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Golibersuch

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[54] **CONTRAST ENHANCEMENT FOR IMMUNOLOGICAL FILM DETECTION**

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[52] **U.S. Cl.** ..... **23/230 B;** 23/253 TP; 424/12

[51] **Int. Cl.²** **G01N 33/16**

[58] **Field of Search** ..... 23/230 B, 253 R, 253 TP; 424/12

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[57] **ABSTRACT**

A method for increasing the sensitivity of immunologic diagnostic slides by increasing the contrast observable between immunologically reacted and unreacted portions thereof. The method is based on the fact that an arbitrary antibody functions as an antigen when introduced into the system of a vertebrate to whom it is a foreign protein. Specifically reactive antibodies to the antibodies so introduced are therefore produced and may be collected. Accordingly, a plurality of specific immunological reactions may be performed in chain fashion. This technique is used in accordance with one embodiment to build a plurality of successive antigen-antibody films on a substrate to provide for contrast between multilayer protein films and a monomolecular layer which is in excess of the contrast obtainable between monomolecular layers and bimolecular layers to thereby provide diagnostic apparatus of increased sensitivity.

3 Claims, 3 Drawing Figures

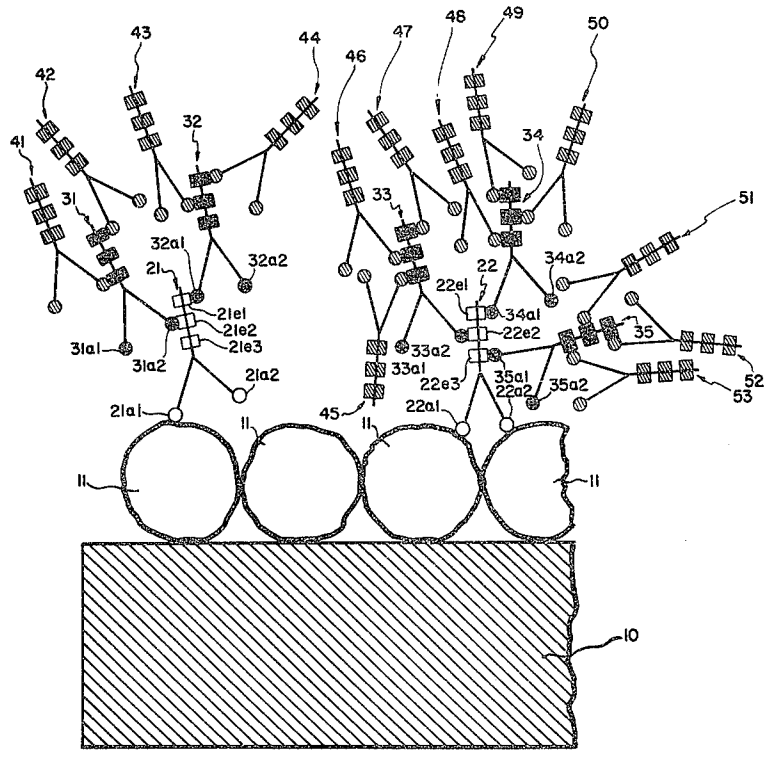

Fig. 1

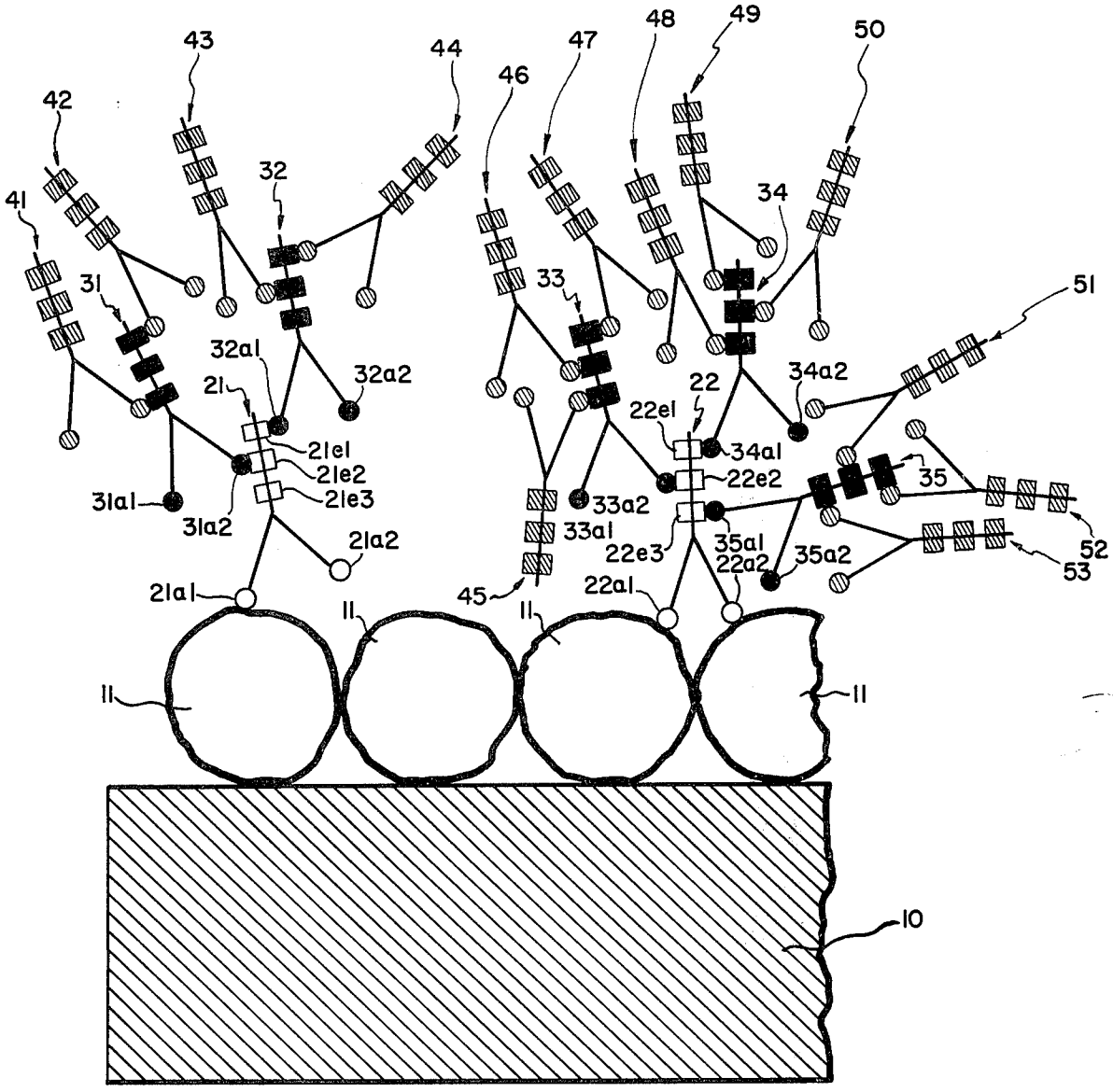
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Fig. 2

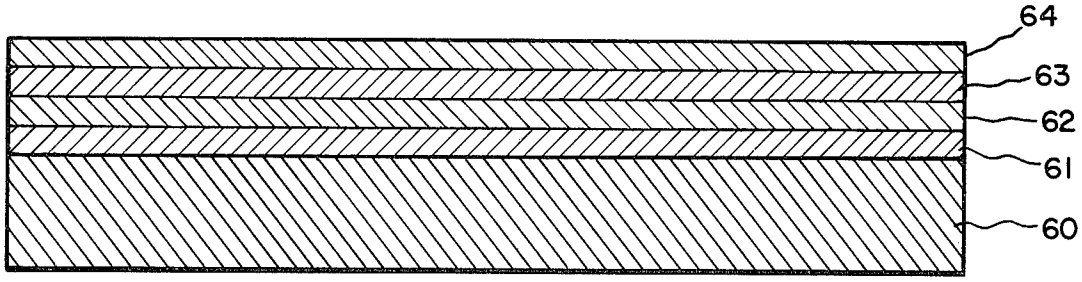
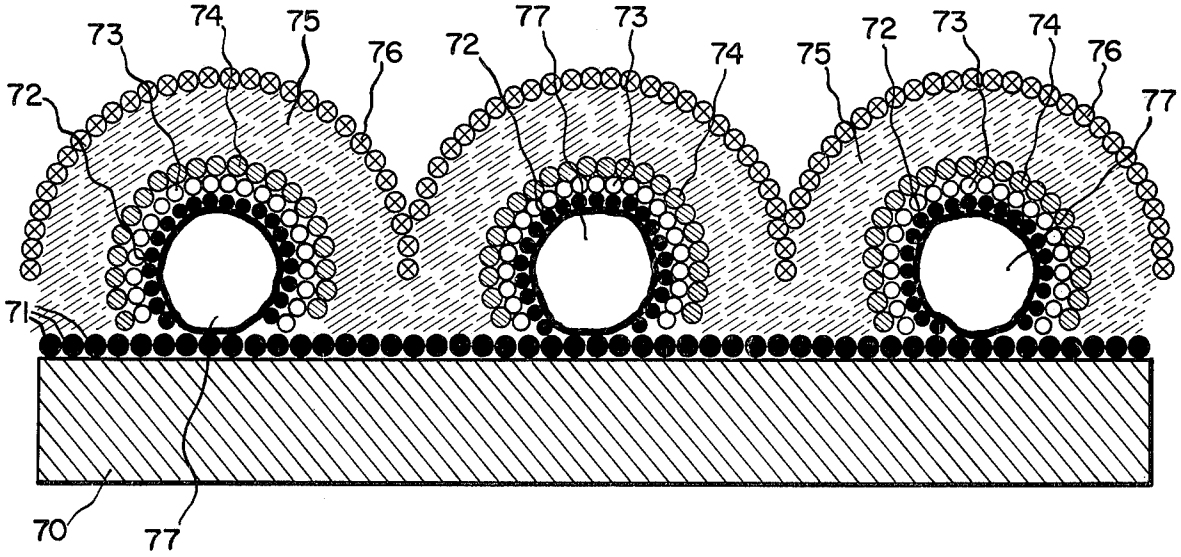


Fig. 3



CONTRAST ENHANCEMENT FOR IMMUNOLOGICAL FILM DETECTION

This invention relates to the immunological detection of protein by detection of immunological complexing between a protein previously absorbed on a surface to form a test slide and the specifically immunologically reactive protein thereto. More particularly, this invention relates to the improvement of contrast observable on such immunologic diagnostic slides by the selective immunologic bonding thereto of additional protein layers.

This application is related to the copending applications of Giaever, Ser. No. 266,278, filed June 26, 1972; Ser. No. 384,113, filed July 30, 1973; and Ser. No. 388,406, filed Aug. 15, 1973 now U.S. Pat. No. 3,853,467. These related copending applications are assigned to the assignee of this invention and are incorporated herein by reference thereto.

The above cited copending applications, Ser. No. 266,278 and Ser. No. 384,113 disclose that an arbitrary protein will adhere to a substrate in a monomolecular layer only and that no other arbitrary protein will adhere to the protein layer. On the other hand, the specifically reacting protein to the first protein absorbed onto the substrate will immunologically bond thereto. In accordance with the teachings of those applications, this discovery is exploited to provide medical diagnostic apparatus in which a substrate having a monomolecular layer of one protein absorbed thereon is used to test suspected solutions for the presence of the specifically reacting protein thereto. If the specifically reacting protein is present in the solution, the slide, after immersion in the solution, has a bimolecular protein layer on the substrate. If the specifically reacting protein be absent from the solution, the slide after immersion, has only the original monomolecular protein layer on the substrate. Optical, electrical, and chemical means for distinguishing between a bimolecular protein layer and a monomolecular protein layer are taught in these related copending applications of Giaever.

In two embodiments of diagnostic apparatus taught in these two related copending applications of Giaever, optical distinction between monomolecular and bimolecular protein layers is made by unaided visual observation of the slide and is further taught therein to provide detection sensitivities which are functions of the structure of the slide and of the absolute thickness of the protein layer or layers thereon. Specifically, the diagnostic slide taught by Giaever to comprise a plurality of metal globules attached to a substrate and having a monomolecular protein layer thereon is taught to have maximum sensitivity for films thinner than 200 Å, while the embodiment taught by Giaever to comprise a gold surface substrate is taught to have maximum sensitivity for films exceeding 30 Å in thickness. The chemical detection embodiment taught by Giaever involves the use of a substrate having a surface of a material which forms a visible amalgam with mercury and depends for its operation on Giaever's discovery that a mercury drop placed atop a bimolecular protein layer requires approximately ten times as long a time to diffuse therethrough as a mercury drop placed atop a monomolecular protein layer. The electrical detection embodiment taught by Giaever involves the use of an electrically conductive substrate material and the placing of an electrode atop the protein layer thereon; the capacitance between the two conductive members is then measured and its value is an indication of the thickness of the dielectric protein film separating them and hence, whether such film is monomolecular or bimolecular. It may, therefore, be seen that the electrical and chemical embodiments taught by Giaever require that the protein to be detected, if present in the solution under test, form a complete second monomolecular layer on the substrate of the diagnostic slide.

The techniques and apparatus taught by Giaever provide for rapid, reliable, and inexpensive detection of immunologically reactive particles. However, in the case of the gold surface substrate, the immunologically reactive particles must exceed approximately 20 Å in diameter. More significantly, some particles, whose detection is highly significant, are present in physiologic specimens only in highly dilute concentrations. In this case, only a small plurality of such particles become bonded to the diagnostic slide and their detectability is limited.

In the special case of large particles found in dilute concentrations, for example, viruses, Giaever has provided, in accordance with the teachings of the above-referenced copending application, Ser. No. 388,406, means for reliable detection of the particles. In this special case, as taught in application Ser. No. 388,406 the test slide includes a layer of an etchable metal underlying a layer of, for example, antibodies to the virus to be detected. A thin layer of non-etchable metal is deposited over the slide and any viruses bonded thereto. After etching, sites at which any viruses have been bonded appear as voids in the slide of sufficient size to be detected by, for example, optical microscopy.

It is known in the immunological arts that antibody molecules function as antigens when introduced into the system of a vertebrate to whom they are foreign proteins. Accordingly, specifically reacting antibodies to a given antibody may be readily produced. This capability has been utilized in the prior art to provide for attachment of fluorescent molecules to immunologically complexed proteins to aid in the detection of particular species of microorganisms. For example, antibodies to human immunoglobulins may be prepared and chemically combined with flourescent organic molecules such as the isothiocyanates. This socalled tagged antibody is then used to render an immunological reaction visible by ultraviolet microscopy. One of the most significant utilities for such antibodies to antibodies known to the prior art is the serological test for syphilis after the method of Coons, now known in the art as FTA-STS procedure. In the FTA-STS procedure, a quantity of Treponema pallidum (T. pallidum) is dried on a slide. The slide is then immersed in a blood specimen. The slide is subsequently immersed in a solution of tagged immunoglobulin. The anti-human immunoglobulin does not bond to the T. pallidum; accordingly, only if the specimen contained antibodies to T. pallidum will the slide fluoresce when observed by ultraviolet microscopy.

Thus, the prior art may be seen to have found utility for a single immunological reaction. However, no utility is known in the prior art for serially occurring immunological reactions.

It is the principal object of this invention to improve the detection sensitivity of immunologic diagnostic slides.

A further object of this invention is to improve such detection sensitivity by building multimolecular layers of specifically reacting proteins on such slides.

It is another object of this invention to improve the detection sensitivity of such slides by bonding additional protein layers thereon in sufficient quantity to insure the presence of at least two additional complete monomolecular protein layers on a surface of such an immunologic diagnostic slide.

Briefly, and in accordance with one embodiment of this invention, an immunologic test slide having as one surface thereof a monomolecular layer of protein which is immunologically reactive with the protein to be detached is immersed in a solution suspected of containing the protein to be detected. After immersion in the suspected solution, the substrate has a bimolecular protein layer thereon consisting of the original monomolecular protein layer and overlying monomolecular layer of the protein to be detected if the protein to be detected was present in the suspected solution. On the other hand, if the suspected solution did not contain the protein of interest, the slide after immersion therein has only the original monomolecular protein layer thereon. The slide is then immersed in a solution of protein which reacts immunologically with the protein of the second monomolecular layer. The slide then has a trimolecular protein layer thereon if the suspected solution contained the protein of interest or a monomolecular layer thereon if the suspected solution did not contain the protein of interest. Accordingly, detection sensitivity of the slide is improved because of the increased contrast between a monomolecular and trimolecular protein layer as opposed to that between a monomolecular and bimolecular protein layer.

The novel features of this invention sought to be patented are set forth with particularity in the appended claims. The invention, together with further objects and advantages thereof, may be understood from a reading of the following specification and appended claims in view of the accompanying drawing in which:

FIG. 1 is a schematic illustration of the attachment of a plurality of protein layers to a substrate.

FIG. 2 is an immunologic test cross-sectional elevation view of a slide having three monomolecular protein layers thereon.

FIG. 3 is a sectional elevation view of a slide prepared in accordance with one embodiment of this invention illustrating the completion of a monomolecular layer on a slide.

FIG. 1 shows a substrate **10** having adsorbed thereon a plurality of arbitrary protein molecules or other particles, such as, for example, viruses or hepatitis associated antigen particles **11** in accordance with the teachings of the aforementioned copending applications of Giaever. A plurality of molecules or other particles **11** are immunologically complexed with their specifically reacting antibody molecules indicated generally at **21** and **22.** Antibody molecules **21** and **22** are illustrated schematically to facilitate an explanation of the structure thereof. For convenience, unless otherwise required by specific context, the initial particle layer is referred to hereinafter as "molecules", it being understood that other particles are also included in the scope of this invention.

The five major classes of antibodies comprise the immunoglobulins designated as IgG, IgM, IgA, IgD, and IgE. The preponderant immunoglobulin is IgG, which constitutes approximately 85 percent of the immunoglobulins in normal human sera and is found in roughly similar preponderance in the normal sera of other vertebrates. An IgG particle comprises two heavy peptide chains and two light peptide chains joined by disulphide bonds to form a generally "Y" shaped antibody molecule. IgG antibody molecules are immunologically divalent; that is to say, each molecule has two antibody active sites. The active sites **21***a*1 and 21*a*2 of molecule 21 and 22*a*1 and 22*a*2 of molecule 22 are located at the extreme ends of the arms of the Y-shaped molecule. The active sites provide for immunologic bonding of the antibody to a molecule of its associated antigen, as shown, for example, in FIG. 1 where active sites 21*a*1, 22*a*1, and 22*a*2 are bonded to a plurality of protein molecules 11. As previously pointed out, antibody molecules also function as antigens when introduced into a vertebrate system to which they are a foreign protein. They accordingly stimulate the production of antibodies specific to the anitbody qua antigen. An antibody to IgG antibody is another IgG antibody, and accordingly also has two active sites. These active sites bond to particular antigenically active sites on the first antibody. The antigenically active sites are known alternatively as antigenic determinants or epitopes. As previously stated, the number of antibody active sites of an IgG antibody is known to be 2. The number of epitopes associated with an IgG molecule is not precisely known but the number of epitopes is known to be at least 2. Epitopes are known to be present on the tail of the Y-shaped molecule and are probably also present on the arms thereof. For the purposes of illustrating the principle of the operation of this invention, three epitopes are shown disposed along the heavy peptide chains of each antibody molecule illustrated in FIG. **1.** The epitopes of antibody molecule **21** are designated as **21***e*1, 21*e*2, and 21*e*3. The epitopes of antibody molecule 22 are designated as 22*e*1, 22*e*2, and 22*e*3.

Thus, the adsorption of molecules **11** onto substrate **10** and the immersion of the resulting immunologic test slide into a solution of antibodies to protein **11** to immunologically bond molecules **21** and **22** thereto constitutes the practice of the aforementioned inventions of Giaever. In accordance with the practice of this invention, a quantity of solution of antibodies of type represented by **21** and **22** is injected into a vertebrate to stimulate the production of antibodies thereto. The antibodies produced are collected by means known in the art, and a solution thereof is provided for use in the improved diagnostic procedure in accordance with this invention. The slide is immersed in a solution of the anti-antibodies and if molecules **21** and **22** are bonded to molecules **11,** a plurality of molecules **31, 32, 33, 34,** and **35** become immunologically bonded to molecules **21** and **22.** If, on the other hand, antibodies of the type of molecules **21** and **22** are absent from the solution into which the slide was first dipped, molecules **31–35** will be absent from the slide after immersion into the solution containing them because molecules **31–35** react specifically with molecules **21** and **22** and will not attach to molecules **11.** Accordingly, at this stage, the procedure in accordance with this invention, the detectability of molecules **21** and **22** is improved in that their presence is determined by distinguishing between a layer of molecules **11** and a layer of molecules **11, 21,** 22, 31, 32, 33, 34, and **35** as opposed to distinguishing between a layer of molecules **11** and a layer of mole-

cules **11, 21,** and **22.** Further, in accordance with this invention, the process may be repeated to add additional protein layers to further increase detection sensitivity, as for example, the selective bonding of molecules **41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52,** and **53** to molecules **31, 32, 33, 34,** and **35.**

Antibody molecules **31, 32, 33, 34,** and **35** have respectively active sites **31***a*1, 31*a*2, 32*a*1, 32*a*2, 33*a*1, 33*a*2, 34*a*1, 34*a*2, 35*a*1, and 35*a*2. Antibody 31 is immunologically bonded to antibody 21 by the attachment of active site 31*a*2 to epitope 21*e*2. Antibody 32 is immunologically bonded to antibody 21 by the attachment of active site 32*a*1 to epitope 21*e*1. Antibody 33 is immunologically bonded to anitbody 22 by the attachment of active site 33*a*2 to epitope 22*e*2; antibody 34 is bonded to antibody 22 by the attachment of active site 34*a*1 to epitope 22*e*1; antibody 35 is bonded to antibody 32 by the attachment of active site 35*a*1 to epitope 22*e*3. As shown on the lelf-hand side of FIG. 1, with regard to the protein chain beginning with antibody 21, each antibody is shown as having two active epitopes. That is, each antibody qua antigen binds two antibodies thereto. Thus, antibody 21 has antibodies 31 and 32 bonded thereto which in turn have antibodies 41, 42, 43, and 44 bonded thereto. This corresponds to the known minimum number of active epitopes per IgG antibody molecule. On the right-hand side of FIG. 1, the protein chain beginning with antibody 22 is illustrated as having three active epitopes per antibody. Thus, it may be seen that the sensitivity improvement obtained in accordance with this invention is proportional to the number of active epitopes per antibody molecule and the number of immersions of the slide into successive antibody solutions.

In one example of sera immunological reactions performed in accordance with this invention, a layer of bovine serum albumin was adsorbed on a substrate. The resulting slide was then immersed in a solution of rabbit anti-serum to bovine serum albumin, after which the slide contained the original bovine serum albumin layer having immunologically bonded thereto a layer of rabbit IgG immunoglobulin antibodes to bovine serum albumin. The slide was next immersed in a solution of goat anti-serum to rabbit IgG, after which the slide contained a layer of bovine serum albumin, a layer of rabbit anti-BSA IgG, and a layer of goat anti-rabbit IgG antibodies. The next step was the immersion of the slide in a solution of rabbit anti-serum to goat IgG to add a fourth layer of rabbit anti-goat IgG molecules to the slide. Clearly, the last two steps of goat anti-serum to rabbit IgG, followed by rabbit anti-serum to goat IgG may be repeated serially for as many operations as desired to thereby build an n-molecular layer protein film.

In another example of the application of this technique, a quantity of hepatitis associated antigen particles was adsorbed on a substrate. The resulting slide was then immersed in a human serum specimen. The slide was next immersed in a solution of rabbit antibodies to human hepatitis antibody. Accordingly, the slide, if the human test serum contained hepatitis antibody, has thereon hepatitis associated antigen having hepatitis antibody immunologically bonded thereto and rabbit anti-human hepatitis antibody immunologically bonded to the hepatitis antibody. On the other hand, if the serum sample were negative, the slide would have only the original hepatitis associated antigen particles thereon. This technique in accordance with this invention was found to significantly improve the detectability of human hepatitis over that obtainable by the bonding of hepatitis antibody to hepatitis associated antigen alone. This example points out that the technique of this invention has particular utility in cases in which the initial protein including particle deposited on the slide is substantially larger than its specifically reacting protein.

FIG. **2** is a sectional elevation view illustrating a multimolecular protein film on a slide. Substrate **60** may, for example, in accordance with the first example cited above, initially have a monomolecular layer **61** of bovine serum albumin adsorbed thereon to constitute an immunologic diagnostic slide. Immersion in rabbit anti-serum to bovine serum albumin will adhere a second monomolecular layer **62** over layer **61** by specific immunologic reaction. Subsequent immersion in goat anti-serum to rabbit IgG will immunologically bond monomolecular layer **63** to layer **62** if layer **62** is present. Similarly, layer **64** comprising rabbit anti-goat IgG is bonded to the slide by immersion in rabbit anti-serum to goat IgG. As indicated above, the process may be continued but since the bonding of layers is a specific immunologic reaction, the process will be irrevocably interrupted if any one layer does not form. Specifically, the structure of FIG. **2** has utility for high sensitivity detection of the protein comprising layer **62.** Layer **61** is attached to slide **60** by adsorption which is a non-specific process. All subsequent processes, however, are specific. Accordingly, if a test specimen contains the specifically reacting protein to the protein of layer **61,** layer **62** forms and an n-molecular protein layer can be formed thereover in accordance with this invention. If, on the other hand, the test specimen does not contain protein immunologically reactive with the protein of layer **61,** layer **62** does not form and no subsequent layers can be formed. Therefore, a protein of interest in a specimen may be detected with any desired degree of sensitivity by distinguishing between a monomolecular protein layer on a substrate and a layer of any desired thickness.

FIG. **3** is a cross-sectional elevation view of a slide illustrating another utility for the technique of this invention. FIG. **3** illustrates the utility of this invention in the detection of proteins which are present in physiologic specimens in concentrations too dilute to provide for the formation of a complete layer on a slide. As an example utilizing specific proteins and not by way of limitation to those proteins, a substrate **70** has absorbed adsorbed a monomolecular layer of human hepatitis antibodies **71.** The resulting slide is immersed in a blood specimen and a plurality of hepatitis associated antigen particles **77** become immunologically bonded thereto if they are present in the specimen. The slide is then immersed in a solution of monkey hepatitis antibody to bond a layer of molecules **72** thereof on such hepatitis associated antigen particles as may be bonded to the slide. The slide is next immersed into a solution of rabbit anti-serum to monkey IgG to bond a layer of molecules **73** thereof over the monkey hepatitis anitbody molecules. It is clear that the layer of molecules **72** could be formed of human hepatitis antibody **71.** However, if this were done, the next layer would have to be anti-human IgG molecules which would bond not only to those antibodies surrounding the hepatitis associated antigen particles **77** but also to the original layer of molecules **71** thereby defeating the purpose of the

technique of this invention. The next immersion of the slide may be, for example, into goat anti-serum to rabbit IgG to form a layer of molecules **74** thereof on the slide. This process is repeated a number of times as indicated generally by the volume shown as **75** until the point is reached at which a last monomolecular protein layer **76** completely fills the spaces between particles **77** on the slide. This embodiment of this invention provides for the formation of a protein layer of at least bimolecular thickness over the entirety of the slide and has particular utility to the chemical and electrical detection methods disclosed in the aforementioned copending applications of Giaever as discussed above.

While this invention has been described with reference to particular embodiments and examples, other modifications and variations will occur to those skilled in the art, in view of the above teachings. Accordingly, it should be understood that within the scope of the appended claims, the invention may be practiced otherwise than is specifically described.

The invention claimed is:

1. A method for improving the detection sensitivity of immunologic diagnostic slides, said slides comprising a substrate having a layer of particles of a first protein adsorbed thereon for specifically reacting immunologically with a second protein, said second protein being the protein to be detected, said method comprising the steps of:

a. contacting said slide with a fluid suspected of containing said second protein to bond a plurality of particles of said second protein to particles of said first protein, and then

b. contacting said slide with a solution of a third protein immunologically reactive with said protein to be detected, said first, second and third proteins all being different from each other.

2. The method of claim **1** further comprising the steps of:

preparing a solution of antibodies to the third protein immunologically reactive with the second protein to be detected; and

c. contacting said slide resulting from step (b) with said solution of antibodies.

3. The method of claim **1** further comprising the steps of:

preparing a series of solutions of antibodies, each solution of said series comprising antibodies to the antibodies of the next preceding solution of the series; and

c. serially contacting said slide resulting from step (b) with each solution of said series.

* * * * *