

[54] REAGENTS AND TESTS FOR SYPHILIS
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424/11; 424/12
[51] Int. Cl. G01n 31/02; G01n 31/22; G01n 33/16
[58] Field of Search..... 424/3, 7, 8, 11, 12, 13

[56] References Cited
UNITED STATES PATENTS
2,111,976 3/1938 Laughlin..... 424/8
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2,301,717 11/1942 Terry..... 424/8
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3,074,853 1/1963 Brewer..... 424/12
3,666,421 5/1972 Price..... 424/12 X
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OTHER PUBLICATIONS

Berger, Chem. Abs., Vol. 38, 1944, pp. 3996-3998.
Berger, Brit. Exptl. Path., Vol. 24, 1943, pp. 252-261.

Primary Examiner—Albert T. Meyers
Assistant Examiner—A. P. Fagelson
Attorney, Agent, or Firm—Frank M. Van Riet

[57] ABSTRACT
An antigen preparation for use in the detection of syphilis and other treponemal diseases comprising an antigen reagent and a class of dyes and a method of using said antigen preparation, are disclosed.
9 Claims, No Drawings

REAGENTS AND TESTS FOR SYPHILIS

BACKGROUND OF THE INVENTION

The use of serological tests for the detection of syphilis and other treponemal diseases has become more and more commonly practiced in recent years. These tests are usually based on an agglutination reaction and are conducted in clinics and doctors' offices preparatory to more extensive diagnosis of the patient.

The most commonly practiced test constitutes the use of a finely divided solid such as charcoal in conjunction with a card having a surface color contrasting to that of the solid material, see U.S. Pat. No. 3,074,853. In practice, the test utilizes a common antigen liquid which is buffered and to which is added the charcoal. A drop of the resultant antigen solution-charcoal suspension is then placed on a test card in admixture with one drop of serum. The card is then shaken and the results are visually interpreted. Additional diagnostic reagents have also recently been patented, see U.S. Pat. Nos. 3,564,089 and 3,600,494, while Lockyer, Brit. J. vener. Dis. (1970), Vol. 46, pages 290-294 sets out a modified Laughlin flocculation test using a Kahn antigen coupled with a scarlet red stain and a Wassermann complement-fixation technique, using a cardiolysin antigen.

The known test methods, while resulting in adequate syphilis detection in most cases, are by no means per-

SUMMARY OF THE INVENTION

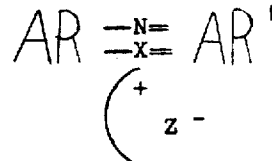
I have now found a novel visual method for the detection of syphilis which has material advantages over other techniques such as the use of charcoal or latex emulsions. Since my system does not utilize solid particles, no light scattering is caused and therefore a more easily visible particle is produced if a positive serum or plasma is used. Therefore, the instant test is more accurate and reliable.

According to my novel test, when my novel antigen reagent is contacted with an antibody-containing serum or plasma, a complex is formed which forms a coacervate with one component of the reagent. A positive test is indicated by colored flocs which are especially noticeable on a light background, e.g., a white card.

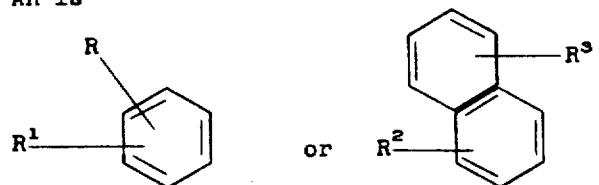
DESCRIPTION OF THE INVENTION INCLUDING PREFERRED EMBODIMENTS

As mentioned briefly above, I have discovered a novel antigen preparation for use in carrying out an agglutination test for syphilis and other treponemal diseases. The antigen preparation consists of two basic ingredients, the first being a common V.D.R.L., U.S.R. or Reiter antigen reagent, known to those skilled in the art and more completely identified in U.S. Pat. No. 3,564,089, mentioned above.

The second component of my novel antigen preparation is a dye compound having the formula



wherein AR is

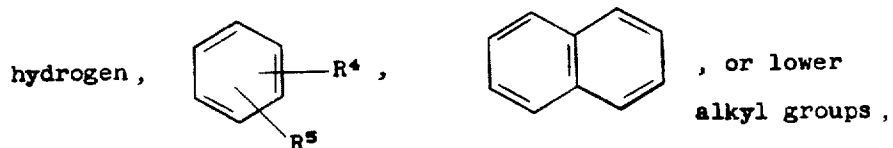


fect and each possesses its own difficulties. The charcoal test, for example, is very difficult to interpret due to the tendency of the positive and negative results to be similar in many instances. Where a definitely positive test is encountered, no difficulty arises, however, in cases where the results are in doubt, further exten-

wherein R and R¹ are, individually, hydrogen, lower alkoxy, lower alkyl, amino, monoalkylamino, dialkylamino or monoarylamino groups, no more than one of R and R¹ being hydrogen and R² is hydrogen, amino or monoarylamino groups, R³ is an SO₃Na group, Z is SO₃ or Cl, X is oxygen, sulfur or N

Y

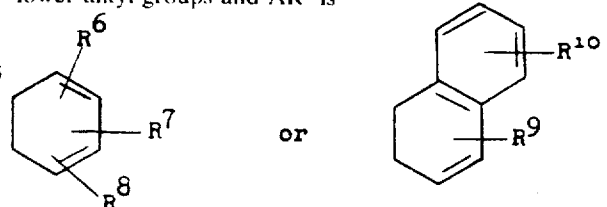
wherein Y is



sive tests must be conducted before a definite conclusion can be drawn. With regard to the scarlet red stain test, the results tend to be nebulous because of the faint pink color of the aggregated particles and the concomitant difficulty in analyzing and interpreting the test.

It has also been well recognized that other commonly used tests have been known to give completely erroneous results in 2.0 percent of the tests run and false positive results in 20 percent of the test cases.

R⁴ and R⁵ are, individually, hydrogen, lower alkoxy or lower alkyl groups and AR¹ is



wherein R⁶ is hydrogen or lower alkyl, R⁷ and R⁸ are, individually, hydrogen, amino, monoarylamino, lower alkyl, dialkylamino, monoalkylamino, hydroxy or nitro groups, no more than one of R⁷ and R⁸ being hydrogen and R⁹ and R¹⁰ are, individually, hydrogen, amino, monoarylamino, monodialkylaminoarylamino, or hydroxy groups, and the zinc double chlorides thereof.

The dye compounds and mixtures are present in my novel antigen preparations in conjunction with solvents, buffers, etc., in a manner as is known in the art. These solvents, buffers etc. per se form no part of the instant invention and are used as described in known antigen systems such as those discussed in the above-identified U.S. patents, hereby incorporated herein by reference.

The components of the novel antigen preparation of my invention are employed in concentrations ranging from about 0.1 percent to about 25.0 percent, by weight, of the standard V.D.R.L., U.S.R. or Reiter antigen and from about 0.0001 percent to about 0.2 percent, by weight, of the dye compounds, said weights being based on the total weight of the resultant mixture including solvents, e.g., water, alcohol, etc., and buffer.

By "standard V.D.R.L. and U.S.R. antigen," as used herein, is meant the compositions set forth and prescribed by the Manual of Tests for Syphilis (1969), U.S. Dept. of Health, Education and Welfare, Public Health Service, National Communicable Disease Center.

A third component may also be present in my novel antigen preparations. This component is preferred but not critical and comprises a flocculating agent. The flocculating agent can be employed in amounts ranging from about 0.0001 to about 0.05 percent, by weight, based on the total weight of the novel antigen preparation of the instant invention, i.e., standard antigen and dye compound, and includes such known flocculants as vinyliminidazoline, acrylamide, acrylic acids, acrylonitrile, styrene, maleic anhydride, etc., polymers and copolymers thereof with each other and other known copolymerizable monomers.

In practice, the flocculating agent aids in the precipitation and coaccervation of the antigen-antibody complex and thereby aids in the visual detection of the positive test.

In preparing the antigen formulations of the present invention, standard V.D.R.L. antigen can be used. The dye compound may be incorporated into an alcoholic solution of the V.D.R.L. antigen or added after the reagent antigen has been prepared in buffer. If a flocculating agent is to be incorporated into the basic preparation, it is also added with slight agitation at this time.

The U.S.R. antigen-dye compound formulation is made by first preparing the V.D.R.L. antigen suspension in buffer. This system is then centrifuged in a stainless steel vessel at approximately 2,000 g for 15 minutes. The fluid is decanted and the sides of the vessel wiped dry. The sediment is suspended in U.S.R. suspending medium which consists of a known and appropriate mixture of monopotassium phosphate, disodium phosphate, merthiolate, choline chloride and ethylenedinitrilotetraacetic acid. The dye compound or compounds may be incorporated into the alcoholic solution of V.D.R.L. antigen or the U.S.R. antigen after its suspension of U.S.R. suspending medium.

When standard Reiter antigen is used, the dye compound is merely added thereto and the resultant antigen preparation is used as such in the test.

The tests utilizing the antigen preparations of the instant invention entail the use of a test card which may or may not have a flocculating agent deposited thereon. The test card should be smooth in order to render the test results easily discernible. Although it has been previously indicated in the prior art that the surface must be wettable, I have found that such is not the case and a wettable surface is merely preferred. The card may be composed of well calendered paper or cardboard and may be absorbable, however, only small degrees of absorbability are preferred. One feature of the use of my novel antigen preparations is that the test may be read for the test card whether the spot is wet or dry and therefore results can be ascertained more rapidly than when using other techniques. The card may also be a laminate of a paper or cardboard base having a water-permeable or water-impermeable material coated thereon such as polyethylene. The paper itself or the coating, however, should be of a color which is contrasting with regard to the color of the dye employed in the test. Basically, any card similar in construction and composition to that disclosed in U.S. Pat. No. 3,074,853 may be used. The flocculating agent may be deposited on the card, if desired, by merely evaporating it from a solution after having placed a solution thereof thereon. The solvent may be either water or an organic material such as methylene chloride, etc. An adhesive may also be added to the card beforehand to insure adequate adhesion of the flocculating agent thereto. A suitable adhesive for this purpose is methyl cellulose. The same flocculating agents as mentioned above with regard to direct addition thereof to the antigen preparation may be used. The flocculating agent is usually deposited in selected areas only of the test card, these areas usually being designated as a circle, etc., the area within which the test is actually conducted. When the flocculating agent is present on the card per se, it is not necessary to add further flocculating agent to the antigen preparation being used to conduct the test.

Tests are usually carried out by shaking the cards after the antigen preparation and serum have been added dropwise thereto. The shaking may be carried out on, for example, a horizontal disc about 10 inches square at about 80-240 strokes per minute with a slight eccentric motion. However, hand shaking is also effective.

The following examples are set forth for purposes of illustration only and are not to be construed as limitations on the instant invention except as set forth in the appended claims. All parts and percentages are by weight unless otherwise indicated.

EXAMPLE I

U.S.R. Test

The antigen used in this test is an alcoholic solution of 0.03 percent cardiolipin, 0.9 percent cholesterol and 0.21 ± 0.01 percent lecithin. This standard V.D.R.L. antigen and the standard buffered saline diluent for the preparation of the reagent V.D.R.L. antigen are commercially available.

Unheated Serum Reagin (U.S.R.) Test

Preparation of U.S.R. Antigen-Dye Compound Reagent

A. EDTA (0.1 M)

Dissolve 3.72 g [(ethylenedinitrilo)tetraacetic acid disodium salt](EDTA) to a volume of 100 ml.

B. Choline Chloride Solution (40 percent)

Dissolve the contents of a 250 g bottle of choline

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chloride in distilled water to a final volume of 625 ml. Filter and store at room temperature.

C. Phosphate (0.02 M), Merthiolate (0.2 percent) Solution

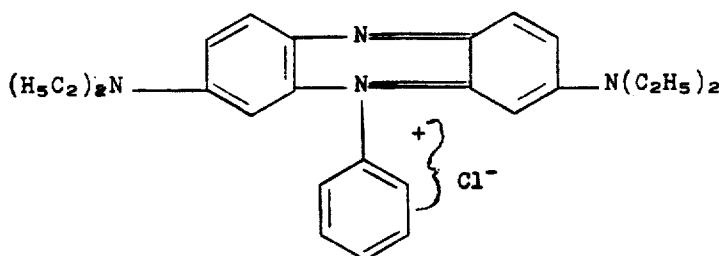
Dissolve 1.42 g Na_2HPO_4 , 1.36 g KH_2PO_4 , 1.00 g merthiolate in distilled water to a final volume of 500 ml. at a pH of 6.9.

Suspending Solution	
Solution A	1 part
Solution B	2 parts
Solution C	4 parts
Distilled Water	1 part

Preparation of the Antigen Suspension

1.6 ml of commercially available V.D.R.L. buffered saline are pipetted to the bottom of a 30 ml flat-bottom, glass-stoppered vessel. Two (2.0) ml of antigen is added drop by drop so that it is added in about 6 seconds directly onto the saline solution. The vessel is then continuously but gently rotated on a flat surface while the antigen is being added. The last drop is blown out without touching the pipette to the saline solution. The vessel is rotated for an additional 10 seconds. Sixteen and four tenths (16.4) ml of standard buffered saline is then added and the vessel shaken (with the top on) bottom to top and back thirty (30) times in about 10 seconds. The resultant product is the V.D.R.L. antigen suspension used below.

The above prepared V.D.R.L. antigen suspension is centrifuged in an angle centrifuge at room temperature at a relative centrifugal force of approximately $2,000 \times g$ for 15 minutes. The supernatant fluid is decanted by inverting the tube away from the side containing the sediment. The inside of the vessel is wiped with a cotton gauze without disturbing the sediment while the tube is held in an inverted position. The sediment is then resuspended in the above suspending solution with a volume equal to that of the original portion of antigen suspension that was centrifuged. If more than one container is used for centrifuging, the contents are pooled and mixed gently. The result is U.S.R. antigen. Seven hundredths (0.07) ml of a 1 percent solution of "Amethyst Violet", having the formula



and identified as Basic Dye C.I. No. 50225, is slowly added to the U.S.R. antigen while the antigen is gently agitated. The result is an antigen preparation ready for testing.

Procedure for the Detection of Reagin Antibody (U.S.R. Test)

The test may be conducted on serum or plasma samples which have not been heated, although heat inactivated samples are satisfactory.

Fifty μl of serum and 1/60 ml of the above antigen preparation are added to a commercially available white test card. The mixture is briefly mixed with a mixing stick or other device. The card is then put on a clinical

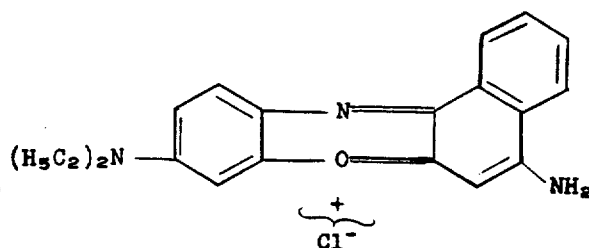
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rotator and rotated for 6 minutes at 130 r.p.m. Dark violet aggregated antigen-dye particles are visible under an incandescent, incident beam of light, denoting a positive syphilis test. A negative reaction is one in which the reagents do not aggregate into discrete particles but remain as a homogenous suspension.

EXAMPLE 2

Antigen Preparation

To the V.D.R.L. antigen suspension (see above) is added 0.04 ml of 0.1 percent alcoholic solutions of "Nile Blue A", having the formula



and designated as Basic Blue C.I. No. 51180. The antigen preparation is now ready for use.

Procedure for the Detection of Reagin Antibody (V.D.R.L. Test)

Plasma is removed from the blood cells of a suspect patient and heated at 56°C . for 30 minutes. Fifty μl of the sample and 1/60 ml of the above V.D.R.L. antigen preparation are placed on a commercially available white test card. After mixing, the card is placed on a clinical rotator and rotated as in Example 1. Aggregated dark blue particles visible under an incandescent, incident beam of light denote a positive syphilis test. A negative reaction is one in which the reagents do not aggregate into discrete particles but remain as a homogenous dispersion.

EXAMPLE 3

An antigen preparation is again produced as in Example 1. The test is conducted in the same manner as the U.S.R. test except that 1 μl of a 0.1 percent aqueous solution of polyvinylimidazoline is added to the test card and dried at room temperature before rotating. The aggregated particles are such that visibility thereof

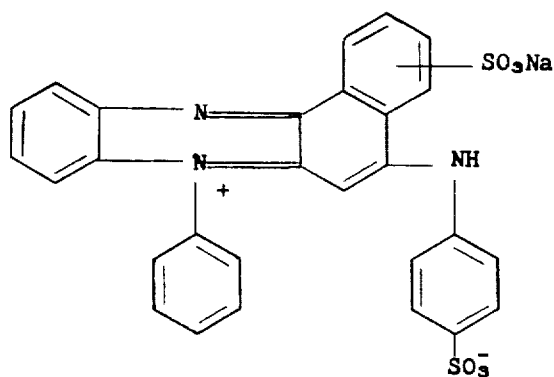
is further enhanced over the card wherein no imidazoline polymer is used.

EXAMPLE 4

An antigen preparation is produced as in Example 2. The test is conducted in the same manner as the V.D.R.L. test except 1 μl of a 0.1 percent aqueous solution of polyvinylimidazoline is added to the test card and dried as in Example 3. Visibility of the aggregated particles is again amplified.

EXAMPLE 5

An aqueous solution of 0.1 percent of "Azocarmine G", having the formula



and designated as Acid Red-C.I. No. 50085, is prepared and 0.5 ml. of it are added to 1.0 ml of Reiter antigen. This constitutes the Reiter-dye compound antigen preparation. One drop of this preparation is placed on a glass slide and one drop of suspect serum is added, followed by brief mixing with a wooden device. The re-

sultant mixture is rotated for 4 minutes at 160 r.p.m. and read under a microscope at 2X power. Fluffy, translucent particles of red intensity appear after standing 1 minute indicating a positive syphilis reaction.

5 By "Reiter antigen," as used herein is meant that such as disclosed by De Alessandro et al., Isolation and Purification of a Protein Antigen of the Reiter Treponema, Amer. Jour. of Syphilis and Gonorrhea, Vol. 37, page 135 et seq. 1953.

10 A typical testing procedure is set forth by Cammefax et al., Reiter Protein Complement Fixation Test for Syphilis, Public Health Reports, Vol. 72, page 335 et seq., 1957.

15 Following the procedures of Examples 1-5, various other dye compounds are substituted for the compounds used therein. The dye compounds correspond to Formula I, above, and, in each instance, show an easily detected, positive syphilis reaction via the presence of aggregated particles. The dye compounds used are set forth in Table I, below.

TABLE I

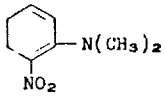
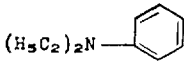
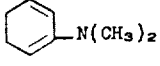
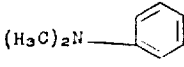
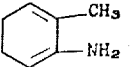
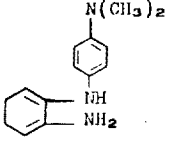
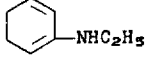
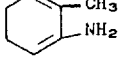
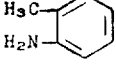
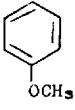
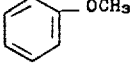
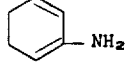
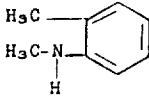
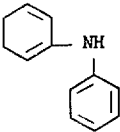
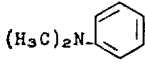
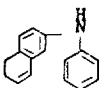
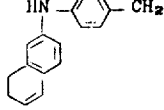
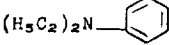
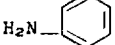
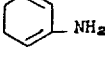
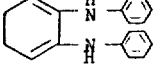
Exam- ple	Antigen and Method of Examination No.	DYE COMPOUND					Name and C.I. No.
		AR	X	Z	Y	AR'	
6	1		S	Cl *	—		Basic Blue 24 C.I. No. 52030
7	2		O	Cl	—		Basic Dye C.I. No. 51185
8	3	do	O	Cl	—		Basic Blue 10 C.I. No. 51190
9	5	do	O	Cl	—		Basic Dye C.I. No. 51195
10	2	do	O	Cl	—		Basic Dye C.I. No. 51205
11	4	do	O	Cl	—		Basic Dye C.I. No. 51210
12	1		S	Cl	—		Basic Dye C.I. No. 52000
13	1	do	S	Cl	—		Basic Dye C.I. No. 52005
14	2		S	Cl	—		Basic Dye C.I. No. 52010
15	2	do	S	Cl *	—		Basic Blue C.I. No. 52015

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Table I—Continued

16	5	do	S	Cl *	—		Basic Green 5 C.I. No. 52020
17	5		S	Cl *	—		Basic Blue 25 C.I. No. 52025
18	1		S	Cl *	—		Basic Blue 17 C.I. No. 52040
19	1	do	N	Cl	H		Basic Dye C.I. No. 50030
20	2	do	N	Cl	C ₂ H ₅		Basic Dye C.I. No. 50035
21	4	do	N	Cl	H		Basic Dye C.I. No. 50040
22	2		N	Cl	CH ₃	do	Basic Dye C.I. No. 50045
23	1		N	Cl			Basic Dye C.I. No. 50050
24	1		N	Cl			Basic Violet 6 C.I. No. 50055
25	1		N	Cl	do		Basic Dye C.I. No. 50150
26	1	do	N	Cl	do		Basic Dye C.I. No. 50155
27	1	do	N	Cl			Basic Dye C.I. No. 50160
28	2		N	Cl	do	do	Basic Dye C.I. No. 50165
29	2		N	Cl			Basic Dye C.I. No. 50200
30	4	do	N	Cl	do		Basic Dye C.I. No. 50204

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Table I—Continued

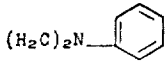
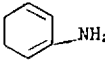
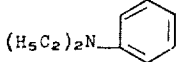
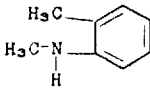
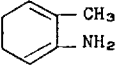
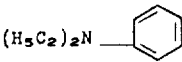
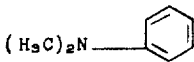
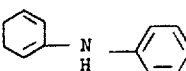
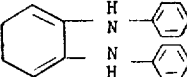
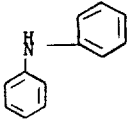
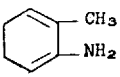
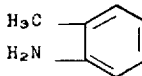
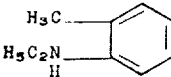
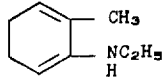
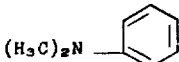
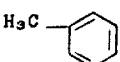
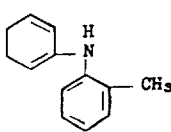
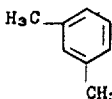
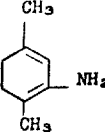
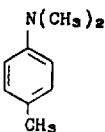
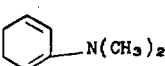
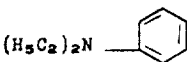
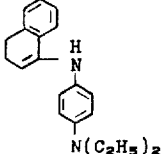
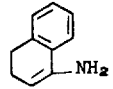
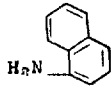
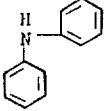
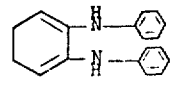
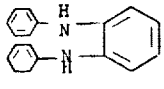
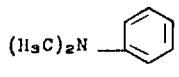
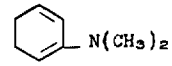
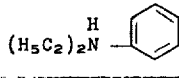
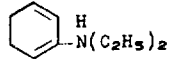
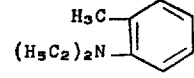
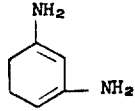
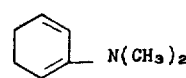
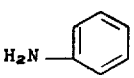
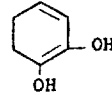
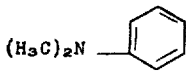
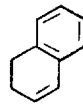
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32	2		N	Cl	do	do	Basic Dye C.I. No. 50206
33	2		N	Cl	do		Basic Dye C.I. No. 50215
34	3		N	Cl	do	do	Basic Dye C.I. No. 50216
35	1		N	Cl	do		Basic Dye C.I. No. 50220
36	1	do	N	Cl	do		Basic Dye C.I. No. 50021
37	4		N	Cl	do		Basic Violet 12 C.I. No. 50235
38	4		N	Cl	do	do	Basic Red 2 C.I. No. 50240
39	4		N	Cl	do		Basic Red 10 C.I. No. 50250
40	2		N	Cl			Basic Dye C.I. No. 50255
41	1	do	N	Cl			Basic Dye C.I. No. 50260
42	1		N	Cl			Basic Dye C.I. No. 50265
43	1		N	Cl			Basic Blue 13 C.I. No. 50306

Table I—Continued

44	5		N	Cl			Basic Red 6 C.I. No. 50375
45	1		N	Cl	do	do	Basic Red 6 C.I. No. 50375
46	1		N	Cl			Solvent Blue 7 C.I. No. 50400
47	2		N	Cl	do	do	Solvent Blue 7 C.I. No. 50400
48	1		O	Cl *	—		Basic Dye C.I. No. 51000
49	2		O	Cl	—		Basic Blue 3 C.I. No. 51004
50			O	Cl	—		Basic Dye C.I. No. 51010
51		do	O	Cl *	—		Basic Dye C.I. No. 51015
52			O	Cl	—		Mordant Violet 35 C.I. No. 51025
53			O	Cl *	—		Basic Blue 6 C.I. No. 51175

* = Zinc double chloride salt

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I claim:

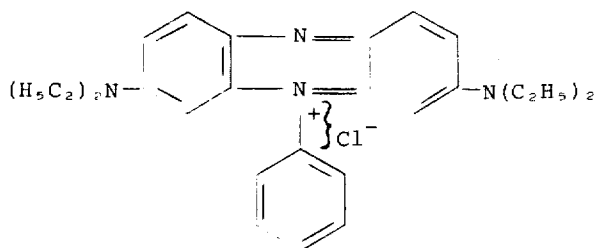
1. An antigen preparation for use in carrying out an agglutination test for syphilis which comprises

- 55 a. 0.1–25 percent by weight of a syphilis antigen reagent,
b. 0.0001–0.2 percent by weight of a dye compound comprising

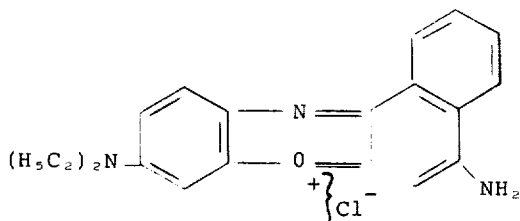
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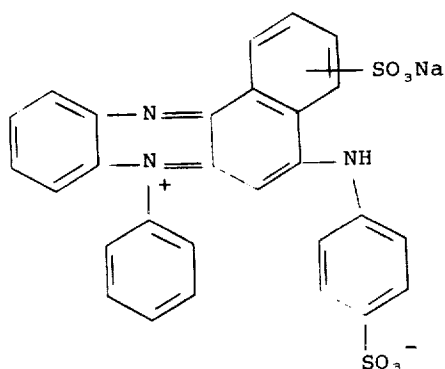


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or



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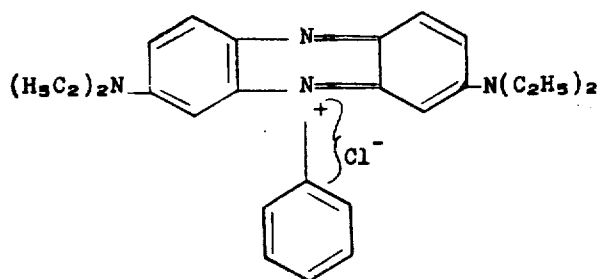
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and

c. a flocculating amount of polyvinylimidazoline.

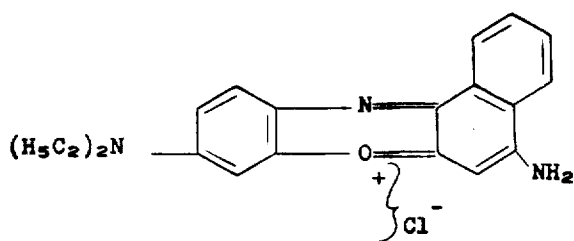
2. The antigen preparation of claim 1 wherein said dye compound has the formula



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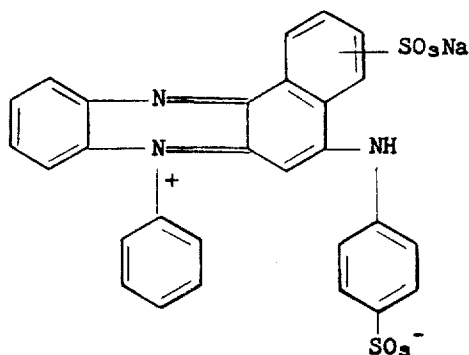
3. The antigen preparation of claim 1 wherein said dye compound has the formula



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4. The antigen preparation of claim 1 wherein said dye compound has the formula

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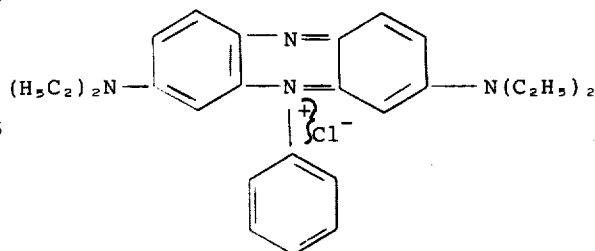
5. A method for carrying out an agglutination test for syphilis on a test card having dried thereon a test spot containing a flocculating amount of polyvinylimidazoline said method comprising contacting said test spot with

1. a test serum or plasma and

2. an antigen preparation comprising

a. 0.1-25 percent by weight of a syphilis antigen reagent and

b. 0.0001-0.2 percent by weight of a dye compound comprising



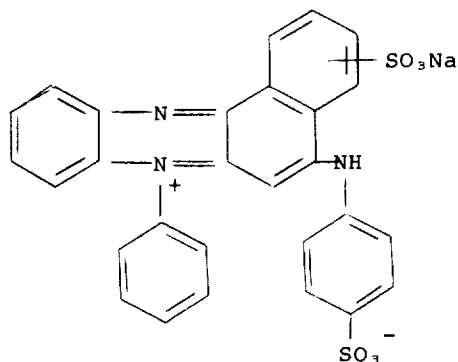
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or

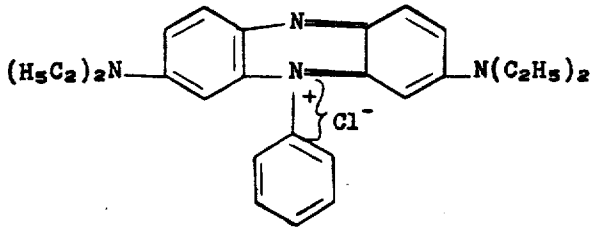


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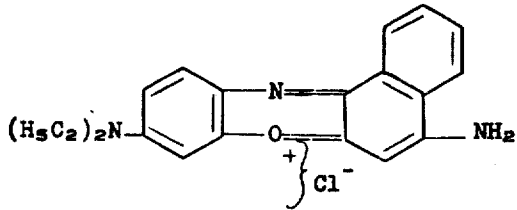
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6. The method of claim 5 wherein said dye compound has the formula

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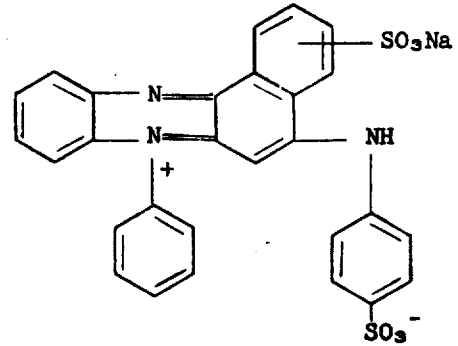


7. The method of claim 5 wherein said dye compound has the formula



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8. The method of claim 5 wherein said dye compound has the formula



9. A method for carrying out an agglutination test for syphilis which comprises mixing a test serum or plasma with the antigen preparation of claim 1.

* * * * *