

PATENT SPECIFICATION

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(54) SPECIFIC BINDING-ADSORBENT ASSAY METHOD AND TEST MEANS

(71) We, MILES LABORATORIES INC., a Corporation organised and existing under the laws of the State of Indiana, U.S.A., of 1127 Myrtle St., Elkhart, Indiana 46514, U.S.A., do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

Background of the Invention

1. Field of the Invention

This invention relates to methods and means for determining a substance in a liquid based on its affinity for a specific binding partner. In one aspect, therefore, the present invention relates to binding assays of the immunological type for determining antigens and haptens or their antibodies. In a preferred embodiment, the present invention relates to radioimmunoassay (RIA) methods and means for detecting hepatitis B surface (HB_s) antigen in serum and plasma samples.

There is an ever increasing interest in improving specific binding assay techniques and test means. Classical RIA techniques involve competition between sample antigen and radiolabeled antigen for binding with a specific antibody. The amount of radioactivity which becomes associated with the antibody is a function of sample antigen concentration and can be determined by precipitating, and thereafter separating, the radioactive antigen-antibody complex, or more conveniently, by using an insoluble form of antibody such as antibody coupled to solid particles or matrices. Recently, several alternative labeling materials have been reported for replacement of radio-isotopes, including enzymes, coenzymes, enzyme substrates, fluorescent molecules, molecules capable of participating in light producing reactions, and enzyme modulators.

There is a particular need for a more advantageous specific binding assay technique for detecting hepatitis B surface (HB_s) antigen in samples of serum or plasma. It is well accepted that a patient can contract hepatitis after receiving a transfusion of blood containing HB_s antigen. Until recently, a wide variety of techniques were used routinely by hospital laboratories and blood banks to detect HB_s antigen in blood drawn for transfusion purposes. Such techniques included immunodiffusion, complement fixation, counterelectrophoresis, latex agglutination, hemagglutination, and RIA. Recently, it has become a requirement in the United States that blood drawn for transfusion be tested for hepatitis by the most sensitive assay techniques available. RIA methods have since become the most widely used for detecting HB_s antigen.

2. Description of the Prior Art

Several specific binding assay techniques have evolved which make use of nonspecific binders or adsorbents. Such specific binding-adsorbent assays fall into two distinct categories:

(1) Use of Adsorbents to Separate Free Label from Bound Label

This category is exemplified by the method described in U.S. Patents Nos. 3,414,383; 3,937,799 and 3,938,953 and is usually applied to the detection of small molecules such as hormones, particularly thyroxine. In these methods, competition

is initiated in solution between sample ligand and labeled ligand for a specific binding partner, such as a hormone-binding-protein or an antibody. Upon completion of the competitive reaction, free labeled ligand is separated from labeled ligand bound to the binding partner by addition of a nonspecific adsorbent for the free ligand.

(2) Use of Adsorbents to Pre-Extract Sample Ligand

In this method, a nonspecific adsorbent is added to the sample to extract the ligand of interest. The adsorbent is then removed from the sample, adsorbed ligand is eluted off of the adsorbent, and the released ligand is placed in competition with labeled ligand for a specific binding partner. U.S. Patents Nos. 3,776,698; 3,816,076 and 3,947,564 are representative of this technique. A variation is described in U.S. Patent No. 3,659,104 for the detection of thyroxine wherein the sample and radiolabeled thyroxine are passed through a column of adsorbent followed by passage of a soluble specific binder, thyroxine binding globulin. The amount of radiolabeled thyroxine eluted from the column is proportional to the amount of thyroxine in the sample.

Nonspecific adsorbents have also been used as sites for competition between sample ligand and labeled ligand, particularly for the determination of hormone uptake values. Application of this technique to the determination of triiodothyronine (T-3) uptake values is described in U.S. Patents Nos. 3,451,777; 3,710,117 and 3,823,001.

In United States Department of Commerce, National Technical Information Service (NTIS) Report No. PB-241,333 dated January 27, 1975 and in U.S. Patent No. 4,020,151 issued April 26, 1977 (based on application Serial No. 658,298 filed February 17, 1976), there are described immunoassay methods for detecting antigens or antibodies on a solid surface. Neither reference described the use of ion-exchange adsorbents under controlled conditions of pH to minimize non-specific adsorption nor the specific detection of HB_s antigen.

Additional background regarding the use of nonspecific adsorbents in analytical techniques, and in specific binding assays in particular, is afforded by the following: *Principles of Competitive Protein-Binding Assays*, ed. Odell and Daughaday, Chapters IX and XI, J.B. Lippincott Company (1972), and *Clinical Chemistry 19(2)*: 146—186 (1973).

Returning to assay methods specifically for detecting hepatitis, all of the present commercially available RIA methods for detecting HB_s antigen employ a two-site immunoradiometric, or so-called "sandwich", approach. In this technique, the serum sample to be tested is incubated with an insolubilized antibody against HB_s antigen (anti-HB_s), such as in the form of anti-HB_s coated on the insides of a test tube or on the surface of plastic beads. A proportion of the insolubilized anti-HB_s becomes bound by sample HB_s antigen if present. The insoluble material is then washed and radiolabeled anti-HB_s added. Following another wash, the amount of radioactivity associated with the insolubilized anti-HB_s is measured as an indication of the presence or absence of HB_s antigen in the sample tested. Exemplary of this RIA approach are the methods described in U.S. Patents Nos. 3,867,517 and 3,949,064.

While relatively specific and sensitive to HB_s antigen, the prior art "sandwich" RIA approaches leave room for improvement in their manufacture and use. The preparation and quality assurance of the insolubilized anti-HB_s is relatively costly and time consuming. Furthermore, the assay procedure requires two lengthy incubation steps of 30 minutes or more, one for the interaction between sample HB_s antigen and the insolubilized anti-HB_s, and the other for interaction with the labeled anti-HB_s. Also, the insolubilized anti-HB_s is relatively unstable and requires special shipping and storage conditions.

An additional disadvantage of the prior art RIA approaches is their inability to detect HB_s antigen in the form of its immune complex with anti-HB_s in the sample tested. The nature of the insolubilized anti-HB_s used in the prior art methods is such that it will bind only free HB_s antigen having free antigen determinants and not HB_s antigen-antibody complex. Thus, any danger of post-transfusional hepatitis due to the presence of HB_s immune complex in the transfused blood would go undetected using the available assay methods.

It is therefore an object of the present invention to provide an improved specific binding assay method and test means employing a nonspecific adsorbent for the substance to be determined, particularly for determining HB_s antigen, in its free state or additionally in the form of its immune complex.

Summary of the Invention

The present invention provides a uniquely convenient method and test means for determining a specific binding substance in a liquid sample employing a nonspecific adsorbent and a labeled form of a specific binding partner to the substance to be determined. The assay method, in general, comprises the steps of:

- (a) contacting a solid adsorbent with the liquid sample to be tested and with a specific binding partner of the substance to be determined, which binding partner is incorporated with a label and which adsorbent is of a type capable of nonspecifically binding with the substance to be determined, such as an ion exchange material; and
- (b) determining the amount of the label which either (i) becomes associated with the adsorbent by binding of the labeled binding partner with adsorbed substance to be determined or (ii) remains unassociated therefrom.

wherein step (a) is performed under conditions favorable to binding between the substance to be determined and the adsorbent, and either the adsorbent is of a type incapable of significantly binding with the labeled binding partner

or step (a) is performed under conditions unfavorable to significant binding between the labeled binding partner and the adsorbent.

By nonspecific binding is meant a general physiochemical attraction, such as of an ionic or hydrophobic (van der Waals) type, occurring between the adsorbent and the substance to be determined.

It is preferable that step (a) be accomplished by (i) contacting the sample with the adsorbent, (ii) optionally separating the adsorbent from the unadsorbed portion of the sample, and (iii) thereafter contacting the adsorbent with the labeled binding partner. However, if desired, the adsorbent may be contacted with the sample and the labeled binding partner substantially simultaneously.

Step (b) may be accomplished by separating from the adsorbent substantially all of the labeled binding partner not associated therewith by binding with adsorbed substance to be determined, and measuring the amount of the label either associated with the adsorbent or separated therefrom. This approach is particularly useful where the label is a radio-active material. The present method offers the ability to obtain a concentration effect by contacting the adsorbent with a volume of the sample several times, preferably 2 times, the volume of the adsorbent. The method is applicable to a wide variety of substances and is particularly suited for the detection of HB_s antigen and anti-HB_s.

The use of an ion exchange material as the adsorbent provides an assay method particularly suited to the determination of water soluble substances having a net positive or negative electrical charge. The labeled binding partner selected has an isoelectric point (pI value) different from that of the substance to be determined. An ion exchange material and pH conditions can then be selected whereby the adsorbent is capable of nonspecifically binding with the labeled binding partner. The pH conditions are selected to be between the pI value of the substance to be determined and that of the labeled binding partner. Then, if the pI value of the substance to be determined is less than that of the labeled binding partner, an ion exchange material of an anionic type is selected. If the relationship between the pI values is reversed, a cationic type of ion exchange material is selected.

As applied to the detection of hepatitis B surface (HB_s) antigen, which has a net negative charge and a pI value of less than about 5, the particularly preferred assay method comprises the steps of:

- (a) contacting the sample to be tested with a solid anion exchange material under conditions of pH between 5 and 8;
- (b) washing the anion exchange material with a liquid having a pH of between 5 and 8;
- (c) contacting the anion exchange material under conditions of pH between 5 and 8 with anti-HB_s incorporated with a label and having a pI value greater than about 8; and
- (d) determining the amount of the label which either (i) becomes associated with the anion exchange material by binding of the labeled anti-HB_s with adsorbed HB_s antigen or (ii) remains unassociated therefrom.

Preferably the anion exchange material is a diethylaminoethyl-substituted polymer, such as DEAE-cellulose, preferably is in a particulate form, and preferably is contained in a flow-through column.

The present invention also provides a particularly advantageous method for detecting anti-HB_s comprising the steps of:

- (a) providing a solid adsorbent having nonspecifically bound thereto HB_s antigen;
- 5 (b) contacting the adsorbent with the sample to be tested and with anti-HB_s incorporated with a label; and
- (c) determining the amount of the label which either (i) becomes associated with the adsorbent by binding of the labeled anti-HB_s to adsorbed HB_s antigen or (ii) remains unadsorbed therefrom,

10 wherein either the adsorbent is of a type incapable of significantly binding with the labeled anti-HB_s or step (b) is performed under conditions unfavorable to significant binding between the labeled anti-HB_s and the adsorbent.

Also provided by the present invention is a method for detecting HB_s antigen in the form of its immune complex with anti-HB_s. Such method comprises the steps of:

- 15 (a) combining the sample to be tested, and immuno-chemical-bond weakening agent, preferably a chaotropic agent such as urea, and a solid adsorbent of a type capable of nonspecific binding with HB_s antigen, preferably an anion exchange material such combination being effected under conditions favourable to such binding;
- 20 (b) separating said bond weakening agent from the adsorbent; and
- (c) determining the HB_s antigen bound to the adsorbent, by contacting the same with an antibody to hepatitis B surface antigen, which antibody is incorporated with a label, and determining the amount of the label which becomes associated with the adsorbent by binding of the labelled antibody with adsorbed hepatitis B surface antigen.
- 25

Brief Description of the Drawing

Figure 1 is a schematic diagram of one form of the present method applied, for illustration purposes, to the detection of an antigen (Ag);

30 Figure 2 is an exploded perspective view of a column test device which may be comprised in test means for carrying out the present method; and

Figure 3 is a graphical representation of the affinity of various adsorbents for HB_s antigen under various conditions of pH.

Description of the Preferred Embodiments

35 The preferred mechanism by which the present assay method may be accomplished is depicted schematically in Figure 1 of the drawing. This figure relates, for illustration purposes only, to the detection of an antigen (Ag) using a labeled antibody (Ab*). Any physical form of the adsorbent (Ad) suggested in the diagram is merely illustrative, since the adsorbent may take on a wide variety of forms as will be described more fully hereinafter.

40 Referring to Figure 1, a sample suspected of containing an antigen of interest (Ag) is brought into contact with the adsorbent (Ad) as illustrated by steps (a) and (b). A proportion of the antigen present in the sample becomes bound to the adsorbent. Excess labeled antibody (Ab*) is then contacted with the adsorbent, as illustrated by steps (c) and (d), resulting in the association of labeled antibody with the adsorbent by binding with adsorbed antigen. As illustrated by step (e), the excess labeled antibody is separated from the adsorbent with the amount of labeled antibody remaining associated with the adsorbent being a direct function of the amount of antigen present in the sample tested. This method may also be adapted to the detection of antibody by substituting labeled antigen for labeled antibody in steps (c) through (e) and by selecting an appropriate adsorbent and assay conditions.

45 The adsorbent must have an affinity for nonspecific binding with antigen while having no substantial affinity for antibody, or *vice-versa* where the method is adapted as described in the preceding paragraph for the detection of antibody.

55 Such a situation can be obtained in one way by selecting an adsorbent of a type which by its physical and/or chemical nature is capable of nonspecifically binding with the substance to be determined (i.e., antigen in the method depicted in Figure 1) but which is incapable under any conditions of significantly binding with the labeled binding partner (i.e., labeled antibody in the method depicted in Figure 1).

60 An example of this situation is the use of an insolubilized lectin, such as Concanavalin A, as the adsorbent in an assay for a carbohydrate-containing antigen, such as a glycoprotein, using a labeled F_{ab} fragment as the specific binding partner.

The glycoprotein would be strongly adsorbed by Concanavalin A, whereas the labeled F_{ab} would be unaffected by its presence.

Another way of selecting the necessary binding relationships between the substance to be determined, its labeled binding partner, and the adsorbent is to select an adsorbent which under certain environmental conditions will strongly adsorb the substance to be determined but will not significantly bind with the labeled binding partner. Such environmental conditions affecting the affinity of the adsorbent include pH, ionic strength or salt concentration, and solvent composition. One example of a situation of this type is the use of a hydrophobic material as the adsorbent in an assay for a material containing hydrophobic groups such as HB_s antigen. Under appropriate conditions of ionic strength, hydrophobic materials such as alkyl-substituted agaroses and ω -amino alkyl-substituted agaroses strongly adsorb HB_s antigen but bind only insignificantly with anti- HB_s . Another example is the use of a cross-linked dextran gel (e.g., Sephadex® gel made by Pharmacia AB, Uppsala, Sweden) as the adsorbent in an assay for aromatic haptens or antigens, such as triiodothyronine or tetraiodothyronine (thyroxine). Under appropriate conditions of pH, the aromatic hapten or antigen is strongly adsorbed by the dextran gel whereas subsequently added labeled binding partner (e.g., antibody to triiodothyronine or thyroxine, or thyroxine binding globulin) is not adsorbed significantly.

In addition to ion exchange materials, which will be discussed below, numerous other adsorbent materials may be used in the context of the present invention. Following is a list of some such useful adsorbents.

Adsorbents

Non-ionic cellulose

e.g., Whatman (Clifton, New Jersey, U.S.A.) types —
 CF-1®, long fiber powder
 CF-11®, medium fiber powder
 CC-31®, microgranular powder
 CC-41®, microgranular powder

("Whatman" is a registered Trade Mark)

e.g., Bio-Rad (Richmond, California, U.S.A.) types —
 Cellex® N-1, powder
 Cellex® 410, powder

Silica gel

e.g., Whatman type—SG 81, loaded paper; or
 Bio-Rad types—Bio-Sil® A or Bio-Sil® HA

Hydroxylapatite (Bio-Rad)

Alumina: acid, base, or neutral types (Bio-Rad)

Alumina C-gamma gel (Bio-Rad)

Calcium phosphate

Hydroxypropyl dextran

e.g., Pharmacia (Piscataway, New Jersey, U.S.A.) type—Sephadex®

LH 20

Dextran (Pharmacia)

Dextran sulfate (Pharmacia)

Alkyl agaroses

e.g., Pharmacia types—octyl-Sepharose® CL-4B or
 phenyl-Sepharose® CL-4B

e.g., Miles Research Products (Elkhart, Indiana, U.S.A.) types— ω -amino
 alkyl agaroses

Lectin-agarose (Miles Research Products)

Poly-L-lysine agarose (Miles Research Products)

Plastics, e.g., polystyrene, polyethylene, and polypropylene.

It will be recognized by one skilled in the art that various adsorbent/substance to be detected/labeled binding partner combinations may be adapted to the assay method and test means of the present invention.

It is particularly preferred to use an ion exchange material as the adsorbent when the substance to be determined is water soluble and has an isoelectric point (pI value) different from that of a labeled specific binding partner. The difference between the pI values of the substance to be determined and the labeled binding partner usually should be at least 0.5 and preferably is greater than 2.0. The isoelectric point of a substance is the pH at which the substance has no net

electrical charge. When in solution, the effective charge of such a substance depends upon the pH of its environment. In a solution having a pH above that of the pI value of a substance, such substance will take on an effective net negative charge. Conversely, such substance will take on an effective net positive charge in a solution having a pH below that of its pI value. The charged substance in solution will have an affinity for an ion exchange material bearing an opposite charge. Thus, a negatively charged substance will be attracted by an anion exchange material and a positively charged substance will be attracted by a cation exchange material. Therefore, in carrying out the present method, the substance to be determined will be adsorbed by the ion exchange material, while the labeled binding partner will not, by selecting an ion exchange material (i) of an anionic type when the pI value of the substance to be determined is less than that of the labeled binding partner, or (ii) of a cationic type when the pI value of the substance to be determined is greater than that of the labeled binding partner; and by performing the assay under conditions of pH between the pI values of the substance to be determined and the labeled binding partner. Following are lists of various ion exchange materials that may be used in accordance with the present invention:

Anion Exchange Materials

| | | |
|----|---|----|
| 20 | Diethylaminoethyl (DEAE) cellulose e.g., Whatman types— DE-1®, floc DE-11®, powder DE-22®, fibrous DE-23®, fibrous | 20 |
| 25 | DE-32®, dry, microgranular DE-52®, wet, microgranular DE-81®, paper e.g., Bio-Rad type—Cellex® D, fibrous | 25 |
| 30 | Diethylaminoethyl (DEAE) agarose e.g., Bio-Rad type—DEAE Biogel® A Diethylamino (DEAE) dextran e.g., Pharmacia type—DEAE Sephadex®, bead | 30 |
| 35 | Aminoethyl-Sepharose® 4B (Pharmacia) Ecteola cellulose e.g., Whatman types— ET-11®, powder ET-41®, powder (high purity) ET-81®, paper e.g., Bio-Rad type—Cellex® E, fibrous | 35 |
| 40 | Triethylaminoethyl (TEAE) cellulose e.g., Bio-Rad type—Cellex® T, fibrous Diethyl-(2-hydroxypropyl)-amino (QAE) cellulose e.g., Bio-Rad type—Cellex® QAE, fibrous | 40 |
| 45 | Diethyl-(2-hydroxypropyl)-amino (QAE) dextran e.g., Pharmacia type—QAE-Sephadex® Benzoylated diethylaminoethyl cellulose e.g., Bio-Rad type—Cellex® BD, fibrous | 45 |

Cation Exchange Materials

| | | |
|----|---|----|
| 50 | Cellulose phosphate e.g., Whatman types— P-1®, floc P-11®, powder P-41®, powder (high purity) P-81®, paper | 50 |
| 55 | Carboxymethyl cellulose e.g., Whatman types— CM-1®, floc CM-11®, powder CM-22®, fibrous CM-23®, fibrous | 55 |
| 60 | CM-32®, dry, microgranular CM-52®, wet, microgranular CM-82®, paper | 60 |

| | | |
|----|--|----|
| | e.g., Bio-Rad type—Cellex® CM, fibrous | |
| | Carboxymethyl dextran | |
| | e.g., Pharmacia type—CM-Sephadex® | |
| 5 | Phosphoryl cellulose | |
| | e.g., Bio-Rad type—Cellex® P, fibrous | 5 |
| | Carboxymethyl agarose | |
| | e.g., Bio-Rad type—CM Biogel® A | |
| | e.g., Pharmacia type—CH-Sepharose® 4B | |
| 10 | Sulphopropyl dextran | |
| | e.g., Pharmacia type—SP-Sephadex® | 10 |

[For further information on the use of ion exchange materials as adsorbents see *J. Amer. Chem. Soc.* 78:756—763 (1956) and *J. Infect. Dis.* 129:371—375 (1974).]

A table illustrating various pairs of specific binding substances having different pI values follows:

| 15 | First Substance of Pair | pI | Second Substance of Pair | pI | 15 |
|----|------------------------------------|-------|-------------------------------------|-----|----|
| | HB _s antigen | 3.5—5 | Anti-HB _s | 7—8 | |
| | Thyroxine-binding-globulin (TBG) | 4.5 | Anti-TBG | 7—8 | |
| 20 | Albumin | 4.5 | Anti-albumin | 7—8 | |
| | IgG | 7—8 | Carbamylated anti-IgG | 5 | 20 |
| | Thyroid-stimulating-hormone (TSH) | 9.5 | Carbamylated anti-TSH | 5 | |
| | Ferritin | 5.4 | Anti-ferritin | 7—8 | |
| | Alkaline phosphatase (AP) | 4.3—5 | Anti-AP | 7—8 | |
| 25 | Fibrinogen | 5.5 | Anti-fibrinogen | 7—8 | 25 |
| | Follicle-stimulating hormone (FSH) | 3.0 | Anti-FSH | 7—8 | |
| | Insulin | 5.6 | Anti-insulin | 7—8 | |
| | Lysozyme | 10—11 | Anti-lysozyme | 7—8 | |
| 30 | Plasminogen | 6.5—8 | Carbamylated anti-plasminogen | 5 | 30 |
| | Prothrombin | 4.6 | Anti-prothrombin | 7—8 | |
| | Thyroglobulin | 4.5 | Anti-thyroglobulin | 7—8 | |
| | Transcobalamin | 6.2 | Anti-transcobalamin | 7—8 | |
| 35 | Thyroxine | 7.5 | Thyroxine-binding-globulin (TBG) or | 4.5 | 35 |
| | | | Anti-thyroxine | 7—8 | |
| | Luteinizing-hormone (LH) | 5 | Anti-LH | 7—8 | |

40 The present invention is applicable to the detection of either or both of the above listed first and second substances by selection of an anionic or cationic exchange material according to the guidelines given herein. 40

45 The adsorbent may take any physical form which allows interaction between its adsorption sites and the corresponding nonspecific binding sites on the substance to be determined and which allows further interaction of the adsorbed substance to be determined with the labeled binding partner. The adsorbent can take a wide variety of shapes of solid insoluble materials, usually of a polymeric nature. A particulate form, such as that of powders, beads, filaments and crystals, is preferred because of the relatively large surface area available; however, sponges, sheets, strips, pads, and other integral matrices and lattices may also be used even to the point of carrying out the assay in a vessel, such as a test tube or plate well, constructed in whole or in part of the adsorbent material. In addition to its large surface area, a particulate form is conveniently dispensable into a test vessel or reaction chamber and is readily useable in a column, flow-through format. 50

55 An illustration of a column test device preferred for use in the present method is provided in Figure 2 of the drawing. The depicted column test device comprises a cylindrical tubular body 11 terminating at one end in a tapered tip portion 12. The body 11 is formed of polypropylene or other suitable material and is provided with valve means, such as a friction fit bottom cap 13, which fits over and removably closes tip portion 12. A bed 14 of the adsorbent, such as an ion exchange material in particulate form, is retained between two porous polyethylene discs 15 and 16 in the lower portion of body 11. A reservoir 17 is provided in body 11 above the disc 15. In Figure 2 a quantity of liquid buffer is shown disposed within the reservoir 17. 60

A removable top cap 18 is provided for sealing the upper end of body 11 to provide an integral closed device for shipping and storage. The packed bed volume is preferably between 0.25 and 2.0 ml. The body 11 is preferably between 5 and 30 mm in diameter and between 50 and 150 mm in height.

5 With reference to the present method in its broadest aspects, it is preferred that the adsorbent be contacted with the test sample, separated from the unadsorbed portions of the sample, and then contacted with labeled binding partner. Separation of the adsorbent from unadsorbed portions of the sample assures removal of possible interferants from the sample which, if allowed to remain during the application of the labeled binding partner, might affect reproducibility by promoting nonspecific binding of the labeled material or otherwise. This separation is conveniently accomplished by washing the adsorbent under conditions favorable to adsorption of the substance to be determined. For example, following the method employing an ion exchange material as the adsorbent, the washing liquid may be a buffer having a pH between the pI values of the substance to be determined and the labeled binding partner. 10 15

The determination of the amount of the label which either (i) becomes associated with the adsorbent by binding of the labeled binding partner with adsorbed substance to be determined or (ii) remains unassociated therefrom may be accomplished in either of two ways. First, where the binding of the labeled binding partner with the adsorbed substance to be determined measurably alters some determinable characteristic of the label, the extent of alteration of such characteristic may be determined directly in the mixture formed upon introduction of the labeled binding partner to the adsorbent, much in the manner associated with "homogeneous" binding assays, with no need to separate "bound" label from "free" label. Such a situation could arise where the label used is an enzyme or coenzyme, or other material having a monitorable characteristic, and the binding of the labeled binding partner and the adsorbed substance to be determined would affect, either by inhibition or enhancement, the enzymatic or coenzymatic activity, or other characteristic, of the label. 20 25 30

In the second method, a separation is accomplished between the adsorbent and substantially all of the labeled binding partner not associated therewith by binding with the adsorbed substance to be determined, and the amount or an alterable characteristic of the label is measured either associated with the adsorbent or separated therefrom. This technique is required where the label is unaffected by binding between the labeled binding partner and the adsorbed substance to be determined, but can also be followed where some characteristic of the label is affected. Examples of the former type of label which are particularly useful in the present method are radioactive materials such as ^3H , ^{131}I and ^{125}I . However, it is contemplated that any of the known labels used in binding assays may be utilized. The separation required by this technique is conveniently accomplished by washing the adsorbent under conditions favorable to adsorption of the substance to be determined and, unless the adsorbent is of a type incapable of significant binding with the labeled binding partner, unfavorable to adsorption of the labeled binding partner. The amount of the label remaining associated with the washed adsorbent or contained in the washings can be related to the presence, or amount, of the substance to be determined in the sample tested by comparison with a negative control, or standard curve, respectively. Other methods can also be used to accomplish the desired separation, including centrifugation and filtration. 35 40 45

50 The present method is especially useful for the detection of hepatitis B surface (HB_s) antigen in liquid samples such as serum or plasma samples. As mentioned previously, various hydrophobic materials can act as adsorbents for HB_s antigen, including certain alkyl-substituted polymers such as ω -aminoalkyl-substituted agaroses. Particularly preferred, however, are ion exchange materials. The pI value of HB_s antigen falls roughly within the range of 3.5 to 5, and for anti- HB_s , roughly around 8. The assay conditions are therefore maintained between 5 and 8, and preferably between 6 and 8, and an anion exchange material is used as the adsorbent. Useful anion exchange materials are diethylaminoethyl-substituted polymers, particularly those having a polysaccharide, e.g., cellulose or dextran, backbone. DEAE-cellulose has been found to be a particularly useful HB_s antigen adsorbent. It is contemplated that a cation exchange material such as carboxymethyl cellulose may be used as the adsorbent if the pI value of the labeled anti- HB_s were altered to a value below that of HB_s antigen. 55 60

A particularly useful method for detecting HB_s antigen in a serum or plasma

sample using an anion exchange material in particulate form and contained in a column involves the steps of:

- (a) introducing the sample to a column of an anion exchange material in particulate form equilibrated in a liquid, such as a buffer, having a pH of between 5 and 8, preferably between 6.5 and 7.5;
- (b) passing through the column a liquid, such as a buffer, having a pH of between 5 and 8, preferably between 6.5 and 7.5, to remove substantially all HB_s antigen not adsorbed to the anion exchange material;
- (c) introducing to the anion exchange material a predetermined volume of a solution of radiolabeled anti-HB_s having a predetermined level of radioactivity;
- (d) incubating the solution of radiolabeled anti-HB_s in contact with the anion exchange material for between 1/2 and 18 hours, preferably between 1 and 3 hours, at a temperature of between 4 and 50°C, preferably between 40 and 50°C;
- (e) passing through the column a liquid, such as a buffer, having a pH of between 5 and 8, preferably between 6.5 and 7.5, to remove substantially all radiolabeled anti-HB_s not associated with the anion exchange material by binding to adsorbed HB_s antigen;
- (f) measuring the radioactivity of the anion exchange material or of the eluate from the next previous step; and
- (g) comparing the radioactivity measured in the next previous step to that measured following the same procedure using a liquid sample containing no significant amount of HB_s antigen in place of the serum or plasma sample.

Examples of buffers referred to in steps (a), (b), and (e) are phosphate buffer, barbital buffer, citrate buffer, tris-(hydroxymethyl)-aminomethane (TRIS) buffer, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) Buffer, and 1,4-piperazine-bis-(ethanesulfonic acid) (PIPES) buffer.

The particular advantages of the present method for detecting HB_s antigen over the prior art methods include the manufacturing advantages of eliminating the need for insolubilized anti-HB_s, which is a significant cost and stability factor in most prior art methods, and the user advantages of a single incubation step, fewer manipulations, storability of adsorbent materials, increased sensitivity, and versatility of detecting HB_s antigen in the form of its immune complex if desired.

In addition to the detection of HB_s antigen, the present method is readily adaptable to the detection of HB_s antigen in the form of its immune complex with anti-HB_s and to the detection of anti-HB_s itself.

The detection of HB_s antigen bound to anti-HB_s, i.e., in the form of its immune complex, is carried out by:

- (a) combining the sample to be tested, an immunochemical-bond weakening agent, and a solid adsorbent of a type capable of nonspecific binding with HB_s antigen; such combination being effected under conditions favourable to such binding;
- (b) separating said bond weakening agent from the adsorbent, such as by washing; and
- (c) determining the HB_s antigen bound to the adsorbent by contacting the same with an antibody to hepatitis B surface antigen, which antibody is incorporated with a label, and determining the amount of the label which becomes associated with the adsorbent by binding of the labelled antibody with adsorbed hepatitis B surface antigen.

By an "immunochemical-bond weakening agent" is meant a substance capable of decreasing the affinity of anti-HB_s for HB_s antigen in the presence of the adsorbent such that at least one antigenic determinant site is made available on the HB_s antigen upon separation of the bond weakening agent and the adsorbent. Such a bond weakening agent may be an acid or an alkali or other materials known for this purpose such as those referred to as chaotropic agents, e.g., urea, guanidine, or thiocyanate. [See *Immunochem.* 1:289—293 (1964); *Biochim. Biophys. Acta* 107:593—596 (1965); and *Arch. Biochem. Biophys.* 116:82—91 (1966).] The combined use of an anion exchange material, preferably DEAE-cellulose, and a urea solution, preferably at a concentration of between 2 and 8 molar, is particularly effective in this method for detecting HB_s antigen immune complex. The amount of HB_s antigen bound to the adsorbent may be determined by any available means but preferably is carried out by adding labeled anti-HB_s and

measuring the amount of label that becomes associated with the adsorbent in the manner discussed previously concerning the detection of HB_s antigen.

Anti-HB_s can be determined by following the general method discussed previously, that is, by contacting a test sample with an adsorbent for anti-HB_s, thereafter with labeled HB_s antigen and determining the amount of label which either becomes associated with the adsorbent or remains unassociated therefrom. Additionally, the availability of strong adsorbents for HB_s antigen, such as ion exchange materials, can be taken advantage of in the detection of anti-HB_s by following the method of:

- (a) providing a solid adsorbent having nonspecifically bound thereto HB_s antigen;
- (b) contacting the adsorbent with the sample to be tested and with anti-HB_s incorporated with a label; and
- (c) determining the amount of the label which either (i) becomes associated with the adsorbent by binding of the labeled anti-HB_s to adsorbed HB_s antigen or (ii) remains unadsorbed therefrom,

wherein either the adsorbent is of a type incapable of significantly binding with the labeled anti-HB_s or step (b) is performed under conditions unfavorable to significant binding between the labeled anti-HB_s and the adsorbent. This method allows the selection of adsorbent, reaction conditions, i.e., pH, temperature and incubation times, and labeled anti-HB_s to be based on the same criteria as discussed at length above for the detection of HB_s antigen.

In general, the test means of the present invention comprises, usually in a kit or test system form, (a) a solid adsorbent of a type capable of nonspecifically binding with the substance to be determined and (b) a labeled form of a specific binding partner of the substance to be determined. The test means may additionally include a liquid buffer for use in performing one or more of the wash steps preferred in carrying out the assay method. The type of adsorbent, the character, e.g., pH, of the buffer, and the type of label are selected according to the criteria described previously relative to the assay method. Where the test means is designed for the detection of HB_s antigen immune complex, it additionally comprises a solution of the appropriate immunochemical-bond weakening agent.

The adsorbent is preferably arranged in a column format, and in such format, is preferably in a particulate form as discussed previously. The column format not only provides a convenient system for introducing sample and labeled binding partner to the adsorbent, for washing the adsorbent, and for counting radioactivity where the label is a radioactive material, but it also provides a means of concentrating on the adsorbent the substance to be determined from the sample. The column format allows a relatively large volume of sample to be passed through or by the adsorbent, thereby effectively concentrating the substance to be determined relative to a later applied labeled binding partner.

Additionally, the effective concentration of the substance to be determined may be increased by first treating the sample to precipitate the substance to be determined, recovering the precipitate, and redissolving it in a smaller volume of solvent. The resulting solution of the substance to be determined can then be applied to the adsorbent and the assay method completed as described herein. For example, in detecting HB_s antigen, one can add polyethylene glycol to a serum sample to precipitate HB_s antigen, centrifuge or filter, and dissolve the HB_s antigen in a small volume of buffer. The resulting solution will contain an effectively greater concentration of HB_s antigen than was present in the serum sample.

It is contemplated that the present assay method and test means may be applied to the qualitative or quantitative determination of any substance for which there is a natural or synthetic specific binding partner, including peptides, proteins, carbohydrates, glycoproteins, steroids, antigenic particles, and so forth. For example, the following materials may be detected using the present assay method and test means: hormones, such as tetraiodothyronine (thyroxine) and triiodothyronine; enzymes, such as lactic dehydrogenase and alkaline phosphatase; therapeutic agents, such as gentamicin and diphenylhydantoin, virus particles, such as HB_s antigen and bacteriophages; whole bacterial cells; and nucleic acids, such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), to name just a few. The present invention is particularly suited to the detection of antigens and haptens, and antibodies thereto, particularly HB_s antigen and anti-HB_s. Various liquid specimens may be analyzed according to the present invention, particularly physiological or biological fluids such as serum, plasma, urine, saliva, and amniotic; cerebral and spinal fluids.

The present invention will now be illustrated, but is not intended to be limited, by the following examples.

EXAMPLE 1
Radioimmunoassay (RI-Ad™) for
Hepatitis B Surface Antigen

This Example describes an assay procedure for the detection of HB_s antigen using an anion exchange material as nonspecific adsorbent. The accuracy of the RI-Ad procedure was assessed against the United States Bureau of Biologics (BOB) Reference Panel No. 3. The sensitivity of the RI-Ad procedure was determined relative to the Ausria® II radioimmunoassay test kit marketed by Abbott Laboratories, North Chicago, Illinois U.S.A.

A. Preparation of Assay Columns

The columns comprised 0.5 milliliter (ml) of settled anion exchange resin, DEAE-cellulose type DE-52, Whatman Inc., Clifton, New Jersey U.S.A., held in a 9.5x75 millimeter (mm) polyethylene column between two porous polyethylene discs. The columns were fitted with top and bottom caps after equilibration with a volume of 0.02M sodium monobasic phosphate: sodium dibasic phosphate buffer (herein referred to as "phosphate" buffer) at pH 6.8, the buffer volume being sufficient to saturate the resin bed and provide about a 1 ml buffer in the reservoir above the bed. The geometry of the column was selected to facilitate the measurement of radiation emitted from the resin bed with conventional gamma counting instrumentation, such as the Gammacord® instrument marketed by the Ames Company Division of Miles Laboratories, Inc., Elkhart, Indiana U.S.A. The general configuration of the complete test column was similar to that depicted in Figure 2 of the drawing.

B. Assay Procedure

1. The top cap was removed and the column placed in a drain rack.
2. A 200 microliter (μ l) volume of the serum or plasma test sample, or a negative control sample, was added to the buffer in the reservoir above the DEAE-cellulose resin bed. The column was swirled to mix the sample and buffer and was then drained by removal of the bottom cap.
3. The resin bed was washed with two consecutive additions of 4 ml of 0.02 M phosphate buffer (pH 6.8).
4. When the column had drained completely, a 200 μ l volume of ¹²⁵I-anti-HB_s [specific activity of labeled anti-HB_s=14–20 microcurie/microgram (μ Ci/ μ g); total activity=approx. 50,000 counts per minute (cpm)] was added to the top of the resin bed and allowed to soak into the resin after which the bottom cap was replaced. [Anti-HB_s used had been raised against HB_s antigen, purified by the method of Ling and Overby, *J. Immunol.* 109:834 (1972), and labeled by the chloramine T method of Hunter and Greenwood, *Nature* 194:495 (1962)].
5. The column was then incubated either (i) for 2 hours at 45°C (the "2 hour/45°C" assay) or (ii) overnight at room temperature, 22°C (the "overnight/RT" assay).
6. The bottom cap was then removed and the resin bed was washed with two consecutive additions of 4 ml of 0.02M phosphate buffer (pH 6.8).
7. When the column had drained completely, the bottom cap was replaced and the column placed in the well of a gamma counting instrument, such as the aforementioned Gammacord instrument, and its radioactivity counted. (In an alternative procedure the washings from previous step 6 are collected and their aggregate radioactivity measured.)
8. A positive sample was determined as one which yielded a level of radioactivity associated with the resin bed greater than (or which yielded a level of radioactivity in the washings less than) a cut-off value calculated as the average cpm of gamma radiation yielded by the resin beds (cpm bound) (or contained in the washings, where the alternative procedure is followed) of at least seven replicates of a negative control plus (or minus, where the alternative procedure is followed) four standard deviations. An example of a calculation of a cut-off value where the resin bed cpm bound is measured is as follows:

Seven replicate samples of a negative control are run separately through the assay procedure and the counts per minute (cpm) of gamma radiation associated with the resin bed (cpm bound) for each run is recorded.

| | Replicate No. | Cpm bound | |
|----|--------------------|------------------|----|
| | 1 | 1459 | |
| | 2 | 1488 | |
| | 3 | 1398 | |
| 5 | 4 | 1444 | 5 |
| | 5 | 1543 | |
| | 6 | 1487 | |
| | 7 | 1387 | |
| | total | 10206 | |
| 10 | average | 1458 | 10 |
| | standard deviation | ±54 | |
| | cut-off | 1458+(4×54)=1678 | |

Therefore, any sample tested which would give a cpm bound greater than 1678 would be considered as positive for HB_s antigen.

15 C. Accuracy—Performance with BOB Panel

Each BOB panel sample was assayed by both the 2 hour/45°C procedure and the overnight/RT procedure. The results appear in Table 1. In the table, the BOB codes stand for the following:

| | BOB Code Letter | Meaning | |
|----|-----------------|---|----|
| 20 | A | contains relatively high level of HB _s antigen detectable by all current methods | 20 |
| | B | contains a lesser level of HB _s antigen detectable by counter electrophoresis, reversed passive latex agglutination, reversed passive hemagglutination, and radioimmunoassay | 25 |
| 25 | C | contains even lesser level of HB _s antigen detectable consistently only by reversed passive hemagglutination and radioimmunoassay | 30 |
| 30 | (C) | contains low level of HB _s antigen occasionally detectable by radioimmunoassay | 35 |
| 35 | D | contains very low level of HB _s antigen not detectable by any current method | 35 |
| | N | a true negative for HB _s antigen | |

The calculations of the cut-off values for the 2 hour/45°C and the overnight/RT procedures were as follows:

| | Cut-Off Value Calculations | 2 hours/45°C | overnight/RT | |
|----|---|--------------|--------------|----|
| 40 | average cpm bound for negative control replicates | 3374 | 5404 | 40 |
| | standard deviation (S.D.) | ±117 | ±123 | |
| | percent coefficient of variation (%C.V.) | 3.46 | 2.27 | 45 |
| 45 | cut-off value (cpm) | 3842 | 5896 | 45 |

TABLE 1

| | Sample Number | BOB Code | Cpm (2 hours/45°C) sample | sample-CO* | Cpm (overnight/RT) sample | sample-CO* | |
|----|---------------|----------|---------------------------|------------|---------------------------|------------|----|
| 50 | 302 | D | 3403 | Neg | 4960 | Neg | 50 |
| | 303 | N | 3481 | Neg | 4478 | Neg | |
| | 304 | C | 8160 | +4318 | 12100 | +6204 | |
| | 306 | A | 22377 | +18535 | 24197 | +18301 | |
| | 311 | N | 3680 | Neg | 4202 | Neg | |
| 55 | 312 | A | 21122 | +17280 | 24095 | +18199 | 55 |
| | 315 | (C) | 3411 | Neg | 5853 | Neg | |
| | 317 | N | 3325 | Neg | 4970 | Neg | |

TABLE 1 (continued)

| | Sample Number | BOB Code | Cpm (2 hours/45°C) | | Cpm (overnight/RT) | | |
|----|---------------|----------|--------------------|------------|--------------------|------------|----|
| | | | sample | sample-CO* | sample | sample-CO* | |
| | 318 | C | 21570 | +17738 | 25880 | +19984 | |
| 5 | 319 | B | 18378 | +14536 | 22731 | +16835 | 5 |
| | 320 | B | 24730 | +20888 | 30057 | +24163 | |
| | 323 | C | 5569 | +1727 | 7721 | +1825 | |
| | 326 | C | 18589 | +14747 | 21773 | +15877 | |
| | 327 | B | 2776 | +23934 | 32403 | +26507 | |
| 10 | 330 | A | 21360 | +17518 | 25155 | +19259 | 10 |
| | 331 | N | 3182 | Neg | 4838 | Neg | |
| | 332 | C | 9993 | +6151 | 16709 | +10813 | |
| | 333 | C | 9891 | +6049 | 13684 | +7788 | |
| | 336 | B | 21471 | +17629 | 29233 | +23337 | |
| 15 | 337 | C | 19724 | +15882 | 23139 | +17243 | 15 |
| | 338 | C | 4793 | +951 | 6672 | +776 | |
| | 343 | (C) | 4606 | +754 | 6236 | +1340 | |
| | 344 | C | 4388 | +546 | 6939 | +1043 | |
| | 349 | D | 3625 | Neg | 6446 | +550 | |
| 20 | 350 | B | 22265 | +18423 | 28162 | +22266 | 20 |

*sample cpm bound minus cut-off (CO) value

The RI-Ad procedure using both incubation conditions correctly identified every BOB panel member having a code of A, B or C. There were no false positives as every N coded BOB panel member yielded a negative result. Additionally, BOB panel member number 343 coded as (C) was picked up as a positive by the RI-Ad procedure under both incubation conditions and even one D coded panel member, number 349, was picked up as positive by the overnight/RT RI-Ad procedure.

D. Sensitivity—Comparison with Ausria II

Three positive BOB panel members (sample numbers 312, 326 and 336) and one negative member (sample number 317) were selected. Dilutions of each were prepared between 1:50 and 1:64,000. Selected dilutions were assayed using the RI-Ad procedure, under both incubation conditions, i.e., 2 hours/45°C (2 hr) and overnight/RT (ON), and using Ausria II kits, Abbott Laboratories, North Chicago, Illinois U.S.A., following the procedure given in the product insert (the insert specifies that a "P/N Ratio" of 2.1 or more represents a positive sample result). The results appear in Table 2. The positive cut-off value calculations for the RI-Ad procedures are given at the bottom of the table. "N.C." represents the average cpm bound for the negative control replicates; "S.D.", standard deviation; and "%C.V.", percent coefficient of variation.

The RI-Ad procedure was shown to be at least as sensitive under both incubation conditions as the Ausria II procedure, and in certain circumstances showed greater sensitivity. For example, the 1:64,000 dilution of panel member number 312 (Code A) was determined as positive using the 2 hour RI-Ad procedure but negative using Ausria II.

| Dilution | BOB #312 (A) | | | TABLE 2 BOB #326 (C) | | | BOB #336 (B) | | | BOB #317 (N) | |
|-----------|----------------|-------------|------------------------|-------------------------|-------------|------------------------|---------------|-------------|------------------------|----------------|-------------|
| | RI-Ad, 2 hr | cpm O.N. | Ausria II P/N Ratio | RI-Ad, 2 hr | cpm O.N. | Ausria II P/N Ratio | RI-Ad 2 hr | cpm O.N. | Ausria II P/N Ratio | RI-Ad, 2 hr | cpm O.N. |
| 1/50 | — | — | — | 5954 | 8235 | 8.3 | — | — | — | — | — |
| 1/100 | — | — | — | 4454 | 5280 | 4.1 | — | — | — | — | — |
| 1/100 | — | — | — | 4454 | 5280 | 2.2 | — | — | — | — | — |
| 1/200 | — | — | — | 3983 | 4220 | Neg | 3880 | 5352 | 10.0 | Neg | Neg |
| 1/400 | — | — | — | 3377 | 3719 | Neg | 3166 | 4161 | 4.8 | Neg | Neg |
| 1/500 | 6238 | 9385 | 58.3 | — | — | — | — | — | — | — | — |
| 1/800 | — | — | — | 3066 | 3351 | Neg | 3090 | 3607 | 2.5 | Neg | Neg |
| 1/1000 | 6210 | 7836 | 43.7 | — | — | — | — | — | — | — | — |
| 1/1600 | — | — | — | 2940 | Neg | Neg | 2924 | 3385 | Neg | Neg | Neg |
| 1/2000 | 5275 | 6590 | 31.3 | — | — | — | — | — | — | — | — |
| 1/3200 | — | — | — | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| 1/4000 | 4390 | 5346 | 21.1 | — | — | — | — | — | — | — | — |
| 1/6400 | — | — | — | — | — | Neg | Neg | Neg | Neg | Neg | Neg |
| 1/8000 | 3725 | 4288 | 10.6 | — | — | — | — | — | — | — | — |
| 1/12800 | — | — | — | — | — | — | Neg | Neg | Neg | Neg | Neg |
| 1/16000 | 3349 | 3774 | 5.7 | — | — | — | — | — | — | — | — |
| 1/32000 | 3102 | 3459 | 3.0 | — | — | — | — | — | — | — | — |
| 1/64000 | 2979 | Neg | Neg | — | — | — | — | — | — | — | — |
| N.C. Mean | 2571 | 3010 | | 2571 | 3010 | | 2571 | 3010 | | 2571 | 3010 |
| S.D. | ±90 | ±97 | | ±90 | ±97 | | ±90 | ±97 | | ±90 | ±97 |
| %C.V. | 3.5 | 3.2 | | 3.5 | 3.2 | | 3.5 | 3.2 | | 3.5 | 3.2 |
| Cut Off | 2931 | 3398 | | 2931 | 3398 | | 2931 | 3398 | | 2931 | 3398 |

1,584,137

Also, the 1:200 and greater dilutions of panel member number 326 (Code C) were determined as positive using both RI-Ad procedures, whereas Ausria II picked up only the 1:100 and lesser dilutions. For the same panel member, the 2 hour RI-Ad procedure gave positive results down to the 1:1600 dilution (a 16 fold greater sensitivity than Ausria II) and the overnight RI-Ad procedure gave positive results down to the 1:800 dilution (an 8 fold greater sensitivity than Ausria II). Further, for panel member number 336 (Code B), both RI-Ad procedures gave positive results down to the 1:1600 dilution, whereas positive results from the Ausria II procedure ceased at the 1:800 dilution (a 2 fold greater sensitivity over Ausria II). All of the dilutions tested for the negative panel member (number 317) were found to be negative.

Example 2

Radioimmunoabsorbent Assay (RI-Ad™) for Hepatitis B Surface Antigen in the Form of Immune Complex with Antibody

This example describes a procedure for detecting HB_s antigen complexed with anti-HB_s, i.e., in the form of its immune complex. In this example the ability to detect HB_s antigen in serum samples containing anti-HB_s, as well as the ability to detect HB_s in the form of its immune complex in contrived samples, is demonstrated.

A. Preparation of Assay Columns

The columns were prepared as in the preceding Example.

B. Assay Procedure

1. With the bottom cap in place and top cap removed, the phosphate buffer in the reservoir was poured from the column and replaced with 1 ml of 8M urea in 0.02M phosphate buffer (pH 6.8).
2. A 200 μ l volume of the serum or plasma test sample, or a negative control sample, was added to the urea/buffer in the reservoir. The column was swirled to mix the sample and buffer/urea and was allowed to stand for 10 minutes before draining by removal of the bottom cap.
3. After draining, the procedure described in Part B of Example 1 was followed, beginning with step 3.

C. Detection of HB_s Antigen in Serum with Endogenous Anti-HB_s

A negative control serum and a commercial (comm.) serum containing a low level of HB_s antigen and anti-HB_s were tested using the above modified RI-Ad procedure with a 2 hour/45°C incubation, the Ausria II procedure mentioned in the previous Example, and the Ausab® procedure (Abbott Laboratories, North Chicago, Illinois U.S.A.—radioimmunoassay for anti-HB_s). The results are given in Table 3.

TABLE 3

| Sample | modified RI-Ad, cpm Sample | Ausria II P/N Ratio | Ausab RIA Units |
|--------------|-------------------------------|------------------------|--------------------|
| neg. control | 3313 | Neg | Neg |
| comm. serum | 4574 | +321 | 477 |

*sample cpm bound minus cut-off value

The data indicates that the modified RI-Ad procedure is capable of detecting HB_s antigen in the presence of endogenous anti-HB_s (verified by Ausab results) whereas the Ausria II procedure could not.

In order to confirm the presence of HB_s antigen in the commercial serum, a centrifugation experiment was performed. A sample of the commercial serum was equilibrated in glycine buffer (pH 2.0) and applied to a 5—20% weight to weight (w/w) sucrose gradient. The sample/gradient mixture was centrifuged at 36,000 revolutions per minute (rpm) for 3 hours. Fractions taken from the bottom part of the tube were found to be positive for HB_s antigen by the Ausria II procedure. This corroborated the presence of HB_s antigen in the commercial serum and clearly established that the HB_s antigen, when separated from anti-HB_s by centrifugation, could be detected by Ausria II whereas it had escaped detection in the commercial serum in which it was complexed with anti-HB_s.

D. Detection of HB_s antigen in the form of its immune complex

A series of contrived test samples were prepared by adding increasing volumes of HB_s antigen positive sera to a constant volume of high titer human anti-HB_s sera. The final volumes were equalized with normal serum negative for both HB_s antigen and anti-HB_s. After incubation for 20 hours, any precipitate which formed was removed by centrifugation and the supernatant saved as the contrived sample. Each contrived sample was tested using the modified RI-Ad procedure with a 2 hour/45°C incubation, the Ausria II procedure, and the Ausab procedure. The results are given in Table 4. The "Ag/Ab Ratios" are the ratios of the volume of HB_s antigen positive serum added to the volume of anti-HB_s positive serum.

The modified RI-Ad procedure detected HB_s antigen in the presence of anti-HB_s to the lowest level of HB_s antigen added (Ag/Ab Ratio=0.2). At the point where anti-HB_s was in excess (Ag/Ab Ratio≤0.4), the Ausria II procedure failed to detect the presence of HB_s antigen. The presence of anti-HB_s, on the other hand, was confirmed in the samples having Ag/Ab Ratios less than or equal to 1.0 by the Ausab procedure; however, the Ausab procedure itself failed to detect anti-HB_s in the presence of excess HB_s antigen (Ag/Ab Ratio≥2.0).

TABLE 4

| Ag/Ab Ratio (v/v) | modified RI-Ad, cpm : sample | sample-CO* | Ausria II P/N Ratio | Ausab RIA Units |
|-------------------|------------------------------|------------|---------------------|---------------------|
| 4.0 | 34654 | +30755 | 107.4 | Neg |
| 3.0 | 34348 | +30449 | 127.9 | Neg |
| 2.0 | 29902 | +26003 | 146.9 | Neg |
| 1.0 | 9143 | +5272 | 11.3 | 16 |
| 0.4 | 5337 | +1438 | Neg | 54×10 ² |
| 0.2 | 5707 | +1808 | Neg | 512×10 ² |
| 0.0 | 3515 | Neg | Neg | 512×10 ² |

*sample cpm bound minus cut-off value

Example 3

Study of Adsorption Characteristics of Various Anion Exchange Materials for HB_s Antigen

The ability of the following anion exchange materials to adsorb HB_s antigen was studied over the pH range 5.0 to 8.0:

- (1) DEAE-cellulose (type DE-52—Whatman Inc., Clifton, New Jersey U.S.A.)
- (2) DEAE-Sephadex® (Pharmacia Fine Chemicals, Piscataway, New Jersey U.S.A.)
- (3) QAE-Sephadex® (Pharmacia)
- (4) DEAE-Biogel® A (Bio-Rad Laboratories, Richmond, California U.S.A.)

A 200 μl volume of ¹²⁵I-HB_s antigen [25 nanograms per milliliter (ng/ml)] was added to each of a series of columns containing the various anion exchange materials at various known pH's. Each column was then washed with two 4.0 ml volumes of phosphate buffer at the corresponding pH. The columns were then counted in a gamma counter instrument. The results appear in graphical form in Figure 3 of the drawings.

An optimum adsorption was observed at a pH of 6.0—6.5 for each of the resins examined. DEAE-cellulose, DEAE-Sephadex, and QAE-Sephadex showed similar maximum adsorption affinities for HB_s antigen, while DEAE-Biogel A had a somewhat lesser affinity for the antigen. Overall, DEAE-cellulose was found to be the resin of preference in the column format because of its advantageous flow rate and minimum tendency to absorb ¹²⁵I-anti-HB_s.

Example 4

Radioimmunoabsorbent Assay (RI-Ad™) for Hepatitis B Surface Antigen (Alternative Procedure)

This example describes an alternative RI-Ad procedure for detecting HB_s antigen wherein the wash step between sample application and addition of labeled anti-HB_s is eliminated. The accuracy of this alternative RI-Ad procedure was assessed against BOB Reference Panel No. 3 (see Example 1).

Each panel member was tested by the RI-Ad 2 hour/45°C procedure described in Example 1 (using a different batch of ^{125}I -anti- HB_s) and by the same procedure without wash step 3. The results are given in Table 5. The cut-off value calculations for two procedures, i.e., "with wash" and "without wash", are given at the bottom of the table. The abbreviations are the same as those used in Example 1. A negative result, i.e., where cpm bound was less than the cut-off value, is indicated by a minus sign. The differences in cpm bound data produced in this Example using the wash step (Table 5) and that produced in Example 1 (Table 1) are due to the use of different batches of ^{125}I -anti- HB_s .

TABLE 5

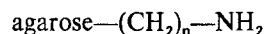
| Sample Number | BOB Code | cpm, bound | |
|---------------|----------|------------|--------------|
| | | with wash | without wash |
| 302 | D | 1865 (-) | 1784 (-) |
| 303 | N | 1898 (-) | 1673 (-) |
| 304 | C | 8010 | 3360 |
| 306 | A | 17136 | 6963 |
| 311 | N | 1734 (-) | 1760 (-) |
| 312 | A | 21200 | 6933 |
| 315 | (C) | 2133 (-) | 1644 (-) |
| 317 | N | 1934 (-) | 1734 (-) |
| 318 | C | 17647 | 8517 |
| 319 | B | 19705 | 6600 |
| 320 | B | 17745 | 4586 |
| 323 | C | 4210 | 2508 |
| 326 | C | 19427 | 8927 |
| 327 | B | 20541 | 6718 |
| 330 | A | 18369 | 6132 |
| 331 | N | 1876 (-) | 1821 (-) |
| 332 | C | 8821 | 3339 |
| 333 | C | 9502 | 4371 |
| 336 | B | 16319 | 5605 |
| 337 | C | 18811 | 11807 |
| 338 | C | 3631 | 2841 |
| 343 | (C) | 3650 | 2802 |
| 344 | C | 2894 | 2722 |
| 349 | D | 2146 (-) | 2482 |
| 350 | B | 16417 | 5251 |
| N.C. Mean | | 1814 | 1939 |
| S.D. | | ±86 | ±98 |
| %C.V. | | 4.7 | 5.05 |
| Cut-Off | | 2158 | 2331 |

This data indicates that elimination of the first wash step does not affect the ability of the RI-Ad procedure to detect as positive samples those BOB panel members having a code of A, B, or C.

Example 5

Use of Hydrophobic Adsorbents in a Radioimmunoabsorbent (RI-Ad) Assay for Hepatitis B Surface Antigen

In this example, a series of ω -amino alkyl-substituted agaroses were evaluated as adsorbents in the RI-Ad procedure described in Example 1. The test columns and assay procedure were as described in Example 1 except that the DEAE-cellulose adsorbent was replaced with various ω -amino alkyl-substituted agaroses and that in step 6 the adsorbent was washed with two consecutive additions of 2 ml of a mixture of tris-hydroxymethylaminomethane:hydrochloride buffer and 0.5M sodium chloride (pH 8.2). Two serum samples, one negative and one positive for HB_s antigen, were tested using a series of columns, each containing a different one of the following materials having the general formula:



where $n=4$ to 10, i.e., the number of carbons in the alkyl side chain.

| | adsorbent | no. of side chain carbons | manufacturer's code no.* | |
|---|-------------------------------|---------------------------|--------------------------|---|
| | ω -amino ethyl agarose | 2 | 62-080 | |
| | ω -amino butyl agarose | 4 | 62-081 | |
| 5 | ω -amino hexyl agarose | 6 | 62-082 | 5 |
| | ω -amino octyl agarose | 8 | 62-083 | |
| | ω -amino decyl agarose | 10 | 62-084 | |

*Research Products Division of Miles Laboratories, Inc., Elkhart, Indiana U.S.A.

10 The results appear in Table 6. 10

TABLE 6

| | no. of side chain carbons in adsorbent | cpm bound (positive sample) minus cpm bound (negative sample) | |
|----|--|---|----|
| | 2 | -93 | |
| 15 | 4 | +70 | 15 |
| | 6 | +358 | |
| | 8 | +1970 | |
| | 10 | +2117 | |

20 The data indicates that ω -amino alkyl-substituted agaroses having alkyl side chains containing greater than 4 carbon atoms are useable adsorbents in the RI-Ad procedure for detecting HB_s antigen. 20

Example 6

Radioimmunoabsorbent Assay (RI-Ad) for Antibody to Hepatitis B Surface Antigen

25 This example describes an assay procedure for the detection of anti-HB_s using an anion exchange material pre-treated with a standardized amount of HB_s antigen. The ability of the RI-Ad procedure to detect anti-HB_s was assessed by comparison with the Ausab radioimmunoassay test kit marketed by Abbott Laboratories. 25

A. Preparation of Assay Columns

30 The columns were prepared as in Example 1. 30

B. Assay Procedure

1. The top cap was removed and the column placed in a drain rack.
2. A 200 μ l volume of HB_s antigen positive reference serum was added to the buffer in the reservoir above the DEAE-cellulose resin bed. The column was swirled to mix the sample and buffer and was then drained by removal of the bottom cap. 35
3. After draining, the resin bed was washed with two consecutive additions of 4 ml of 0.02M phosphate buffer (pH 6.8).
4. A 200 μ l volume of the test sample, or a negative control sample, was then layered onto the top disc and allowed to flow into the resin bed. 40
5. The bottom cap was replaced and the column incubated for 2 hours at 45°C. 40
6. The bottom cap was removed and a 200 μ l volume of ¹²⁵I-anti-HB_s was added to the top of the resin bed and allowed to flow into the resin bed. The bottom cap was then replaced and the column incubated an additional 2 hours at 45°C. 45
7. The bottom cap was removed and the resin bed was washed with two consecutive additions of 4 ml of 0.02M phosphate buffer (pH 6.8).
8. When the column had drained completely, the bottom cap was replaced, and the column counted in a gamma counting instrument. 50

C. Comparison with Ausab Procedure

55 A series of serum samples positive for anti-HB_s were tested by the above RI-Ad procedure and using Ausab kits, Abbott Laboratories, following the procedure given in the product insert. Anti-HB_s titer was determined by both methods for each sample. Following the RI-Ad procedure, titer was defined as the greatest dilution of a sample resulting in inhibition of cpm bound greater than four standard deviations from a negative control mean (see Example 1). Following the Ausab 55

procedure, titer was determined in RIA units according to the method given in the product insert. The results are given in Table 7.

| | | TABLE 7 | | |
|----|---------------|--------------------|-------------------|----|
| | Sample Number | RI-Ad Titer | Ausab Titer | |
| 5 | 1 | 1.15×10^3 | 158×10^3 | 5 |
| | 2 | 2.2×10^3 | 72×10^3 | |
| | 3 | 6.0×10^2 | 382×10^3 | |
| | 4 | 1.4×10^3 | 16×10^4 | |
| | 5 | 1.05×10^3 | 352×10^3 | |
| 10 | 6 | 1.2×10^3 | 412×10^3 | 10 |
| | 7 | 6.0×10^2 | 262×10^3 | |
| | 8 | 1.0×10^2 | 292×10^3 | |
| | 9 | 4.8×10^2 | 262×10^3 | |
| | 10 | 6.8×10^2 | 262×10^3 | |
| 15 | 11 | 8.5×10^2 | 183×10^3 | 15 |
| | 12 | 5.2×10^2 | 183×10^3 | |
| | 13 | 2.4×10^3 | 235×10^3 | |
| | 14 | 6.2×10^2 | 208×10^3 | |
| | 15 | 5.2×10^2 | 235×10^3 | |
| 20 | 16 | 3.8×10^2 | 112×10^3 | 20 |

The data confirmed the ability of the RI-Ad procedure to detect the presence of anti-HB_e comparable with the Ausab procedure.

WHAT WE CLAIM IS:—

1. A method for determining a specific binding substance in a liquid sample, comprising the steps of:
 - (a) contacting said sample with (1) a specific binding partner of the substance to be determined, which binding partner is incorporated with a label and which labeled binding partner has a pI value different from that of said substance to be determined, and (2) a solid ion exchange adsorbent (i) of an anionic type when the pI value of said substance to be determined is less than that of said binding partner, or (ii) of a cationic type when the pI value of said substance to be determined is greater than that of said labeled binding partner; and
 - (b) determining the amount of the label which either (i) becomes associated with the adsorbent by binding of the labeled binding partner with adsorbed substance to be determined or (ii) remains unassociated therefrom, wherein step (a) is performed under conditions of pH between the pI value of the substance to be determined and that of said labeled binding partner.
2. A method as in Claim 1 wherein step (a) is accomplished by (i) contacting the sample with the adsorbent and (ii) thereafter contacting the adsorbent with the labeled binding partner.
3. A method as in Claim 2 wherein, after the sample has been contacted with the adsorbent, the said adsorbent and the unadsorbed portion of the liquid sample are separated before the adsorbent is contacted with the labeled binding partner.
4. A method as in Claim 3 wherein said separation step is accomplished by washing the adsorbent under conditions of pH between the pI value of the substance to be determined and that of said labeled binding partner.
5. A method as in Claim 1 wherein said adsorbent is contacted with the sample and the labeled binding partner substantially simultaneously.
6. A method as in Claim 1 wherein step (b) is accomplished by (i) separating from the adsorbent substantially all of the labeled binding partner not associated therewith by binding with adsorbed substance to be determined, and (ii) measuring the amount of the label either associated with the adsorbent or separated therefrom.
7. A method as in Claim 6 wherein the said separation step is accomplished by washing the adsorbent so that the amount of the label either associated with the washed adsorbent or contained in the washings can be measured.
8. A method as in any one of Claims 1 to 7 wherein said label is a radioactive material.
9. A method as in any one of Claims 1 to 8 wherein the difference between the pI value of the substance to be determined and that of the labeled binding partner is greater than 0.5.

10. A method as in Claim 9 wherein the difference between the pI value of the substance to be determined and that of the labeled binding partner is greater than 2.0.

11. A method as in any one of Claims 1 to 10 wherein the said substance to be determined is an antigen or a hapten and the said specific binding partner thereof is an antibody.

12. A method as in any one of Claims 1 to 10 wherein the said substance to be determined is hepatitis B surface antigen.

13. A method as in any one of Claims 1 to 10 wherein the said substance to be determined is an antibody to hepatitis B surface antigen.

14. A method as in any one of Claims 1 to 13 wherein the ion exchange material is in a particulate form and is contained in a column.

15. A method for detecting hepatitis B surface antigen in a liquid sample, comprising the steps of:

(a) contacting said sample with a solid adsorbent of a type capable of nonspecifically binding with hepatitis B surface antigen;

(b) contacting the adsorbent with an antibody to hepatitis B surface antigen, which antibody is incorporated with a label; and

(c) determining the amount of the label which becomes associated with the adsorbent by binding of the labeled antibody with adsorbed hepatitis B surface antigen,

wherein steps (a) and (b) are performed under conditions favorable to binding between hepatitis B surface antigen and the adsorbent, and

either the adsorbent is of a type incapable of significantly binding with the labeled antibody under any conditions

or step (b) is performed under conditions unfavorable to significant binding between the labeled antibody and the adsorbent.

16. A method as in Claim 15 wherein the said adsorbent is a hydrophobic material.

17. A method as in Claim 16 wherein the said hydrophobic adsorbent is an ω -amino alkyl-substituted agarose having 4 to 10 carbon atoms in the alkyl group.

18. A method as in Claim 15 wherein the said adsorbent is an ion exchange material and wherein steps (a) and (b) are performed under conditions of pH between the pI value of hepatitis B surface antigen and that of the labeled antibody.

19. A method as in Claim 18 wherein the said labeled antibody has a pI value greater than that of hepatitis B surface antigen and wherein the said ion exchange material is of an anionic type.

20. A method as in Claim 19 wherein the said anionic type exchange material is a diethylaminoethyl-substituted polymer.

21. A method as in Claim 20 wherein the polymer is a polysaccharide.

22. A method as in Claim 19 wherein the said anionic type exchange material is a diethylaminoethyl-substituted cellulose.

23. A method as in any one of Claims 19 to 22 wherein the pI value of the labeled antibody is greater than 8.0 and wherein steps (a) and (b) are performed under conditions of pH between 6.0 and 8.0.

24. A method as in Claim 18 wherein said labeled antibody has a pI value less than that of hepatitis B surface antigen and wherein the said ion exchange material is a cationic type.

25. A method as in any one of Claims 15 to 24 wherein the said label is a radioactive material.

26. A method for detecting hepatitis B surface antigen in a sample of serum or plasma, comprising the steps of:

(a) introducing said sample into a column of an anion exchange material in particulate form equilibrated in a liquid having a pH of between 5 and 8;

(b) passing through the column a liquid having a pH of between 5 and 8 to remove substantially all hepatitis B surface antigen not adsorbed to the anion exchange material;

(c) introducing to the anion exchange material a predetermined volume of a solution of radiolabeled antibody to hepatitis B surface antigen having a predetermined level of radioactivity;

(d) incubating the solution of radiolabeled antibody in contact with the anion exchange material for between 1/2 and 18 hours at a temperature of between 4 and 50°C;

(e) passing through the column a liquid having a pH of between 5 and 8 to

- remove substantially all radio-labeled antibody not associated with the anion exchange material by binding to adsorbed hepatitis B surface antigen;
- (f) measuring the radioactivity of the anion exchange material or of the eluate from the next previous step; and
- 5 (g) comparing the radioactivity measured in the next previous step to that measured following the same procedure using a liquid sample containing no significant amount of hepatitis B surface antigen in place of said serum or plasma sample. 5
27. A method for detecting hepatitis B surface antigen in the form of its immune complex with antibody in a liquid sample, comprising the steps of: 10
- (a) combining said sample, and immunochemical-bond weakening agent, and a solid adsorbent of a type capable of nonspecific binding with hepatitis B surface antigen such combination being effected under conditions favorable to such bonding; 10
- (b) separating said bond weakening agent from the adsorbent; and 15
- (c) determining the hepatitis B surface antigen bound to the adsorbent by contacting the same with an antibody to hepatitis B surface antigen, which antibody is incorporated with a label, and determining the amount of the label which becomes associated with the adsorbent, by binding of the labelled antibody with adsorbed hepatitis B surface antigen. 20
28. A method as in Claim 27 wherein the said adsorbent is an anion exchange material.
29. A method as in Claim 28 wherein the said anion exchange solid adsorbent material is DEAE-cellulose.
- 25 30. A method as in Claim 27, 28 or 29 wherein the said bond weakening agent is a chaotropic agent. 25
31. A method as in Claim 30 wherein the said chaotropic agent is urea, guanidine, or thiocyanate.
32. A method as in Claim 30 wherein the said chaotropic agent is urea.
- 30 33. A method as in Claim 32 wherein urea is present at a concentration of between 2 and 8 molar. 30
34. A method for detecting an antibody to hepatitis B surface antigen in a liquid sample, comprising the steps of:
- (a) providing a solid adsorbent having nonspecifically bound thereto hepatitis B surface antigen; 35
- (b) contacting the adsorbent with said sample and with an antibody to hepatitis B antigen, which antibody is incorporated with a label; and 35
- (c) determining the amount of the label which either (i) becomes associated with the adsorbent by binding of the labeled antibody to adsorbed hepatitis B surface antigen or (ii) remains unadsorbed therefrom, 40
- wherein either the adsorbent is of a type incapable of significantly binding with the labeled antibody or step (b) is performed under conditions unfavorable to significant binding between the labeled antibody and the adsorbent. 40
35. Test means for determining a specific binding substance in a liquid sample comprising 45
- (a) a labeled specific binding partner for said substance having a pI value different from that of said substance; and 45
- (b) a solid ion exchange adsorbent
- (i) of an anionic type when the pI value of said substance to be determined is less than that of said labeled binding partner, or 50
- (ii) of a cationic type when the pI value of said substance to be determined is greater than that of said labeled binding partner. 50
36. Test means as in Claim 35 which additionally comprises a liquid for washing said adsorbent having a pH between the pI value of said substance to be determined and that of said labeled binding partner. 55
37. Test means as in Claim 35 or 36 wherein said specific binding partner is labeled with a radioactive material.
38. Test means as in Claim 35, 36 or 37 wherein said ion exchange material is in a particulate form and is contained in a column.
- 60 39. Test means for detecting hepatitis B surface antigen in a sample of serum or plasma, comprising 60
- (a) a solid anion exchange material;
- (b) labeled antibody to hepatitis B surface antigen having a pI value greater than about 8; and
- 65 (c) a wash liquid having a pH of between 5 and 8. 65

40. Test means as in Claim 39 wherein the said antibody is labeled with a radioactive material.

41. Test means as in Claim 39 or 40 wherein the said anion exchange material is in a particulate form.

5 42. Test means as in Claim 41 wherein the said particulate anion exchange material is contained in a column. 5

43. Test means as in any one of Claims 39 to 42 wherein the said anion exchange material is a diethylaminoethyl-substituted polymer.

10 44. Test means as in Claim 43 wherein the polymer is a polysaccharide.

45. Test means as in any one of Claims 39 to 42 wherein the said anion exchange material is a diethylaminoethyl-substituted cellulose. 10

46. Test means as in any one of Claims 39 to 45, capable of detecting hepatitis B surface antigen in the form of its immune complex with an antibody, which additionally comprises a solution of an immunochemical-bond weakening agent.

15 47. Test means as in Claim 46 wherein the said anion exchange material is DEAE-cellulose. 15

48. Test means as in Claim 46 or 47 wherein the said bond weakening agent is a chaotropic agent.

20 49. Test means as in Claim 48 wherein the said chaotropic agent is urea, guanidine, or thiocyanate. 20

50. A method as claimed in Claim 1, 15, 26, 27 or 34, substantially as described in any one of the foregoing Examples 1, 2, 4, or 5.

51. Test means as described in Claim 35 or 39, substantially as hereinbefore described.

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DETECTION OF ANTIGEN

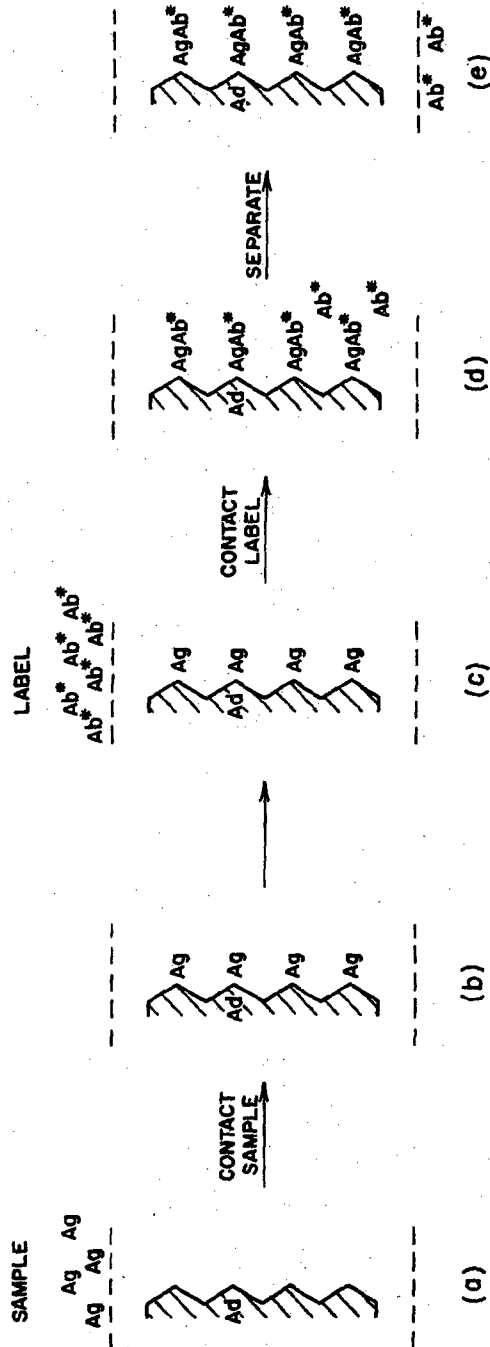


FIG. 1

1584137

COMPLETE SPECIFICATION

3 SHEETS

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Sheet 2*

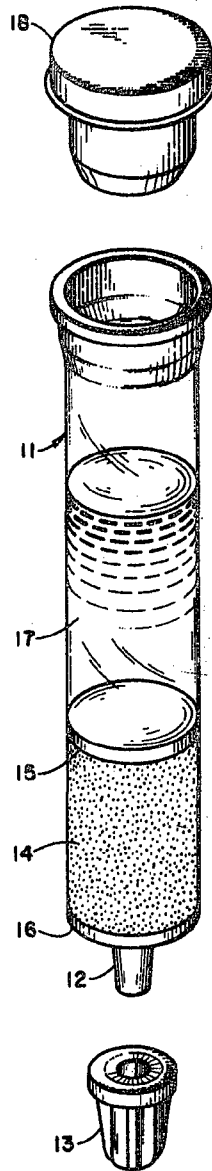


FIG. 2

1584137

COMPLETE SPECIFICATION

3 SHEETS

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Sheet 3

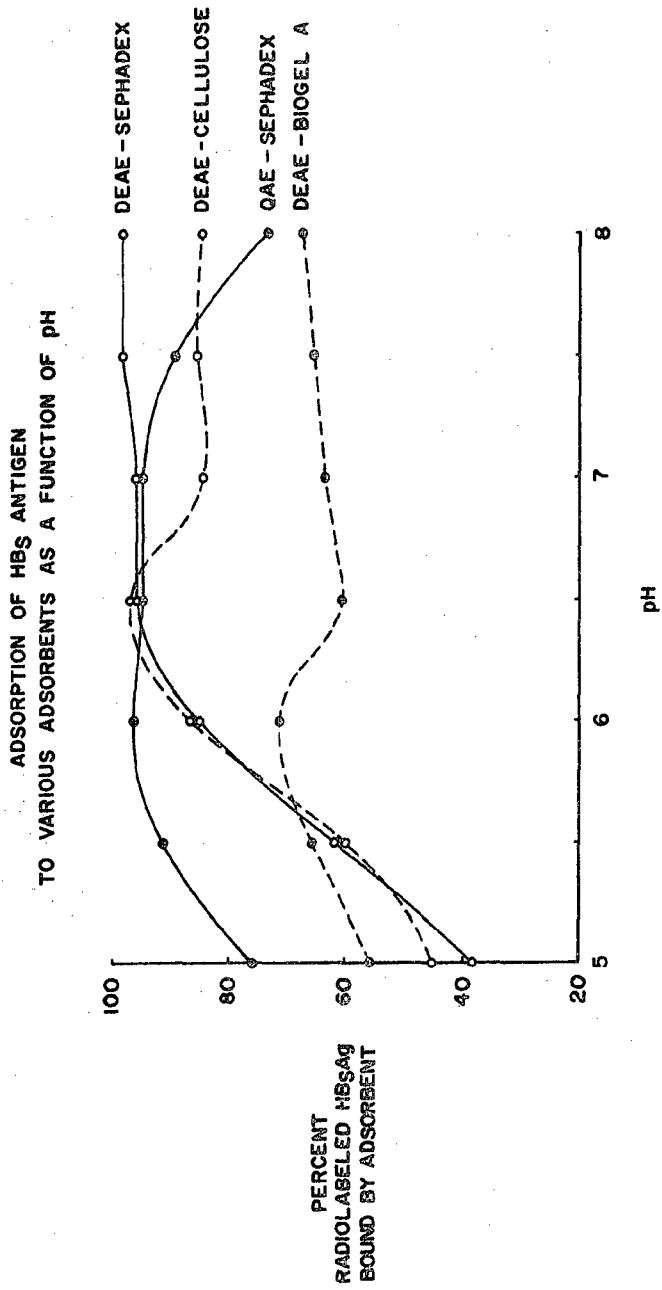


FIG. 3