PATENT SPECIFICATION (11) 1 588 253

1 588 253

(21) Application No. 18340/77 (22) Filed 2 May 1977

(31) Convention Application Nos. 682807 (32) Filed 3 May 1976 in
682808
682830

(33) United States of America (US)

(44) Complete Specification Published 23 Apr. 1981

(51) INT. CL.[3] G01N 33/54
C08B 11/14 11/145 37/02 37/12

(52) Index at Acceptance
G1B BR
C3A 4C1
C3H 202 203 220 242 H1
C3U 2C1 2C5 2CX 3C 4C2 4CX 4L
C3Y A110 B120 B135 F101

(19)

(54) IMMUNOASSAY PROCEDURE AND COMPOSITE THEREFOR

(71) We, BECKMAN INSTRUMENTS, Inc., 2500 Harbor Boulevard, Fullerton, California, United States of America, a corporation organized and existing under the laws of the State of California, United States of America, 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:

This invention pertains to a method of separating free from bound fractions in an immunoassay procedure and to an immunochemical composite for use therein.

Solid phase radioimmunoassay (RIA) has become popular because the system in which both the antigen-antibody reaction and the separation of free and bound antigen can be achieved in a single step results not only in a simple and rapid RIA, but also eliminates a number of handling and other errors which are inherent in other separation techniques. Catt et al., *Biochem. J., 100*: 31c (1966), originally used as solid phase materials powdered polymers bearing reactive thiocyanate groups (–N=C=S) capable of forming covalent linkages with antibodies. Antibodies coupled to cyanogen-bromide-activated dextran and cellulose particles came into vogue as a result of the work of Wide, Porath, and Axen [Wide et al., *Biochem. Biophys. Acta, 130:* 257 (1966), Axen et al., *Nature* (Lond.) *214*: 1302 (1967), and Wide, *Acta Endocrinol.* (Copenhagen) Suppl. No. *142*: 207 (1969)]. Alternatively, Ternynck et al., *F.E.B.S. Letts.* 23: 24 (1972), used glutaraldehyde as a two-step bifunctional reactant to couple antibodies to the amide groups of polacrylamide.

It has been well established that the efficiency of affinity adsorbents increases considerably when hydrocarbon spacers are introduced to separate the ligand from the solid matrix [Cuatrecasas et al., *Proc. Nat. Acad. Sci. U.S., 61:* 636 (1968)]. The spacer is thought to increase the flexibility and mobility of the ligand allowing unhindered access of the protein to the ligand. Armed with this knowledge, Cambiaso et al., *Immunochem., 12*: 273 (1975), coupled gamma globulin fractions to glutaraldehyde-activated aminohexyl derivatives of Sepharose-4B, as shown in Figure 1, and showed this procedure to produce useful immunosorbents.

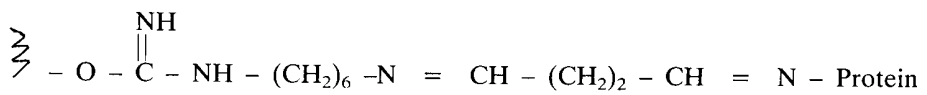


Figure 1

It remains to be established whether purified antibodies or antibody containing gamma globulin fractions coupled to Sepharose by this procedure will prove more efficient for antigen fixation than the same antibodies coupled to Sepharose by means of the cynogen bromide method. In this respect, Bolton et al., *Biochemica et Biophysica Acta, 329*: 318 (1973), reported that recovery of antibody activity tends to be higher on cyanogen bromide activated solid preparations of antisera to haptens and small peptides than to similar solid phase preparations of antisera to large molecular weight protein hormones (see Figure 2).

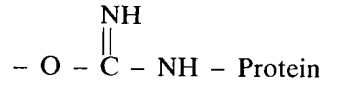


Figure 2

Although it may seem reasonable that solid phase preparations of antisera to large molecules produced by procedures similar to Cambiaso et al., supra, might yield a better recovery of antibody activity than those solid preparations produced without a spacer arm, there exist two disadvantages in the chemical procedures used at this time to covalently link antibodies to solid matrices. First, in many cases, the exact nature of the chemical reactions are not well established and, secondly, the antibodies immobilized may be bound to the solid matrix in a random manner and thus the possibility of the coupling involving an "essential" amino acid residue is increased.

As far back as 1959, Ludwig et al., *Abst. 135th Meeting Am. Chem. Soc.,* 44c (1959), reported on the reaction of substituted imido esters with typical α and ε-amino groups of glycylglycine and ε-amino-caproic acid to form amidines (see Figure 3).

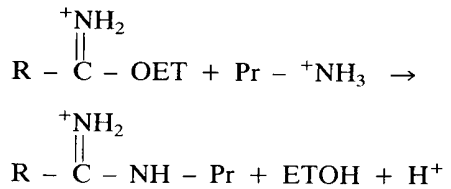


Figure 3

These authors stated that the reaction proceeds rapidly in an aqueous solution near neutral pH even at 1°C., and under these conditions the imidoester reagent does not react with model compounds containing sulfhydryl groups, phenolic groups, imidazole groups, or peptide bonds. Further, they noted that the net charge of the peptide is unchanged through most of the pH range, the amidine function having a pk near 12.

In 1963 Wofsky et al., *Biochem.*, *2*:104 (1963), exhaustively admidinated various proteins and found the reaction to be very specific for lysine residues. Furthermore, they reported that extensive admidination produces remarkably few detectable effects on the biological activity of antibodies. They concluded that lysine is not critically involved in the reactive sites of any of the antibodies examined (anti-bovine serum albumin (anti-BSA), anti-benzene-arsonate, anti-D-benzoylaminophenyl acetate, anti-DNP, anti-β-lactoside and anti-SIII). SIII means biuronic acid repeating unit of a Type III pneumococcal polysacchoride — see Wofsky et al.

Later in 1966, Dutton et al., *Biochem. and Biophys. Res. Comm., 23*:730 (1966), extensively modified anti-DNP antibodies with the crosslinking agent diethylmalonimidate dihydrochloride and similarly reported quantitative retention of antibody activity (see Figure 4).

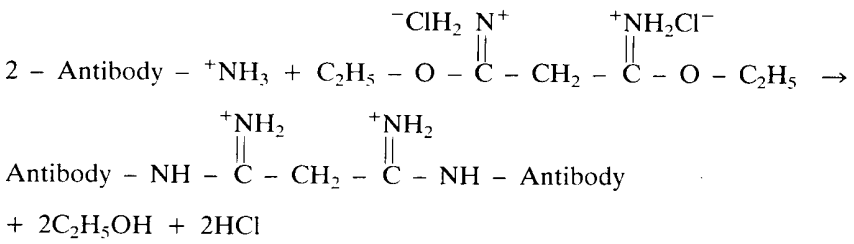


Figure 4

Although the work of Ludwig et al., Wofsky et al., and Dutton et al. has been around for what would in scientific circles be recognized as a considerable number of years, no one has transplanted their work into the art of immunoassay procedures. One reason for this may be that skilled artisans prefer not to use charged derivatized matrices. For example, Wilchek et al., *Molecular and Cellular Biochemistry* Vol. 4, No. 3, p. 181 (1974), report that positively charged derivatized matrices cause non-specific adsorption and therefore are not desirable to be used in immunoassay procedures. Therefore, those skilled in the art of immunoassay procedures may have considered the employment of the above imidoester reagents undesirable in that said reagents would produce positively charged solid phase immunochemical composites.

The end-point of any competitive binding analysis involves determining the relative proportion of antigen (or hapten) that is free and antigen (or hapten) that is bound to the saturable binder. Current basic separation techniques involve differential migration of bound and free fractions (i.e., paper chromatoelectrophoresis, gel filtration), adsorption methods (i.e., charcoal, silicates), fractional precipitation (i.e., ammonium sulfate, polyethylene glycol), and a double antibody method. According to Ratcliffe, *Br. Med. Bull., 30*:32 (1974), an ideal separation technique should fulfill the following criteria:

A. It must completely separate bound and free fractions with a wide margin for error in the conditions used for separation.

B. It must be simple, quick, cheap, and use reagents and equipment that are readily available.

C. It should be unaffected by plasma or serum. Ratcliffe further notes that for general clinical application all manipulations should be performed in a single tube, be suitable for automation, and be applicable to a wide range of antigens or haptens (i.e., small peptides and steroids as well as large molecular weight proteins).

Although the double antibody method for separation of free and bound fractions in radioimmunoassay (RIA) systems is currently one of the most widely employed separation techniques and under optimum conditions satisfies most of the criteria mentioned above, it does possess certain inherent disadvantages. See Ratcliffe, supra; Seki et al., *Endocrinol. Japan, 20:*121 (1973); and Koninckx et al., *Acta Endocrinol., 81*:43 (1976). Since carrier protein must be added, large quantities of selected precipitating antibody are required and thus the method is expensive. It requires considerable length of time for the immunoprecipitation reaction to reach equilibrium (24 to 48 hours at 4°C.). Finally, due to the possibility of aspecific interferences by factors present in the serum, the conditions of the assay must be meticulously evaluated before establishing an assay system.

One of the main disadvantages in the use of solid phase primary antibodies in RIA systems is that as a result of the loss of antibody titer and avidity which often occurs during the coupling step, one must develop longer, more expensive and more complicated systems (i.e., sequential assays). (Zeltner et al., *Clin. Chem., 20*:5 (1974)). Recently, Den Hollander et al., *J. Immunol. Methods, 1*:247 (1972), developed a new separation method employing second antiserum coupled to an insoluble matrix by use of cyanogen bromide and called the separation method the double antibody solid phase (DASP) method. Although the titer and avidity of the second antibody is most certainly reduced in these solid preparations, the primary antibody reaction remains unaltered and thus one of the main disadvantages of solid phase systems is circumvented. In fact, the DASP method possesses the following advantages over the more conventional soluble double antibody method of separation of free and bound fractions:

A. Since solid preparations of precipitating antibody require little or no carrier protein, less second antibody is required to precipitate the first antibody.

B. The DASP method requires less time for complete separation of free and bound fractions.

C. Aspecific interferences by factors present in the serum are totally absent with the DASP precipitation if one works in the area of excess second antibody. In fact, once optimal conditions for precipitation of the immune complex are established, no frequent reassessment is required.

For all the pluses one obtains with solid preparations of precipitating antibody, there remains one mechanical disadvantage common to all solid phase assays. It is necessary in all solid phase assays to agitate the reactants continuously with the additional extra task of first stoppering the assay and then centrifuging and unstoppering them prior to the washing step. Chan et al., *Ann. Clin. Biochem., 12*:173 (1975), coupled primary antibodies to cyanogen bromide activated Sephadex G25 (Trade Mark), ultrafine, bead-formed, dextran gel (less than 10 μ particle size) and reported that with small incubation volumes (300 to 400 microliters) it is possible for the antibody reaction to proceed without the need for vertical rotation or any other means of continuous agitation.

Today antibodies have been covalently attached to a variety of solid matrices such as agarose, glass, polyacrylamide, and even iron oxide powder. Siegel et al., *J. Clin. Endocrinol Metab., 37*:526 (1973), Weetall, *Chem. Abst., 77*:18064y (1972), Moore et al., *Steroids, 20*:199 (1972), and Hersh et al., *Clinica Chemica Acta, 63*:69 (1975).

Although in principle first antibody linked to a solid phase represents a simple and versatile separation procedure, there are a number of disadvantages with this approach. The two questions one must answer in regard to solid phase systems using primary antibodies are, first, the possible loss of antibody activity (titer) with consequent waster of valuable antisera and, secondly, the possible reduction of sensitivity which may result with solid systems as compared with those which can be achieved using the same antisera in an aqueous solution. As noted above, Bolton et al., supra, reported that recovery of antibody

activity tended to be higher in solid preparations of antisera to hapten and small peptides than to similar solid preparations of antisera to large molecular weight protein hormones. Likewise, antisera to haptens covalently coupled to solid matrices show little or no less in assay sensitivity when compared to the uncoupled antibody system whereas there was a dramatic loss of sensitivity when antibodies to large protein hormones were tested. These authors concluded that any loss of assay sensitivity resulting from the use of chemically attached antisera is probably caused by steric hindrance of the larger antigens.

Therefore, one of the major disadvantages against the use of solid preparations of primary antibody in RIA systems is that this method is not universal. (The approach cannot be implemented equally well for large and small molecules). Other disadvantages which apply to all primary solid phase RIA procedures are one must pipette an accurate amount of antibody from an insoluble suspension of gel, the assay mixture must be mechanically agitated to assure proper mixing, and centrifugation is usually required to separate the bound and free fractions.

The use of Sephadex columns in syringes to bind a mixture of labeled I^{125}-thyroxine (T_4) and T_4 released from serum proteins by alkali, followed by a subsequent protein binding analysis with a fixed, limiting amount of thyroid binding globulin (TBG) on each column has recently been introduced. Seligson et al., *Clin. Chem. Acta, 38*:199 (1972) and Alexander et al., *Clin. Chem., 20*:553 (1974). In this procedure, the Sephadex column also serves to separate free T_4 from the T_4-TBG complex. Similar tests have been developed for radiolabeled triiodothyronine (T_3) in which an antibody to T_3 is used as the competitive protein binder. Alexander et al., *Clin. Chem., 20*:1353 (1974). In both assays the Sephadex column serves to bind the mixture of antigen and label while other serum constituents are not retained by the gel. Next, a fixed amount of competitive protein binder is incubated in the void volume of the column during which the antisera and label redistribute between the column and the binder Elution with buffer removes the antigen binder complex while the free antigen remains attached to the Sephadex column. Although this approach is readily automated, this system requires the accurate loading of equal amounts of gel in all columns. Thus, one must continuously stir the Sephadex on a magnetic stirrer while transferring the suspension with a graduated pipette.

Boguslaski et al., *Analytical Chemistry*, Vol. 47, No. 9, 1583 (1975), have recently reported on a column radioimmuno-assay for the determination of digitoxin (mw 765) which employs a column of immobilized primary antibody which acts both as a reaction chamber and separation device. Similar primary antibody column RIA's have been reported for vitamin B12 (mw 1,355), Boguslaski et al., *Clinica Chemica Acta,* 62:349 (1975), and insulin (mw~ 6,000), Davis et al., *Clinica Chemica Acta, 66*:379 (1976). In view of the work of Bolton et al., supra, this method is not universal in that assay sensitivity for large molecules (e.g., TSH having a molecular weight of 30,000) would be limited due to the properties of solid phase antibodies against large molecules.

It has now been discovered that immunochemical composites containing positively charge imidoestes as coupling agents are excellent means for separating free from bound fractions in an immunoassay procedure.

It has also been discovered that immunochemical composites containing a finely divided derivatized polysaccharide matrix wherein the polysaccharide matrix has an average wet maximum dimension of 1 to 18 μ and also containing positively charged imidoesters which covalently couple said finely divided, derivatized, polysaccharide matrix to an antibody are excellent means for separating free from bound fractions without the need for vertical rotation of any other type of continued agitation. Further, such immunochemical composites display an amount of activity which far exceeds the activity displayed by the immunochemical composites prepared by Chan et al.

It has been discovered that a universal solid phase column system for the separation of free and bound fractions can be obtained when one uses immunochemical composites containing a derivatized polysaccharide matrix and also containing positively charged imidoesters which covalently couple the derivatized polysaccharide matrix to antibodies.

According to the present invention there is provided an immunochemical composite for separating free from bound fractions in an immunoassay procedure, comprising a derivatized polysaccharide matrix covalently coupled to an antibody by a bifunctional coupling agent of the formula

$$H_{2c+1}C_cO - \overset{{}^{+}NH_2}{\overset{\|}{C}} - (CH_2)_n - \overset{{}^{+}NH_2}{\overset{\|}{C}} - OC_cH_{2c+1}$$

wherein n is an integer from 1 to 6 and e is 1 or 2.

Further according to the present invention there is provided a method of separating free

from bound fractions in an immunoassay procedure, comprising contacting a solution with the immunochemical composite defined above.

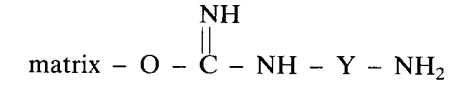
The polysaccharide matrix may be selected from cellulosic polymers, dextran polymers, agarose, and derivatives thereof.

In one embodiment of the invention, the polysaccharide matrix moiety of the immunochemical composite has an average unit maximum dimension of 1 to 18 μ. The polysaccharide matrix can be, for example, a matrix having a plurality of hydroxyl groups attached thereto, or a derivative thereof. Preferred polysaccharide matrices include cellulosic polymers, dextran polymers, agarose, and derivatives thereof. Cellulosic polymers and derivatives thereof are the poly-saccharide matrices of choice.

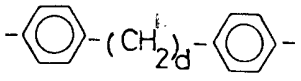
In one embodiment of this invention, the polysaccharide matrix is finely divided and has an average wet maximum dimension of 1 to 18 μ, preferably 10 to 15 μ. In this embodiment, the polysaccharide matrix may be spherical, linear, or have any other geometric configuration, provided that its average wet maximum dimension (diameter, side, length) is as described above. Several types of polysaccharide matrices are commercially available in this finely divided form, for example, Sephadex brand, bead-formed, dextran gel is available in several grades having a dry particle diameter of 10 to 40 μ as well as less than 10 μ. It is also possible to reduce the polysaccharide matrices average wet maximum dimension by chemical techniques, for example by hydrolysis. This can be done by contacting the polysaccharide matrix with an acidic solution, e.g., a 3 to 10 N solution of hydrochloric, sulfuric, or other suitable acid for a sufficient period of time, e.g., 2 to 24 hours. The acidic mixture is then neutralized with a basic solution, e.g., a 3 to 10 N solution of sodium or potassium hydroxide, and subsequently washed and dried by standard techniques.

The polysaccharide matrices can be activated by any suitable method known to those skilled in the art. Exemplary reagents suitable for activating the polysaccharide matrix include cyanogen halide, epihalohydrin, haloacetyl halides, and divinyl-sulphone. See Patty, *Industrial Hygiene and Toxicology*, Vol. 2, p. 634, Interscience, New York, N.Y. (1949), Axen et al., *Nature* (Lond.), *214*:1302 (1967), Rosner et al., *Biochem.*, *14*:4813 (1975), Jagendorph et al., *Biochimica et Biophysica Acta, 78*:516 (1963), and Porath et al., *Nature New Biol.*, *238*:261 (1972), said publications being incorporated herein in toto by reference. Preferably, a cyanogen halide or an epihalohydrin reagent is used to activate the polysaccharide matrix. More preferably, the polysaccharide matrix is activated by an epihalohydrin reagent or mixture thereof and most preferably, the polysaccharide matrix is activated by epichlorohydrin.

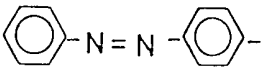
An α,ω-diaminospacer can then be coupled to the above activated polysaccharide matrix via one of the α,ω-diaminospacer's amino groups thereby forming a derivatized polysaccharide matrix. To illustrate this point, if the polysaccharide matrix has been activated by a cyanogen halide reagent, the derivatized polysaccharide matrix will have the formula



wherein the matrix is a polysaccharide matrix as defined above and wherein Y is a spacer. Exemplary spacers include $-(CH_2)_m-$, $(CH_2)_b - NH - (CH_{2c}) -$,

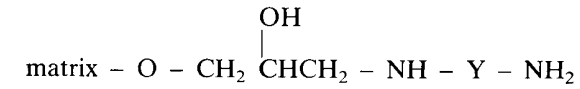


, and ,



wherein m is an integer from 1 to 12, preferably 4, 5 or 6, wherein b and c independently are integers from 1 to 6, preferably 2 or 3, and wherein d is an integer from 1 to 10, preferably 2, 3 or 4. Preferably, Y is $-(CH_2)_m-$.

As a further illustration of a derivatized polysaccharide matrix, if the polysaccharide matrix has been activated by an ephihalohydrin reagent, the derivatized polysaccharide matrix will have the formula

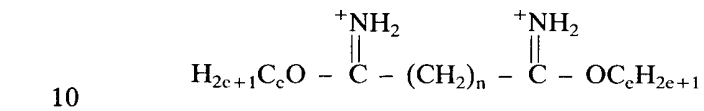


wherein the matrix and Y are as defined above.

The antibody to which the derivatized polysaccharide matrix is covalently coupled can be either a primary antibody or a secondary antibody. Since this invention's sole requirement

is that the antibody possess a lysine residue, virtually all primary and secondary antibodies can be covalently coupled to the derivatized polysaccharide matrix because all antibodies possess such lysine residues. Preferably, the antibody is a secondary antibody.

The crux of this invention is the use of imidoesters as the coupling agent for the immunochemical composite. The imidoester preferably has the general formula



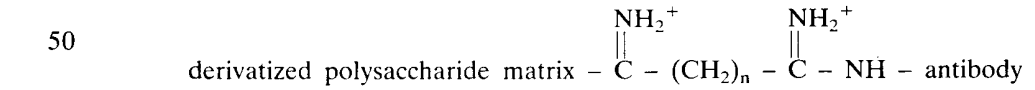
wherein n is an integer from 1 to 6, preferably 4, 5 or 6, and wherein e is 1 or 2. The use of these imidoesters enables one to attach antibodies covalently to solid supports through known chemical reactions which immobilize both primary and secondary antibodies through their lysine residues which in most instances are not essential for immunological activity. Further, the presence of a positively charged matrix does not cause adverse nonspecific adsorption onto the immunochemical composite.

The immunochemical composites of this invention can be prepared, for example, in accordance with the following general procedure. An activating reagent is contacted with the desired polysaccharide matrix in a solution having a desirable pH. The pH can be in a general range from about 7.5 to about 10.0 with the particular pH being dictated by the activating reagent and polysaccharide matrix being used. The reaction can be allowed to proceed at room temperature. The activating reagent is allowed to remain in contact with the polysaccharide matrix for a sufficient period of time, from about 5 minutes to 5 hours, to enable the matrix to become activated. The excess activating reagent is removed from the activated polysaccharide matrix by washing said matrix with a suitable medium, e.g., water, buffer, (e.g., sodium bicarbonate), etc. The activated matrix is then suspended in a suitable medium, e.g., an aqueous solution of dimethylformamide. The desired α,ω-diaminospacer is then added to the suspended activated polysaccharide matrix and the reaction is allowed to proceed for about 1 to 10 hours at room temperature. The excess α,ω-diaminospacer is removed from the derivatized polysaccharide matrix by washing said matrix with a suitable medium, e.g., a solution of dimethylformamide, followed by a washing with a suitable buffer, e.g., a sodium bicarbonate buffer. After this double washing procedure, the derivatized polysaccharide matrix is suspended in a suitable buffer, e.g., a sodium bicarbonate buffer.

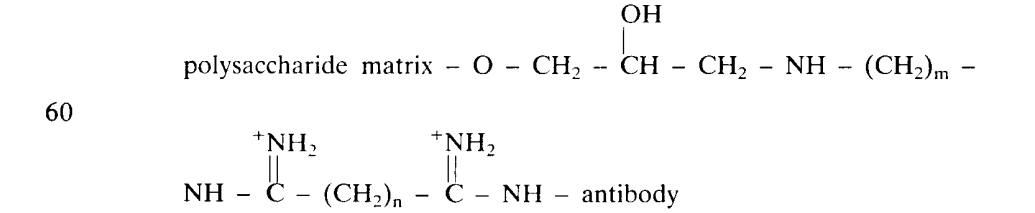
The bifunctional coupling agent or mixture thereof is dissolved in a basic solution at about 4°C. If necessary, the pH is adjusted to about 8 to 9. The suspended derivatized polysaccharide matrix is then contacted with the dissolved bifunctional coupling agent and the mixture is rotated at about 4°C. for 1 to 5 hours.

After removing the excess bifunctional coupling agent, the coupled derivatized polysaccharide matrix is suspended in a mixture containing a suitable buffer, e.g., a sodium bicarbonate buffer, and a primary or secondary antibody function. The mixture is rotated for about 10 to about 24 hours in a cold environment. The immunochemical composite is then thoroughly washed with a suitable buffer, e.g., a sodium bicarbonate buffer, and then suspended in a suitable buffer having a pH of about 8, e.g., a barbital buffer containing about 0.1% gelatin.

An example of an immunochemical composite of this invention and as prepared by the above general procedure has the schematic structure



wherein derivatized polysaccharide matrix, n, and antibody are as defined above. The preferred immunochemical composite has the formula



wherein polysaccharide matrix, m, n and antibody are as defined above.

The immunoassay procedure of this invention entails contacting a solution containing free and bound fractions with an immunochemical composite of the invention via techniques well known to those skilled in the art of immunoassay and thereby separating the free from the bound fractions. See Weir, "Immunology for Undergraduates", Churchill Livingstone, Edinbrugh, U.K. (1973) and Ratcliffe, *British Medical Bulletin 30*:32 (1974), said publications being incorporated herein in toto by reference.

In one preferred embodiment, the immunoassay procedure entails a method in which continuous agitation and thereby the need to stopper the tubes is eliminated. See Chan et al., *Ann. Clin Biochem., 12*:173 (1975), said publication being incorporated herein in toto by reference. In another preferred embodiment, the immunoassay procedure is a solid phase column immunoassay procedure employing an immunochemical composite of the present invention via techniques well known to those skilled in the art of immunoassay, and thereby separating the free from the bound fractions. See, supra, Ratcliffe, supra, Boguslaski et al., *Clinica Chemica Acta, 62*:349 (1974), Boguslaski et al., *Analytical Chemistry,* Vol. 47, No. 9, 1583 (1975), and Davis et al., *Clinica Chemica Acta, 66*:379 (1976), said publications being incorporated herein in toto by reference. Preferably, the immunoassay procedures of the instant invention are RIA procedures whose techniques are also well known to those skilled in the art. See Skelley et al., "Radioimmunoassay", *Clinical Chemistry,* Vol. 19, No. 2, 146 to 186 (1973), said publication being incorporated herein in toto by reference.

Another method of this invention is a suspension procedure such as those described above, except that the composite used therein is not the novel immunochemical composite mentioned above and described in more detail below but an immunoassay reagent comprising a finely divided, polysaccharide matrix covalently coupled to a secondary antibody via any of the various techniques known to those skilled in the art. Although this latter method does not have the degree of efficacy found in the other above described methods of this invention (see examples and discussion below), nevertheless, it is a marked improvement and possesses distinct advantages over the previously-proposed primary antibody methods such as that of Chan et al.

In previous-proposed suspension methods, the primary antibody is attached to a matrix, thereby adversely interfering with the primary antibody-large antigen biological reaction. Also, these methods require an accurate addition of gel (i.e., composites comprising primary antibodies bound to matrices) which creates a constant source of error. However, suspension methods of this invention which use secondary antibodies attached to matrices eliminate or mitigate both of the above described problems.

In general, the immunochemical composites of this invention can be employed in a solid phase column immunoassay procedure in accordance with the following procedure. The immunochemical composites are placed into any column or other suitable reaction vessel, preferably pre-washed with a suitable buffer, thereby forming apparatus for separating free from bound fractions via a solid phase column immunoassay procedure. To minimize costs as well as to improve the flow rate through said column, it is preferred also to add to said column nonactivated polysaccharide matrices of various sizes. All loaded columns will have an inherent hold up volume (i.e., the maximum amount of fluid which can be retained by the column without eluting any of said fluid).

If one desires to use the novel chemical composites of this invention which contain secondary antibodies in a solid phase column immunoassay procedure, one could preferably perform the primary immunoassay reaction outwith the column. When the primary immunoassay is performed outwith the column it can be performed in any suitable volume, preferably a final volume of 400 λ. The primary immunoassay reaction can take 0.5 to 5 hours, preferably 0.5 to 1 hour, the exact time being dictated by the assay being performed. An aliquot of said reaction mixture approximately equal to said packed column's hold up volume is then added to the column and incubated on the solid phase secondary antibody column for a period of 0.5 to 1 hour, the exact time again being dictated by the assay being performed. After the incubation period, the column is washed with an appropriate amount of suitable buffer, said amount being sufficient to separate the free from bound fractions (that is being at least equal to the column's hold up volume). The column, which now contains only the bound fraction, is then capped and counted.

The reaction time for incubation of the columns can be cut very short by using an excess of bound second antibody and one can even elute immediately through the solid phase second antibody columns if an excess of second antibody is used.

The hereinbefore described immunoassay procedure employing this invention's solid phase second antibody columns can therefore be separated into three basic steps: incubation of primary antibody reaction at a station removed from the column, application of an aliquot of the reaction mixture to the solid phase second antibody column, and elution of the free fraction with buffer immediately of after a minimal incubation period on the

column.

The above described solid phase second antibody procedure is simple, easily automated, precise, and versatile. Thus, both assays for large molecules (e.g. TSH) and small haptens (digoxin, T_4, T_3) can be performed using this invention's novel immunochemical composites. Furthermore, one obtains completely separate bound and free fractions with a wide margin for error in the conditions used for the separation. The method does not interfere with the primary binding reaction, is relatively inexpensive, and makes use of equipment and reagents that are readily available.

With respect to small molecules, the solid phase second antibody columns may be employed in more than one mode. The columns may be loaded with primary antibody, labeled antigen (or hapten), and sample and both the primary reaction and solid phase antibody reaction allowed to proceed simultaneously in the void volume of the column. After a given period of time, the columns are eluted with buffer to separate free from bound fractions.

Another approach is to incubate the primary antibody on the solid phase second antibody column and thus produce primary antibody columns on which the primary antibody is biologically bound to the covalently attached second antibody. Label and sample are then incubated on the columns which serve as a device for separating free and bound fractions.

The immunochemical composites of this invention can contain a primary antibody covalently coupled to a derivatized polysaccharide matrix.

An embodiment of this invention uses a solid phase column immunoassay procedure wherein the composite is not the immunochemical composite described above but an immunoassay reagent comprising a polysaccharide matrix covalently coupled to a secondary antibody via any of the various techniques known to those skilled in the art. Although this latter method does not have the degree of efficacy found in the other methods of this invention, nevertheless it is a marked improvement and possesses distinct advantages over the previously-proposed solid phase primary column procedures. Although there is a loss in antibody activity (titer) when a second antibody is covalently coupled to a derivatized polysaccharide matrix, consumption of second antibody is nevertheless reduced because large quantities of carrier globulin are not required as in the case of the double antibody procedure in the liquid state. Another advantage of an immunoassay procedure employing the solid phase second antibody columns is that this method alleviates the problem of accurately dispensing a precise amount of second antibody from a suspension of gel because in this method one need only have a minimal amount of second antibody. Levels above this preset minimal amount have no effect on the sensitivity or accuracy of the results obtained from this invention's novel method.

Embodiments of the present invention will now be described by way of illustration in the following Examples.

EXAMPLE 1

Epichlorohydrin (3 ml) was added to a mixture of 6 gms of microcrystalline cellulose Type 50 (50 μ average particle size) in 30 ml of 1N sodium hydroxide with vigorous stirring at room temperature. After 2 hours, the excess epichlorohydrin was removed by washing with 1 liter of water. The washed activated cellulosic matrix was then suspended in 60 ml of a 50% aqueous solution of dimethylformamide. To this suspended activated matrix was added 0.85 gms of 1,6-hexanediamine. The reaction was allowed to proceed with stirring for 2 hours at room temperature and then the excess 1,6-hexanediamine was removed by washing with 1 liter of a 50% aqueous solution of dimethylformamide. After washing with 1 liter of 0.1M sodium bicarbonate, the derivatized cellulosic matrix was suspended in 0.1M sodium bicarbonate to give a 1:1 mixture of derivatized matrix to sodium bicarbonate.

Dimethyladipidate (DMA; 0.735 gm; 3 m moles) was dissolved in 0.6 ml of cold 5N sodium hydroxide solution with stirring at 4°C. After the addition of cold 0.1 M sodium bicarbonate the pH was adjusted to 8.5 with 1N sodium hydroxide. To this solution was added 6 ml of 0.1 M sodium bicarbonate containing 0.8 to 1.0 gram of derivatized cellulose and the mixture was rotated at 4°C. for two hours.

After the removal of excess dimethyl adipimidate, the coupled derivatized cellulosic matrix was suspended in 9 ml of 0.1M sodium bicarbonate (4°C.) and 1.1 ml of goat antirabbit gamma globulin fraction (42.82 mg/ml) in 0.1M sodium bicarbonate (4°C.) and the mixture was rotated in a cold room. The immobilized second antibody was then thoroughly washed with 0.1M sodium bicarbonate and finally suspended in 10 ml of barbital buffer, pH 8.0, containing 0.1% gelatin.

Another immunochemical composite was also prepared according to the procedure of Example 1 except that the bifunctional coupling agent used was dimethyl suberimidate dihydrochloride (DMS) instead of the DMA of Example 1.

EXAMPLE 2

Microcrystalline cellulose type 50 (1 gm) was added to a solution of 1.0 gm of cyanogen bromide in water at room temperature. The pH of the mixture was immediately adjusted to about 11.0 with 2N sodium hydroxide and maintained at this pH for 6 to 12 minutes by the controlled addition of 2N sodium hydroxide. After the pH stabilized at about 11, the mixture was allowed to stand an additional 5 to 10 minutes before the activated cellulosic matrix was washed with 1.1 liters of 0.1M sodium bicarbonate at 4°C. to remove the excess cyanogen bromide.

The thoroughly washed activated cellulosic matrix was then suspended in 10 ml of 0.1M sodium bicarbonate and about 47 mgms of goat antirabbit gamma globulin in 1.3 ml of 0.1M sodium bicarbonate was added thereto. The suspension was then mixed at room temperature overnight.

The following day the immunochemical composite was washed with 600 ml of 0.1M sodium bicarbonate and 100 ml of barbital buffer (pH 8.0) containing 0.1% gelatin. Finally, the composite was suspended in about 10 ml of the above gelatin containing barbital buffer.

EXAMPLE 3

Solid phase preparations of activated microcrystalline cellulose as prepared in examples 1 and 2 were titered against ^{125}I-labeled rabbit gamma globulin as follows:

200λ – barbital buffer pH 8.0 containing 3.5% BSA
100λ – ^{125}I-labeled rabbit gamma globulin containing 0.1% normal rabbit serum
100λ – barbital buffer pH 8.0
200λ – solid phase second antibody preparation

Each tube was incubated at room temperature for 1/2 hour with shaking and subsequently centrifuged for 20 minutes at 1,000 × g.

The units of activity were calculated from the largest dilution, i.e., titer, of solid phase second antibody that resulted in maximal binding of the labeled antigen. The formula used to calculate the units of activity is as follows:

$$\frac{\frac{1}{\text{titer}}}{\text{sample size}} \times \text{total volume of solid phase antibody preparation} = \text{Units of Activity}$$

wherein the sample size in our example is 200λ (0.2 ml) and wherein the total volume of solid phase secondary antibody preparation is 10 ml. Since these titers were done in the presence of 100 λ of 0.1% normal rabbit serum, one actually looks for the largest dilution of solid phase second antibody capable of binding about 1 microgram of rabbit gamma globulin. The results of these calculations are listed in Table I. As Table I clearly depicts, the titer of the goat anti-rabbit antisera coupled to the DMA derivatized cellulose was far superior to the corresponding cyanogen bromide (CNBr) coupled preparation of microcrystalline cellulose.

EXAMPLE 4

Derivatized cellulose as prepared in Example 2 and the DMS version of Example 1 were titered against ^{125}I-thyroxine in the presence of about 1 microgram of rabbit gamma globulin as follows:

20λ – barbital buffer pH 8.0 containing 3.5% BSA
100λ – ^{125}I-thyroxine in barbital buffer pH 8.0 containing 2% BSA
100λ – rabbit antiserum against thyroxine at a dilution of 1 per 1,000 in barbital buffer pH 8.0.
200λ – solid phase second antibody preparation.

Each tube was incubated at room temperature for about 1/2 hour with shaking and subsequently centrifuged for 20 minutes at 1000 × g. The precipitates were then suspended in 1.0 ml of barbital buffer pH 8.0 containing 2% BSA and centrifuged for 20 minutes at 1000 × g. In these experiments the labeled thyroxine is immunologically bound to its specific antibodies which are present as a fraction of about 1 microgram of rabbit gamma globulin. Thus, in this example, one is able to indirectly measure the unit of second antibodies bound to the cellulose matrix by calculating the largest dilution of solid phase second antibody preparation to give the maximal binding of labeled-thyroxine.

Table I also depicts the results of this data. As Table I clearly shows, the DMS experiments compare favorably to the DMA data and both preparations far surpass their corresponding cyanogen-bromide controls. Similar improvements in the amount of units of activity recovered also result when the immunochemical composites of this invention contain primary antibodies instead of secondary antibodies.

Therefore, this invention's positively charged immuno-chemical composites containing bifunctional imidoesters as coupling agents must be considered a marked improvement over previously-proposed immunochemical composites used in immunoassay procedures.

TABLE I

	Support	Protein concentration of antibody containing gamma globulin fraction	Titer	Units of activity recovered	Percent increase in activity recovered
*A.	1 gram cyanogen bromide activated microcrystalline cellulose	47 mg of goat anti-rabbit gamma globulin	1/2.3	115	---
A.	1 gram dimethyl adipidimate activated microcrystalline cellulose	47 mg of goat anti-rabbit gamma globulin	1/16.6	850	739
**B.	1 gram cyanogen bromide activated microcrystalline cellulose	40 mg of goat anti-rabbit gamma globulin	1/3.2	160	---
B.	1 gram dimethyl suberimidate activated microcrystalline cellulose	40 mg of goat anti-rabbit gamma globulin	1/16	800	500

*A – label ^{125}I-rabbit gamma globulin
**B – label ^{125} I-thyroxine

EXAMPLE 5

Microcrystalline cellulose (9 gm) type 50 (50 μ average particle size) was added to 30 ml of a 6N hydrochloric acid solution and the mixture was stirred for a period of 4 hours at room temperature. After the 4 hour reaction time, the mixture was neutralized with a 6N solution of sodium hydroxide and the hydrolized cellulose was washed with 1,200 ml of water. The packed cellulose was further washed with 300ml of methanol followed by 100 ml of diethyl ether. The gel residue was suspended in 100 ml of ether and dried under reduced pressure.

Example 5 was repeated several times with the sole modification being the reaction time. The reaction time was varied from 4 hours to periods of 6, 24 and 64 hours. A microscopic examination disclosed that the microcrystalline cellulose Type 50 which had been hydrolyzed for 4 and 6 hours had an average wet length of approximately 15 to 20 μ and those which had been hydrolyzed for 24 and 64 hours had an average wet length of approximately 10 to 12 μ.

EXAMPLE 6

Epichlorohydrin (3 ml) was added to a mixture of 6 gms of microcrystalline cellulose Type 50 in 30 ml of 1N sodium hydroxide with vigorous stirring at room temperature. After 2 hours, the excess epichlorohydrin was removed by washing with 1 liter of water. The washed, activated, cellulosic matrix was then suspended in 60 ml of a 50% aqueous solution of dimethylformamide. To this suspended, activated matrix was added 0.85 gms of 1,6-hexanediamine. The reaction was allowed to proceed with stirring for 2 hours at room temperature then the excess 1,6-hexanediamine was removed by washing with 1 liter of a 50% aqueous solution of dimethylformamide. After washing with 1 liter of 0.1m sodium bicarbonate, the derivatized, cellulosic matrix was suspended in 0.1M sodium bicarbonate to give a 1:1 mixture of derivatized matrix to sodium bicarbonate.

Dimethylsuberimidate (DMS; 0.80 gm; 3 m moles) was dissolved in 0.6 ml of cold 5N sodium hydroxide solution with stirring at 4°C. After the addition of cold 0.1 M sodium bicarbonate, the pH was adjusted to 8.5 with 1N sodium hydroxide. To this solution was added 6 ml of 0.1M sodium bicarbonate containing 0.8 to 1.0 grams of derivatized cellulose and the mixture was rotated at 4°C. for 2 hours.

After the removal of excess DMS, the coupled, derivatized, cellulosic matrix was suspended in 9 ml of 0.1M sodium bicarbonate (4°C.) and 1.3 ml of goat antirabbit gamma globulin fraction (36 to 38 mg/ml) in 0.1M sodium bicarbonate (4°C.) and the mixture was rotated in a cold room. The immobilized, secondary antibody was then thoroughly washed with 0.1M sodium bicarbonate and finally suspended in barbital buffer, pH 8.0, containing 0.1% gelatin, to give a final volume of 25 ml.

Example 6 was repeated wherein the sole modification was the size of the microcrystalline cellulose used. The various other sizes of microcrystalline cellulose employed were microcrystalline cellulose Type 20 and microcrystalline cellulose Type 50 which had been hydrolyzed for 4 hours, 6 hours, 24 hours, and 64 hours.

EXAMPLE 7

Microcrystalline cellulose type 50 (1 gm) was added to a solution of 1.0 gm of cyanogen bromide (CNBr) in water at room temperature. The pH of the mixture was immediately adjusted to about 11.0 with 2N sodium hydroxide and maintained at this pH for 6 to 12 minutes by the controlled addition of 2N sodium hydroxide. After the pH stabilized at about 11, the mixture was allowed to stand an additional 5 to 10 minutes before the activated, cellulosic matrix was washed with 1.1 liters of 0.1M sodium bicarbonate at 4°C. to remove the excess CNBr.

The thoroughly washed, activated, cellulosic matrix was then suspended in 10 ml of 0.1m sodium bicarbonate and about 47 mgm of goat antirabbit gamma globulin in 1.3 ml of 0.1M sodium bicarbonate was added thereto. The suspension was then mixed at room temperature overnight.

The following day the immunochemical composite was washed with 600 ml of 0.1M sodium bicarbonate and 100 ml of barbital buffer (pH 8.0) containing 0.1% gelatin. Finally, the composite was suspended in the above gelatin containing barbital buffer to give a final solution of 25 ml.

Example 7 was repeated wherein the sole modification was the size of the microcrystalline cellulose used. The various other sizes of microcrystalline cellulose employed were microcrystalline cellulose Type 20 and microcrystalline cellulose Type 50 which had been hydrolyzed for 4, 6, 24, and 64 hours.

EXAMPLE 8

Second antibody solid preparations of microcrystalline cellulose Type 50 as prepared in Examples 6 and 7 were titered against ^{125}I-thyroxine in the presence of about 1 μg of rabbit gamma globulin as follows:

20λ – barbital buffer pH 8.0 containing 3.5% BSA
100λ – ^{125}I-thyroxine in barbital buffer pH 8.0 containing 2% BSA
100λ – rabbit antisera against thyroxine at a dilution of 1/1000 (containing about 1 μg rabbit IgG)
200λ – solid phase second antibody preparations

In experiments in which the reactants were agitated, each tube was incubated at room temperature for one-half hour with shaking and subseqently centrifuged for 20 minutes at 100 × g. The precipitates were suspended in 1.0 ml of barbital buffer, pH 8.0, containing 3.5% BSA and recentrifuged for 20 minutes at 1000 × g. However, in the experiments in which the reactants were not agitated, each tube was incubated at 37°C. for one-half hour and before centrifugation 1 ml of barbital buffer, pH 8, containing 3.5% BSA was added. Each tube was subsequently centrifuged at 1000 × g for 20 minutes.

In both of these experiments the labeled thyroxine is immunologically bound to its specific antibodies which are present as a fraction of approximately 1 microgram of rabbit gamma globulin. Thus, one is able to indirectly measure the units of second antibody bound to the different cellulose matrices by calculating the largest dilution of each of the solid phase precipitating antibody preparations to give maximal binding of labeled thyroxine.

The units of activity were calculated as described above except that the total volume of solid phase secondary antibody preparation is 25 ml. The results of these calculations are listed in Tables II and III. As Tables II and III clearly depict, the titer of the goat antirabbit antisera coupled to the DMS derivatized finely divided cellulose was far superior to the corresponding CNBr coupled preparation of finely divided, microcrystalline cellulose. In fact, the titer of said DMS derivatized, finely divided cellulose which underwent no shaking was even superior to the shaken CNBr coupled preparations.

TABLE II

Support	Protein concentration of antibody containing gamma globulin fraction	Titer shaking	Units of activity recovered shaking	Titer nonshaking	Units of activity recovered nonshaking	% Units non-shaking Units shaking
1 gram CNBr activated microcrystalline cellulose Type 50	47 mg of goat anti-rabbit gamma globulin	1/1.6	212	0	0	0
1 gram CNBr activated microcrystalline cellulose Type 20	" "	1/3.7	462	1/1.6	200	43.29%
1 gram CNBr activated 4 hr. acid hydrolyzed microcrystalline cellulose Type 50	" "	1/5	625	1/2.9	362	57.92%
1 gram CNBr activated 6 hr. acid hydrolyzed microcrystalline cellulose Type 50	" "	1/5	625	1/2.9	362	57.92%
1 gram CNBr activated 24 hr. acid hydrolyzed microcrystalline cellulose Type 50	" "	1/5	625	1/3.4	425	68.00%
1 gram CNBr activated 64 hr. acid hydrolyzed microcrystalline cellulose Type 50	" "	1/5.5	688	1/3.4	425	61.77%

TABLE III

Support	Protein concentration of antibody containing gamma globulin fraction	Titer shaking	Units of activity recovered shaking	Titer nonshaking	Units of activity recovered nonshaking	% Units nonshaking / Units shaking
1 gram DMS activated microcrystalline cellulose Type 50	49 mg of goat anti-rabbit gamma globulin	1/5	750	1/2	250	33.33%
1 gram DMS activated microcrystalline cellulose Type 20	" "	1/11.5	1438	1/5.1	638	44.37%
1 gram DMS activated 4 hr. acid hydrolyzed microcrystalline cellulose Type 50	" "	1/11.5	1438	1/7.0	875	60.85%
1 gram DMS activated 6 hr. acid hydrolyzed microcrystalline cellulose Type 50	" "	1/11.5	1438	1/7.0	875	60.85%
1 gram DMS activated 24 hr. acid hydrolyzed microcrystalline cellulose Type 50	" "	1/11.5	1438	1/7.0	875	60.85%
1 gram DMS activated 64hr. acid hydrolyzed microcrystalline cellulose Type 50	" "	1/11.5	1438	1/7.0	875	60.85%

TABLE IV

Support	*Shaking* Units of Activity by DMS / Units of Activity by CNBr	*Nonshaking* Units of Activity by DMS / Units of Activity by CNBr	Units of Activity by DMS nonshaking / Units of Activity by CNBr shaking
Microcrystalline Cellulose Type 50	$\frac{750}{212} = 3.54$	$\frac{250}{0} =$ Indefinite	$\frac{250}{212} = 1.18$
Microcrystalline Cellulose Type 20	$\frac{1438}{462} = 3.11$	$\frac{638}{200} = 3.19$	$\frac{638}{462} = 1.38$
4 hr. acid hydrolyzed Microcrystalline Cellulose Type 50	$\frac{1438}{625} = 2.30$	$\frac{875}{362} = 2.42$	$\frac{875}{625} = 1.40$
24 hr. acid hydrolyzed Microcrystalline Cellulose Type 50	$\frac{1438}{625} = 2.30$	$\frac{875}{425} = 2.06$	$\frac{875}{625} = 1.40$
64 hr. acid hydrolyzed Microcrystalline Cellulose Type 50	$\frac{1438}{625} = 2.09$	$\frac{875}{425} = 2.06$	$\frac{875}{625} = 1.27$

TABLE V

		Control Sera								
	Assay Time at 37°C.	Digoxin Beckman Control Serum	Thyroxine Beckman Control Serum	Trioodo-thyronine Beckman Control Serum	TSH Beckman Control Serum	TSH Beckman Control Serum Diluted 1/2	Lederle I	Lederle II	Ortho I	Ortho II
Digoxin (ng/ml)	1/2 hr.	2.75 (2.0-2.7)*	---	---	---	---	1.44 (.94-1.78)	>6.0	.62 (.6-1.2)	4.25 3.2-5.2)
Triiodothyronine (ng/dl)	2 hr.	---	86 (90-110)	222 (165-225)	85 (70-90)	---	116 (98-118)	474 (409-457)	---	---
Thyroxine (μg/dl)	1/2 hr.	---	12.3 (12-20)	---	5.0 (6-7)	---	7.2 (6.4-9.6)	16.1 (13.6-22)	---	---
Human Thyrotropin Stimulating Hormone (micro international units/ml)	6 hr.	---	---	---	77.0 (40-75)	35.2	3.7 (3-4)	2.6 (2.3-2.95)	7.2 (3.9-7.9)	32.2 (23-39)

*Values in ( ) are those obtained with the conventional double antibody method

Example 8 was repeated except that the size of the microcrystalline cellulose used was varied. This variation, as above, entailed the use of microcrystalline cellulose Type 20 and microcrystalline cellulose Type 50 which had been hydrolyzed for 4, 6, 24, and 64 hours. The results of this data is also listed in Tables II and III.

An examination of Table II reveals several things. in the column entitled "Units of Activity Recovered Shaking," since the vial is being shaken, all matrix sizes should be equally mixed and equally suspended and therefore the exposure of the CNBr covalently bound second antibody to the antigen is the same. However, it is readily apparent that the matrix size does influence the biological activity of the CNBr covalently coupled second antibody. This column indicates three basic groups, namely, microcrystalline cellulose Type 50, microcrystalline cellulose Type 20, and microcrystalline cellulose Type 50 which has been hydrolyzed for 4 hours or more. The basic thrust of said column is that one cannot recover more activity by shaking a CNBr activated matrix than that recovered using a microcrystalline cellulose Type 50 which had been hydrolyzed for 4 hours.

In the column entitled 'Units of Activity Recovered Nonshaking,' suspendability of a particle, and therefore, matrix size, comes into play as well as the biological activity of the covalently coupled second antibody. Four subdivisions are found in this column, namely, microcrystalline cellulose Type 50, microcrystalline cellulose Type 20, microcrystalline cellulose Type 50 which has been hydrolyzed for 4 and 6 hours, and microcrystalline cellulose Type 50 which has been hydrolyzed for 24 and 64 hours, with maximum biological activity being found in the last group.

A similar comparison of Table III indicates the striking improvement of the immunochemical composites of this invention over the immunochemical composites of Table II. The column entitled "Units of Activity Recovered Shaking" has two basic subgroups instead of the three which appear in Table II. Namely, the microcrystalline cellulose Type 50 differs from microcrystalline cellulose Type 20 and smaller. This differential implies that one cannot obtain more biological activity than is recovered when using a microcrystalline cellulose Type 20. However, upon examining the column entitled "Units of Activity Recovered Nonshaking," one sees that the column is divided into three groups, namely microcrystalline cellulose Type 50, microcrystalline cellulose Type 20, and microcrystalline cellulose Type 50 which has been hydrolyzed for either 4, 6, 24 or 64 hours. One can conclude, therefore, than when one uses a microcrystalline cellulose or similar polysaccharide having a maximum dimension of 18 μ or less, the size of the matrix is not a limiting factor in determining the amount of activity recovered. Further, one can see that although one does not obtain the degree of activity present when shaking, the nonshaking units of activity recovered using any of the present immunochemical composites far exceed the amount of units of activity recovered shaking using an antibody directly coupled to a matrix via the CNBr process. The reason for this vast improvement is that the present composites effectively position the antibody at a distance removed from the matrix surface and thereby decrease the effect and influence of the matrix size on the biological reaction. Besides removing the antibody from the matrix surface, this invention also provides for the antibody to be coupled to a derivatized matrix via a coupling agent which, as noted above, does not in most instances adversely affect said antibody's immunological activity.

In addition to the above, the improved efficacy of the present immunological chemical composites enables one to use larger matrix sizes in a nonagitation procedure thereby making it possible for one to employ a centrifugation device with lower g's. The ability to use a centrifuge having a lower g rating is significant in that one working with the present immunological composites can now use conventional lab centrifuges and thereby avoid the high expense one would otherwise have to incur if he wished to practice a prior art method such as that of Chan et al.

As Table IV dramatically depicts, one obtains about a 27 to 40% increase using the immunochemical composites of this invention in a nonagitated procedure over the shaking procedure wherein the antibody is directly coupled to a matrix via the CNBr process.

Table V compares the efficacy of employing the present immunochemical composites with the conventional previously-proposed double antibody method in various immunoassay tests. As Table V indicates, the results obtained using the method and immunochemical composites of this invention compare favorably with respect to the prior art method using said conventional double antibody method. It should be noted that the RIA of thyroxine via the conventional double antibody method takes about 2 hours whereas an RIA employing the immunochemical composites of this invention takes but a half hour.

EXAMPLE 9

Epichlorohydrin (3 ml) was added to a mixture of 6 gms of microcrystalline cellulose Type 50 (50 μ average particle size) in 30 ml of 1N sodium hydroxide with vigorous stirring at room temperature. After 2 hours, the excess epichlorohydrin was removed by washing

with 1 liter of water. The washed activated cellulosic matrix was then suspended in 60 ml of a 50% aqueous solution of dimethylformamide. To this suspended activated matrix was added 0.85 gms of 1,6-hexanediamine. The reaction was allowed to proceed with stirring for 2 hours at room temperature and then the excess 1,6-hexanediamine was removed by washing with 1 liter of a 50% aqeuous solution of dimethylformamide. After washing with 1 liter of 0.1M sodium bicarbonate, the derivatized cellulosic matrix was suspended in 0.1M sodium bicarbonate to give a 1:1 mixture of derivatized matrix to sodium bicarbonate.

Dimethylsuberimidate (DMS; 0.80 gm; 3 m moles) was dissolved in 0.6 ml of cold 5N sodium hydroxide solution with stirring at 4°C. After the addition of cold 0.1 M sodium bicarbonate the pH was adjusted to 8.5 with 1N sodium hydroxide. To this solution was added 6 ml of 0.1M sodium bicarbonate containing 0.8 to 1.0 grams of derivatized cellulose and the mixture was rotated at 4°C. for 2 hours.

After the removal of excess DMS, the coupled derivatized cellulosic matrix was suspended in 9 ml of 0.1M sodium bicarbonate (4°C.) and 1.3 ml of goat antirabbit gamma globulin fraction (36 to 38 mg/ml) in 0.1M sodium bicarbonate (4°C.) and the mixture was rotated in a cold room. The immobilized second antibody was then thoroughly washed with 0.1M sodium bicarbonate and finally suspended in barbital buffer, pH 8.0, containing 0.1% gelatin, to give a final volume of 25 ml.

EXAMPLE 10

Procedure for the preparation of columns

Prewash each empty column with 1 ml of a barbital buffer containing 0.1% gelatin and 2% BSA. Wash in separate tubes both fibrous cellulose and microcrystalline cellulose Type 50. Spin down the cellulosic fractions and make 1:1 mixtures (by volume) of fibrous cellulose/buffer and microcrystalline cellulose/buffer with said buffer being a barbital buffer containing 0.1% gelatin. Then add equal volumes of the fibrous cellulose and microcrystalline cellulose mixtures to give a final mixture which can be used as a support for all columns. Each column contains the equivalent of 1.4 ml of the above mixture plus 200 λ of the proper dilution of solid phase precipitating antibody. This gives a hold up volume of 300 µl. Next, each loaded column is prewashed with 1 ml of said barbital buffer containing 0.1% gelatin and 2% BSA. The columns are then capped for storage.

EXAMPLE 11

The primary RIA is done in a 400 λ final volume at a space remote from the column. The primary reaction can be performed for 0.5 to 5 hours, preferably 0.5 to 1 hour. An aliquot of the primary reaction mixture (300λ) is then applied to the solid phase second antibody column. This mixture is allowed to incubate for a period of 0.5 to 1 hour. The solid phase column is then washed with 1 ml to 3 ml of said barbital buffer containing 0.1% gelatin and 2% BSA to separate the free from bound fractions. The column which now contains only the bound fractions is then capped and counted.

Data obtained by performing various tests in accordance with the general procedures outlined in the above Examples is shown in Table VI.

TABLE VI

	37° Primary Assay Time Remote from Column, hr.	Room Temperature Incubation Time on Column, hr.	*Control Sera* Lederle I	Lederle II	Thyroxine Beckman Control Serum	Beckman Control Serum	TSH Beckman Control Serum Diluted 1/2
Digoxin (ng/ml)	1/2	1/2	1.23 (0.8-1.3)*	3.48 (3-3.8)	---	---	---
Thyroxine (μg/dl)	1/2	1/2	8.33 (6.4-9.6)	17.37 *(13.6-22)*	20.05 (12-20)	7.58 (6-7)	---
Triiodo thyronine	2	1/2	110.60 (98-118)	517.57 (409-457)	106.70 (90-110)	93.45 (70-90)	
Human Thyrotropin Stimulating Hormone (micro international units/ml)	5	1	---	---	---	59.36 (40-75)	30.92

*Values in ( ) are those obtained with the conventional double antibody method.

TABLE VI (Continued)

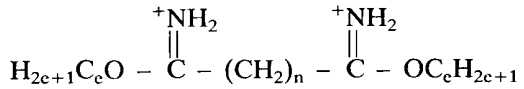
	37° Primary Assay Time Remote from Column, hr.	Room Temperature Incubation Time on Column, hr.	Triiodo Thyronine Beckman Control Serum	*Control Sera* Ortho I	Ortho II
Digoxin (ng/ml)	1/2	1/2	---	---	---
Thyroxine (µg/dl)	1/2	1/2	---	---	---
Triiodo thyronine ng/dl)	2	1/2	245.06 (165-225)	---	---
Human Thyrotropin Stimulating Hormone (micro international units/ml)	5	1	---	7.95 (3.9-7.9)	31.63 (23-39)

Table VI compares the efficacy of employing immunochemical composites of this invention in a solid phase column assay with the conventional prior art double antibody method in various immunoassay tests. As Table VI indicates, the results obtained using the present solid phase column method and immunochemical composites of this invention compare favorably with respect to the prior art method using said conventional double antibody method.

In addition to possessing all the advantages of solid phase immunoassay procedures, the solid phase column immunoassay procedure of this invention eliminates the need for centrifugation and also lends itself to automation.

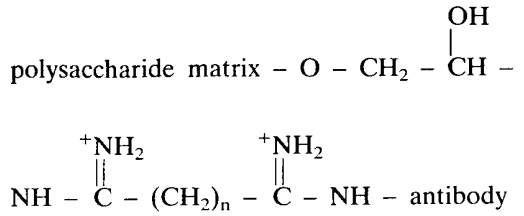
WHAT WE CLAIM IS:

1. An immunochemical composite for separating free from bound fractions in an immunoassay procedure, comprising a derivatized polysaccharide matrix covalently coupled to an antibody by a bifunctional coupling agent of the formula



wherein n is an integer from 1 to 6 and e is 1 or 2.

2. A composite as claimed in claim 1, having the formula



wherein m is an integer from 1 to 12.

3. A composite as claimed in claim 2, wherein m is 4, 5 or 6.

4. A composite as claimed in claim 2 or 3, wherein n is 4, 5 or 6.

5. A composite as claimed in any one of claims 1 to 4, wherein the polysaccharide matrix is selected from cellulosic polymers, dextran polymers, agarose, and derivatives thereof.

6. A composite as claimed in any one of claims 1 to 5, wherein the polysaccharide matrix has an average wet maximum dimension of 1 to 18 μ.

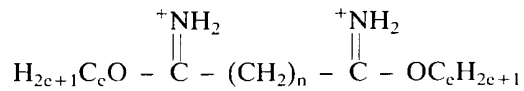
7. A composite as claimed in claim 5 or 6, wherein the polysaccharide matrix is a cellulosic polymer of a derivative thereof having an average wet maximum dimension of 10 to 15μ.

8. A composite as claimed in any one of the preceding claims, wherein the antibody is a secondary antibody.

9. A composite as claimed in any one of the preceding claims, wherein the derivatized polysaccharide matrix comprises an activated polysaccharide matrix coupled to an α,ω-diamino-spacer through one of the α,ω-diaminospacer's amino groups.

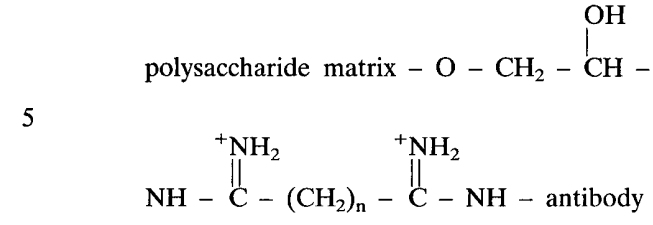
10. An immunochemical composite for separating free from bound fractions in an immunoassay procedure, substantially as hereinbefore described with reference to any one of the Examples.

11. A method of separating free from bound fractions in an immunoassay procedure, comprising contacting a solution with an immunochemical composite which comprises a derivatized polysaccharide matrix covalently coupled to an antibody by a bifunctional coupling agent of the formula



wherein n is an integer from 1 to 6 and wherein e is 1 or 2.

12. A method as claimed in claim 11, wherein the immunochemical composite has a formula



wherein m is an integer from 1 to 12.

13. A method as claimed in claim 12, wherein m is 4, 5 or 6.

14. A method as claimed in claim 11 or 12 or 13, wherein n is 4, 5 or 6.

15. A method as claimed in any one of claims 11 to 14, wherein said polysaccharide matrix is selected from cellulosic polymers, dextran polymers, agarose, and derivatives thereof.

16. A method as claimed in any one of claims 11 to 15, wherein said polysaccharide matrix has an average wet maximum dimension of 1 to 18μ.

17. A method as claimed in claim 15 or 16, wherein said polysaccharide matrix is a cellulosic polymer or a derivative thereof having an average wet maximum dimension of 10 to 15μ.

18. A method as claimed in any one of claims 11 to 17, wherein said antibody is a secondary antibody.

19. A method as claimed in any one of claims 11 to 18, wherein a tube containing said composite remains essentially motionless during an incubation step.

20. A method as claimed in any one of claims 11 to 19, wherein the method is a solid phase column immunoassay procedure.

21. A method as claimed in claim 20, wherein a primary immunoassay reaction is performed outwith said column and an aliquot of the reaction mixture is subsequently incubated on a solid phase second antibody column.

22. A method as claimed in claim 21, wherein an excess of the second antibody is present on the solid phase column.

23. A method as claimed in claim 20, wherein a solid phase second antibody column is loaded with primary antibody, labeled antigen and sample, so that a primary reaction and a solid phase antibody reaction proceed simultaneously in the void volume of the column.

24. A method as claimed in claim 20, wherein primary antibody is incubated on a solid phase second antibody column thereby producing a primary antibody column and wherein labeled antigen and sample are incubated on said primary antibody column.

25. A method as claimed in any one of claims 11 to 24, wherein said immunoassay procedure is a radioimmunoassay procedure.

26. A method of separating free from bound fractions in an immunoassay procedure, substantially as hereinbefore described with reference to any one of the Examples.

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Printed for Her Majesty's Stationery Office, by Croydon Printing Company Limited, Croydon, Surrey, 1981.
Published by The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.