be marked or complete.

(14) Examine the patient's tubes and ASO control serum tubes for hemolysis and record the highest dilution of serum showing NO hemolysis when viewed in good light. Compare this tube with the Red Cell Control (tube #13) to determine the absence of hemolysis.

e. **Reporting Results.** Before valid results can be reported, the Red Cell Control (tube #13) must show no hemolysis; the Streptolysin Control (tube #14) must show marked to complete hemolysis; and the ASO control serum must give the expected results. If the above conditions are met, report the reciprocal of the highest dilution of serum to show NO hemolysis as the ASO titer in Todd units as indicated from table 9–2. NOTE: The unit value of each tube is figured WITHOUT the dilution due to the addition of the Streptolysin Reagent and the red cell suspension.

f. **Sources of Error:**

(1) The Streptolysin Reagent must be rehydrated and used within 10 min of rehydration, or it may be refrigerated for up to 2 hours before addition to the test.

(2) Dirty glassware may cause inaccurate results. Detergents may lyse the red cells.

Table 9–2. Antistreptolysin-O Test (Complete).

Table 9-2. Antistreptolysin-O Test (Complete)

Tube Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Serum Dilution	1:10		1:100					1:500					CONTROLS	
Diluted Serum, ml	0.8	1.2	1.0	0.8	0.6	0.4	0.3	1.0	0.8	0.6	0.4	0.2	-	-
Ve Buffer, ml	1.2	0.8	0.5	0.2	0.4	0.6	0.7	0.5	0.2	0.4	0.6	0.8	1.5	1.0
SHAKE TUBES GENTLY TO MIX														
Streptolysin "Reagent", ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-	0.5
SHAKE GENTLY TO MIX, THEN INCUBATE AT 37°C FOR 15 MINUTES														
% Red Cell Suspension, ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
SHAKE GENTLY TO MIX, INCUBATE AT 37°C FOR 15 MINUTES (SHAKING AFTER 15 MINUTES), CENTRIFUGE, AND READ														
Final Serum Dilution	1:13	1:20	1:100	1:125	1:166	1:250	1:333	1:500	1:625	1:833	1:1,250	1:2,500	Red Cell Control	Strep-tolysin Control
Todd Units	12	50	100	135	166	250	333	500	625	833	1250	2500		

(3) For maximum accuracy, use a new pipet to prepare and dispense each of the preliminary serum dilutions.

(4) Bacterially contaminated sera may have elevated ASO titers.

(5) Sera must be free of hemolysis.

(6) All controls must give the expected results before valid results on patient's sera can be reported. A higher than expected titer of the control serum might suggest that insufficient (or impotent) streptolysin-O reagent or insensitive red cells were used; a lower than expected titer might suggest too much streptolysin-O reagent or hypersensitive red cells were used.

g. **Discussion:**

(1) If the screen test is used and all seven tubes are free of hemolysis, the serum will have to be retested using the complete 13-tube test to determine the actual titer.

(2) Repeating the ASO titer at biweekly intervals for a month or so following a streptococal infection is generally more informative than a single test, although a single low or elevated titer may be useful.

(3) The Todd unit for reporting ASO titers is defined as the amount of serum that neutralizes 2.5 minimum hemolytic doses of streptolysin-O. An International Unit (IU), based on a standard reference serum, has also been proposed. One IU is essentially identical to one Todd unit.

(4) In addition to the ASO titration method presented in this manual, many other modifications, all based on the same principle, are available. Some of these methods use normal serial dilutions of serum, while others use semimicro and microtitration methods.

9-5. Interpretation of Results. Although there is no firmly established normal value for the ASO test, titers of 166 or less Todd units are generally found in apparently healthy individuals who have not had a recent streptococcal infection. In addition, titers of less than 166 can usually be used to exclude rheumatic fever in which the average titer is about 500 units. However, the titer of a single specimen is less significant than a two tube or greater rise or drop in titer when compared with a previous specimen, because a few normal individuals may have a very high titer and a number of factors may affect the ASO titer at a given time. Titers may be affected by an individual's immunological competence and age as well as the number and nature of streptococcal infections.

a. ASO titers usually start to rise about a week after the onset of the streptococcal infection, but in the milder types of infections, titers fall to normal by about 2 months after resolution of the illness. Titers tend to remain higher after multiple streptococcal infections and following rheumatic fever and acute poststreptococcal glomerulonephritis although in severe cases of glomerulonephritis, the ASO titer may drop to very low levels late in the disease.

b. The age of the individual must also be considered in interpreting ASO results. At birth, the titer is similar to the mother's because of transplacentally transferred antibodies. Titers remain low for a couple of years and reach adult levels in about 10 years. Titers may be lower in old individuals due to decreased antibody production with old age.

Chapter 10

C-REACTIVE PROTEIN

10-1. Significance of C-Reactive Protein. C-reactive protein (CRP) is a globulin found in the serum and certain other body fluids during the acute stages of several inflammatory and necrotic conditions. CRP is so-called because it was originally found that the serum of patients with pneumococcal pneumonia contained a globulin (CRP) that precipitated a solution of the somatic C-polysaccharide of pneumococci. However, although CRP reacts like an antibody, it is not an antibody. Later, CRP was found to be produced in a wide variety of acute inflammatory diseases.

a. **Diseases Producing CRP.** The detection of CRP in the serum has been most widely applied in following the progress of rheumatic attacks associated with poststreptococcal rheumatic fever. In addition, the protein is usually present very soon after myocardial infarctions and in patients with wide-spread malignancies. CRP is also present in a large percentage of cases of active rheumatoid arthritis, as well as many bacterial infections. As a rule, viral infections do not stimulate the production of CRP, but hepatitis, mumps, and varicella are notable exceptions. Even immunizations, such as typhoid, and injections of penicillin may result in the production of detectable levels of CRP. In short, any inflammatory condition may stimulate the production of CRP.

(1) CRP appears in the serum within about 24 hours of the onset of the inflammatory process and generally disappears with the resolution of the acute phase of the inflammation. The appearance and persistence of CRP essentially parallel the increase in the erythrocyte sedimentation rate (ESR) during inflammation, but the CRP is a better indicator of inflammation because the ESR is affected by anemia, pregnancy, and several other factors, while the CRP is not. Unfortunately, CRP may disappear from the serum before the inflammation has actually subsided; in these cases, the ESR may be more useful.

(2) Although the actual amount of CRP produced during an inflammatory episode cannot be correlated directly with the severity of inflammation, measuring the CRP in serial serum specimens from the same patient provides useful information for prognosis and following therapy. In general, a decreasing amount of CRP with time indicates a favorable prognosis. Except in those cases where CRP may disappear before complete resolution of the inflammation, a negative CRP indicates an absence of inflammation and necrosis because CRP is not present in normal individuals.

b. **Tests for CRP.** Original tests for CRP were based on the phenomenon that led to the discovery of CRP; that is, the precipitation of pneumococcal C-polysaccharide by CRP in the serum. However, purified CRP was found to be highly antigenic in rabbits, and present tests use CRP antiserum from immunized rabbits or other animals in one of two basic methods—precipitin tests in capillary tubes or agglutination of latex particles that have been coated with CRP antiserum.

(1) Latex Tests for CRP. Latex particles coated with anti-CRP antibodies agglutinate when mixed with patient's serum containing CRP. Latex tests can be performed on serially diluted serum in test tubes. In addition, at least two manufacturers produce slide test kits that use this principle. These slide tests require only a few minutes to perform a qualitative test as opposed to 2 hours by the capillary precipitin test. In addition, these slide tests may be performed on serially diluted serum to obtain the CRP titer. However, procedures for CRP slide tests vary significantly from manufacturer to manufacturer. Therefore, to help assure that the kit manufacturer's instructions will be followed, no latex CRP procedures will be presented in this manual.

(2) Capillary Precipitin Tests for CRP. Another type of test for CRP involves the precipitation of CRP by anti-CRP antiserum. These tests are performed in capillary tubes, and except for minor variations between antiserum manufacturers' instructions, the test procedure is fairly constant. The remainder of this chapter is devoted to this test.

10-2. Capillary Precipitin Test for CRP.

a. **Principle.** Equal volumes of patient's

serum and anti-CRP antiserum are mixed in a glass capillary tube. The tube is incubated in a vertical position at 37°C. Formation of a precipitate in 2 hours is qualitative evidence of the presence of CRP in the patient's serum. Semiquantitative results may be obtained by measuring the height of packed precipitate after the tube has been allowed to stand overnight at room or refrigerator temperature.

b. **Reagents and Equipment:**

(1) Patient's serum, fresh, clear, collected after at least 4 hours of fasting.

(2) C-reactive protein antiserum (CRPA), available from several commercial sources. Store at 4°–10°C.

(3) CRP-positive control serum, available commercially.

(4) Capillary tubes, 90 to 100 mm × 0.8 mm inside diameter (I.D.), or as specified by the antiserum manufacturer, preferably dry-heat sterilized.

(5) Gauze or paper wipes.

(6) Plasticine or modeling clay.

(7) Incubator, 37°C.

(8) Refrigerator, 4°–10°C.

c. **Preliminary Steps:**

(1) Reconstitute the CRP antiserum and control serum according to manufacturer's instructions. Store reconstituted sera at 4°–10°C.

(2) Have the patient fast at least 4 hours. Collect serum following venipuncture or capillary puncture. Centrifuge the serum if particulate matter is present; clear serum must be used. Perform the test as soon as convenient, because refrigerated serum may give false positive reactions.

d. **Procedure:**

(1) Draw about 3 cm of CRP antiserum into a capillary tube and wipe the tube. See step 1 of figure 10–1.

(2) Draw an equal volume (about 3 cm) of patient's serum into the tube. NOTE: The patient's serum must be in contact with the antiserum, and both must be free of bubbles. See step 2 of figure 10–1.

(3) Mix the contents by letting the sera flow back and forth in the tube several times. See step 3 of figure 10–1.

(4) Wipe all serum and finger prints from the tube, form an air space at the bottom of the tube, and insert the tube vertically into plasticine (or modeling clay) so that the bottom meniscus is above the plasticine. See step 4 of figure 10–1.

(5) Repeat the above steps for each patient's serum and the positive control serum.

(6) For QUALITATIVE results, incubate the tubes at 37°C for 2 hours and examine the tubes in front of a light source for the presence (or absence) of a precipitate.

(7) For SEMIQUANTITATIVE results, incubate the tubes at 37°C for 2 hours and examine the tubes in front of a light source for the presence (or absence) of a precipitate. Refrigerate POSITIVE tests at 4°–10°C (or room temperature, if specified by manufacturer) overnight and measure the height (in mm) of the column of precipitated protein. NOTE: At times, the precipitate will fail to completely settle to the bottom of the tube. In these tubes, measure the height of each layer of precipitate and add the results together.

e. **Reporting Results.** If the positive control serum gives the expected results, report the results as follows:

(1) Qualitative Test:

(a) Precipitate present—Positive.

(b) No precipitate present—Negative.

(2) Semiquantitative Test:

(a) No precipitate present—Negative.

(b) Less than 1 mm—Trace.

(c) 1 mm—1+.

(d) 2 mm—2+.

(e) 3 mm—3+.

(f) 4 mm or more—4+. NOTE: As an alternative, readings greater than 4+ may be reported, such as 6 mm—6+, and so forth.

f. **Sources of Error:**

(1) Fresh serum must be used because refrigerated serum may produce false positive results.

(2) Free calcium is required for this test; therefore, plasma is unsatisfactory.

(3) The reactivity of CRP antisera may vary between manufacturers and even from lot to lot. Therefore, be sure to use a single lot of antiserum in following the progress of a given patient.

(4) Sterilized capillary tubes are recommended for this test to prevent confusion in differentiating precipitation from bacterial growth.

(5) Clear serum must be used.

10–3. Interpretation of Results. The presence of C-reactive protein in the serum indicates unspecified acute inflammatory or necrotic activity. In general, the CRP is positive in the same conditions that cause an elevated erythrocyte sedimentation rate (ESR), but the CRP usually appears before the ESR becomes abnormal. In addition, the CRP may become undetectable before the ESR returns to normal. In fact, the CRP may revert to

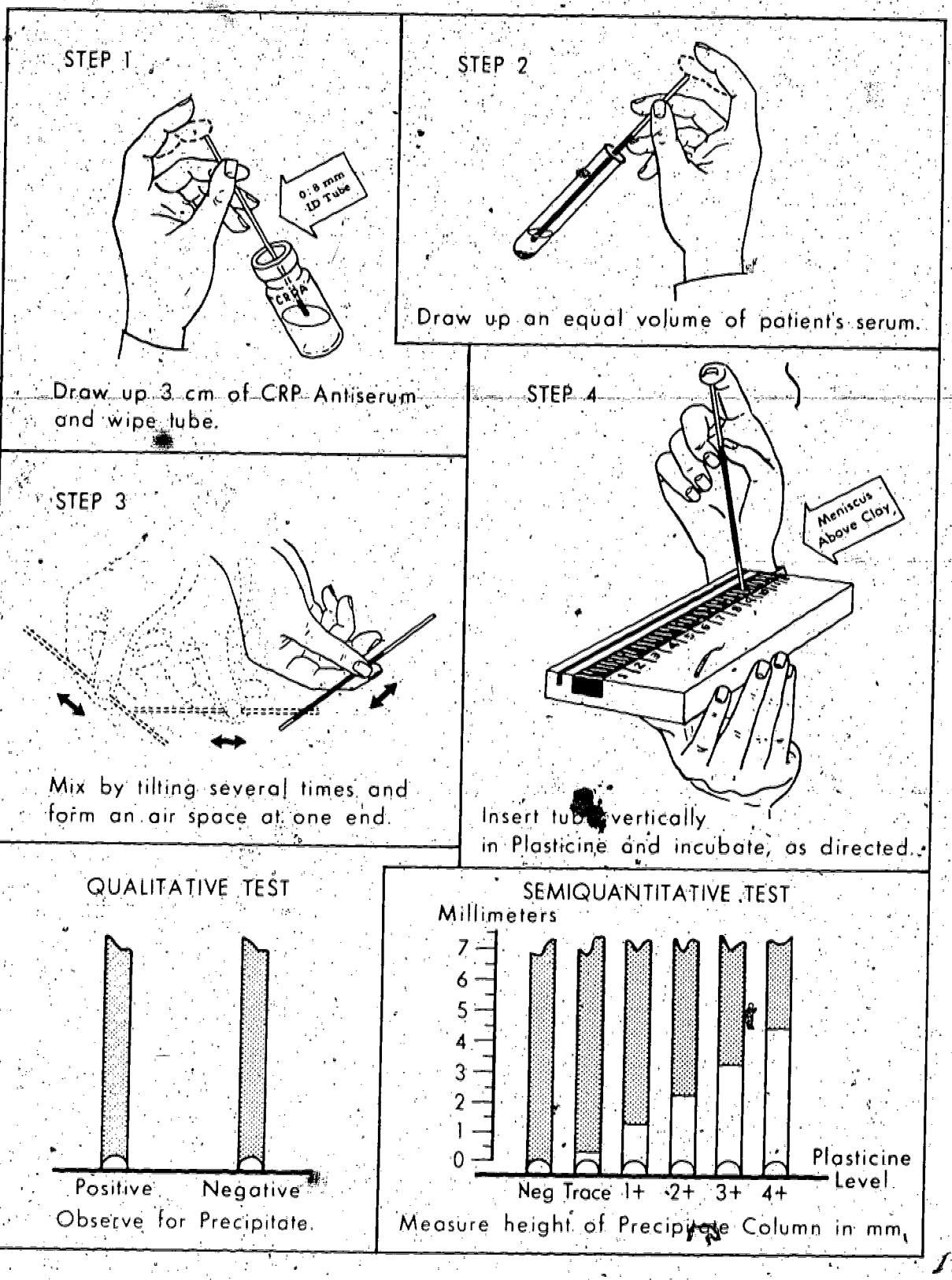


Figure 10-1. C-Reactive Protein Precipitin Test.

negative before the inflammatory process has completely resolved. Therefore, caution must be used in interpreting the results from patients with a negative CRP and an elevated ESR.

a. Semiquantitation of CRP, either by serially diluting the serum for latex tests or measuring the height of the column of the precipitate in the capillary precipitin tests, does not necessarily parallel the severity of a given inflammatory process. However, semiquantitation has proven useful in following the course of the attack and the effectiveness of therapy. For example, a progressively decreasing CRP level suggests a favorable prognosis. Occasionally, after a successful course of cortisone therapy, CRP may reappear in the serum when the cortisone injections are stopped, but the CRP tests revert to negative in a few days.

b. CRP is nearly always present soon after the onset of acute rheumatic attacks of rheumatic fever and following myocardial infarctions. Acute rheumatoid arthritis and malignancies are other inflammatory diseases that usually produce CRP. In addition, most bacterial infections will stimulate the production of CRP, but viral infections rarely will. Hepatitis, mumps, and varicella are examples of viral diseases that do regularly produce CRP. At times, even immunizations and injections of antibiotics will stimulate the production of detectable amounts of CRP. The important consideration in interpreting results of CRP tests is the multitude of conditions that can cause a positive test.

Chapter 11

TESTS FOR RHEUMATOID ARTHRITIS

11-1. Significance of Rheumatoid Factors:

a. **The Disease.** Rheumatoid arthritis is a chronic inflammatory disease of unknown etiology affecting the joints and synovial membranes. Early symptoms of joint pain and muscular stiffness may occur at any age with females more frequently affected than males. These early symptoms are not unique to rheumatoid arthritis so several other diseases must be ruled out in the differential diagnosis. Later in the disease, subcutaneous nodules are frequently noted over bony prominences. The disease may ultimately progress to severe skeletal deformity and complete immobilization of the affected joints.

b. **Rheumatoid Factors.** Depending on the test method used, about 80 percent of classical cases of rheumatoid arthritis have anti-gamma-globulin antibodies in their serum. These antibodies are mainly against IgG and react with the gamma globulin from several species of animals, as well as from the patient. Because these antibodies are most frequently associated with rheumatoid arthritis, they are called rheumatoid factors or, simply, RF.

(1) Rheumatoid factors were originally thought to be a single macroglobulin related to IgM immunoglobulin, but they are now known to be, in fact, IgM immunoglobulin along with small quantities of IgG and IgA. The presence of rheumatoid factors in IgM, IgG, and IgA is further evidence that these factors are antibodies. In effect, they are antibodies against antibodies.

(2) Unfortunately, the mere presence of rheumatoid factors in the serum does not confirm a diagnosis of rheumatoid arthritis, because a varying percentage of several other diseases may produce detectable levels of so-called rheumatoid factors. In addition, a negative test for rheumatoid factors does not rule out the possibility of rheumatoid arthritis, because about 20 percent of actual cases have negative tests. The presence or absence of rheumatoid factors also varies with the age of the patient, with children less apt to produce the factors, while older individuals are more frequently positive—even in the absence of rheumatoid arthritis. Despite these variations in production, serotests for the detection of rheumatoid factors in the serum are useful in the diagnosis of rheumatoid arthritis. Detecting increases in the titer of these factors is especially significant.

c. **Tests for Rheumatoid Factors.** Several types of tests have been developed for the detection of rheumatoid factors in the serum. Some of these tests, such as those involving radioimmunoassay, are very complex and, therefore, will be impractical for the average clinical laboratory. Most of the practical tests are based on the passive agglutination of either erythrocytes or inert particles coated with gamma globulin from animals or humans. The erythrocytes most frequently used are either fresh or preserved sheep cells, while polystyrene latex particles are the most commonly used inert particles. In general, the latex tests are more sensitive (more false positives with other diseases) than the erythrocyte tests, but a positive erythrocyte test is more likely to indicate rheumatoid arthritis. Examples of both types of tests are included in this manual. Most of these tests are available commercially in kit form.

11-2. Latex Slide Screening Test:

a. **Principle.** A 1:20 dilution of patient's serum in glycine-saline buffer diluent is mixed on a slide with a suspension of globulin-coated polystyrene latex particles. Agglutination (macroscopic clumping) of the latex particles indicates the presence of rheumatoid factors in the patient's serum.

b. **Reagents and Equipment.** Available in kit form from several sources. NOTE: Be sure to follow the manufacturer's instructions.

(1) Patient's serum, fresh or frozen.
(2) Glycine-saline buffer diluent.
(3) Latex-globulin reagent.
(4) Control sera, positive and negative.
(5) Test tubes, 12 × 75 mm.
(6) Pipets, 1 ml serological and transfer.
(7) Glass slides.
(8) Applicator sticks or toothpicks.

c. **Preliminary Steps.** In a test tube, prepare

an approximate 1:20 dilution of the patient's serum by adding 1 drop of serum to 1 ml of glycine-saline buffer diluent and mixing gently. Do NOT dilute the control sera. Frozen sera should be at room temperature before testing.

d. **Procedure:**

(1) Place 1 drop of diluted patient's serum on a section of the slide.

(2) Place 1 drop each of UNDILUTED positive and negative control sera on separate sections of the slide.

(3) Gently resuspend the latex-globulin reagent and add 1 drop to each serum.

(4) Using a separate applicator stick for each serum, mix each serum thoroughly with the latex-globulin reagent and spread each mixture over an area about the size of a quarter.

(5) Tilt the slide from side to side for 1 minute and observe for macroscopic clumping (agglutination) of the latex particles.

e. **Reporting Results:** If the positive and negative control sera give the expected results, report the results of the patients' sera as follows:

(1) No visible clumping—Negative.

(2) Partial clumping—Weakly Reactive or Weekly Positive.

(3) Complete clumping—Reactive or Positive.

f. **Sources of Error:**

(1) Store reagents and perform procedures according to manufacturers' instructions. Do not allow the latex-globulin reagent to freeze.

(2) All glassware must be scrupulously clean, because tests for rheumatoid factors are very sensitive to pH and ionic changes. Spontaneous agglutination can occur if the pH falls to the isoelectric point of the gamma globulin (about 6.6); therefore, a pH of 8.2 must be maintained.

(3) Failure to dilute the patient's serum before testing may result in false-negative results due to the prozone phenomenon. Some kit manufacturers recommend heat-inactivated serum (56°C for 30 min) to reduce the incidence of prozoning.

(4) Do not read the test after the time period specified by the manufacturer because drying of the reactants may resemble agglutination.

11-3. Eosin-Latex Slide Screening Test:

a. **Principle.** Patient's serum (or whole blood) is mixed with a drop of eosin solution on a slide. Then, a drop of a suspension of uncoated latex particles is mixed with the serum-eosin mixture. Spontaneous agglutination of the latex particles indicates the presence of rheumatoid factors in the serum. The addition of the eosin solution before the latex particles reduces the incidence of false-positive tests.

b. **Reagents and Equipment.** Available commercially in kit form.

(1) Patient's serum, fresh or frozen.

(2) Positive control serum.

(3) Eosin reagent.

(4) Latex suspension.

(5) Glass slides.

(6) Toothpicks or similar stirrers.

(7) Light source.

c. **Preliminary Steps:**

(1) When a new batch of reagents is first opened, check them for proper storage by mixing 1 drop of eosin reagent with 2 drops of latex reagent on a slide. Rock the slide over a light for 3 minutes. A smooth red suspension indicates satisfactory reagents.

(2) Collect the patient's serum. Freeze the serum if it cannot be tested within a reasonable time. If whole blood is to be used, it must be used WITHOUT anticoagulant.

d. **Procedure:**

(1) Place 1 drop of UNDILUTED patient's serum (or 3 drops of whole blood WITHOUT anticoagulants) on a slide.

(2) Place 1 drop of positive control serum in another section of the slide.

(3) Add 1 drop of eosin reagent to each serum and mix each with a separate stirrer.

(4) Gently resuspend the latex reagent and add 2 drops to each serum-eosin mixture. Mix well and spread each mixture over an area about 1 inch square.

(5) Rock the slide gently for 3 minutes and examine over a light for agglutination of the latex particles.

e. **Reporting Results.** If the positive control serum gives the expected results (agglutination), report the results of the patients' sera as follows:

(1) No agglutination—Negative.

(2) Agglutination—Positive.

f. **Sources of Error.** In general, the same sources of error listed in paragraph 11-2f also apply to this test, with the following additions and exceptions:

(1) UNDILUTED serum must be used in this test. Whole blood without anticoagulants may also be used.

(2) The eosin reagent must be added to the serum before the latex suspension is added. False-positive results frequently occur if this order is reversed.

11-4. Latex Macroscopic Tube Test:

a. **Principle.** A suspension of globulin-coated latex particles is added to serially diluted serum in test tubes. After incubation at times and temperatures specified by the kit manufacturers, the reciprocal of the highest dilution of serum to cause agglutination of the latex particles is reported as the titer of rheumatoid factors in the serum.

b. **Reagents and Equipment.** Available commercially from several sources.

(1) Patient's serum, fresh.
(2) Latex-globulin reagent, as specified.
(3) Buffer diluent, as specified.
(4) Test tubes, 12 × 75 mm.
(5) Pipets, serological, 1 ml.
(6) Test tube rack.
(7) Incubator, 37° or 56°C, as specified.
(8) Refrigerator, 2°-10°C, if needed.
(9) Centrifuge, if needed.

c. **Preliminary Steps:**

(1) Collect the patient's serum. Freeze the serum if it cannot be tested within a reasonable time. Frozen serum should be at room temperature for testing.

(2) If required, prepare the buffer diluent as specified by the manufacturer.

(3) If the latex particles require sensitization with globulin, prepare this reagent. NOTE: Some manufacturers suggest that the sensitization procedure be started on the day before the test is to be performed.

d. **Procedure:**

(1) Place 10 labeled test tubes in a rack.

(2) Pipet 1.9 ml of buffer diluent into the first tube and 1 ml into the remaining nine tubes.

(3) Pipet 0.1 ml of serum into the first tube, mix the contents, and transfer 1 ml of serum-buffer mixture to the second tube. NOTE: For greatest accuracy, this pipet may be discarded and a new one used for each succeeding tube.

(4) Mix the contents of the second tube and transfer 1 ml to the third tube, repeating this procedure through the ninth tube, from which 1 ml is discarded. The tenth tube is the control and receive no serum.

(5) Add 1 ml (or 1 drop, if specified) of latex-globulin reagent to each tube and shake the rack to mix the contents.

(6) Incubate the tubes as specified by the latex-globulin manufacturer.

(7) If specified, centrifuge the tubes for 3 minutes at 2,300 rpm.

(8) Gently agitate the tubes while observing for macroscopic agglutination of the latex particles. Record the highest dilution to show agglutination. This procedure is outlined in table 11-1.

e. **Reporting Results.** Report the reciprocal of the highest serum dilution to show agglutination as the titer. The titer does NOT include the dilution of the serum caused by adding the latex-globulin reagent.

f. **Sources of Error.** The sources of error listed in paragraph 11-2f apply to this test, but special attention must be paid to the specific manufacturer's instructions due to the many minor (but significant) variations available.

11-5. Sensitized Sheep Cell Slide Test:

a. **Principle.** Patient's serum is mixed with stabilized sheep erythrocytes that have been coated (sensitized) with gamma globulin. Agglutination of the erythrocytes indicates the presence of rheumatoid factors in the patient's serum.

Table 11-1. Latex Macroscopic Tube Test for Rheumatoid Factors.

Table 11-1. Latex Macroscopic Tube Test for Rheumatoid Factors.

Tube Number	1	2	3	4	5	6	7	8	9	10
	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	Discard 1 ml	
Buffer Diluent, ml	1.9	1	1	1	1	1	1	1	1	1
	1 milliliter of:									
Serum, ml	0.1	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	None (Control)
Latex Globulin, ml or drop, as indicated	1	1	1	1	1	1	1	1	1	1
SHAKE TO MIX; INCUBATE, AS INDICATED BY MANUFACTURER; CENTRIFUGE; AND READ FOR AGGLUTINATION.										
Final Serum Dilution	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Control

b. **Reagents and Equipment.** Available commercially in kit form.

(1) Patient's serum, fresh or frozen.
(2) Positive and negative control sera.
(3) Sensitized sheep erythrocyte reagent.
(4) Saline, 0.9%.
(5) Glass or paper slides.
(6) Capillary tubes, calibrated, with bulb.
(7) Stirrers.

c. **Preliminary Steps:**

(1) Collect the patient's serum. Freeze the serum if it cannot be tested within a reasonable time. Allow frozen sera to warm to room temperature and mix thoroughly before testing.

(2) Allow all reagents and control sera to warm to room temperature before testing.

(3) When a new lot of sensitized sheep erythrocyte reagent is opened, test the reagent with the positive and negative control sera according to the procedure below as a check on the suitability and proper storage of the reagent.

d. **Procedure:**

(1) With the calibrated capillary, transfer a measured amount of patient's serum (or control serum, during the reagent check) to a slide.

(2) Resuspend the sensitized sheep erythrocyte reagent and add 1 drop to each serum.

(3) Using a clean stirrer for each serum, mix each serum with the reagent and spread the mixture.

(4) If paper cards are used, rock the card for 30 seconds and let it stand undisturbed and examine for agglutination at the end of 2 minutes. With glass slides, rock the slide for 2 minutes and examine for agglutination.

(5) If no agglutination with the patient's serum occurs, the test is reported as—Negative.

(6) If agglutination occurs, prepare a 1:10 dilution of serum in 0.9% saline (1 drop of serum and 9 drops of saline) and repeat the test.

e. **Reporting Results.** If the control sera give the expected results, report the results as follows:

(1) No agglutination with undiluted serum—Negative.

(2) Agglutination with undiluted serum only—Positive, Undiluted Only. NOTE: This result may also be due to heterophile antibodies as well as low-titered rheumatoid factors, such as may be seen with a number of diseases other than rheumatoid arthritis.

(3) Agglutination with both undiluted and diluted serum—Positive.

f. **Sources of Error.** The sources of error listed in paragraph 11-2f also apply to this test, with the following additions and exceptions:

(1) Do not freeze the erythrocyte reagent because hemolysis may occur.

(2) Be sure to bring all reagents and sera to room temperature before testing.

(3) The possibility of the presence of heterophile antibodies must be considered in all positive tests.

(4) Undiluted, unheated serum at room temperature must be used for this test.

11-6. **Interpretation of Results.** These tests for rheumatoid factors should not be considered to be specific for the diagnosis of rheumatoid arthritis. In fact, only about 80 percent of the cases of classical rheumatoid arthritis produce detectable levels of rheumatoid factors, with the actual percentage varying from test to test. In addition, significant percentages of several other diseases produce these factors. However, these tests are still useful when their limitations are considered in interpreting results.

a. **Sensitivity and Specificity of Tests.** In general, the latex tests are more sensitive detectors of rheumatoid factors than the tests using erythrocytes. By contrast, the erythrocyte tests have fewer false-positive results so that positive erythrocyte tests more likely indicate rheumatoid arthritis than a positive latex test does.

(1) False-Negative Tests. About 20 percent of apparently classical cases of rheumatoid arthritis fail to produce detectable levels of rheumatoid factors when tested by the usual tests. Although the exact cause of false negatives is unknown, it has been found that these patients have a predominance of IgG antiglobulins rather than IgM as seen in positive patients. It has been suggested that these false negatives really are a different disease entity.

(2) False-Positive Tests. Most of the diseases that cause false-positive tests for rheumatoid factors are chronic diseases. As a rule, only a certain percentage of these cases will be positive, and usually only weakly positive. Some of these diseases are subacute bacterial endocarditis (SABE), syphilis, Hansen's disease, sarcoidosis, tuberculosis, cancer, and liver diseases, such as cirrhosis and viral hepatitis. In addition, most types of leukemia cause false positives, with some types about 100-percent positive. The false positives found in lupus erythematosus (LE) are interesting, because a significant number of cases of rheumatoid arthritis, in turn,

cause false-positive serotests for LE. In general, false-positive tests for rheumatoid factors might be expected in any disease that results in a hypergammaglobulinemia.

b. **Interpretation of Titration Tests.** The minimum significant titer of rheumatoid factors varies from kit manufacturer to manufacturer, with some suggesting 1:20 (or 1:40) or greater on unheated serum as significant and 1:160 or greater on heated serum. Therefore, the manufacturer's instructions must be consulted in interpreting these tests. However, regardless of the test method used, the height of the titer of rheumatoid factors cannot be correlated directly with the severity of the disease, but a rise in titer helps confirm a diagnosis of rheumatoid arthritis. Titers generally persist for the life of the patient. Those diseases that cause false-positive screening tests for rheumatoid factors usually have low titers in titration tests. Frequently, high titers are found in apparently normal individuals who have an increased tendency for developing rheumatoid arthritis in the future. The use of titration tests may also be useful in identifying patients with hyperviscosity of the blood due to hypergammaglobulinemia.

Chapter 12

TESTS FOR LUPUS ERYTHEMATOSUS

12-1. Significance of Antinuclear Antibodies:

a. **The Disease.** Systemic lupus erythematosus (SLE) is a connective-tissue (collagen) disease affecting primarily young women. The cause of the disease is unknown, but patients appear to have a genetic predisposition to the disease. Viral involvement has also been postulated. Early symptoms of weight loss, anemia, and arthritis are shared by several other diseases, such as rheumatoid arthritis and others. The course of SLE may vary from a mild, chronic condition to a rapidly fatal course with involvement of the kidneys, liver, lungs and other organs.

b. **Antinuclear Antibodies.** Patients with SLE (and other collagen diseases) produce a variety of antibodies that appear to be directed against antigens from the patient's own body. Antibodies against the patient's erythrocytes, leukocytes, thrombocytes, and other body cells have been detected. Some of these antibodies are directed against the nuclei of the patient's cells as well as nucleated cells from other species. Although the role of these antibodies in the disease is unknown, three of these antinuclear antibodies or factors are important in the tests for SLE.

(1) Antinucleoprotein Antibodies. These antibodies are produced against the DNA-histone complex of nuclei in a variety of diseases, including SLE. They react with the patient's nuclei as well as normal human nuclei and nuclei from other species of animals. Antinucleoprotein antibodies take part in the so-called LE-cell phenomenon, in which phagocytosis of altered nuclear material occurs. Tests for the demonstration of LE cells are covered in the hematology manual. In addition to the LE-cell phenomenon, these antibodies are also important in the serotests to be covered in this manual.

(2) Anti-DNA Antibodies. These antibodies do not appear to have a role in the LE-cell phenomenon, but their presence in the serum is usually associated only with the SLE, and especially the acute phases of the disease. These anti-DNA antibodies react with either free DNA or with DNA in the DNA-histone complexes of nuclei. They are important in the fluorescent antinuclear antibody test.

(3) Antinucleolar Antibodies. These antibodies appear to be more frequently produced in scleroderma than in SLE. As their name implies, these antibodies react with the nucleoli of nuclei.

c. **Tests for SLE.** The classical test for SLE is the demonstration of LE cells. In addition, two types of serologic tests have proven practical in the diagnosis of SLE. These serotests are important because only about 75 percent of SLE patients ever produce the typical LE cells, and the identification of these LE cells requires highly trained personnel. One type of serotest is based on the agglutination of nucleoprotein-coated latex particles and another on the detection of antinuclear antibodies by the indirect fluorescent antibody technique. In addition, complement fixation and several other serotests have been used in this disease.

(1) Latex Test for SLE. The most simple (but the least sensitive) test for SLE is the latex agglutination test using latex particles coated with nucleoprotein. Although a positive latex test is usually indicative of SLE, unfortunately, the test is only positive in about one-third of the cases of SLE. Despite these limitations, the speed and simplicity of this test have made it useful.

(2) Fluorescent Antinuclear Antibody (ANA) Test. Nearly 100 percent of cases of SLE and lower percentages of several other diseases produce one or more of the antinuclear antibodies. These antibodies are detectable by reacting the patient's serum with nucleated cells and detecting the antigen-antibody reaction following the addition of fluorescein-labeled, antihuman globulin. Although equipment for fluorescent microscopy is required for this test, this is the best screening test available for SLE. The titer of antinuclear antibodies may also be determined by this method.

12-2. Latex Slide Test for Antinucleoprotein Antibodies:

a. **Principle.** Patient's serum is mixed on a slide with a suspension of nucleoprotein-

coated latex particles. Agglutination of the latex particles indicates the presence of antinucleoprotein antibodies in the serum.

b. **Reagents and Equipment.** Available commercially in kit form:

(1) Patient's serum, fresh or frozen.

(2) Latex-nucleoprotein reagent, with dropper.

(3) Positive and negative control sera.

(4) Capillary tubes, provided with kit.

(5) Glass slides.

(6) Toothpicks or similar stirrers.

c. **Preliminary Steps.** Collect the patient's serum. Freeze the serum if the test cannot be performed in a reasonable length of time. Allow the serum to warm to room temperature and mix thoroughly before testing.

d. **Procedure:**

(1) With the capillary tubing provided, place 1 drop of patient's serum (and each control serum) on a section of the slide.

(2) Gently, but thoroughly, resuspend the latex reagent and add 1 drop to each serum.

(3) Thoroughly mix the serum with the latex and spread the mixture over an area about 20 × 40 mm.

(4) Tilt the slide for 2 minutes and examine for agglutination against a dark background with reflected light.

e. **Reporting Results.** If the control sera give the expected results, report the results of the patient's sera as follows:

(1) No agglutination—Negative.

(2) Any agglutination—Positive.

f. **Sources of Error:**

(1) The quantities of serum and latex reagent are critical so be sure to use only the capillaries and droppers provided with the kit.

(2) Drying of the latex reagent on the slide may resemble agglutination.

(3) The serum must be thoroughly mixed with the latex reagent for valid results.

g. **Discussion:**

(1) False-positive results with this test are rare, but an occasional patient with rheumatoid arthritis may be positive. In addition, other collagen diseases might be expected to be positive.

(2) False-negative results occur in about two-thirds of the cases of SLE. These cases have to be diagnosed by other criteria, such as the LE-cell test or the fluorescent antinuclear antibody test.

12-3. Fluorescent Antinuclear Antibody (ANA) Test:

a. **Principle.** Patient's serum is incubated on a slide or coverslip containing cultured, nucleated cells of human or animal origin. After incubation, the excess serum is washed off and fluorescein-labeled, antihuman globulin (conjugate) is incubated on the cells. After this incubation, the excess conjugate is washed from the cells and the nuclei are examined on a fluorescent microscope. The presence of antinuclear antibodies in the patient's serum is indicated by fluorescence of the nuclei, with the specific type of antibody present producing a characteristic type of fluorescence.

b. **Reagents and Equipment.** Available commercially in kit form.

(1) Patient's serum, fresh or frozen.

(2) Tissue culture cells, human or animal, on slides or coverslips.

(3) Fluorescein-labeled, antihuman globulin (conjugate), diluted according to manufacturer's instructions.

(4) Phosphate-buffered saline (PBS), pH 7.4, 10 × concentrate or powdered forms are available.

(5) Glycerol.

(6) Mounting medium, 1 part of 1× PBS and 9 parts of glycerol.

(7) Positive and negative control sera.

(8) Glass slides.

(9) Test tubes, 12 × 75 mm.

(10) Pipets, pasteur-type.

(11) Diamond-point pencil.

(12) Absorbent tissue.

(13) Moist chamber, such as a plastic slide box (tightly covered) or a petri dish with wet filter paper or gauze.

(14) Incubator, 37°C.

(15) Fluorescent microscope with UV light source, appropriate filters, and a brightfield (or darkfield) condenser.

(16) Immersion oil, low fluorescence.

c. **Preliminary Steps:**

(1) If required, reconstitute the conjugate according to the manufacturer's instructions. This stock conjugate may be dispensed in small aliquots in tightly capped containers and stored frozen at −20°C or lower until needed. Immediately prior to use, prepare the working dilution of the conjugate as specified.

(2) Make a 1:10 dilution of patient's serum in 1× PBS (1 drop of serum to 9 drops of 1× PBS). Some kit manufacturers recommend a 1:20 dilution of serum.

(3) Dilute the control sera according to manufacturer's instructions.

(4) Remove the required number of tissue culture slides or coverslips from the preservative and gently, but thoroughly, wash off the preservative with cold running water

followed by 4 rinses with 1× PBS. Store the slides in 1× PBS until ready to use. If the slides or coverslips require cutting to smaller sizes, wipe them dry on the underside only, score them with a diamond-point pencil in the desired size, and gently break along the scored lines. Do NOT allow the cell surface to dry during these operations. Replace the slides in 1× PBS until ready to use.

d. **Procedure:**

(1) Remove tissue culture slides from the PBS, wipe the undersides, and place the slides (cells uppermost) on corks or rubber stoppers in the moist chamber.

(2) Cover the entire surface of each slide with the appropriate 1:10 dilution of patient's serum and appropriately diluted control sera.

(3) Replace the lid on the moist chamber and incubate at 37°C for 30 minutes, tilting the slides every 5–10 minutes.

(4) After incubation, rinse the slides 4 times with 1× PBS, wipe the undersides, and return them to the moist chamber.

(5) Cover the entire surface with the working dilution of conjugate.

(6) Replace the lid on the moist chamber and reincubate at 37°C for 30 minutes, tilting the slides every 5–10 minutes.

(7) After incubation, rinse the slides 4 times with 1× PBS, shake off excess PBS, and wipe the underside dry. The slides may also be rinsed a couple of times with water.

(8) Mount the slide or coverslip with a small amount of mounting medium (PBS-glycerol) on the cell surface.

(9) Examine the cell nuclei for fluorescence in the thinner areas of the cell sheet with a fluorescent microscope at 100 to 400× using a brightfield or darkfield condenser. Some suggested exciter and barrier filters are as follows:

(a) Exciter Filters. Either a BG 12 or AO 702 filter may be used.

(b) Barrier Filters. Several types of barrier filters may be used. Some of these are OG 1, AO 724, AO 1124, B and L Y-8, and Zeiss 50/–(II/0).

(10) Record the type of nuclear fluorescence, if present, for each patient according to the following scheme:

(a) Peripheral (Ring). The periphery of the nucleus fluoresces more brightly than the rest of the nucleus.

(b) Homogeneous (Solid). The nucleus fluoresces uniformly throughout.

(c) Speckled. Several small spots of intranuclear fluorescence.

(d) Nucleolar. Only nucleoli (one to a few/nucleus) fluoresee.

e. **Reporting Results.** If positive and negative control sera give the expected results, report the results of the patient's serum as follows:

(1) No nuclear fluorescence—Negative.

(2) Nuclear fluorescence observed—Positive, along with type of fluorescence. Also preferably with the titer.

f. **Sources of Error:**

(1) Incomplete washing of the cells following incubation with the patient's serum or conjugate may result in excessive nonspecific fluorescence. High titers of rheumatoid factors also cause nonspecific fluorescence. A final wash with water before mounting and reading the slides may help alleviate some of this problem. In addition, examination of thinner areas of the cell layer will be easier to interpret. Increasing the working dilution of the conjugate may become necessary.

(2) Failure to dilute the patient's serum may result in false-positive results due to antinuclear antibodies produced in other connective tissue diseases, such as rheumatoid arthritis and scleroderma.

(3) Allowing the cells to dry during the procedure may cause nonspecific fluorescence. The incubation stages must be performed in a moist atmosphere to prevent drying of the serum or conjugate on the slide.

(4) The preservative must be completely washed from the tissue culture slides before testing.

(5) Incomplete washing of the slides following incubation with the patient's serum may also produce false-negative results by interfering with the reaction of the conjugate with the nuclear-coated globulins.

(6) Prolonged exposure to light (especially UV light) will rapidly diminish the fluorescence of the nuclei. Therefore, if the cells cannot be examined immediately after the test is completed, storage of the slides in the dark at 2°C to 8°C for no longer than 2 days will usually still yield valid results. Be sure to carefully check the controls in these instances.

(7) The fluorescein-labeled antihuman globulin (conjugate) loses potency on repeat freezing and thawing. Dispensing and freezing the stock conjugate in batch-sized aliquots is recommended. The working dilution of conjugate should not be frozen, but it may be stored at 2°C to 8°C for a few weeks. Potency of the working dilution is lost rapidly—more rapidly, if frozen.

(8) Positive control sera have been found

to decrease in titer, even if frozen. Prolonged storage of serum should be discouraged.

g. **Discussion.** Ideally, all positive tests should be titered, because a high or rising titer is most likely due to SLE. Titers are accomplished by testing serial-twofold dilutions of the patient's serum in 1× PBS, starting with 1:20 through about six tubes. For best results, identical measured quantities of each dilution should be tested.

12-4. Interpretation of Results:

a. **Latex Slide Test.** Positive results with this test usually indicate the presence of antinucleoprotein antibodies produced in SLE. Other diseases rarely cause positive results with this test although other connective-tissue disorders, such as rheumatoid arthritis, might be positive. Unfortunately, the simplicity of this test is overshadowed by the fact that only about one-third of the cases of SLE will cause this test to be positive. This high specificity for SLE, but low sensitivity, must be considered when interpreting test results.

b. **Fluorescent Antinuclear Antibody (ANA) Test.** The ANA test is the method of choice as a screening test for SLE, because nearly 100 percent of the cases that are not on prolonged steroid therapy will have a positive test. A rising ANA titer also helps to pinpoint SLE as the most probable cause of the rise, but low titers may be found in several other conditions. Performance of the test on diluted serum helps to rule out some of these false positives. Additional valuable information on the cause of a positive ANA test can be obtained by careful observation of the type of nuclear fluorescence present.

(1) Peripheral Fluorescence. This type of fluorescence is due primarily to anti-DNA antibodies. Not all cases of SLE produce anti-DNA antibodies, but if present, they are most likely due to SLE.

(2) Homogeneous Fluorescence. This type of fluorescence is due to antinucleoprotein antibodies. This fluorescence can mask other types of reactions, but if present, it usually indicates SLE or severe cases of rheumatoid arthritis.

(3) Speckled Fluorescence. This type of fluorescence is due to reactions with nuclear products that are not DNA or nucleoprotein. The antigens may be RNA-protein complexes in the nuclei. SLE may give this type of fluorescence, but it is most frequently seen in conditions, such as rheumatoid arthritis, chronic liver disease, systemic sclerosis (scleroderma), subacute bacterial endocarditis, and, potentially, any collagen disease.

(4) Nucleolar Fluorescence. This type of fluorescence is due to antigens present in the nucleoli of the nuclei. It is produced most frequently in systemic sclerosis, but may also be present in significant percentages of cases of SLE and rheumatoid arthritis.

CHAPTER 13

TESTS FOR PREGNANCY

13-1. Significance of Human Chorionic Gonadotropin (HCG):

a. **Introduction.** Human chorionic gonadotropin (HCG) is a hormone that is produced by the placenta during pregnancy and found in several body fluids, including blood and urine. It is also produced by certain rare tumors associated primarily with the reproductive organs of both males and females. With the exception of these rare tumors, pregnancy is the only condition to consistently produce HCG. The detection of HCG in urine or serum provides the basis for pregnancy tests.

b. **Types of Tests for HCG.** Until the past decade, pregnancy tests were based on the effects of HCG on the reproductive systems of various animals. Bioassay was necessary, because no other practical laboratory tests were available that were sensitive enough to detect the minute amounts of HCG found in the blood or urine of pregnant women. Over the years, rats, mice, rabbits, and a variety of frogs and toads were used with varying degrees of success. However, following the isolation and purification of HCG and the discovery that it was antigenic in rabbits, immunologic tests for pregnancy have essentially replaced the use of animals. Except for a radioimmunoassay test for HCG, nearly all of the immunologic tests for pregnancy presently in use are based on the inhibition of agglutination of either latex particles or erythrocytes that have been coated with HCG. A wide variety of these tests are available commercially in kits.

(1) In most of these tests, the patient's urine is, first, mixed with known HCG antiserum from rabbits. The next step is to add the HCG-coated latex particles or erythrocytes to the mixture. If HCG is present in the urine, the HCG antiserum will react with this urinary HCG, thereby inhibiting subsequent agglutination of the latex particles of erythrocytes. Conversely, agglutination indicates the absence of urinary HCG—or a negative pregnancy test.

(2) In addition, many of these tests can be performed on serially diluted serum or urine to provide quantitative HCG results in international units (I.U.) per 24 hours or per liter. The I.U. is based on the activity of an aliquot of a chorionic gonadotropin standard maintained by the World Health Organization.

c. **Sensitivity of Pregnancy Tests.** Aside from the obvious requirement to maintain an animal colony, the main disadvantage of animal tests for HCG is the variable sensitivity of these animals in detecting HCG. Sensitivity varies even between animals of the same species and also with the seasons of the year in some species. Immunologic HCG tests, on the other hand, are more readily standardized and more sensitive than animal tests. For example, radioimmunoassay can detect as little as 15 I.U. of HCG/liter, while the erythrocyte tests have a sensitivity of about 1,000 I.U./liter. Even the relatively insensitive latex tests, which have a sensitivity of 2,000 or more I.U./liter, can detect smaller amounts of HCG than can most animal tests. The actual sensitivity of each test kit is specified by the manufacturer, but a sensitivity of about 1,000 I.U./liter permits early diagnosis of pregnancy without being so sensitive that the small quantities of pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), in the urine cause a significant number of false positives.

d. **HCG in Pregnancy.** Pregnancy tests with a sensitivity of about 1,000 I.U./liter will first become positive about 4–7 days after the expected starting date of the first-missed menstrual period. These tests are nearly always positive within 3 weeks. In normal pregnancies, HCG titers on first morning urines rise rapidly and peak at about 100,000 I.U./liter in about 70 days after the last menstrual period. Then, titers fall and level off at about 25,000 for the duration of the pregnancy. Rapidly falling titers or titers of less than 5,000 usually precede abortions. Low titers of HCG are also frequently seen in ectopic pregnancies. With normal deliveries, these pregnancy tests usually revert to negative in about a week. Persistence of HCG after delivery may indicate a pathological condition.

e. **HCG in Other Conditions.** High titers of HCG, that persist more than a hundred days after a missed menstrual period, may indicate the development of a hydatidiform mole or, more rarely, choriocarcinoma. Complete removal of a mole should result in pregnancy tests becoming negative in about a week. However, a persistently positive pregnancy test a week later may indicate either incomplete removal of the mole or an accompanying choriocarcinoma. In addition, teratomas of the testes and ovaries frequently cause positive pregnancy tests due to HCG production.

13–2. Latex Agglutination Inhibition Slide Tests. Many manufacturers produce pregnancy test kits based on latex agglutination inhibition. In selecting the kit to use, be sure to consider the sensitivity of the method in the decision. As a rule, latex pregnancy tests are less sensitive than hemagglutination tests in detecting HCG. Because of the number of manufacturers producing pregnancy test kits based on latex agglutination inhibition, the procedure and information in this paragraph will be presented in general terms only. For specific instructions, refer to the manufacturers' instructions.

a. **Principle.** Patient's urine is mixed on a slide with HCG antiserum, usually from rabbits. Then a drop (or two) of a suspension of HCG-coated latex particles is mixed with the urine-antiserum mixture, and the mixture is spread over an area prescribed by the manufacturer. The slide is rotated manually and examined within 2 minutes for agglutination. If HCG is present in the urine, the HCG antiserum is neutralized and agglutination of the HCG-coated latex particles does not occur—a positive pregnancy test. Conversely, if no HCG is present in the urine, the antiserum is not neutralized, and agglutination occurs—a negative pregnancy test.

b. **Reagents and Equipment.** Except for the items needed for urine collection, all items are usually supplied with the test kit:

(1) Patient's urine, fresh or frozen.

(2) Human chorionic gonadotropin antiserum (rabbit). Refrigerate, but do not freeze.

(3) HCG-coated latex suspension. Refrigerate, but do not freeze.

(4) Known positive and negative urines, collected locally and stored frozen in small aliquots.

(5) Pipets, disposable, size as specified.

(6) Glass slide, provided.

(7) Toothpicks or similar stirrers.

(8) Light source, preferably fluorescent.

c. **Preliminary Steps:**

(1) Collect the patient's urine. First morning specimens are preferred due to concentration of the HCG, but random samples are usually acceptable, if positive. Refrigerate or freeze speciments that cannot be tested in a few hours. Specimens should be at room temperature and well mixed for testing. Turbid urines may be centrifuged, and the supernate tested.

(2) Allow all reagents to warm to room temperature before testing.

(3) Test newly opened reagents with known positive and negative urines.

d. **Procedures.** Be sure to follow manufacturers' instructions if at variance with the following steps:

(1) Using the pipets provided with the kit, place 1 drop of urine on a section of the slide provided with the kit.

(2) Add 1 drop of HCG antiserum to the urine, mix thoroughly with a stirrer, and rotate the slide for 30 seconds.

(3) Using the dropper provided with the reagent, add 1 drop (or 2, if specified) of HCG-coated latex suspension to the urine-antiserum mixture. Mix thoroughly with a stirrer and spread the urine-antiserum-latex mixture over the entire designated area of the slide.

(4) Rotate the slide as specified and observe for agglutination of the latex particles under a direct fluorescent light at the end of 2 minutes.

e. **Reporting Results:**

(1) Agglutination within 2 minutes—Negative.

(2) No agglutination within 2 minutes—Positive.

f. **Sources of Error:**

(1) Freezing, as well as prolonged storage at room or higher temperatures, can destroy the reactivity of the reagents, so refrigerate them.

(2) Failure to allow reagents and urine to warm to room temperature before testing may result in false-positive (no agglutination) results.

(3) Blood or protein in the urine may inhibit agglutination of the latex particles, thereby causing false-positive results.

(4) Do not use reagents beyond the expiration date indicated by the manufacturer.

(5) Do not mix reagents from different manufacturers or even lot numbers of the same manufacturer.

(6) Care must be exercised in interpreting results of agglutination inhibition tests, because, unlike most serologic test results, a

positive result is indicated by no reaction and vice versa.

(7) Reagents must be thoroughly mixed for valid results.

(8) All glassware must be scrupulously clean and free of detergents for valid results.

(9) Drying of the reagents on the slide may simulate agglutination.

(10) Slides must be examined within the time specified by the manufacturer.

(11) Patient's serum must NOT be tested by this method. Use a hemagglutination inhibition method.

(12) First-morning urine specimens will yield more positives than random specimens due to concentration of the HCG.

g. **Discussion:**

(1) Known positive and negative urine specimens may be tested periodically as reagent controls. These urines may be dispensed in small aliquots and stored frozen. Once thawed, the aliquots should be discarded.

(2) Also available commercially is a kit based on latex agglutination inhibition with the antiserum and HCG-coated latex dried on cards. This kit requires water as the only reagent, in addition to urine. Another advantage of this test is that no refrigeration is required.

(3) Other latex tests for pregnancy, based on the direct agglutination of antiserum-coated latex particles, are available commercially. In these tests, urinary HCG agglutinates the latex particles; consequently, agglutination indicates pregnancy (the presence of HCG).

13-3. Hemagglutination Inhibition Tests. Because of the number of manufacturers producing pregnancy kits based on this principle, the procedure and information in this paragraph will be presented in general terms only. In selecting the kit to use, be sure to consider the sensitivity of the method in the decision.

a. **Principle.** Diluted patient's urine (or specially treated serum) is mixed with liquid or freeze-dried HCG antiserum in a test tube. Then HCG-coated erythrocytes are added, or freeze-dried erythrocytes may have been included with the freeze-dried antiserum. The erythrocytes are allowed to settle, undisturbed, for 2 hours. Inhibition of hemagglutination, as evidenced by the formation of a ring of settled cells, indicates the presence of HCG in the urine—a positive pregnancy test. Conversely, hemagglutination, as evidenced by the formation of a uniform mat of settled cells, indicates a negative test.

b. **Reagents and Equipment:**

(1) Patient's urine, fresh or frozen, or properly treated serum.

(2) HCG antiserum, liquid or freeze-dried in combination with HCG-coated erythrocytes.

(3) HCG-coated erythrocytes, suspension or freeze-dried in combination with the antiserum.

(4) Diluent, as specified.

(5) Test tubes, size as specified. Tests using freeze-dried reagents are performed in the reagent containers.

(6) Pipets, serological or as provided.

(7) Test tube rack, preferably with mirrow.

c. **Preliminary Steps:**

(1) Collect patient's urine according to paragraph 13-2c. If required, dilute the urine using the diluent and quantities specified by the manufacturer. If serum is to be tested, treat the serum as specified by the kit manufacturer.

(2) Test newly opened reagents with known positive and negative urines.

d. **Procedure.** The procedure outlined below is given in general terms only. Be sure to follow manufacturer's instructions.

(1) Add the specified volume of urine, diluted urine, or treated serum to the appropriate test tube.

(2) Add diluent, if required.

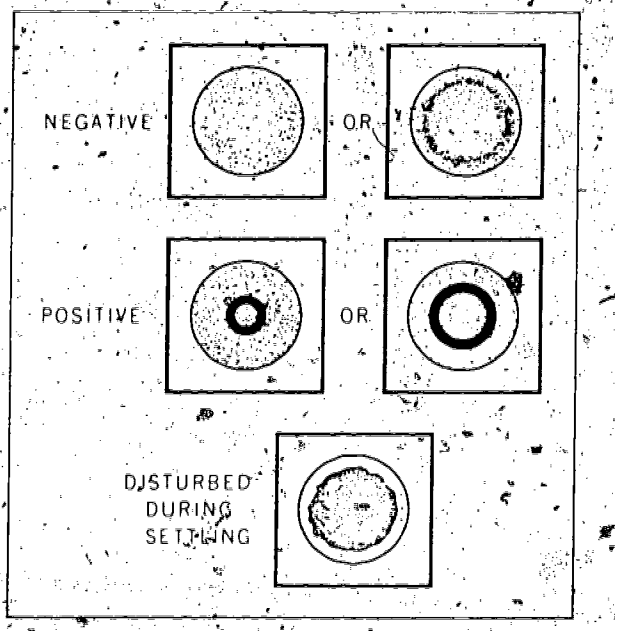


Figure 13-1. Reading Hemagglutination Inhibition Tests for HCG.

(3) With liquid reagents, add the specified amount of antiserum FOLLOWED by the specified amount of thoroughly resuspended, HCG-coated erythrocyte suspension.

(4) Gently mix the contents of the tubes by shaking and place the tubes in a rack. NOTE: Tubes must be exactly vertical.

(5) Let the tubes stand for 2 hours at room temperature in a place where they will be undisturbed by vibration and excessive heat.

(6) At the end of 2 hours, examine the tubes from the bottom (or with the aid of a mirrored rack) for agglutination as evidenced by the formation of a thin mat of settled erythrocytes covering the entire rounded bottom of the tube. Formation of a ring of settled erythrocytes indicates inhibition of hemagglutination. Tubes that have been disturbed during the settling period may show a ragged-edge layer of cells. Examples of possible reactions are depicted graphically in figure 13–1.

e. **Reporting Results:**

(1) Formation of a well-formed ring of cells (hemagglutination inhibition)—Positive.

(2) No ring (or a thin, jagged ring) of cells (no hemagglutination inhibition)—Negative.

f. **Sources of Error.** In general, the sources of error listed for the latex tests in paragraph 13–2f also apply to these tests. The following exceptions and additions should be noted:

(1) Use only test tubes specified by the manufacturer.

(2) The tubes must be exactly vertical and undisturbed during the 2-hour settling period.

(3) Be sure to use the diluent specified by the manufacturer.

(4) As a rule, urinary proteins do not interfere with these tests as much as with latex tests.

13–4. Quantitative HCG Tests. Titration of urinary HCG output is usually performed on 24-hour urine specimens with results reported in I.U. excreted/24 hours. As an alternative, first morning specimens may be tested and reported in I.U./liter of urine. Both latex and hemagglutination tests have been applied to quantitating HCG by serially diluting urine and testing each dilution as an individual specimen. The highest dilution to yield positive results is then used, along with the test sensitivity specified by the manufacturer and the volume of urine, to calculate the number of international units of HCG excreted/24 hours (or per liter on first morning specimens). For specific test procedures, follow the manufacturer's instructions.

13–5. Interpretation of Results. Most requests for pregnancy tests will require only a positive or negative result. Although the actual meaning of a positive or negative test varies with the sensitivity of the test procedure being used, most hemagglutination pregnancy tests will be positive when HCG concentrations exceed about one international unit/ml (1,000 I.U./liter) of urine or serum, but latex tests are much less sensitive. These same tests are negative at HCG concentrations below the sensitivity specified by the manufacturer.

a. **Normal Pregnancies.** Immunologic pregnancy tests usually become positive within the first week after the first-missed menstrual period and remain positive throughout the course of uncomplicated pregnancies. Occasionally, tests may revert to negative later in the pregnancy when HCG levels fall. After delivery, the tests usually become negative within a week. Persistance of a positive test more than a week after delivery most likely indicates a pathological condition, such as hydatidiform mole, choriocarcinoma, or gonadal teratomas.

b. **Ectopic Pregnancies.** Pregnancy tests are frequently negative during these pregnancies.

c. **Threatened Abortions.** Although pregnancy tests cannot be used as a reliable indicator of fetal death, falling HCG titers or titers of less than 3,000 I.U./24 hours (5,000/liter on first-morning urines) usually indicate inevitable abortion.

d. **Hydatidiform Moles.** These intrauterine tumors are usually associated with very high titers of HCG. These titers must be differentiated from the transient peak seen in normal pregnancies. Occasionally, these tumors are accompanied by low, but detectable, HCG titers. Following complete removal of the mole, pregnancy tests should revert to negative in about a week. In fact, a persistently positive test following attempted removal of the tumor may indicate either incomplete removal or, more seriously, choriocarcinoma.

e. **Choriocarcinoma.** These HCG-producing tumors may develop in conjunction with normal pregnancy, incomplete abortion, or hydatidiform mole. HCG titers are generally very high in these tumors, but these titers must also be differentiated from the transient peak seen in normal pregnancy.

f. **Ovarian and Testicular Teratomas.** These relatively rare tumors also produce HCG. Consequently, the usual pregnancy test may be requested—even on males.

g. **False Negatives.** Due to the high sensitivity (about 1 unit of HCG/ml) of immunologic pregnancy tests, false-negative tests are rare. However, if urine specimens are collected too early in the pregnancy, negative results may be obtained. In addition, threatened abortions and ectopic pregnancies are frequently associated with very low levels of HCG. Testing of first morning urines will help minimize the occurrence of false negatives.

h. **False Positive.** Positive pregnancy tests due to HCG produced by hydatidiform moles, choriocarcinoma, and related tumors should not be considered as false positive. However, occasional high urinary levels of the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), may cause false positives. Although the sensitivity of immunologic pregnancy tests are adjusted to preclude most false positives, care must be exercised in interpreting pregnancy tests on urine collected during ovulation (high LH) and menopause (high FSH). In addition, blood or protein in the urine may cause false-positive results by inhibiting agglutination of the HCG-coated latex or erythrocyte suspension.

Chapter 14

TESTS FOR RUBELLA ANTIBODIES

14-1. Significance of Rubella Titers:

a. **The Disease.** Rubella, also called German or 3-day measles, is usually an insignificant, contagious viral infection. In addition, a single infection usually confers lifelong immunity. Although the disease may reach epidemic proportions, especially in children and young adults, symptoms and complications are usually minimal. However, if the infected patient happens to be a pregnant woman in the early stages of pregnancy, the effect on her developing fetus can be disastrous.

b. **Congenital Rubella.** An infected pregnant female can transmit rubella to the fetus during a time usually limited to the first trimester of pregnancy. Frequently, these pregnancies result in spontaneous abortions, but if the fetus should be carried to term, a high percentage of infected infants will be born with various abnormalities. The earlier in the pregnancy that infection occurs, the greater the chance of fetal malformations—up to 80 percent, if infection occurs during the first month of pregnancy. Any organ system of the fetus may be affected. Some of the end results may be low birth weight; failure to thrive; eye involvement resulting in chorioretinitis, glaucoma, or cataracts; central nervous system involvement resulting in cerebral palsy or retardation; deafness; and malformations of the heart. The incidence of severe fetal abnormalities is so high that therapeutic abortions are usually warranted when a pregnant woman contracts rubella during the first trimester. The detection of susceptiblity to rubella and the identification of infected pregnant females is usually the responsibility of the serologist.

c. **Immunity and Susceptibility.** As mentioned above, a single rubella infection usually confers lifelong immunity to reinfection. In addition, an immune pregnant woman passively transfers antibodies (and short-lived immunity) of rubella to her developing fetus. This immunity usually lasts for about 6 months after birth. By then the maternal antibodies in the infant have fallen to a low enough titer for the infant to be susceptible to rubella. Unfortunately, about 15 percent of pregnant women will not be immune to rubella and, therefore, cannot transmit antibodies to the fetus. It is these women that are susceptible to rubella and have the high risk of delivering malformed infants. These women must be identified, especially if they are exposed to (or actually contract) rubella early in gestation, because therapeutic abortions may be indicated.

d. **Prevention of Rubella.** There is no practical treatment available for rubella. Therefore, all approaches (short of therapeutic abortion) for minimizing the problems of congential rubella have been directed toward preventing the disease. Two main methods have been used—immunizations and injections of gamma globulin.

(1) Immunizations. In recent years, attempts have been made to reduce exposure of pregnant females to rubella cases by mass immunizations of children with an attenuated rubella vaccine. Although the vaccine seems to prevent clinical cases of rubella, immunized individuals may actually cause a greater danger to nonimmune pregnant females. It has been found that immunized persons may still have subclinical cases and transmit virus to a now unsuspecting pregnant female and others. The whole question of the effectiveness of mass immunization in preventing congential rubella is still being studied. However, immunization of susceptible, nonpregnant females of childbearing age is usually indicated, but birth control must be practiced for 2 months following immunization as a precaution against complications due to the use of the attenuated vaccine.

(2) Prophylaxis with Gamma Globulin. If injected before the patient is exposed to rubella, large quantitites of gamma globulin (antibodies) will prevent clinical cases of rubella for a short time. Unfortunately, however, pregnant females given massive doses of gamma globulin may still develop a viremia, and fetal infections may occur even though the mother shows no signs of illness.

e. **Tests for Rubella Antibodies.** Several serologic tests have been developed to determine an individual's susceptibility to rubella. In addition, unless the virus can be isolated and identified (a time-consuming project requir-

ing specialized techniques), these serologic tests may be used in the diagnosis of clinical or subclinical cases of the disease. Some of these rubella antibody tests are complement fixation, indirect fluorescent antibody, neutralization (various types), and viral hemagglutination inhibition tests. The only tests to be covered in this manual are two modifications of the hemagglutination inhibition test.

(1) Antibodies in Clinical Rubella. Detectable levels of rubella antibodies are usually present at about the same time as the rash appears in rubella. Titers reach a peak in a week or two, and with most of the serologic tests, the titers stay fairly constant for life. In the hemagglutination inhibition test, a titer of 1:10 (1:8/some authors) or greater usually indicates a past rubella infection or immunization and, most important, lifelong immunity to reinfection. Individuals with titers of less than 1:10 (or 1:8) are usually susceptible to rubella. If a patient has not been immunized, a fourfold or greater rise in titer is indicative of active disease; however, since the titers rise so rapidly, the first serum specimen must be collected very early in the disease in order to demonstrate a rise in titer.

(2) Antibodies in Congenital Rubella. The serodiagnosis of congenital rubella is difficult, but as a rule, if the antibody titer of the infant at about 6 months of age is the same as it was at birth, congenital rubella is likely. By 6 months of age, the titer of rubella antibodies received from the mother should be very low so that a significant titer of antibodies will most likely have been produced by an infected infant. Another method is to detect IgM rubella antibodies in the infant. Only IgG antibodies are acquired from the mother so the presence of IgM rubella antibodies in the infant indicates congenital rubella infection. The detection of IgM involves testing the infant's serum before and after treatment with 2-mercaptoethanol which breaks down IgM antibodies. A drop in titer after 2-mercaptoethanol treatment indicates congenital rubella.

14-2. Hemagglutination Inhibition (HAI) Test, Microtitration Method:

a. **Principle.** There are three main steps in the performance of this test:

(1) Antigen Standardization. Known rubella virus is serially diluted with microtitration equipment in the appropriate buffer. Then, a suspension of pigeon, goose, human group O, or 1-day-old chick erythrocytes is added. The rubella virus has the ability to agglutinate these cells in the cold. The highest dilution of virus to produce complete agglutination of the erythrocyte is considered to be one hemagglutinating (HA) unit. Four HA units are used in the test proper.

(2) Adsorption of Nonspecific Inhibitors from Serum. All sera contain substances (mainly beta-lipoproteins) that nonspecifically inhibit hemagglutination by the rubella virus. Since the HAI test is based on the inhibition of hemagglutination by anti-rubella antibodies (specific inhibition), all nonspecific inhibitors must be adsorbed from the serum before testing. In addition, some sera may contain agglutinins against the erythrocytes to be used in the test. These agglutinins must also be adsorbed from the serum before the test is performed. Two basic methods for the removal of nonspecific inhibitors are in use, with some serologists preferring the kaolin method, and others preferring the heparin-manganous chloride method. Both methods will be presented in this manual.

(a) Kaolin Method. Nonspecific inhibitors are absorbed from the serum at room temperature by the addition of a suspension of kaolin. After the kaolin (and attached inhibitors) is sedimented by centrifugation, a suspension of adsorption erythrocytes is incubated with the supernate in the cold. After incubation, the mixture is centrifuged again. After heat inactivation the supernate is ready for testing in the HAI test. As an alternative, the serum may be inactivated before kaolin treatment.

(b) Heparin-Manganous Chloride Method. In this method, nonspecific inhibitors are precipatated with a mixture of heparin and manganous chloride followed by the erythrocyte suspension to remove hemagglutinins against the test cells from the serum, if present. The supernate is tested in the HAI test without heat inactivation.

(3) Hemagglutination Inhibition Test. Using microtitration equipment, the supernate prepared from the kaolin or heparin-manganous chloride adsorption is serially diluted in the appropriate buffer. Then, rubella virus (four HA units) is added to each dilution and the mixture incubated in the cold. Following incubation, a suspension of erythrocytes is added to each dilution. The presence of antirubella antibodies in the patient's serum is indicated by the inability of the rubella virus to agglutinate the indicator erythrocytes. If all control sera and retitration of the rubella HA antigen give the expected reactions, the antibody titer of the patient's serum is reported as the highest

serum dilution to completely inhibit hemagglutination by the virus:

b. **Reagents and Equipment.** The reagents for this test are available commercially from several sources in kit form. Be sure to follow manufacturers' instructions at all times. For best results, use only reagents from a single manufacturer in a given test run. Do not substitute. Do not use reagents beyond the expiration date.

(1) Rubella HA antigen.

(2) Buffered diluent, as specified by the kit manufacturer. Usually dextrose-gelatin-veronal (DGV) or HEPES buffer is used.

(3) Indicator erythrocytes, 0.25 to 0.5% suspension.

(4) Adsorption erythrocytes, 50% suspension. Some manufacturers combine these with the nonspecific inhibitor adsorbent.

(5) Nonspecific inhibitor adsorbent, either

(a) Kaolin suspension or

(b) Heparin-manganous chloride.

1. Sodium heparin, 5000 units/ml.

2. $MnCl_2$, 1 molar solution.

(6) Patients' sera and control sera, as follows:

(a) High-titered positive.

(b) Low-titered positive.

(c) Negative (less than lowest dilution tested).

(7) Water bath, 56°C.

(8) Refrigeration, 4°C (range 2°C–8°C).

(9) Centrifuge, refrigerated, 4°C.

(10) Microtitration equipment. Complete kits are available commercially. The following items are needed:

(a) "V" plates, permanent or disposable.

(b) Pipet droppers, 25-microliter (0.025 ml).

(c) Microdiluters, 25-microliter (0.025 ml).

(d) Delivery testers, 25-microliter.

(e) Plastic sealing tape, roll.

(f) Test reading mirror, optional but helpful.

(11) Test tubes, 10 or 12 × 74 mm.

(12) Beakers, 250 ml.

(13) Cotton swabs.

(14) Burner, Fisher or Bunsen.

(15) Pipet, serological, 0.1, 0.2, and 1 ml.

(16) Test tube rack.

c. **Preliminary Steps:**

(1) Use of Microtitration Equipment:

(a) Pipet Dropper:

1. Fill pipet and hold it vertically over the wells of the microtitration plate to disperse drops.

2. Drops that miss the well may be wiped up with a cotton swab.

(b) Microdiluter Preparation:

1. Wash diluter in running water, rinse in distilled water, and allow to dry. Diluters may also be flamed and cooled to room temperature.

2. Prewet diluter (calibrated portion only) in just enough diluent (or water) in a beaker to cover the calibrated portion. Do not wet shaft.

3. Fill diluter by rotating handle.

4. Test diluter on the delivery test. If the diluter fails to dispense the proper quantity continue prewetting step and retest later.

(c) Serial Dilution with Microdiluters:

1. Put appropriate diluent in wells of microtitration plate according to test procedure.

2. Fill pretested diluter in fluid (serum or virus suspension) to be serially diluted.

3. Place loaded diluter in appropriate well WITHOUT touching sidewalls of the well.

4. Rotate diluter in diluent for 4 sec.

5. Transfer diluter to next well and repeat steps 3 and 4 until serial dilution is complete.

(2) Rubella HA Antigen Standardization. This standardization needs to be performed only once on each lot of antigen, but the dilution of virus is confirmed each time the hemagglutination inhibition (HAI) test is performed on a batch of sera.

(a) Make a 1:4 dilution of rubella antigen in cold (4°C) buffer (0.1 ml of antigen to 0.3 ml of buffer). Mix and incubate at 4°C for 15 min before using.

(b) Place 0.025 ml of buffer in each of nine wells of a microtitration "V" plate.

(c) With a 0.025 ml diluter, serially dilute 0.025 ml of the 1:4 antigen dilution through the first eight wells.

1. Discard 0.025 ml from well #8.

2. Well #9 is the cell (erythrocyte) control and receives no rubella antigen.

3. This dilution pattern results in a twofold dilution starting with 1:8 in the first well. This is the dilution used to determine the antigen titer. Subsequent additions of diluent and erythrocytes are not considered in the dilution.

(d) Add 0.025 ml of cold buffer to each well.

(e) Incubate at 4°C for 15 min.

(f) GENTLY resuspend the indicator erythrocytes (0.25 to 0.5% suspension) and

Table 14–1. Rubella Antigen Standardization.

Table 14-1. Rubella Antigen Standardization.

Well Number	1	2	3	4	5	6	7	8	9
	0.025 ml	0.025 ml	0.025 ml	0.025 ml	0.025 ml	0.025 ml	0.025 ml	0.025 ml	Discard 0.025 ml
Buffer, ml	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
	0.025 milliliters of:								
Rubella HA Antigen, ml	1:4 (From Separate Tube)	1:8	1:16	1:32	1:64	1:128	1:256	1:512	None (Cell Control)
Buffer, ml	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Indicator Erythrocytes, ml	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Total Volume, ml	0.075	0.075	0.075	0.075	0.075	0.075	0.075	0.075	0.075
Dilution of HA Antigen	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Cell Control

add 0.025 ml to each well. The performance of the HA procedure is shown graphically in table 14–1.

(g) Mix by gently tapping plates and cover each plate by stacking the plates or with plastic sealing tape.

(h) Incubate plates at 4°C (2° to 8°C) for 1 to 2 hr or until cell control (well #9) shows a button of cells at the bottom of the well. Do not incubate longer than needed. Readings are made by examining the plates from the bottom or with a reading mirror.

(i) Record the highest dilution of rubella antigen to produce COMPLETE hemagglutination. This dilution contains one HA unit. See figure 14–1 for a typical example of HA antigen standardization result.

(j) Calculate the dilution of rubella antigen that will contain four HA units by dividing the dilution observed in step (i) by 4. This is the working dilution (4 HA units) to be used in the HAI test on the patient's serum. EXAMPLE: If a 1:128 dilution of virus is the highest to produce complete agglutination (see figure 14–1), 128/4 = 32 or a 1:32 dilution of virus will contain 4 HA units, the concentration to be used in the HAI test.

(3) Adsorption of Nonspecific Inhibitors From Serum. Two methods for the removal of nonspecific inhibitors from serum are in use. Use the method specified by the kit manufacturer. Do not interchange steps in the procedures between methods.

(a) Kaolin Method:

1. Inactivate all sera (including patient's, high-positive, low-positive, and negative controls) for 30 min at 56°C. As an alternative, inactivation may be performed on the supernate after the adsorption steps.

2. Add 0.4 ml of kaolin suspension to 0.1 ml of each inactivated serum in properly labeled test tubes.

3. Mix by shaking and incubate for 20 min at room temperature.

4. At the end of the incubation period, centrifuge all tubes at 4°C for 20 min at 2000 rpm.

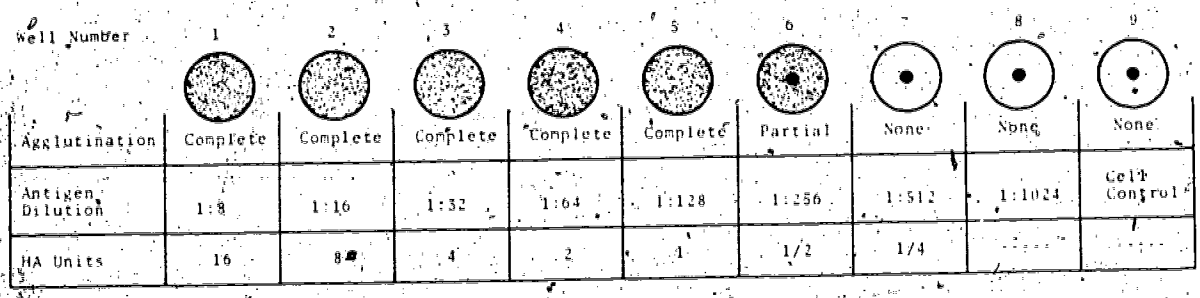


Figure 14–1. Example, Rubella HA Antigen Standardization Results.

5. Leave the supernate in the tubes and add 0.05 ml of adsorption erythrocytes (50% suspension) to each supernate and shake the tubes.

6. Incubate all tubes at 4°C for 1 hr with periodic shaking.

7. Centrifuge all tubes at 4°C for 20 min at 2000 rpm.

8. Without disturbing the sedimented erythrocytes and kaolin, carefully collect and save the supernate. These adsorbed sera are ready for testing immediately or may be held for a day or two at 2° to 8°C. The adsorbed sera are considered to be at a 1:5 dilution.

(b) Heparin-Manganous Chloride Method:

1. Determine the volume of heparin-manganous chloride solution needed to add 0.1 ml/serum to be tested, including high-titered positive, low-titered positive, and negative controls. Combine equal volumes of heparin and manganous chloride solutions to yield the required volume.

2. Add 0.1 ml of serum to 0.2 ml of cold buffer in a test tube and mix by shaking. NOTE: Sera are NOT heat inactivated for this method.

3. Add 0.1 ml of heparin-manganous chloride mixture to each tube and mix by shaking.

4. Incubate tubes for 15 min at 4°C.

5. After incubating, add 0.1 ml of adsorption erythrocytes (50% suspension) to each tube, remix and incubate for 1 hr at 4°C.

6. Centrifuge tubes at 4°C for 20 min at 2000 rpm.

7. Without disturbing the sedimented erythrocytes and precipitate, carefully collect and save the supernates. These adsorbed sera are ready for testing immediately or may be held for a day or two at 2° to 8°C. The adsorbed sera are already diluted 1:5 by this method and are ready for testing WITHOUT heat inactivation. If a precipitate forms during storage of the treated serum, recentrifuge.

d. Procedure:

(1) Confirmation of Antigen Dilution. This confirmation of the dilution of antigen containing 4 HA units must be performed at the same time as each batch of HAI tests.

(a) Standardize the rubella HA antigen according to the procedure in paragraph 14-2c(2) and calculate the dilution of antigen containing 4 HA units.

(b) Determine the quantity of 4-unit antigen needed for the batch of HAI tests to be performed. Each patient's serum and control serum will require 0.125 ml of 4-unit antigen; in addition, the confirmation of the antigen dilution will require 0.05 ml of 4-unit antigen.

(c) Dilute just enough rubella HA antigen for the batch of tests to a concentration of 4 HA units with cold buffer. Let stand at 4°C for 15 min before testing.

(d) Prepare six wells of a microtitration "V" plate and put 0.025 ml of cold buffer in wells #2 through #6.

(e) Add 0.025 ml of properly diluted rubella antigen (4 HA units) to wells #1 and #2. Using a microdiluter and starting with well #2, serially dilute the antigen through well #5.

1. Discard 0.025 ml of antigen-buffer mixture from well #5.

2. Well #6 is the cell (erythrocyte) control and receives no antigen.

3. This dilution scheme has 4 HA units in well #1, 2 HA units in well #2, 1 HA unit in well #3 and so forth.

(f) Add 0.025 ml of cold buffer to each well.

(g) Gently resuspend the indicator erythrocytes (0.25 to 0.5% suspension) and add 0.025 ml to each well. NOTE: This addition of erythrocytes should be made at the same time as erythrocytes are added to the HAI tests.

(h) Mix by gently tapping plates and cover each plate by stacking the plates or with plastic sealing tape.

(i) Incubate plates (at same time as HAI tests) at 4°C (2° to 8°C) for 1 to 2 hours or until the cell control (well #6) shows a button of cells at the bottom of the well.

(j) For valid results, this confirmation of the antigen should show agglutination through well #3 (one HA unit), and the cell control must be negative. However, results may also be considered valid if the test indicates that at least two HA units of rubella antigen is used. Two HA units would be indicated by agglutination in wells #1 and #2. Antigen dilutions not producing agglutination through well #2 (preferably well #3) must be restandardized. The procedure for the confirmation of the antigen dilution is shown graphically in table 14-2.

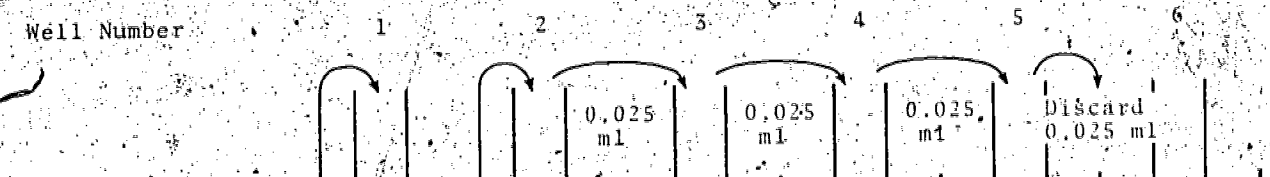
(2) Hemagglutination Inhibition (HAI) Test, Microtitration Method:

(a) Adsorb nonspecific inhibitors from the patient's sera and all control sera by either the kaolin or heparin-manganous chloride method described in paragraph 14-2c(3). This treatment makes a 1:5 dilution of the sera.

(b) Prepare seven wells in "V" plates for

Table 14-2. Confirmation of Rubella Antigen Dilution.

Table 14-2. Confirmation of Rubella Antigen Dilution.



Buffer, ml	None	0.025	0.025	0.025	0.025	0.025
	0.025 milliliters of:					
4 HA unit, Standardized Antigen, ml	4 HA Units (From Separate Tube)		Well #2	Well #3	Well #4	None (Cell Control)
Buffer, ml	0.025	0.025	0.025	0.025	0.025	0.025
Indicator Erythrocytes, ml	0.025	0.025	0.025	0.025	0.025	0.025
Total Volume, ml	0.075	0.075	0.075	0.075	0.075	0.075
Final Dilution, HA Units	4	2	1	1/2	1/4	Cell Control

the first serum to be tested and six wells for each additional patient's serum. Also, prepare six wells for each of the following adsorbed control sera:

1. High-titered positive.
2. Low-titered positive.
3. Negative.

(c) Add 0.025 ml of cold buffer to wells #1 through #5 and 0.05 ml to wells #6 and #7. NOTE: All reagents must be at 4°C for testing.

(d) Add 0.025 ml of adsorbed (1:5 dilution) serum to wells #1 and #6.

(e) Serially dilute 0.025 ml from well #1 through well #5. Repeat the dilution for each serum.

1. Discard 0.025 ml of serum-buffer mixture from well #5. This dilution pattern results in a twofold dilution starting with 1:10 in the first well. Subsequent additions of diluted rubella antigen and erythrocytes are not considered in the dilution.

2. Well #6 is the serum control for the detection of erythrocyte agglutinins that may not have been completely removed from the serum during the adsorption steps. One of these is needed for each serum to be tested.

3. Well #7 is the cell (erythrocyte) control. Only one of these is needed for each batch of tests, but more may be used, if desired.

(f) Add 0.025 ml of diluted (4 HA units) rubella HA antigen to wells #1 through #5 only. Wells #6 and #7 receive no antigen.

(g) Mix the contents by gently tapping the edge of the plate and cover each plate by stacking the plates or with plastic sealing tape.

(h) Incubate the plates at 4°C for 1 hr or as recommended by the kit manufacturer.

(i) After incubation, gently resuspend the indicator erythrocytes (0.25 to 0.5% suspension) and add 0.025 ml to all seven wells.

(j) Mix again by gently tapping the plates, cover the plates, and incubate at 4°C for 1 to 2 hrs or until the cell control (well #7) shows a button of cells at the bottom of the well. Do not incubate longer than needed. NOTE: Perform this incubation at the same time as the antigen dilution confirmation is incubated.

(k) If all controls and the confirmation of the antigen dilution indicate valid results, record the highest dilution of patient's serum to produce COMPLETE INHIBITION of agglutination of the erythrocytes as the rubella HAI titer. For valid results, the following conditions must be met:

1. Confirmation of Antigen Dilution. At least wells #1 and #2 (and ideally also #3) must show agglutination thus indicating that the antigen dilution used in the HAI test contained at least 2 HA units (two wells

Table 14-3. Rubella Hemagglutination Inhibition Test.

Table 14-3. Rubella Hemagglutination Inhibition Test.

Well Number	1	2	3	4	5	6	7
	0.025 ml	0.025 ml	0.025 ml	0.025 ml	Discard 0.025 ml		
Buffer, ml	0.025	0.025	0.025	0.025	0.025	0.05	0.05
	0.025 milliliters of:						
Adsorbed Serum, 1:5, (Supernate), ml	Supernate (From Separate Tube)	1:10	1:20	1:40	1:80	Supernate (From Separate Tube)	None (Cell Control)
4 HA Unit, Standardized Antigen, ml	0.025	0.025	0.025	0.025	0.025	None	None
Incubation	Incubate at 4°C for 1 hr.						
Indicator Erythrocytes, ml	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Total Volume, ml	0.075	0.075	0.075	0.075	0.075	0.075	0.075
Dilution of Serum	1:10	1:20	1:40	1:80	1:160	Serum Control	Cell Control

agglutinated) or ideally 4 HA units (three wells agglutinated). In addition, the cell control (well #6) must show no agglutination.

2. High and Low-titered Control Sera. Inhibition must be within ± 1 well of expected values. Results more than one well from expectation should be considered invalid and the test examined for possible sources of error.

3. Negative Control Serum. No inhibition. If inhibition of agglutination is noted, incomplete removal of nonspecific inhibitors should be suspected.

4. Serum Controls (well #6). No agglutination. Agglutination indicates incomplete removal of erythrocyte agglutinins from the serum. Another aliquot of serum should be adsorbed by the selected method (kaolin or heparin-manganous chloride), but with the addition of 0.2 ml of adsorption erythrocytes instead of the 0.1 ml normally used.

5. Cell Controls (well #7). No agglutination. Agglutination would indicate autoagglutination and unacceptable erythrocytes.

(1) The HAI procedure is shown graphically in table 14-3 and a typical example of HAI test results is shown in figure 14-2.

e. **Reporting Results:**

(1) Rubella Antibodies Detected. Report the highest dilution of serum to produce COMPLETE inhibition of hemagglutination as the titer.

(2) No Rubella Antibodies Detected. When none of the dilutions of serum inhibit hemagglutination by rubella virus, report results as "less than 1:10," the lowest dilution actually tested.

f. **Sources of Error:**

(1) All reagents must be stored and used at 4°C, unless otherwise specified. The rubella HA antigen and the erythrocytes are especially sensitive to temperature changes.

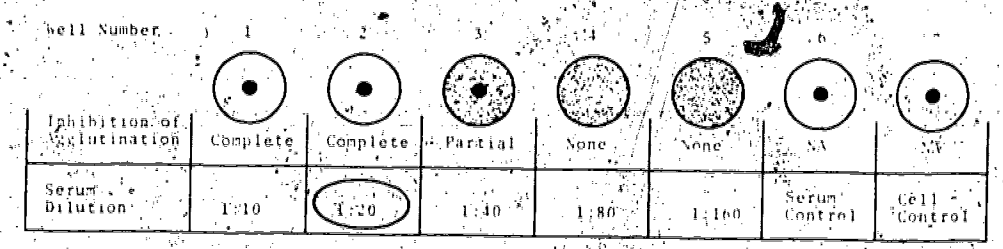


Figure 14-2. Example, Rubella HAI Results.

Titers may also vary with different incubation temperatures.

(2) The antigen dilution must be confirmed at the same time each batch of HAI tests is performed to assure that 4 HA units (minimum—2 HA units) of HA antigen is used in the test.

(3) Do not use reagents beyond the expiration dates.

(4) Do not substitute reagents from various manufacturers.

(5) Hemolysis of indicator erythrocytes may be caused by failure to inactivate serum or dirty glassware.

(6) If the test is to be used to detect a rise in titer, the first serum specimen must be collected before or very shortly after the rash appears, because titers reach a peak very rapidly.

g. **Discussion.** This test may also be performed in 10 to 12 × 75 mm test tubes, but the serial dilutions are slower to perform and much larger reagent quantities (up to eight times as much) are required. The tube method should be performed by substituting 0.2 ml of each reagent for each 0.025 ml used in the microtitration method. In addition, a large aliquot (0.4 ml) of adsorbed serum (supernate) will be required so double all quantities in the adsorption steps. Readings and controls are used as in the microtitration method.

14-3. Interpretation of Results:

a. **Detecting Immunity or Susceptibility to Rubella.** A hemagglutination inhibition (HAI) titer on a single serum sample of 1:10 (1:8 according to some) or greater usually indicates immunity to reinfection, while no titer indicates susceptibility. A single rubella infection usually confers a lifelong significant antibody level, as well as lifelong immunity. In addition, immunization with attenuated rubella virus may produce immunity and apparently significant antibody titers, but unfortunately, some of these individuals, that would be expected to be immune, may still contract subclinical cases of rubella and transmit the virus to nonimmune persons. If the virus is transmitted to a nonimmune pregnant female, the effects on her developing fetus, as described in paragraph 14-1, can be disastrous. Consequently, the detection of nonimmune (susceptible) women of child-bearing age, followed by their immunization before pregnancy, could significantly reduce these hazards.

b. **Detecting Congenital Rubella.** Antibodies that persist at fairly constant levels in the infant after about six months of age usually indicate congenital rubella. At this age, antibodies acquired from the mother should have fallen to low levels.

c. **Detecting Clinical Rubella.** If the rubella virus cannot be isolated and identified from suspected cases, a fourfold or greater rise in hemagglutination inhibition (HAI) titer is significant. However, if therapeutic abortions are at stake, additional tests, such as complement fixation and fluorescent antibody, are recommended. In addition, the first serum specimen must be collected early in the disease, because the HAI titers rise rapidly and reach a peak in a few weeks. As an alternative, a serum sample might be stored from all nonimmune pregnant women and used as a baseline serum in suspected exposures or cases.

Chapter 15

TESTS FOR IMMUNOGLOBULINS AND COMPLEMENT

SECTION A—IMMUNOGLOBULINS

15-1. Significance of Immunoglobulins. Following electrophoresis of serum proteins, only the gamma globulin fraction is found to contain significant antibody activity. When the gamma globulin is injected into certain animals, the animals produce antibodies against the different antigens making up the gamma globulin. By this method, it has been found that gamma globulin is actually made up of five antigenically distinct globulins. These five globulins are referred to collectively as immunoglobulins, with the individual immunoglobulins designated as IgG, IgM, IgA, IgD, and IgE. For practical purposes these immunoglobulins may be considered to be synonymous with antibodies; therefore, alterations in the serum levels of immunoglobulins can have a bearing on the immunity and health of an individual. In fact, either an abnormally high or low serum level of a given immunoglobulin may indicate pathology. These immunoglobulin levels in the serum are estimated by using serologic methods, hence the inclusion of these methods in this manual.

a. **Production of Immunoglobulins.** All immunoglobulins are produced by lymphocytes in response to antigenic stimuli, such as pathogenic and saprophytic microorganisms, as well as other foreign substances that might gain entry into the body. In fact, except for the IgG received transplacentally from the mother, a newborn infant is essentially devoid of immunoglobulins unless it has contracted an intrauterine infection during pregnancy.

(1) The IgG level in the newborn is the same as that of its mother. Consequently, the infant has passively acquired immunity to essentially the same diseases as its mother; the infant is also susceptible to the same diseases. However, the IgG level of the infant falls to low levels in a few months, but during this time, the infant's immunologic system is maturing and commencing to produce all classes of immunoglobulins. By the middle teens, the IgG level is usually back up to the congenitally acquired level present at birth. In normal adults, this IgG comprises about 75 percent (by weight) of the total immunoglobulins.

(2) The other immunoglobulins—that is, IgM, IgA, IgD, and IgE—do not appear to cross the placental barrier in significant quantities. IgM may be produced before birth if the fetus should contract an intrauterine infection, but in general, all antibody (immunoglobulin) production starts after birth. Along with IgG, all of these immunoglobulins normally reach adult levels by the midteens. By that time, about 21 percent (by weight) of immunoglobulins will be IgA, seven percent—IgM, and less than one percent each of IgD and IgE.

b. **Characteristics of Immunoglobulins.** In addition to their antigenic differences, the five classes of immunoglobulins also vary considerably in their production and reactivity. For example, on the first stimulation by an antigen, most of the antibodies produced are IgM, but on a second contact with the antigen, mainly IgG is produced. Although both IgM and IgG may be produced in response to the same antigen, the reactivity of these two classes in serologic tests also varies significantly. IgG antibodies have been found to be especially effective as precipitating antibodies and complement fixing antibodies but less efficient than IgM in agglutination and hemolysis reactions. On the other hand, IgA, IgD, and IgE appear to have few implications in serologic reactions. The reactivity of the five classes also varies in the body. IgG, IgM, and IgA are the main classes that are active in immunity, while IgE appears to be most significant in allergic conditions. In addition to its presence in serum, IgA is also found in many body secretions, such as tears, urine, and bronchial secretions, where it most likely has an additional role in immunity. IgD is the only class of immunoglobulin whose function is essentially unknown.

c. **Immunoglobulin Deficiencies.** The body's inability to produce adequate quantities of one or all immunoglobulins may be inherited or acquired. Some infants may have hereditary agammaglobulinemia with production of all five classes severely depressed, or dysgammaglobulinemia with just one or two classes

low and the others normal. Many of these infants become very susceptible to even the most common infections as soon as the maternal IgG levels have fallen. More frequently, a hypogammaglobulinemia is seen, where one or all classes of immunoglobulins may be moderately, but significantly, depressed. These individuals are also more susceptible to infections than normal. These low levels of immunoglobulins may be due to delayed maturation of the immunologic system of the infant or associated with other conditions. Severe malnutrition, nephrosis, and malignant lymphomas, such as lymphocytic leukemia and Hodgkin's disease, are just some of the conditions that can result in hypogammaglobulinemia and eventually affect the health of the individual. Identification of these individuals is important so that appropriate prophylactic measures can be practiced to prevent infection. A few diseases are characterized by below-normal levels of a single immunoglobulin.

d. **Immunoglobulin Excesses.** Several conditions may result in the overproduction of one or more of the immunoglobulins. For example, viral hepatitis usually shows excessive production of all immunoglobulin classes while another liver disease, Laennec's cirrhosis, shows only IgA and IgG increased. In contrast, hepatomas result in depressed levels of IgM. Systemic lupus erythematosus (SLE) is another disease associated with a general increase in serum immunoglobulins, but especially of IgG. In addition, several diseases are accompanied by significantly high levels of a single immunoglobulin. Examples are African sleeping sickness caused by *Trypanosoma gambiense* and a high IgM, hay fever and asthma—IgE, and multiple myeloma with very high levels of either IgG or IgA and rarely, IgM, IgD, or IgE. As can be seen from the diseases discussed above, abnormally high immunoglobulin levels may be just as significant as low levels.

e. **Tests for Immunoglobulins.** There are several immunologic tests for detecting the presence or absence of immunoglobulins, but the only practical laboratory method for estimating the concentration of the individual immunoglobulins is immunodiffusion. More specifically, single radial immunodiffusion is used. In this method, monospecific antibody produced in animals against one of the five classes of immunoglobulins is incorporated into the layer of agar gel. Then, the patient's serum (containing the immunoglobulin) is put in a well cut into the agar and the system is incubated, usually at room temperature, but the temperature depends on which test is being performed. The immunoglobulin diffuses into the antibody-containing agar and a ring of precipitate forms at the zone of optimum proportions of the patient's immunoglobulin (antigen here) and antibody. The diameter of the precipitate ring is proportional to the concentration of the particular immunoglobulin in the patient's serum. By simultaneously testing known standard reference sera, a quantitative result can be reported for the patient's immunoglobulin level.

(1) Several manufacturers make kits which include prepared agar plates, reference sera, and everything needed to perform these tests. Kits are available for all five classes of immunoglobulins as well as several other biologically significant proteins that may be found in serum and other body fluids in certain disease states. In addition, complement, to be discussed in the next section of this chapter, is also assayed by single radial immunodiffusion with all equipment available in kit form.

(2) Another very sensitive method for assaying small quantitites of these proteins is through the use of radioimmunoassay (RIA), but this method is beyond the capability of the average laboratory.

15–2. Immunodiffusion Tests for Immunoglobulins.

a. **Principle.** Patient's serum is put in a well cut into monospecific-antibody-containing agar gel in plates (usually commercially prepared). Simultaneously, three or four reference sera of known concentrations of the particular immunoglobulin under study are placed in other appropriately labeled wells. The plates are covered tightly and incubated according to the times and temperatures specified for the immunoglobulin. As the immunoglobulin diffuses into the antibody-containing agar, a ring of precipitate forms at the point of optimum proportions of antigen (immunoglobulin) and antibody. The diameter of the precipitate ring is proportional to the concentration of the specific immunoglobulin in the patient's serum. By plotting the diameter of the precipitate ring of each reference serum on the linear scale against its known immunoglobulin concentration on the logarithmic scale of two- or three-cycle semilogarithmic graph paper, the concentration of immunoglobulin in the patient's serum can be determined. NOTE: This reference curve MUST be prepared for each batch of tests to compensate for variations in incubation temperatures, humidity, agar gel lot

variations, and other daily variables that might affect precipitate diameters.

b. **Reagents and Equipment:**

(1) Patient's serum.

(2) Reference sera, three or four different concentrations for each desired immunoglobulin.

(3) Immunodiffusion plates, various sizes with precut wells are available commercially.

(4) Moist chamber, such as a tightly covered jar or plastic bag containing wet gauze or filter paper.

(5) Capillary pipets or microliter syringes.

(6) Graph paper, two- or three-cycle semilogarithmic.

(7) Incubator, 37°C, needed for certain, but not all, immunodiffusion tests.

(8) Calibrated magnifying device for measuring ring diameters. Available commercially or, as an alternative, a dissecting microscope with an eyepiece reticle or stage micrometer may be used.

c. **Preliminary Steps:**

(1) Collect the patient's serum. Fresh serum is preferred, but frozen serum, that has not been repeatedly frozen and thawed, may be used.

(2) Remove immunodiffusion plates from the refrigerator. Plates containing visible moisture should be left open at room temperature for a few minutes before proceeding with the test.

d. **Procedure:**

(1) Identify wells for each patient's serum and reference serum. If more than one plate is required for the batch of tests, only one complete series of reference sera needs to be tested, but add one of the reference sera to each additional plate used in the batch.

(2) With a capillary pipet, CAREFULLY fill each well with the appropriate serum to exactly the top of the agar when viewed from the side. This is best accomplished by introducing the filled pipet to the bottom of the well and slowly withdrawing the pipet as the well fills. Do not overfill, underfill, introduce bubbles with the serum, or touch the sidewalls of the well with the pipet as these procedures may result in distortions in the precipitate rings or invalid results. NOTE: With practice, the capillary pipets may be filled to the exact point to accurately and rapidly fill the wells. If may be advantageous to mark this point, once it is determined, on new pipets. Since well sizes vary between kits, be sure to doublecheck filled wells.

(3) Cover the plates, put them into the moist chamber, and incubate as specified by the kit manufacturer. NOTE: If room temperatures fluctuate outside of the range of 20° to 25°C, use a constant temperature incubator.

(4) After incubation, measure and record the outside diameters (to the nearest 0.1 mm) of the precipitate rings. NOTE: Slightly oblong rings may be measured by averaging the diameters of the shortest axis and longest axis. Grossly irregular rings should be discarded, and the test repeated. Faint rings may be enhanced by dipping plates in 7.5 percent acetic acid for a minute or two and washing gently with cold water.

(5) Prepare a reference curve by plotting the ring diameter (or average diameter) of each reference serum on the linear (horizontal) scale against its immunoglobulin concentration on the logarithmic (vertical) scale of two- or three-cycle semilogarithmic graph paper. For ease in reading unknown results, use as much of the paper as possible. Be sure to start labeling the bottom line of each logarithmic cycle with 1, 10, 100, or 1000 mg% as appropriate, and NOT with "zero". See figure 15-1 for an example of a reference curve. DO NOT use this curve.

(6) Draw a straight line to best fit the three or four reference points.

(7) Read the concentrations of the patient's sera from the reference curve. NOTE: If more than one plate is used and the value of the reference serum (one must be tested with each additional plate) does not fall on or very close to the reference curve, draw a line through the observed value parallel to the original curve to compensate for the variation in the individual plate. Then, read all patient's sera for that plate from the altered reference curve.

e. **Reporting Results.** Results may be reported in mg/100 ml of serum, or the recently proposed "Units of Activity" or International Units. In addition, the normal values for the test should be included on the report, because normals vary from kit manufacturer to manufacturer. In fact, ideally normal values should be developed by each laboratory, but one manufacturer's products and another's normal values should not be interchanged—they're probably significantly different.

f. **Sources of Error:**

(1) Use fresh serum for most reliable results. IgD appears to be especially altered by storage.

(2) Plates incubated longer than indicated by the manufacturer may yield invalid results. The diameters of the precipitate rings are proportional to the concentration

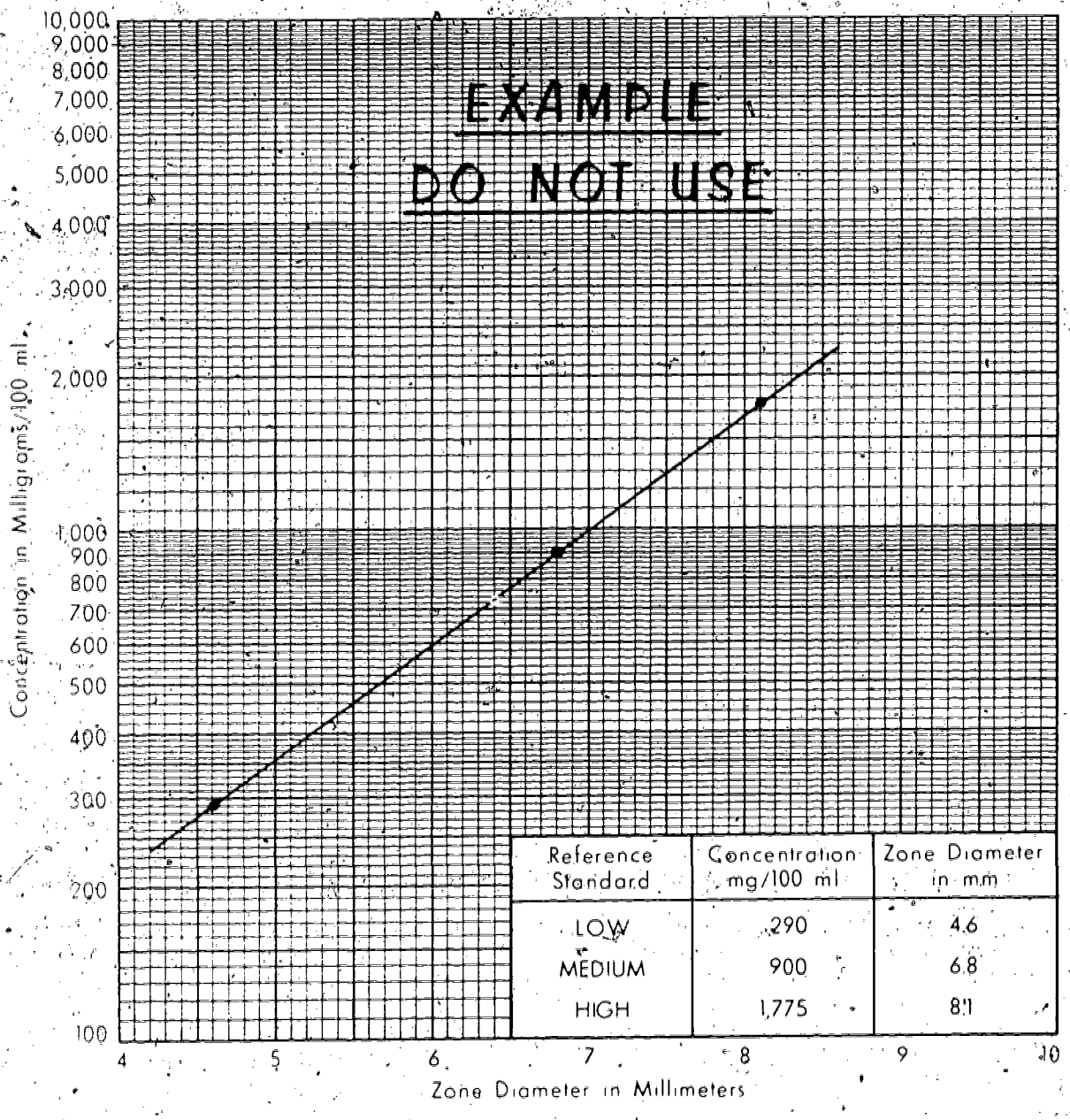


Figure 15–1. Example, Reference Curve for IgG.

of immunoglobulin at a given point in time, and the reactions are not allowed to go to completion.

(3) A complete reference curve must be prepared for each batch of tests to compenstate for daily variations in test conditions. In addition, but sure to test one reference serum in each additional plate used in a batch to detect individual variations between plates.

(4) For most valid results, the values of unknown sera should fall within the range of concentrations of the reference sera. Several manufacturers make kits for the detection of low levels of immunoglobulins. Excessively high levels of immunoglobulins may be assayed by first diluting the serum with isotonic saline and then multiplying the observed results by the appropriate dilution factor.

(5) Be sure to use constant-temperature incubators if temperatures fluctuate.

(6) Wells must be filled as specified, or invalid results may result.

g. **Discussion.** With minor variations in incubation times and temperatures, single radial

immunodiffusion tests are available commercially for all five immunoglobulins as well as the following proteins: albumin, transferrin, haptoglobin, ceruloplasmin, alpha-2-macroglobulin, complement C'3 (covered in the next section of this chapter) and a few others. In addition, some of the tests have been made more sensitive through the use of radioisotopes.

15-3. Interpretation of Results. Normal values of serum immunoglobulins vary over a wide range from individual to individual—even among apparently normal adults. More significantly, the age of the individual must be considered when interpreting results of these tests, because all immunoglobulins, except IgG, are essentially nonexistent at birth, but reach adult levels by the midteens or later. The exception, IgG, is at adult levels at birth, but then falls to low levels in a few months only to begin rising again to eventually reattain adult levels by the midteens.

a. Another important consideration in interpreting the results of these tests is that normal values vary, depending on which manufacturer's test kits were used. Ideally, each laboratory should develop its own normal values for each age and immunoglobulin. However, if developing normal values is not feasible for a given laboratory, the normal values of the specific kit manufacturer must be followed. Do not mix normal values between manufacturers; they might vary significantly.

b. Depending on the immunoglobulin and the age of the patient being tested, either an abnormally high or low serum level might indicate a pathologic condition. Whether a result is normal or abnormal must be decided in light of the normal values used by the individual laboratory. Consequently, the conditions listed below for each immunoglobulin are considered to be associated with a relative increase or decrease, the individual results must be analyzed with reference to the normals established by the individual laboratory.

(1) Conditions With Generalized Alterations of Immunoglobulin Levels:

Increased	Decreased
Infectious hepatitis	Nephrosis
Lupus erythematosus,	Malnutrition
Rheumatoid arthritis	Agammaglobulinemia, hereditary
	Hypogammaglobulinemia, transient or acquired
	Protein-losing enteropathy
	Malignant lymphomas

(2) Conditions With Altered IgG Levels:

Increased	Decreased
IgG-myeloma	Mongolism
Hyperimmunization	
Microbial infections	
Laennec's cirrhosis	

(3) Conditions With Altered IgM Levels:

Increased	Decreased
IgM-myeloma (rare)	Hepatomas
Trypanosomiasis	
Congenital infections (at birth)	
Waldenstrom's macroglobulinemia	

(4) Conditions With Altered IgA Levels:

Increased	Decreased
IgA-myeloma	Some normal individuals
Laennec's cirrhosis	Ataxia telangiectasia
	Gastrointestinal disease

(5) Conditions With Altered IgD Levels:

Increased	Decreased
IgD-myeloma (rare)	Importance unknown

(6) Conditions With Altered IgE Levels:

Increased	Decreased
IgE-myeloma (rare)	Importance unknown
Hay fever	
Allergic asthma	
Eczema	
Ascaris infestation	

SECTION B—COMPLEMENT

15-4. Significance of Complement. Complement (C') is a collective term for a group of eleven serum proteins that react sequentially in the presence of certain antigen-antibody complexes. Some of the components of C' are heat labile; thus heat inactivation of serum is a simple means of destroying complement activity for serologic tests where C' might interfere with results. In reactions with some antigen-antibody complexes, all eleven C' components may be involved; in others, only a few of the eleven are needed. Several types of antigen-antibody complexes can deplete one or more of the C' components from serum. In effect, antigen-antibody reactions inactivate complement as readily as heat does. This depletion of complement by antigen-antibody complexes is the basis for the complement fixation test described briefly in chapter 2. In addition to complement fixation, the activities of C' and its components have been studied in relation to several in vitro and in vivo situations. Several diseases are known that show alterations in either the activity or serum level of C' or its components. The detection of these variations is primarily by single-radial immunodiffusion.

a. **Lytic Activities of Complement.** One of the most widely studied functions of C′ is its role in the lysis of certain cells that have been reacted with specific antibody. For example, many gram-negative bacteria may be agglutinated by antibody alone, but complement, along with antibody, amy cause bacteriolysis. Antibodies and C′ are also involved in the in vitro lysis of several types of tumor cells and erythrocytes. In fact, hemolysis of antibody-coated erythrocytes has been used as an assay method for total C′ activity. The correlation of these in vitro C′ activities with similar in vivo activities is difficult, especially with regard to the role of C′ in immunity. However, C′ does seem to be involved in certain acquired hemolytic anemias, such as paroxysmal cold hemoglobinuria. In these cases, C′ activity in the serum drops significantly.

b. **Nonlytic Activities of Complement.** In addition to its lytic activities, C′ also takes part in several other antigen-antibody reactions. Some of these reactions would appear to have a significant effect on the immunity of an individual. For example, C′ has been found to be active in opsonization, immune adherence, and chemotaxis, all of which help promote the phagocytosis of invading microorganisms and other foreign particles. Increased permeability of blood vessels is another C′ associated observation that may enhance an individual's ability to combat disease by allowing antibodies and other antimicrobial factors to diffuse more readily to the invasion site.

c. **Diseases With Altered Complement Levels.** Total C′ activity has been studied in a wide variety of diseases, but very few of these diseases have abnormal C′ levels consistently enough to be useful diagnostically. Several infectious diseases have been found to have low total C activities late in the disease, but there are other more specific and practical means for diagnosing these infections. Although kits are available commercially for assaying total C′ activity, these tests have not gained wide acceptance. The problem is that a low total C′ level does not identify the abnormal component (or components), and frequently an individual component may be significantly abnormal while total C′ activity appears normal. In addition, the test must be performed immediately after the serum is collected. By contrast, the individual C′ component levels have been studied in only a few diseases, and C′3 is the only component for which the practical laboratory test is commercially available. C′3 is assayed by single radial immunodiffusion—the same test used for immunoglobulins in the previous section of this chapter. Although deficiencies in other C′ components have been related to certain diseases, only C′ deficiencies are covered in this manual.

(1) Acute Poststreptococcal Glomerulonephritis. Low serum levels of C′3 have been found in this disease. The low levels are most likely due to the immunologic reaction of antibodies and C′3 with tissues of the kidneys, especially the glomeruli. Both gamma globulin and C′3 have been found in glomerular tissue in this disease.

(2) Systemic Lupus Erythematosus (SLE). The low serum levels of C′3 in SLE is most likely due to the similar immunologic factors as discussed with acute poststreptococcal glomerulonephritis. The depression of C′3 levels becomes especially evident in SLE with renal involvement. C′3 levels may return to normal as the renal lesions heal. Sequential testing provides a method for following the severity of renal involvement in both SLE and acute poststreptococcal glomerulonephritis.

(3) Bacteremia by Gram-Negative Bacilli. A lower than normal level of C′3 is noted in severe cases of bacteremia caused by certain gram-negative bacilli. The depression of C′3 is especially correlated with a poor prognosis, such as a patient in shock or about to die.

(4) Chronic Liver Diseases. Diseases, such as chronic viral hepatitis and alcoholic cirrhosis, may have low C′3 levels, but levels return to normal with resolution of the disease.

(5) Graft Rejections. Low C′3 levels have been observed in severe cases of organ-transplant rejections. A progressively decreasing C′3 suggests a poor prognosis for retention of the graft.

15–5. Immunodiffusion Test for Complement C′3. This test is the same as the one given for immunoglobulins in paragraph 15–2, except that the agar gel contains antibody against complement C′3. Special emphasis should be made to use fresh serum for this test. Ideally, serum should be separated from the clot and tested immediately, but frozen serum may be used. All sources of error and other facets of the test have been covered in paragraph 15–2.

15–6. Interpretation of Results. Each laboratory should develop its own range of normal values for C′3, or as an alternative, should be sure to use the values specified by the kit

manufacturer. C'3 test results are more useful when the test is performed serially as the disease progresses. See paragraph 15-4c for diseases with altered C'3 levels.

Chapter 16

MISCELLANEOUS USES OF SEROLOGIC TESTS

16-1. Introduction. Although the most frequently performed serologic tests have been covered in the previous chapters of this manual, serologic tests for several other infectious and noninfectious diseases and conditions deserve at least brief mention. Some of these tests are applicable to relatively limited parts of the world; others are cosmopolitan in their potential use. Due to the requirement for sophisticated equipment and techniques, many of these tests are practical only for larger laboratories. However, some of these tests are within the capability of even the smallest clinical laboratory, but the decision to provide the test must be made in light of the number of requests for the test, the critical nature of a prompt result, the prevalence of the disease under study, and associated factors. This decision is made easier, because many of these tests are available commercially and have relatively stable reagents.

16-2. Tests for Bacterial and Spirochetal Infections. Most bacterial infections can be effectively diagnosed by isolating and identifying the causative organism, while in spirochetal infections, this is difficult or, as in syphilis, impossible. Even in diseases where isolation of the organism is possible, serologic tests can provide a valuable adjunct to diagnosis. In addition to the agglutination tests for certain bacterial and rickettsial diseases covered in chapter 5 and the tests for syphilis in chapter 6, serologic tests may prove useful in several other diseases of this type.

a. **Gonorrhea.** A recently developed latex test appears to be especially useful for the detection of chronic cases, such as asymptomatic females—the most difficult cases to diagnose by cultural methods.

b. **Leptospirosis.** Most serologic tests for this disease are based on the microscopic or macroscopic observation of agglutination of either live or formalinized spirochetes. Others that have been reported are fluorescent antibody, complement fixation, and latex tests.

c. **Melioidosis.** Although the causative organism of this disease can usually be readily isolated and identified by competent technicians, complement fixation and bacterial agglutination tests have proven useful in chronic and subclinical cases.

d. **Tuberculosis.** Although the immunologically based skin tests for tuberculosis are widely used, serologic tests seem to have limited application. Some of the tests that have been tried are: complement fixation, immunodiffusion, indirect hemagglutination, and agglutination tests using coated latex, bentonite, or kaolin particles.

16-3. Tests for Parasitic Infections. Most parasitic infections are detected by microscopic examination of clinical specimens for the parasite or some stage in its development, such as eggs or larvae. As a rule, these infections do not stimulate the production of antibodies unless the parasite invades the tissue. Another factor limiting the application of serologic tests in these diseases is that very few parasites stimulate the production of antibodies that are specific for the parasite. Consequently, only a few parasitic diseases have practical serologic tests.

a. **Amebiasis.** The diagnosis of extraintestinal amebiasis, especially amebic liver abscess, is a complicated problem. A wide variety of serologic tests have been applied to this problem, with varying degrees of success. Some of these tests are indirect hemagglutination, capillary precipitin, immunodiffusion, fluorescent antibody, latex, complement fixation, and immunoelectrophoresis tests. In addition, an intradermal skin test has been used.

b. **Malaria.** Because accurate speciation of malarial parasites is imperative, competent clinical microscopy may never be replaced as a diagnostic method for this disease. However, two types of serologic tests, fluorescent antibody and indirect hemagglutination, have been applied to the disease, but cross-reactivity between species (even nonhuman species of malarial parasites may be used as the antigen) limits the usefulness of these tests in clinical cases.

c. **Schistosomiasis.** Detecting the presence of blood flukes by finding their characteristic eggs in feces or urine provides the most definitive information in this disease. How-

ever, especially in light infections, chances of finding the eggs are frequently so low that a wide variety of serologic tests have been developed to aid in diagnosis. Several different antigens have been used in these tests, with some tests using antigens made from miracidia, cercariae, or the adult worms. Some of the tests that have been used are: complement fixation, fluorescent antibody with cercaria as the antigen, indirect hemagglutination, charcoal agglutination, precipitin, cercarial agglutination, immunoelectroadsorption, miracidial immobilization, and others. Some of these tests are species specific, while most just indicate the presence of unspecified blood flukes. There are also skin tests for this disease.

d. **Toxoplasmosis.** This disease is usually diagnosed by serologic means. Two types of antibodies are produced—persisting antibodies and short-lived ones. One of the tests for persisting antibodies is the classical Sabin-Feldman dye test; others are fluorescent antibody and indirect hemagglutination tests. Tests for short-lived antibodies, such as in newborn infants, are complement fixation, fluorescent antibody for IgM, and immunodiffusion tests. In addition, a skin test is available.

e. **Trichinosis.** This intramuscular parasitic infection is another disease that is diagnosed almost exclusively by serologic means. Consequently, a multitude of serologic tests have been developed for this disease. One of the more widely accepted tests has been the agglutination of antigen-coated bentonite particles. More recently, a test similar to the RPR card tests for syphilis has been described. In this card test, the antigen is adsorbed on cholesterol-lecithin crystals in a charcoal suspension. Some of the other tests that have been used in this disease are: complement fixation, precipitin, indirect hemagglutination, fluorescent antibody, and a variety of agglutination tests using particles, such as latex, collodion, and others. In addition, skin tests, which remain positive for years, are available.

16-4. Tests for Mycotic Infections. In general, fungi are not highly antigenic, and when they are, cross-reactivity severely limits the usefulness of serologic tests. However, a few serologic tests have been used in mycoses, mainly the systemic mycoses. The most useful test has proven to be immunodiffusion, but other methods apply to certain diseases. One of the more recently developed tests is the application of immunoelectroosmophoresis (IEOP) to the detection of fungal antibodies in the patient's serum. In addition, skin tests are available for most of the systemic mycoses, but these tests may be negative in proven cases.

a. **Blastomycosis.** In addition to the tests described above, complement fixation has been used for this disease, but a high percentage of proven cases have negative tests. Even in positive tests, cross-reactivity must be ruled out.

b. **Coccidioidomycosis.** Complement fixation and precipitin tests are additional tests that have been applied to this disease. A rising or falling complement fixation titer has special diagnostic and prognostic significance.

c. **Cryptococcosis.** In addition to the usual tests for cryptococcal antibodies in a patient's serum, a latex test, using antibody-coated latex particles, has been developed to detect cryptococcal antigens in the patient's serum or CSF. The tests for antibodies are based on fluorescent antibody, indirect hemagglutination, and whole-yeast-cell agglutination. By comparing changes in titers of both antigen and antibody tests, valuable information is gained.

d. **Histoplasmosis.** In addition to immunodiffusion and skin tests, some of the tests that have been used in diagnosing histoplasmosis are fluorescent antibody, latex agglutination, and complement fixation tests. Care must be taken in interpreting the results of these serotests performed on patients that have had skin tests, because skin tests may stimulate the production of detectable levels of antibodies.

16-5. Tests for Viral Infections. The definitive diagnosis of viral diseases relies almost exclusively on immunologic methods at some stage in the diagnosis. Even if the causative virus is isolated from clinical material, identification of virus is usually accomplished by immunologic means. Complement fixation tests are available for the majority of viral diseases. In certain viral diseases, tests based on fluorescent antibodies, neutralization or inhibition of viral hemagglutination may be used—or combinations of these methods. Unfortunately, except for detecting susceptibility to infection, such as to rubella during pregnancy, and in a few critically important diseases, the diagnosis of viral diseases usually has only academic or epidemiologic value, because a differential diagnosis rarely can be completed before the patient has either expired or is well on his way to recovery. In addition to rubella, which is

covered in chapter 14, a couple of viral diseases deserve brief mention.

a. **Rabies.** Because prompt initiation of immunization following bites by rabid animals is imperative, fluorescent antibody techniques for the detection of the rabies virus in the brains of suspected rabid animals have proven to be a valuable adjunct to microscopic demonstration of Negri bodies. Neutralization and complement fixation tests have been used to determine antirabies titers of immunized individuals and as an index of rabies activity in animal populations.

b. **Influenza.** In outbreaks of suspected influenza, isolating the causative virus and performing serologic tests on the first few cases of the outbreak are usually recommended. These tests help to specifically identify the strain of virus causing the outbreak, as well as to simplify the diagnosis of subsequent cases, without the requirement of testing all patients. For individual cases, testing for sera collected during the acute and convalescent stages of the disease will provide the most useful information. The serologic tests most frequently used for influenza are based on inhibition of viral hemagglutination, but complement fixation tests may also be used.

16-6. Tests for Noninfectious Conditions. Several of the serologic tests in this manual are for noninfectious conditions, such as pregnancy in chapter 13, lupus erythematosus in chapter 12, and others. Two other applications of immunologic tests will be described briefly.

a. **Hashimoto's Thyroiditis and Related Disorders.** A high percentage of individuals with Hashimoto's thyroiditis and primary myxedema, as well as lesser percentages of cases with certain other thyroid disorders, produce antibodies against antigens from their own thyroid glands. Antibodies may be produced against microsomal antigens or thyroglobulin from the gland. Most of the practical serologic tests are directed toward detection of thyroglobulin antibodies. Some of these tests are immunodiffusion, indirect hemagglutination, latex agglutination, and fluorescent antibody tests. As with any biological system, these tests may be positive in the absence of disease or negative when disease is evident, but high titers are usually significant.

b. **Fibrinogen Concentration.** Although more definitive tests for this clotting factor are available in the chemistry and hematology manuals, a latex agglutination test is also available for estimating fibrinogen concentrations.

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SUMMARY OF REVISED, DELETED, OR ADDED MATERIAL

This manual revises and updates serologic procedures; emphasizes the use of new reagents and procedures; and presents background information and procedures in a completely format.

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GLOSSARY

Acute Phase: The early stage of an infectious disease in which the symptoms, including elevation of temperature, are manifest. With most infectious diseases this phase includes the first 3–5 days after initial onset of illness.

Agglutination: The collection into clumps of the cells or particles distributed in a fluid.

Agglutinins: Antibodies which cause agglutination or clumping of bacteria or other cells in a suspension.

Antibody: A specific globulin which is produced in the body in response to the presence of an antigen and which reacts with the antigen in some observable way.

Antigen: Any substance which, when introduced into the blood or tissues, stimulates the formation of antibodies.

Antinuclear Antibodies: A collective term for antibodies against nuclear components of cells, frequently produced in systemic lupus erythematosus and other collagen diseases.

Antistreptolysin-O Titer: The level in the blood or tissues of specific antibodies against the streptolysin-O hemolytic factor which is produced by certain streptococci.

Beef Erythrocyte Antigen: Substance contained in beef erythrocytes which is capable of absorbing the heterophile antibodies produced as a result of infectious mononucleosis and serum sickness.

Cardiolipin Antigen: Substance composed of extract from fresh beef hearts combined with lecithin and cholesterol. This antigen is used in flocculation tests for syphilis.

Cardiolipin Microflocculation: A flocculation test for syphilis involving the reaction of syphilitic reagin with the cardiolipin antigen. The visible reaction appears as the aggregation of antigen particles to form floccules of varying size.

Chorionic Gonadotropin: A hormone produced by the placenta during pregnancy and by certain tumors.

Cold Agglutinins: Agglutinins which react optimally at low temperatures. Specifically, certain agglutinins present in some cases of primary atypical pneumonia.

Complement: A thermolabile, nonspecific protein substance in normal blood serum which, with specific antibodies, causes lysis of cells and other phenomena.

Complement Fixation: When antigen unites with its specific antibody, complement, if present, is taken into the combination and becomes inactive or fixed. Its presence or absence as free active complement can be shown by adding sensitized blood cells or blood cells and hemolytic antibodies to the mixture. If free complement is present, hemolysis will occur; if not, no hemolysis will be observed.

Control: A controlled system used to test the correctness of observations. In serological testing, controls are generally set up to check conditions and/or reagents.

Convalescent Phase: That stage of an infectious disease which immediately follows the cessation of clinical symptoms. In most infectious diseases this period is generally considered to be 10–14 days after the onset of illness.

C-Reactive Protein: A protein, not normally present in human blood, which appears in a wide variety of inflammatory conditions. It is characterized by its ability to react visibly with the C-polysaccharide somatic substance of the pneumococci.

Differential Heterophile: A test designed to differentiate between the three types of heterophile antibodies.

Febrile Agglutinins: Agglutinating antibodies produced by the body in response to various fever-producing organisms. Examples are antibodies directed against the causative agents of typhoid fever, paratyphoid, tularemia, undulant fever, and typhus.

Flocculation: A phenomenon in which particles dispersed in a medium combine into discrete, usually visible aggregates.

Forssman Antibody: One of the heterophile antibodies which is naturally present in the blood of man in low titer.

Guinea Pig Kidney Antigen: A substance used in the differential heterophile test. It absorbs the Forssman and serum sickness antibodies. The infectious mononucleosis antibody is absorbed to a much lesser degree or not at all.

Hapten: That portion of an antigenic molecule or antigen complex that determines its immunological specificity. It usually does not stimulate antibody formation by itself, but reacts specifically in vivo and in

vitro with the antibody once it is formed.

Hemagglutination: The agglutination (sticking together) of red blood cells.

Hemagglutination-Inhibition: The prevention of agglutination of red blood cells by specific antibodies.

Hemagglutinin: A substance which causes the agglutination of red blood cells.

Hemolysin: An antibody capable of causing the lysis or dissolution of blood cells.

Heterophile Antibodies: A group of antibodies having an affinity for antigens found in a wide range of organisms. All heterophile antibodies have the common property of agglutinating sheep erythrocytes.

Immunity: The acquired or inherited resistance of an individual to infection.

Immunoglobulins: A collective term for the serum proteins that possess antibody activity.

Inactivation: The process by which the activity of serum complement is nullified. The usual means is to heat the serum to 56°C for 30 minutes.

Infectious Mononucleosis: An acute infectious disease characterized by a sudden onset and acute course, with fever and inflammatory swelling of the lymph nodes, especially those of the cervical region. There is a moderate leukocytosis due almost entirely to abnormal mononuclear cells.

Influenza: An acute infectious respiratory disease of viral origin, characterized by sudden onset, fever, and respiratory symptoms. It derives its name from the fact that in ancient times it was believed to be due to the bad "influence" of the stars.

In Vitro: Within a glass; observable in a test tube.

In Vivo: Within the living body.

Lupus Erythematosus: One of the collagen diseases, ranging from a mild skin eruption to a rapidly fatal systemic disease, frequently accompanied by the production of antinuclear antibodies and LE cells.

Lyophilization: The creation of a stable preparation of a biological material by rapid freezing and dehydration of the frozen product under high vacuum.

Lysin: An antibody which has the power of causing dissolution or lysis of cells.

Lysis: The destruction or dissolution of cells by the action of a specific lysin.

Neutralization: An antigen-antibody reaction in which the reactive effect of a particular antigen is nullified by a specific antibody.

Postzone Reaction: A weak or irregular antigen-antibody reaction occurring when a great excess of antigen is exposed to a serum containing a relatively low titer of antibody.

Precipitation: An antigen-antibody reaction in which a soluble antigen is caused to settle out by the action of its specific antibody.

Presumptive Heterophile: A test to determine the presence of increased amounts of heterophile antibodies.

Prozone Reaction: A negative or weak antigen-antibody reaction occurring when serum containing an extremely high titer of antibody is exposed to a relatively small quantity of antigen.

Reagin: An antibody-like substance produced by the body in response to certain types of tissue invasion and destruction. It is found in varying small amounts normally, but is usually increased in syphilis, malaria, and certain other diseases.

Reticuloendothelial System: Cells of the body that show a common phagocytic behavior toward foreign particles. This group includes endothelial and reticular cells of the spleen, lymph, liver, and bone marrow. These cells are considered to be the primary site of antibody production.

Rheumatic Fever: A disease, probably infectious, associated with the presence of hemolytic streptococci in the body. Beginning with an attack of sore throat or pharyngitis, there develop chilliness, rapid rise of temperature, prostration, and painful inflammation of the joints.

Rheumatoid Arthritis: A disease of the joints that may lead to immobilization of the affected joints and severe deformity.

Rheumatoid Factors: Antibodies that react with gamma globulins, frequently produced in rheumatoid arthritis and similar diseases.

Rubella: A viral exanthematous disease, also called German or 3-day measles.

Serial Dilution: Progressively higher dilutions of a substance arranged in a definite sequence or series.

Serology: The branch of biology which concerns itself with antigens and antibodies and their relationships.

Serum Sickness Antibody: Specific antibody produced in response to a foreign serum, especially if an illness results from the introduction of the foreign serum.

Specificity: The special affinity of antigens for their corresponding homologous antibodies.

Streptolysin-O: An oxygen-labile, hemolytic

factor produced by certain streptococci.

Titer: An expression of the highest dilution of a substance which contains sufficient antibody to give a visible reaction.

Virus: A parasitic microorganism, smaller than most bacteria, and capable of multiplication only within a living susceptible host cell.

Weil-Felix Reaction: The diagnostic agglutination of Proteus OX bacteria by the sera of typhus fever cases due to the presence of an antigen in the bacteria common to that found in the causative rickettsial organisms.

Widal Test: A procedure designed to detect antibodies, if present, against the causative organisms of typhoid fever.

PARTIAL LIST OF MANUFACTURERS OF SEROLOGIC REAGENTS

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INDEX

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