HINTON FLOCCULATION TEST FOR SYPHILIS
EQUIPMENT AND GLASSWARE

A. General Equipment

1. Centrifuge with tachometer.
2. Racks, test tube.
3. Water baths, one 37°C and one 56°C.
4. Kahn shaking machine (275 - 285 oscillations per minute, with 1 1/2 inch stroke), or other shaker. A Cenco Meinze laboratory shaker is satisfactory for small laboratories.
5. Interval timer.
6. Maximum and minimum thermometer.

B. Glassware

1. Test tubes, 100 x 11½ mm. O.D. (manufactured by Kimble and described as 45060/S73) *
2. Pipettes, Serological, deliver to the tip.
   1.0 ml. graduated in hundredths.
   5.0 ml. graduated in tenths.
   10.0 ml. graduated in tenths.
3. Flasks, Erlenmeyer, with inverted V-shaped ridge in bottom that produces two semicircular compartments (so-called Hinton flasks).
   125 ml.
   250 ml.
4. Flasks, Erlenmeyer, glass-stoppered.
   500 ml.
   1 liter
5. Graduated cylinders.
   100 ml.
   250 ml.
   500 ml.
   1 liter
6. Filter funnels, fluted, 200 mm.

*Tubes 100 x 13 mm. O.D. are not as desirable as the small ones because with the weakly-positive serums the reactions are much more difficult to read.

HINTON TEST

REAGENTS

1. Hinton Indicator. (See page , "Preparation of Hinton Indicator").
2. 5% sodium chloride solution.
   a. Weigh 5 gms. of previously dried sodium chloride (A.C.S.).
   b. Add sodium chloride to 100 ml. of freshly distilled water and heat solution in an autoclave at 15 pounds pressure for 15 minutes.
   c. Store salt solution in glass-stoppered bottles at room temperature. This solution may be used for three weeks
3. 50% solution of glycerine.
   a. Mix equal volumes of Baker and Adamson's Glycerin (Reagent) and distilled water. This solution keeps indefinitely.

PREPARATION OF SERUM

1. Remove serums from clots by centrifuging and pipetting or decanting.
2. Heat the serums in the 56°C. water bath for 30 minutes. Serums should not be heated before the day of testing. If it is necessary to retest a specimen, use serum freshly separated from the clot if available.
3. Recentrifuge any specimen in which visible particles have formed during heating.

PREPARATION OF GLYCERINATED HINTON INDICATOR

1. Pipette one part of Hinton Indicator into one compartment of a Hinton flask.

   (Note: Not less than 1 ml. or more than 5 ml. of Hinton Indicator should be mixed at one time.)

2. Pipette 0.6 part of 5% sodium chloride solution into the other compartment of the flask. Care should be used in pipetting the salt solution into the flask in order to avoid premature mixing of these solutions.
3. Mix by shaking the flask very rapidly from side to side for one minute.
4. Let the mixture stand exactly five minutes.
5. Add 13.4 parts of 5% salt solution and shake thoroughly.
6. Add 15 parts of 50% glycerol solution and shake thoroughly until the suspension is homogenous.
7. Store in a glass-stoppered bottle or flask in the refrigerator. This suspension, referred to as glycerinated indicator solution, remains usable for at least three weeks.

STANDARD HINTON TEST WITH SERUM

1. Arrange test tubes (100 x 11 mm. O.D.) in suitable racks so that there is one tube for each serum to be tested and for positive and negative serum controls. Number tubes to correspond to the identifying number of serums.
2. Pipette 0.5 ml. of each heated serum into its corresponding tube.

   (Note: Occasionally very strongly-positive serums will elicit a negative reaction when 0.5 ml. of serum is employed as the testing quantity. When this type of reaction is suspected, 0.1 ml. serum should also be tested in addition to the 0.5 ml. quantity of serum.)
3. Pipette 0.5 ml. of the glycerinated Hinton Indicator into each serum tube.

   (Note: Flask containing glycerinated Hinton Indicator should

be shaken when taken from refrigerator. Remove quantity of glycerinated indicator needed and return flask to cold storage immediately.)

4. Shake rack of tubes on shaking machine for 5 minutes.

5. Remove the rack from the shaking machine and place in 37°C. water bath for 16 hours.

(Note: The water bath must not be covered during this period. The bath should be equipped with a maximum and minimum thermometer, and the temperature should not fall below 34°C. nor rise above 39°C. for reliable tests.)

READING AND REPORTING

1. Place a shaded cylindrical fluorescent electric bulb 18 or more inches long in front of a darkened background. The bulb should be about 10 - 14 inches above the level of the eyes and 5 - 7 inches in front of them.

2. Remove each tube from the rack carefully without disturbing contents.

3. Hold the tube at a 45° angle in front of the eyes and on the level with them.

4. Look for clarification of the fluid and for presence or absence of a ring of white flakes or white coarse granules at the meniscus.

5. Lift the tube about 10 inches above the eyes, tilt it so that it is almost parallel with the bulb, and, while slightly agitating the tube, look through it towards the darkened background to determine the presence or absence of flocculation and record positive or negative findings.

Positive: Those reactions demonstrating white flakes or white coarse granules at the meniscus, and definite flocculation when tubes are gently shaken.

Negative: Those reactions demonstrating absence of ring or

band of floccules, and no flocculation or granularity when tubes are gently shaken. Occasionally a non-granular scum at the meniscus may be caused by faulty technique. Hemolyzed serums bacterially contaminated frequently produce a whitish ring which is strongly adherent to tube.

6. Centrifuge all tubes in which clear-cut negative or positive readings cannot be made at 2000 r.p.m. for 5 minutes.
7. Remove tubes from centrifuge and read as described in No. 5, recording only doubtful and negative findings as follows:

   Doubtful: Those reactions demonstrating coarse granulation at the meniscus and definite flocculation when tubes are gently shaken.

   Negative: All specimens failing to react as described under "Doubtful" above.

8. Report those specimens as "unsatisfactory" which are hemolyzed or bacterially contaminated, unless the reaction is strongly positive.

RAPID MINIOR TEST WITH SERUM

1. Heat fresh serum for 3 minutes at 60°C.
2. Arrange test tubes (100 x 11½ mm. O.D.) in suitable racks so that there is one tube for each serum to be tested and for positive and negative serum controls.
3. Pipette 0.5 ml. of serum to be tested into properly numbered test tube.
4. Add 0.5 ml. of glycerinated indicator to each serum tube and shake on shaking machine for 10 minutes.
5. Remove rack of tubes from shaking machine and place in the 37°C. water bath for 20 minutes.

6. Remove tubes from water bath and centrifuge tubes at 2000 r.p.m. for 10 minutes.
7. Remove tubes from centrifuge without agitating contents.
8. Read each tube as described under "Reading and Reporting".
9. Report in accordance with the following outline:

Positive: Those reactions demonstrating plainly visible flakes at the meniscus, and well-marked flocculation when tubes are gently shaken.

Negative: Those reactions demonstrating absence of ring or band of floccules at the meniscus, and no flocculation when tubes are gently shaken.

Unsatisfactory: Those specimens hemolyzed or bacterially contaminated, unless the reaction is strongly positive.

(Note: Despite very close agreement between the results by the Standard Technique and the Rapid Technique, it is best to place the tubes in the refrigerator until such a time as they can be placed in a water bath for sixteen hours and then read as Standard tests.)

PREPARATION OF STOCK INDICATOR

A cholesterolized alcoholic extract of beef heart muscle is employed. Bacto-beef heart, prepared by the Difco Laboratories, Detroit, Michigan, may be used.

1. Weigh 100 grams of beef heart powder and transfer to a one liter glass-stoppered Erlenmeyer flask.
2. Add 400 ml. pure anesthesia ether and shake thoroughly by hand for 10 minutes.

3. Allow powder to settle, then pour off ether through filter paper, discarding this and all other ether extractions.

4. Scrape moist powder from filter paper and return to original flask.

(Note: Do not allow main portion of extracted tissue to dry between extractions.)

5. Add 400 ml. pure anesthesia ether to the moist powder and repeat extractions as before.

6. Repeat ether extractions for a total of five extractions.

7. Remove powder from flask, dry thoroughly, and weigh.

8. Place dry powder in a glass-stoppered bottle with 95% ethyl. alcohol, using 5 ml. of alcohol for each gram of powder.

9. Extract for three days at room temperature, and shake contents of flask vigorously by hand 3 times each day.

10. Filter alcoholic extract through fat-free filter paper into a clean glass-stoppered flask.

11. Add 0.4 gm. of cholesterol (Merck's C.P.) to every 100 ml. of alcoholic extract.

12. Warm stock indicator (cholesterolized alcoholic extract) in a 37°C. water bath until the cholesterol is in solution.

13. Stock indicator should be tested against an indicator found to be wholly satisfactory as determined by both clinical and serologic means.

(Note: Occasionally, adjustment of the lipoidal concentration is necessary for the preparation of a satisfactory stock indicator. A procedure has been described whereby lipoidal concentration can be changed as may be required.[1])

1. Harris, Ad; Rosenberg, A.; Bossak, H.N.: Standardization of Kinton Indicator; Ven. Dis. Inform., 23:263-265, 1942.

QUANTITATIVE TEST

For each specimen arrange a series of eight clean test tubes in a rack.

Prepare serial dilutions of serum, :0,1:2,1:4,1:8,1:16,1:32,1:64, 1:128, as follows:

Pipet 0.5 cc of physiologic salt solution into each of the last seven tubes, leaving the first tube empty.

Then pipet 0.5 cc of inactivated serum into the first tube and 0.5 cc of serum into the second tube. Mix the contents of second tube by drawing the fluid into the pipette and expelling it several times.

Transfer 0.5 cc to the third tube, mix and transfer 0.5 cc to the fourth tube. Repeat this procedure for the remaining four tubes and then discard 0.5 cc of the contents of the eighth tube.

Add 0.5 cc of glycerinated indicator to each tube and proceed according to technique of Standard test.

The results are reported according to dilution.

Any degree of positivity is recorded as positive. It is very similar to the method of reporting as recommended by Harris.*

* J. of Ven. Dis. Inform., Vol. 28, Nov. 1947, No.11

INDICATORS PREPARED FROM CARDIOLIPIN

Stock Hinton Indicators have been prepared from cardiolipin and lecithin manufactured according to the direction of Pangborn, and the tests performed with these indicators thus far have been more accurate than those performed with indicators made from extracts of beef heart. Our experience has been confined to the use of three batches of lecithin and cardiolipin prepared in Dr. Pangborn's laboratory and one batch prepared in the Lederle Laboratories. Until cardiolipin and lecithin are obtainable commercially of specified concentration and potency, it will be impossible to give a brief description of how to adjust the amounts of these substances for optimum results. The stock indicator prepared from cardiolipin, lecithin, and cholesterol is diluted exactly as it is if a dilute indicator were being prepared from the standard stock Hinton indicator.

DAVIES-HINTON FLOCCULATION TEST
for
SPINAL FLUID

REAGENTS.

1. Glycerinated Hinton Indicator (See Page ____, PREPARATION OF GLYCERINATED HINTON INDICATOR).

2. Hinton negative human serum.

   a. Select one of more clear Hinton negative serums and retest in accordance with the RAPID HINTON TEST technic, (See page____), employing the following two indicated quantities.

   Tube 1.-0.5 ml. serum and 0.5 ml. glycerinated Hinton indicator
   Tube 2.-0.1 ml. serum and 0.5 ml. glycerinated Hinton indicator

   (Note: When large numbers of spinal fluids are tested it is convenient to pool, Seitz filter, merthiolate (1-10,000), and perform Rapid Hinton test as above. Store at 8-10°C. for not more than 3 weeks. Avoid the use of cloudy serum.)

3. Gum acacia, 20 percent solution.

   a. Put in a 4-oz. bottle* 20 gms. of white powdered gum acacia (USP).

   b. Add 100 ml. of 3 percent salt solution in distilled water.

   c. Place bakelite cap on bottle, but do not screw on.

   d. Place bottle in the autoclave and maintain for 15 minutes at 15 lbs. of pressure.

   e. Remove from autoclave, screw cap on bottle, shake well to completely dissolve the acacia and maintain in a sterile condition.

   * Flint glass bottles with bakelite screw caps with vinylite liners are very satisfactory for the preparation of the acacia solution.

PRELIMINARY TEST OF HINTON NEGATIVE SERUM-GUM ACACIA SOLUTION MIXTURE.

1. For every ten spinal fluids to be tested mix 5 ml. Hinton negative serum with 5 ml. 20 percent gum acacia.

2. Perform a rapid test as follows:

   a. Into a tube, pipette 0.6 ml. of 0.85 percent sodium chloride solution, 0.2 ml. of freshly mixed acacia-serum mixture, 0.2 ml. glycerinated Hinton indicator and mix well.

   b. Place tube in a 37°C. water bath for 30 minutes.

   c. Centrifuge tube at 2000 r.p.m. for 5 minutes.

   d. A satisfactory acacia-serum mixture yields a negative reaction.

PREPARATION OF SPINAL FLUID.

Centrifuge and decant spinal fluid. Fluids which are visibly contaminated with bacteria are unsatisfactory for testing. A centrifuged bloody spinal fluid should be reported as unsatisfactory if it yields a positive reaction.

SPINAL FLUID FLOCCULATION TEST.

1. Arrange test tubes (100 x 11½ mm. O.D.) in 4 rows so that there are 4 tubes (one behind the other) for each spinal fluid to be tested and for positive and negative spinal fluid controls. Number tubes to correspond to the identifying number of each fluid.

2. For each specimen pipette 0.6 ml. of spinal fluid into correspondingly numbered tube in first row, 0.4 ml. into tube

in second row, 0.2 ml. into tube in third row, and 0.1 ml. into tube in last row.

3. Add 0.2 ml. of acacia-serum mixture to every tube.
4. Add 0.2 ml. of glycerinated Hinton indicator to every tube.
5. Shake racks of tubes vigorously until contents become completely homogeneous.
6. Place racks of tubes in a 37°C. water bath for 16 hours.
7. Remove all tubes from water bath and centrifuge at 2000 r.p.m. for 5 minutes.

READING AND REPORTING.

1. Remove tubes gently from centrifuge without disturbing contents.
2. Before a suitable artificial light, (see READING AND REPORTING of Hinton tests), tap each tube gently at the base while holding it near the top.
3. Report as POSITIVE all reactions that show definite floccules dispersing downward from the meniscus in either tube.
4. Recentrifuge all other tubes at 2000 r.p.m. for 5 minutes.
5. Remove tubes from the centrifuge and examine with tapping as previously described.
6. Report as follows:

POSITIVE: Definite floccules dispersed downward from the meniscus in either tube.

DOUBTFUL: Questionable flocculation in either tube.

NEGATIVE: Absence of flocculation with a ground glass appearance.

DAVIES-RENTON MICROFLOCCULATION TEST

In order to facilitate carrying out this test, specimens of blood should be collected in special rubber-capped glass collection tubes which are then placed in labeled glass tubes that measure approximately 100 mm. in length and 15 mm. in diameter, such tubes being ordinarily called Wassermann tubes. The collection tubes are made of glass tubing and measure 60 mm. in length by about 2.5 mm. in inside diameter. Over each end there should be a flexible rubber cap such as is used to stopper small bottles of vaccine or serum.* The capacity of the tube is about 0.3 cc.

Number the Wassermann tubes containing the specimens and place them in a serologic rack. Take each specimen in its collection tube from the rack, remove the rubber cap from one end, and loosen the clot from the wall of the tube with the stylet of an 18-gauge needle or with some other stiff wire. Replace the rubber cap on the collection tube and return it to the numbered Wassermann tube; centrifuge at high speed for 10 minutes. Examine each specimen so handled to see if the serum is clear and well separated from its clot; if the serum is not clear, recentrifuge for another 10 minutes. If this still does not clear it, make a note of the condition of the serum. Place the rack containing the clear specimens (clot downwards) in a serologic water bath kept at 56° C., preferably for 30 minutes, or if in a hurry 20 minutes, having first filled the Wassermann tubes with water from the water bath. Next, remove the rack from the water bath, pour the water from the tubes, remove the cap from the serum end of each collection tube and notch it just above the junction of clot and serum with a file.

*These rubber caps may be obtained from the West Company, 1117 Shackamaxon St., Philadelphia, Pa. Specify No.3 vial stoppers in No. 68 Stock.

Hold the collection tube horizontally, break it, and discard the part of the tube that contains the clot. Proceed with the part containing the clear serum as follows:

1. Drain into another glass collection tube enough serum so that the length of the column of serum is about 2.5 cm., and drain into a second similar glass tube enough serum so that the column is 0.5 cm. to 1.0 cm. long.

2. With a capillary pipette, add to the first tube glycerinated Hinton indicator equal to the amount of serum in the tube as estimated by the combined length of the column of serum and indicator. Care should be taken not to allow air to separate the serum and Hinton indicator, which would prevent mixing.

The capillary pipette consists of a piece of glass tubing about 10 or 12 cm. long and 1 cm. in diameter. One end is tapered to a capillary tip about 1 mm. or less in diameter; the other end is capped with a rubber bulb. Provided no serum is drawn into the capillary pipette, it need not be rinsed between specimens.

3. To the second tube, with the same capillary pipette, add diluted Hinton indicator equal to 5 times the amount of the serum as estimated by the combined length of the column of serum and indicator.

4. Mix the serum and the Hinton indicator in each tube by tilting the liquid toward alternate ends of the tube 10 times.

5. Cap both ends of the two collection tubes, return one to the original numbered Wassermann tube and the other to a Wassermann tube correspondingly numbered, and place the rack in a 37° C. serologic bath for 16 hours, having first filled the Wassermann tubes with water from the bath.

6. Remove the rack from the water bath and centrifuge the tubes for 5 minutes at approximately 2,000 revolutions per minute.

7. Read the results with a low-power objective of the microscope. The light should be cut down by lowering the condenser so that aggregates at the meniscus can be seen readily. Moreover, the stage of the microscope should be tilted at an angle of about 30° from the horizontal and the tube placed under the lens so that the meniscus is uppermost. Readings are designated as follows:

POSITIVE, if there are definite, discrete, compact clumps at the meniscus in either tube. Gently thumping of the tube may help float the clumps into view.

DOUBTFUL, if there are a few small clumps at the meniscus. In such cases, the clumps should be broken up by thumping the tube with finger and the tube recentrifuged for 3 minutes. The test is reported "doubtful" if small clumps are again visible at the meniscus and "positive" if large, compact clumps are present in either tube.

NEGATIVE, if there are no clumps at all. Amorphous, cloudy, granular material at the meniscus should be interpreted as negative.

UNSATISFACTORY, if badly hemolyzed or contaminated specimens give no marked flocculation in either tube.

The technic described above requires about 0.1 cc. of serum; smaller amounts of serum (0.05 cc. or more) may be handled in the same way in capillary tubes,* measuring 10 or 11 cm. in length and 1.25 to 1.5 mm. in inside diameter, except that the tubes are sealed with a small gas flame instead of rubber caps.

* Capillary tubes of these specifications may be obtained from Friedrich & Dimmock, Millville, N.J.