FURTHER STUDIES ON THE EFFECT OF SOME ANTI-COAGULANTS UPON SERO-DIAGNOSTIC TESTS FOR SYPHILIS

II. HEPARIN, THYMOL-FLUORIDE, ISOTONIC OXALATE, AND CONCENTRATED CITRATE SOLUTION*

BY

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Ample evidence is available that body fluids other than serum contain antibodies (Coleman and Appleman, 1953; Kanter and Appleton, 1940; Klauder and Kolmer, 1921). Occasionally (Biro, 1947) these fluids reveal antibodies more frequently and sooner after infection of the bone marrow in syphilis. Plasma also has an antibody content (Addis, 1912; Cowie, 1909; Gurd, 1912), although some anti-coagulants interfere with the measurement of syphilitic reagin. However, upon the removal of the fibrinogen, some anti-coagulants have no appreciable effect upon the serologic tests. Blood treated with potassium oxalate in optimal concentration can, therefore, be used as well as serum in these tests.

The effects of the treatment of blood on the serodiagnostic tests for syphilis using the following anti-coagulants were studied: heparin, thymol-sodium fluoride mixture, an isotonic potassium and ammonium oxalate mixture, and a concentrated sodium citrate solution. It was observed that clotted blood often resists haemolysis of the erythrocytes by bacterial lysis, enzymatic action, or physical forces, considerably longer than when a sample of the same blood drawn at the same time and treated with potassium oxalate salts sufficient to prevent coagulation. Potassium oxalate, although not added to the blood-taking tubes with sterile technique, will usually be sterile upon bacterial culture, and it is doubtful if the salt itself introduces microorganisms into the blood.

It seems possible that because erythrocytes of oxalated blood are not bound up in a fibrin clot, they present a greater surface for chemical or physical damage, or that the oxalate interferes with the release of some of the antibodies from lymphocytes. Either the anti-coagulant prevents the normal release from the leucocytes or, in interrupting the clotting mechanism, some step essential for antibody release does not occur.

Fleck and Murczynska (1949) reported a phenomenon termed "leukergy" in which citrated blood in disease caused a clumping of leucocytes into homogenous groups. Leukergy lasts 4 days; it is more frequent in infectious diseases and it is not directly related to a phagocytosis. Other theories are that the lymphocytes secrete antibodies and that leukergy is not just a liberation upon cytolysis (Grabar, 1950), or that the lymphocytes form antibodies (Dougherty, Chase, and White, 1944, 1945; Ehrich and Harris, 1942).

It is important to know whether the plasma titre of reagin antibody is at the same level as the serum titre, and also the effects of the various anti-coagulants upon the antibody levels and their measurement.

Method

The VDRL slide, the standard Kahn, and the cardiolipin Mazzini flocculation tests were used. Evidence that more than one type of antibody can be identified and measured is plentiful (D'Alessandro and Dardanoni, 1953; Eagle and Hogan, 1940; Rein and Kostant, 1949). For this reason the Kolmer complement-fixation test was included. These sero-diagnostic tests were performed as described in the "Manual of Serologic

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Tests for Syphilis” (1949). For comparative purposes of the strength of the reactions, the VDRL test was rated in plus signs as in the Mazzini flocculation test.

Plasma specimens were prepared with the following anti-coagulants: heparin, concentrated sodium citrate, a thymol-lactose-sodium fluoride mixture (Sander, 1923), and an isotonic mixture of potassium and ammonium oxalate (Heller and Paul, 1934).

The heparin and 0.2 ml. of a 25 per cent. solution of sodium citrate were contained in a sealed tube, a Vacutainer.* This concentrated solution minimized the dilution effects and gave a final concentration of 0.05 g. sodium citrate per 5 ml. blood. Vacutainers with 5 mg. thymol and 50 mg. sodium fluoride were used. To these tubes containing the dried powder were added 5 ml. blood; 6 mg. powdered ammonium oxalate and 4 mg. potassium oxalate were placed likewise in Vacutainers designed for 5 ml. blood.

Blood specimens were collected in the usual manner from a prominent vein of the arm. A portion of the sample was allowed to clot in a clean dry test tube and the remainder was added to the tubes containing the anti-coagulant. The plasma was removed after a 5-minute period of centrifugation and then heated for 30 min. at 56°C. At the conclusion of the sensitization period, each sample was centrifuged for 10 min. at 3,000 revolutions per minute. The supernatant, thus cleared of precipitated fibrinogen, was decanted into another tube and then employed as a serum would be in the various sero-diagnostic tests.

Results

The results are summarized in Tables I, II, III, and IV.

Using heparin (Table I) it can be seen that the tests on the plasma appear slightly more sensitive.

### Table I

COMPARISON OF RESULTS IN 22 TREATED CASES OF SERUM AND HEPARIN PLASMA

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Serum Kahn</th>
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*Vacutainer was supplied by the Becton-Dickinson Company, Rutherford, New Jersey.

This is true in the flocculation tests even excluding all differences of only one plus degree. In the series studied there was no loss of specificity and heparin was anticomplementary in the complement-fixation tests. Reports that heparin is unsuitable for Wassermann reactions or agglutination tests (Diggs, 1952) must be related to the individual procedures.

Techniques for the precise titration of complement (Brooks, 1920), measurement of complement fixation in small volumes (Eckert, Reeve, and Beard, 1953), or the refined spectrophotometric standardization of complement for fixation tests (Kent, Bukantz, and Rein, 1946; Mayer, Eaton, and Heidelberger, 1946) undoubtedly could not be successfully performed on plasma treated with heparin. Table I reveals that the agreement in the Kolmer test is good in frank high-titre luetic sera as well as in negative cases. In the treated Cases 11, 12, 14, and 15, the heparin plasma appears to be anticomplementary. The destruction of complement by heparin can be easily demonstrated by reducing the amount of complement in the Kolmer tests. At a reduced level in negative tests negative serum Kolmer reactions are obtained, whereas the heparin plasma Kolmer reactions will all be doubtful or weakly positive, indicating that complement has been destroyed by the heparin.

Table II shows that sodium citrate tends to make the tests more sensitive. This increased...
sensitivity is seldom rated over one plus degree. However, wherever there is a difference in the grading of a plasma and the corresponding serum, it is always the plasma that has reacted more strongly. There does not appear to be any unusual distribution between complement-fixation tests and flocculation tests. The more concentrated (0.1 g. per ml.) sodium citrate, which minimizes the dilution effect, does increase the precipitation in the flocculation test. This was seen only in treated cases, however; twenty negative cases were uniformly negative. Anticomplementary reactions were not increased.

**Table III**

COMPARISON OF RESULTS IN 35 TREATED CASES OF SERUM AND PLASMA TREATED WITH THYMOL-
FLUORIDE MIXTURE

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Serum Kahn</th>
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<th>Serum VDRL Slide</th>
<th>Serum Kolmer</th>
<th>Fluoride Kahn</th>
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The results of the treatment of blood with thymol-fluoride mixture are summarized in Table III. There is more precipitation in the flocculation procedures. The increase in positive plasma reactions using the Kolmer technique (Cases 19, 20, and 22) over similar reactions when using serum would indicate that the anti-coagulant in plasma is anticomplementary. In twenty negative cases at least five revealed precipitation in the flocculation tests. Four of these represented doubtful tests with the VDRL when using plasma and one was rated at one plus with the Mazzini test when using plasma. There is a further disadvantage in the use of this thymol-sodium fluoride mixture as an anti-coagulant. Unless the plasma is removed within 24 hrs after the collection of the blood a definite haemolysis occurs, even at icebox temperature (12° C). This in turn makes the flocculation tests more difficult to read. It is of interest to note that this anti-coagulant interferes with some enzyme activities. It cannot be used in urea nitrogen determinations that depend upon urease (Roe, Irish, and Boyd, 1927). However, this feature is advantageous in glucose determinations (Bowman and Enterline, 1954). It has been shown that sodium fluoride inhibits the enzyme enolase in the glycolytic pathway (Baldwin, 1952).

**Table IV**

COMPARISON OF RESULTS IN 21 TREATED CASES OF SERUM AND ISOTONIC OXALATE PLASMA (HELLER AND PAUL MIXTURE)

<table>
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<tr>
<th>Case No.</th>
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<th>Serum VDRL Slide</th>
<th>Serum Kolmer</th>
<th>Isotonic Kahn</th>
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The results of comparison of serum with isotonic oxalate plasma in 21 treated cases are shown in Table IV. In general, it appears that agreement is quite good, certainly with frank positives as it was with twenty additional negative cases. In the treated cases the serum flocculation tests agree with the plasma tests, revealing a slight tendency towards greater sensitivity. In the treated cases with the complement-fixation test there is less agreement. Quite frequently the plasma appears more sensitive, but it also is occasionally less sensitive. In one case the plasma was anticomplementary whereas the serum was not. These equivocal results are rather difficult to interpret without larger sampling, and more studies will be required on larger series of cases to ascertain if this represents a true increase in anticomplementary results.
ANTI-COAGULANTS IN SERO-DIAGNOSTIC TESTS FOR SYphilIS

Discussion

When blood samples that are later to be employed in sero-diagnostic tests for syphilis are treated with anti-coagulants, it is as well to remember that altering ionic content of a serum also changes the reaction in various flocculation tests (Burdon and Bromberg, 1930; Burdon, 1932). Substitution of bivalent ions produces positive tests, while substitution of univalent ions produces negative tests (Breazeale, Reuss, and Pierce, 1946). Kimball and Kabler (1948) have shown that chloride ions may interfere in complement-fixation tests, or may affect the flocculation tests (Green and Shaughnessy, 1942; Kline, 1942). On the other hand it has been shown that sodium oxalate in amounts up to 0.001 g. per ml. blood has no effect on complement-fixation tests (Watanabe, 1919). Potassium oxalate in optimal concentrations has been shown to give, with the plasma so obtained, results comparable to serum in sero-diagnostic tests for syphilis (Coleman and Appleman, 1954), and calcium ion appears to protect complement from spontaneous inactivation (Lepow, Pillemer, and Ratnoff, 1953). Ecker, Castro, and Seifter (1945) report that strong electrolytes, such as chloride, bromide, and iodine with sodium, potassium, or ammonium in final dilution above 2 per cent., will increase the sensitivity of a serum; and that the concentration of electrolytes can be raised to a degree that will decrease the normal inhibiting factor present in fresh serum which is usually destroyed by heating at 56° C. for 30 min. In the results reported here the potassium level never reached a concentration sufficient to have a measurable effect in the tests used, but where both ammonium and potassium ions were present, the precipitation tests did appear to be somewhat more sensitive. Using thymol-sodium fluoride the flocculation tests were more sensitive, but this apparently causes a loss of specificity. This conclusion should be tempered by the fact that only a few sera were tested. There is in this plasma, as well as in the sodium citrate plasma, an greater increase in the final concentration of sodium ion.

The results indicate that heparin plasma also tends to be more sensitive in the flocculation tests, and that heparin destroys complement to a minor degree. This concurs with the work of Wising (1937), who reports that calcium and sodium heparins destroys complement in dilutions of 10\(^{-6}\). In the Kolmer test, using proper controls, this is usually no problem, as there is sufficient excess of complement. More serious objections to heparin are that good samples are not always available and that it is not readily soluble as a powder. The relatively colourless nature of the material when in a dry film on the walls of a blood-taking tube can also be disadvantageous.

It has been noted that the natural heparin concentration in the blood may be quite different in various individuals (Ziff and Chargaff, 1940). It has been demonstrated recently (Freeman, Engelberg, and Dudley, 1954) that perhaps heparin is normally present in the blood in amounts considerably greater than was previously thought. The potency of heparin is not precisely correlated to the sulphur content; yet the removal of the sulphur radicle inactivates its action as an anti-coagulant (Wolffson and McNeely, 1945). Sulphur may play a part in the haemolytic activity of complement. Yamakawa (1943) reports that complement depends upon a redox system containing sulphhydryl groups. A further effect of heparin as an anti-coagulant is that the blood calcium is left in solution in a heparinized preparation (Holt, 1931), whereas with many of the other anti-coagulants the calcium is precipitated or bound in a weakly dissociated compound. This excess calcium is then available in levels approaching that of normal serum to add its stabilizing effect to the calcium. In an electrophoretic study of the effect of heparin on human plasma proteins (Chargaff, Ziff, and Moore, 1941), it was noted that a globulin fraction moving with the mobility of gamma globulin was not attacked by heparin. It was conjectured that the acidic groups of the anti-coagulant molecule serve to arrange the albumin molecules around it. Perhaps this sparing action of the gamma globulin is the reason sero-diagnostic tests and other antibody estimations can be done frequently on heparin plasma.

From the evidence presented it would appear that the anti-coagulants certainly do not interfere with the release of antibody. It would seem that nothing in the “step-wise” completion of the clotting mechanism is needed for the appearance in plasma of reagin of syphilis at levels equivalent to serum as measured by the tests used.

The results would indicate that many anti-coagulants apparently make sero-diagnostic tests more sensitive. Further studies of larger numbers of blood samples are needed to determine if this represents an increase in sensitivity without a loss of specificity.

Summary

(1) The level of reagin antibodies of syphilis as measured by the standard Kahn, Mazzini, VDRL slide, and Kolmer tests in blood treated with heparin, sodium citrate, thymol-fluoride.
mixture, and potassium-ammonium oxalate mixture, has been shown to be equivalent to that in serum.

(2) The addition of these anti-coagulants appears to make the flocculation tests slightly more sensitive.

(3) The completion of the clotting mechanism is not required for the release of these antibodies.

(4) Some of the anti-coagulants destroy complement to a small but capricious degree.

The authors are indebted to the Health Department, Los Angeles City, and to the South East District Health Center where patients were made available for the study.

REFERENCES


Further Studies on the Effect of Some Anti-Coagulants upon Sero-Diagnostic Tests for Syphilis: II. HEPARIN, THYMOL-FLUORIDE, ISOTONIC OXALATE, AND CONCENTRATED CITRATE SOLUTION

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