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THE HINTON AND DAVIES-HINTON TESTS FOR SYPHILIS

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Introduction

In the detection of syphilis the Hinton and Davies-Hinton tests are of special value because of (1) the volume of output by the regular method; (2) quick results in emergencies by the rapid method; (3) the small amounts of blood required by the Davies-Hinton microflocculation test; (4) easy examination of spinal fluids by the Davies-Hinton test.

HINTON TEST FOR SYPHILIS. THIRD MODIFICATION

This test requires precision in execution because consistently accurate results cannot be obtained if minor variations in technic are allowed.

Equipment

1. Test tube racks. To simplify numbering and pipetting serums, these racks should be constructed to hold 10 or 20 tubes in a row.
2. Serum tubes 100 mm long, with an approximately uniform inside diameter of 10 mm.
3. A water bath for inactivating serums.

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* The reliability of this test in comparison with others is authoritatively shown in a series of papers by the American Committee on the Evaluation of Serodiagnostic Tests for Syphilis. This Committee has acted impartially. Nevertheless, its figures and statements have been subject to biased interpretations by individual writers. Therefore, a careful study of these original reports is highly desirable in selecting serologic tests for syphilis. [1,2,3,4].

4. A Wassermann bath or a bacteriologic warm air incubator. The former is preferred because by its use the test is somewhat more sensitive. The water in the bath should be changed frequently to prevent a deposit from sticking to the outside of the tubes.
5. A centrifuge with a speed of over 2,000 revolutions per minute.
6. A maximum and minimum thermometer.
7. Graduated 100-cc and 250-cc cylinders to measure the reagents.
8. Dropping pipettes with rubber bulbs of about 5 cc capacity for drawing off serums.
9. Serologic pipettes of 1.0 cc capacity, graduated in tenths to the tip, to measure serums, and 5-cc or 10-cc serologic pipettes to measure reagents.
10. Thick-walled Erlenmeyer flasks[5] of 125-cc or 250-cc capacity, with an inverted V-shaped ridge blown in the bottom, for mixing glycerinated indicator. This ridge produces two semicircular compartments, each of which holds 3 to 5 cc in flasks with a capacity of 125 or 250 cc. These flasks (figure I) are not listed in any of the catalogues; ours are especially made by Macalaster Bicknell Company, of Cambridge, Mass.

(INSERT PICTURE OF FLASK AS FIGURE I)

Preparation of Stock Solutions

Stock indicator. -- Extract dried, ground, beef-heart muscle (Bacto beef heart, dehydrated, Difco Laboratories) by putting 100 grams* of the powder and 400 cc of anesthesia ether in a wide-mouthed glass-stoppered bottle and shaking thoroughly by hand for 10 minutes. Allow the bottle to stand 5 to 10 minutes so that the solid material may settle out. Then pour as much of the ether as possible through filter paper into an Erlenmeyer flask without pouring out any large quantity of the solid material. Scrape the solid material from the filter paper into the bottle for further extractions. Do not allow the main portion of extracted tissue to dry between extractions. Discard the filtrate in the Erlenmeyer flask. Make five separate extractions in all, using 400 cc of fresh ether and a new filter paper for each. After the final extraction let the tissue dry on the filter paper. Obtain the net weight of this dried residue of ether-insoluble constituents. Place this dried residue in a glass-stoppered bottle with 95 percent ethyl alcohol, using 5 cc of alcohol to each gram of residue. Extract for 3 days at room temperature (17° to 20° C.), shaking the contents of the bottle vigorously by hand for 5 minutes three times each day. Remove the tissue by filtering into a graduated cylinder; measure the alcoholic extract and transfer to a glass-stoppered bottle. Add cholesterol (using 0.4 gm to each 100 cc.) and warm at 37° C. in an incubator or water bath, occasionally shaking, until the cholesterol has dissolved. A solution thus prepared is called stock indicator (antigen).

* The extraction in one operation of larger or smaller amounts of the powder has appeared to yield an inferior reagent.

Warning is given that the best results can be assured only if a stock indicator as prepared above has proved to be accurate in comparative tests with an indicator that is wholly satisfactory as determined by both clinical and serologic means. Materials designated for this test by the Difco Laboratories of Detroit, Michigan, have met this critical requirement.

> The stock indicator should not be stored in a refrigerator, for chilling will precipitate the cholesterol; if, by inadvertent chilling, the cholesterol should precipitate, it must be redissolved (by heating in a water-bath at 37° C.) Stock indicator kept in colorless, glass-stoppered bottles at room temperature for more than 2 years has given as good results as that freshly prepared.

Five percent salt solution. -- Prepare a 5 percent solution of sodium chloride (C.P.) in sterile distilled water, and add 1.0 gm of salicylic acid (C.P.) to each 4500 cc.

> The salicylic acid helps to preserve the potency of glycerinated indicator (described later).

Fifty percent solution of glycerol. -- Prepare by mixing equal volumes of Baker & Adamson's Glycerin (Reagent)* and sterile distilled water.

> The 5 percent salt solution and the 50 percent glycerol solution keep indefinitely.

Glycerinated indicator. -- Directions for mixing this should be followed strictly. Thirty cc is the smallest and 150 cc the largest amount that can be satisfactorily prepared at one time. If larger quantities are required, two or more batches should be pooled. If kept in a refrigerator at a temperature of about 8° C., it will remain unimpaired in strength one month and sometimes longer. This stability of the

* This is an especially pure redistilled glycerin.

glycerinated indicator is extremely advantageous for emergency work and for small laboratories.

Prepare the glycerinated indicator as follows. -- Pipette one part of the cholesterinized heart extract into one compartment of the Erlenmeyer flask (with the inverted V-shaped ridge), and 0.8 part of the 5 percent salt solution into the other.

Use great care, when pipetting the salt solution into the flask to avoid admixture of the two solutions. A 125-cc flask is suitable for the preparation of 30 to 60 cc of glycerinated indicator and a 250-cc flask for 90 to 150 cc.

Mix by shaking the flask very rapidly from side to side for one minute. Let the mixture stand exactly 5 minutes. Without further delay add 13.2 parts of the 5 percent salt solution and shake thoroughly. Finally, add 15 parts of the 50 percent glycerol solution and shake until the suspension is homogeneous.

Performing the Tests

1. Centrifuge the blood, if necessary, to aid in separating the serum from its clot; with a long dropping pipette remove the serum (free from blood cells) and deliver into an appropriately labelled serum tube.

To avoid contamination of one serum by another, after each serum has been drawn off, thoroughly rinse the dropping pipette at least three times with sterilized physiologic salt solution. To minimize bacterial contamination, after every 20 serums have been drawn off empty the washing bottle (about 200 cc capacity) and fill it with fresh salt solution.

2. Heat the serums in the inactivating bath at 55° C. for 30 20 minutes. Be sure that the level of the water is above the level of every serum and that the temperature is kept at 55° C. or 56° C. throughout inactivation. Errors may result if this varies even one or two degrees. Serums should be inactivated on the day of testing. If it is necessary to retest a specimen, use serum freshly separated from the clot.

3. Select all serums that show (a) hemolysis, manifested by redness greater than is produced when 0.1 cc of blood is dissolved in 3.0 cc of distilled water; (b) bacterial contamination, shown by cloudiness; or (c) marked opacity from other causes. Place in a separate rack and test, as soon as possible, according to the rapid method (page ?). This will avoid further deterioration, which by decreasing the sensitiveness of the test and increasing the opacity of the medium makes the test harder to read.

4. Set up the racks with one properly numbered serum tube for each remaining specimen. Tubes should be clear and clean. *

5. With a 1.0 cc pipette, measure 0.5 cc of each heated (inactivated) serum into the tube that has been labelled for it. (Use a separate pipette for each serum.)

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* To clean the tubes, rinse them thoroughly as soon after use as possible with tap water, and then fill each with a warm solution of 5 gm of sodium hydroxide in 1,000 cc of tap water; allow them to stay in this solution for about two hours and wash thoroughly with hot water to remove the alkali. This process usually removes (without the aid of a test-tube brush) any deposit which may have remained from previous use.

For routine purposes one tube is sufficient for each test. If, however, this test is negative and there is reason to suspect syphilis, it is desirable to retest the specimen using 0.1 cc in one tube and 0.5 cc in the other. We have found that in approximately one syphilitic out of 200, the second tube, containing the 0.1 cc of serum, gives a positive reaction, while the first, which contains the 0.5 cc of serum, gives a negative reaction.

If less than ten tests are to be made at one time, use positive and negative controls.

6. Compare the appearance of each pipetted serum with that in the tube from which it was taken. This is to make sure that there has been no error in pipetting or in labelling the tubes.

7. Not more than thirty minutes before incubation, add with a clean 10 cc pipette 0.5 cc of glycerinated indicator to each serum.

8. Pipette 0.5 cc of the same indicator and 0.5 cc of the 5 percent salt solution into an empty serum tube. This serves as a control, the use of which will be indicated later (page 16).

9. Incline the rack to an angle of about 45° and then shake by thrusting it quickly upward and forward, then downward and backward with sufficient speed to cause the fluid to travel halfway up the tubes and make small bubbles. At least 3 minutes of shaking are required for accurate results. If there are enough tests, a shaking machine is desirable.

10. Place the rack in the Wassermann bath or incubator at 37° C. and let it remain for 16 hours (conveniently from 5 p.m. to 9 a.m.), or in the warm air incubator for 18 hours. Longer incubation makes the tests increasingly hard to read. Do not agitate the tubes before reading.

When the racks containing the tests are removed from the bath or incubator, record the readings shown by the bath or incubator thermometer

as well as those of the maximum and minimum thermometer. The temperature should not fall below 34° nor rise above 39° C.

Reading the results. -- The tests should be read within an hour after the incubation has ended. To read them, sit in front of a window but do not face the sunlight. (The light must be good, and for this reason suitable artificial light must be provided on dark days or at night.) In order to determine whether or not there is clearing of the fluid and a ring or band of white flakes or white coarse granules at the meniscus, lift each tube carefully from the rack and hold it at the level of the eye; slant it at an angle of about 45° and view it in the direction of a darkened background on either side of a window or of a suitably placed light. While still viewing it at the same angle, slowly roll the tube between the fingers; this will make a faint ring visible. Finally, gently shake the tube and look for flocculation, evidenced by agglutinated masses or by faint granularity.

The reactions are read and reported as positive (without indicating the intensity of the reaction), negative, doubtful, and unsatisfactory.

> Such reports do not confuse physicians by implying that the intensity of the reaction is related to the clinical condition of the patient. [6]

Positive reactions (recorded +). -- At or a little above the level of the meniscus there is a ring or band, approximately 0.2 to 1.5 mm wide, of white coarse granules or flakes of lipoids, slightly to moderately but not strongly adherent to the wall of the tube.* By gentle shaking,

* Hemolyzed serums that are also bacterially contaminated frequently produce a whitish ring which is strongly adherent to the tube.

the ring or band is loosened and the particles scattered so that they are visible as agglutinated masses in a clear fluid or as somewhat coarse granules in a cloudy field.

Negative reactions (recorded -). -- Usually there is at most only slight clearing but no ring, band, or floccules. However, with some indicators, the negatives may show a "scummy" nongranular deposit of lipoids on the wall of the tube extending from the meniscus downward for from 1 to 3 mm.

Doubtful reactions (recorded ±). -- Centrifuge for 10 minutes at high speed (about 2,000 revolutions a minute) (1) those tubes which on gentle shaking showed only slight granularity beyond that observed in the control tube (containing 0.5 cc of indicator and 0.5 cc of the 5 percent salt solution) and (2) those tubes which showed only a slightly flaky or slightly granular ring. If, as a result of the centrifuging, there is definite clearing, and on top a thin layer of lipoids which shaking breaks up into fine flakes or coarse granules, the test is recorded as ± and reported as doubtful; if these changes have not taken place, it is reported as negative.

Rapid Method

This procedure may be used whenever there is urgent need for a quick report, for example, in testing donors for transfusion and in the diagnosis of suspected primary or secondary syphilis. Although this method has not been officially evaluated it is, according to our experience, from 98 percent to 99 percent as accurate as the regular method.

Technic of rapid method. -- Treat the specimens selected under No. 3 (page 6) as indicated under directions 4, 5 and 6 (page 6). Then add 0.5 cc of the glycerinated indicator to each and shake according to direction 9 (page 7); place in Wassermann bath at 37° for twenty minutes or in a warm air incubator at 37° for one hour; centrifuge at about 2,000 revolutions a minute for 10 minutes, read and record as follows:

Positive (recorded +) if there are plainly visible flakes at the top of the fluid, and a well-marked flocculate (precipitate) is seen on shaking.

Unsatisfactory if the specimen is hemolyzed or bacterially contaminated, unless the reaction is strongly positive. This interpretation is necessary, because even the moderately hemolyzed or bacterially contaminated serum of a known syphilitic usually gives a negative reaction.

Negative (recorded -) if centrifuging has caused no change, provided the specimen shows no decided hemolysis, (see under 3, page 6) or no marked bacterial contamination.

Tubes that show only very fine floccules or none at all after the centrifuging should be well shaken and placed in the water bath for 16 hours at 37° C. or in the incubator for 18 hours, after which the reading and interpretation are made as if the tests had been conducted in the routine manner.

DAVIES-HINTON MICROFLOCCULATION TEST*

In order to facilitate carrying out this test[7], specimens of blood should be collected in special rubber-capped glass collection tubes[8] which are then placed in labelled glass tubes that measure approximately 100 mm long and 15 mm diameter, such tubes being ordinarily called Wassermann tubes. The collection tubes are made of glass tubing and measure 80 mm long by 2.5 to 3 mm inside diameter. Over each end fits a flexible rubber cap such as is used to stopper small bottles of vaccine or serum. The capacity of this tube is about 0.3 cc.

Laboratory Procedure. -- Number the Wassermann tubes containing the specimens and place them in a suitable 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, and if this still does not clear it, the specimen is unsuitable for the test. Place the rack containing the clear specimens (clot downwards) in a suitable serologic water bath for 30 minutes at 56° C., having first filled

* This test in its single official evaluation[2] in 1936 stood highest, and when done for the sake of comparison in our own laboratory in the 1937-38 evaluation, it was 100 percent specific and 0.5 percent more sensitive than the regular Hinton test.

the Wassermann tube with water from the water bath. Next, remove the rack from the water bath, pour the water off the tubes by inclining the rack, remove the cap from the serum end of each collection tube and notch the tube just above the junction of clot and serum with a file such as is used to notch an ampule of arsphenamine. Hold the collection tube horizontally, break it and discard the part of the tube that contains the clot. Handle the part with the clear serum as follows:

1. Drain into one glass tube (similar to the collection tube described above) 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.

> 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 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. Care should be taken not to allow air to separate the serum and Hinton indicator, which would prevent mixing.

3. To the second tube, with the same capillary pipette, add diluted Hinton indicator equal to about five 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 or 15 times.

5. Cap both ends of each tube, return them to the original numbered Wassermann tubes in the rack, and immerse in a 37° water bath for 16 hours, having first filled the Wassermann tubes with water from the bath.

6. Remove the rack from the water bath, 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. Gentle 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 a 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.

Negative, if there are no clumps at all. Amorphous, cloudy material at the meniscus should be disregarded.

> 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 long 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. Inactivation at 56° C. may be reduced to 20 minutes, and incubation at 37° to 30 minutes without apparent loss of accuracy.

DAVIES-HINTON FLOCCULATION TEST OF CEREBROSPINAL FLUID[9]

Although this test has not been officially evaluated, it has, in our experience, proved to be far easier to perform and more efficient than the Wassermann[10] and those flocculation tests with which we have compared it. At present it is being used by the Massachusetts Department of Public Health.

Specimens of spinal fluid for this test should not be cloudy because of bacterial contamination, which is likely to give falsely negative tests, nor admixed with more than a trace of blood, because in rare instances an excess of blood can cause a falsely positive reaction. These facts must be borne in mind when interpreting results, for even if the specimen has been cleared by centrifuging, these statements still hold.

The test requires the following materials:

1. Glycerinated Hinton indicator, which may be made in sufficient quantity to last a month, provided it is stored in the refrigerator at a temperature of 8° to 10° C.

2. Gum acacia, 20 percent, which is prepared by diluting two parts of 30 percent gum acacia,* with one part of physiologic salt solution. This should be kept in a refrigerator and discarded when it becomes cloudy.

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*The gum acacia (containing 4.5 percent of sodium chloride) may be purchased from the Eli Lilly Co. in 100 cc ampules.

3. Hinton-negative human serum, which may be obtained by pooling serums that remain after performing the routine serologic tests. Select for this pooling only Hinton-negative serums which are clear (without hemolysis) and which have been inactivated at 55° C. for 30 minutes. The rather remote possibility of a zonal effect may be ruled out by testing the pooled serum in the following amounts:

Tube 1. 0.5 cc serum and 0.5 cc Hinton indicator

Tube 2. 0.1 cc serum and 0.5 cc Hinton indicator

Tube 3. 0.1 cc serum and 1.0 cc Hinton indicator

Tube 4. 0.1 cc serum and 2.0 cc Hinton indicator

If all of these tubes show a negative Hinton test, the pooled serum is suitable for use and should be passed through a Berkefeld "N" filter and collected under sterile conditions in rubber-stoppered bottles, so that each contains not more than 3 days' supply. These should be kept in a refrigerator at 8° to 10° C. Pooled serum should be kept no longer than 3 weeks and should be thrown away sooner if it becomes cloudy. Old serum is likely to give falsely negative reactions.

Laboratories that test only a few spinal fluids at a time may find it more convenient to prepare only enough serum for the day by selecting one or two of the clearer Hinton-negative serums of that day and retesting by the "rapid" method in the four amounts indicated above. The rapid method consists in shaking the four tubes containing the serum and Hinton indicator for 3 minutes, then placing them in a serologic bath at 37° C. for 30 minutes, centrifuging them for 10 minutes at high speed (about 2,000 revolutions per minute) and then reading the results. If all the tubes are negative, the serum is suitable for testing spinal fluid.

Procedure

In a suitable rack set up two tubes (one behind the other), measuring 10 mm x 100 mm, for each spinal fluid, and two tubes for controls. Label each tube.

Pipette 0.6 cc of the first spinal fluid into the first tube of the first row and the same amount of it into the corresponding tube of the second, and continue in this way with each specimen of spinal fluid. Pipette 0.6 cc of physiologic salt solution into each control tube.

Mix the 20 percent gum acacia with the previously tested clear Hinton-negative human serum in equal parts; for example, if ten spinal fluids are to be examined, 1.5 cc of the serum and 1.5 cc of the 20 percent gum acacia mixed together will be enough to make up for these specimens. Pipette 0.2 cc of this mixture into each tube of the first row (include control) and then pipette 0.2 cc of Hinton indicator into these same tubes.

> If several spinal fluids are to be examined at one time, the freshly mixed acacia-serum mixture may be added to an equal amount of Hinton indicator just prior to use, and 0.4 cc of this mixture pipetted into each tube of the first row.

Into each tube of the second row, including the control, pipette 0.2 cc of the acacia-serum mixture and 0.6 cc of Hinton indicator.

> If so desired, one part of the acacia-serum mixture may be added to three parts of Hinton indicator just prior to use, and 0.8 cc of this mixture measured into each tube of the second row.

Thoroughly and vigorously, either by hand or with a shaking machine, shake the rack containing the tests, so that the contents are completely homogeneous.

Incubate in a water bath at 37° C. for 16 hours, taking care that the water level is slightly above that of the contents of the tubes. Do not allow water to drop into the tubes.

Centrifuge at approximately 2,000 revolutions per minute; then read the tests in front of a window or a suitable artificial light. Hold the tube at the top with one hand and tap near the bottom with a finger of the other hand. By such a procedure, any floccules at the meniscus are dispersed downward and are easily visible.

Report the results as follows:

Positive, if in either tube there is flocculation that is definitely visible. Positive tubes should not be centrifuged a second time, because this may change the reaction to negative on truly positive specimens.

Doubtful, if either of the tubes shows questionable flocculation which centrifuging a second 5 minutes does not amplify.

Negative, if the original ground-glass appearance persists in both tubes. Each negative tube should be centrifuged a second time for 5 minutes, read again and reported as negative if the ground-glass appearance persists; otherwise it should be reported as positive or doubtful, depending upon the visibility of the floccules.

Unsatisfactory, if the tubes containing spinal fluid that was originally turbid from bacterial contamination show no clearly visible particles but a cloudiness that is distinctly greater than that in the control tube, or if the test is positive in the presence of contamination with more than a trace of blood.

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