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# IMMUNOLOGICAL REAGENT AND PROCESS FOR MAKING SAME

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Int. Cl. G01n 31/22
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## ABSTRACT OF THE DISCLOSURE

Immunologically specific reagents are prepared by chemically linking the immunological counterpart of the material to be detected to a cellulosic derivative in particulate form and then physically adsorbing onto the immunological counterpart an acidic indicator material. Hematoxylin, fluorescein, and titan yellow are usable as indicator materials. When a fluid sample containing the immunological material to be detected comes in contact with the immunologically specific reagent, the indicator material shows one of two visible effects, separation from the particles or fixation to the particles. The specific type of visible effect is determined by the particular immunologic counterpart employed. For each reaction a control fluid sample which does not contain the immunological material under test shows an opposite reaction. A chromatographic technique can be used to display the visible effects.

## BACKGROUND OF THE INVENTION

This invention relates to an immunologically specific reagent which can be employed to obtain a visual readout for immunological reactions.

Most of the prior art immunological tests are based upon the principle of agglutination or inhibition of agglutination in which the formation of visually discernible aggregates of particles is the ultimate visible effect. The aggregates of particles form different patterns depending upon the presence or absence of the immunological material material under test. These patterns are occasionally difficult to interpret, particularly for persons who have not had adequate experience with such tests. Other immunological testing is even more complex, requires additional supporting laboratory materials and is generally not used on a routine basis by medical technicians. These types of testing involve such procedures as gel diffusion and electrophoresis followed by gel diffusion.

It has now been discovered that an immunologically specific reagent which gives an easily discernible readout can be formed from a plurality of particles, each having a core of a cellulosic derivative in particulate form chemically linked through covalent bonding to the immunological counterpart of the material to be detected, there being physically adsorbed onto said immunological counterpart an acidic indicator material such as hematoxylin, fluorescein, and titan yellow. Upon contact with a fluid sample containing the immunologic material under test the indicator material will either be released from the particle or will become affixed thereto through chemical action, the specific phenomenon depending upon the particular immunologic counterpart employed. The actual testing can be easily carried out by utilizing a control fluid sample which does not contain the immunologic material under test, since the sample will give the opposite phenomenon.

It is, therefore, an object of the present invention to provide an immunological reagent for detecting an immunologic material of the type described wherein a plurality of particles are employed each of which includes an immunologic counterpart of the material to be detected and onto which counterpart an indicator material is physically adsorbed, said indicator being released or affixed by the presence of the immunologic material under test.



Another object of the present invention is to provide a process for producing the immunological reagent described herein.

## SUMMARY OF THE INVENTION

The invention may best be described by reference to the process for producing the immunological reagent described herein. A fluid suspension of a cellulosic derivative in particulate form, the particles of which have reactive groups on the surfaces thereof, is contacted with the immunological counterpart to the immunological material which is to be detected. The immunologic counterpart then chemically reacts by the formation of covalent bonds with the cellulosic derivative. Next an indicator material is physically adsorbed onto the chemically bound immunological counterpart. The physical adsorption of the indicator material can be carried out in a fluid suspension similar to that employed for the initial reaction. The indicator materials hematoxylin, fluorescein, and titan yellow can be employed in a fluid suspension. The cellulosic derivative which is preferred is an aminobenzyl cellulose such as p-aminobenzyl cellulose or p-aminomethoxybenzyl cellulose.

Prior to physically adsorbing the indicator material onto the immunologic counterpart of the particles it is preferable to block the reactivity of any of the reactive groups which have not been reacted with the immunological counterpart. This can be done by contacting the particles with a blocking dye such as beta-naphthol. Such a blocking dye is not necessary when the indicator material also functions as a blocking dye, as in the case of flourescent which both physically adsorbs to the immunologic counterparts and blocks the reactivity of any unreacted aminobenzyl groups.

Both antigens and antibodies can be employed as the immunological counterpart materials which are chemically reacted with the cellulose derivative. Thus both antigens and antibodies can be detected by the immunological reagent of this invention. Immunological materials which are particularly preferred are human myoglobin, human hemoglobin, human chorionic gonadotropin and their antibodies. The class of antibody materials (gamma-globulin molecules modified to have integral reactive sites is represented by these and other chemically equivalent antibodies.

Other immunological counterpart materials allow similar observable effects when chemically bound to the cellulosic derivative particles of the present invention. The following are exemplary of such other immunological materials: bovine serum albumin, human serum albumin, ovalbumin, blood group A and B antigens, unmodified gamma-globulins, beta-globulins, beta-lipoprotein, and alpha-globulins of the human plasma fraction, anti-C-reactive protein derived from either the goat or the sheep, diphtheria antitoxin, tetanus antitoxin, human transferrin, thyro-globulin, trichinella antigen and similar antigenic materials of either pathogenic or natural organisms, leutininzing hormone and insulin.

The process described above can be further illustrated by reference to the following reaction equations for each of the steps. In these equations "Part." has been used for the cellulosic derivative in particulate form which has reactive groups thereon, "Ag" has been used for an exemplary antigen material, the word "indicator" has been used for the indicator material which is either hematoxylin, fluorescein, or titan yellow. The first step of chemically reacting the cellulosic derivative in particulate

form with the immunological counterpart (Ag) can be illustrated by the following equation:

(1) $\text{Part.} + \text{Ag} \rightarrow \text{Part.}-\text{Ag}$

Any unreacted groups of the initial reactive groups on the cellulosic derivative particles can be blocked by a blocking dye according to the following equation:

(2) $\text{Part.}-\text{Ag} + \text{blocking dye} \longrightarrow \underset{\text{blocking dye}}{\text{Part.}-\text{Ag}}$

The prepared particles which have the immunological counterpart attached thereto through covalent bonds can then be reacted with one of the above indicator materials according to the following equation:

(3) $\underset{\text{blocking dye}}{\text{Part.}-\text{Ag}} + \text{indicator} \longrightarrow \underset{\text{blocking dye}}{\text{Part.}-\text{Ag . indicator}}$

When used to detect an antibody (Ab), i.e., the immunologic material is specific to the antigen of the prepared immunological reagent, the following equation describes the reaction when the indicator is released:

(4) $\underset{\text{blocking dye}}{\text{Part.}-\text{Ag . indicator}} + \text{Ab} \longrightarrow \underset{\text{blocking dye}}{\text{Part.}-\text{Ag . Ab}} + \text{indicator}$

In order to demonstrate the reaction a control may be run according to the following equation:

(5) $\underset{\text{blocking dye}}{\text{Part.}-\text{Ag . indicator}} + \text{normal rabbit serum (NRS)} \longrightarrow$ No change

When the indicator becomes affixed to the particle by reason of contact between the antibody being sought and the prepared particle the following equation describes the reaction:

(6) $\underset{\text{blocking dye}}{\text{Part.}-\text{Ag . indicator}} + \text{Ab} \longrightarrow \underset{\text{blocking dye}}{\text{Part.}-\text{Ag . indicator . Ab}}$

The corresponding reaction for the control test fluid wherein the indicator is released from the particle is illustrated by the following equation:

(7) $\underset{\text{blocking dye}}{\text{Part.}-\text{Ag . indicator}} + \text{NRS} \longrightarrow \underset{\text{blocking dye}}{\text{Part.}} + \text{Ag . NRS} + \text{indicator}$

Thus, in the indicator release situation illustrated by Equation 4, the indicator is released when the immunological material being detected (Ab) contacts the immunological reagent. When the test sample does not contain the Ab the indicator is not released and there is no change as illustrated by Equation 5. In the case of the indicator fixation situation when the Ab contacts the reagent, there is no change since the indicator becomes affixed, as illustrated by Equation 6, and does not release from the Ag on the particle. On the other hand when the prepared particle is contacted with a control sample such as normal rabbit serum (NRS) the rabbit serum interacts with the Ag to form a complex which then allows the indicator to be released as shown by Equation 7.

When an antibody type immunological reagent is to be made up according to Equations 1–3 the Ag is changed to Ab to denote an antibody. Equations 4–7 can be changed by substituting Ag for Ab, and by substituting Ab for Ag in each occurrence in order to illustrate the two different reactions of release and fixation of the indicator with their corresponding control equations for this type of reagent.

An effective means by which the release or the fixation of the indicator material can be visually observed is provided by placing a spot of the immunological reagent on a carrier strip of a bibulous material such as a piece of filter paper or chromatography paper, contacting an edge portion of the strip with a test sample and allowing the sample to be drawn into the bibulous carrier by capillary action. In the case of release of the indicator, such release will be shown by a moving front of the indicator material. There will be no change of the colored area formed by the reagent in the case of fixation. Another way of using bibulous carrier strip with the immunological reagent is to place the spot of the reagent on the carrier strip, and to then place a small quantity of the test sample directly on the reagent area after which an end of the strip can be contacted with a standard chromatographic fluid, such as a saline solution, which moves along the carrier by capillary action and gives a visual indication similar to that observed when test sample fluid is used alone.

The cellulosic derivative particles are preferably made by reacting a purified cellulose with a nitrobenzyl chloride in an alkaline medium, and then treating with sodium hydrosulfite in order to form p-aminobenzyl cellulose particles. The p-aminobenzyl groups can then be made reactive to immunological materials by reacting the particles with nitrous acid ($HNO_2$) which can be conveniently formed by using a mixture of sodium nitrite ($NaNO_2$) and a mineral acid such as hydrochloric acid (HCl). These reagents cause the production of diazonium groups, and the process is referred to as diazotization. The diazotized particles can be directly coupled to immunological materials. Likewise, the blocking dye employed in the process of making the reagent as illustrated by Equation 2, above, reacts directly with any of the unreacted aminobenzyl groups whether they are diazotized or not. It is presumed that all of the amino groups are diazotized so that the blocking dye reacts only with diazonium groups.

As mentioned above, when fluorescein is employed as the indicator material it also functions as a blocking dye for any unreacted diazonium groups. Hence, for this indicator material no blocking dye is technically needed, and Equations 2 and 3 above can be combined into a single equation. However, a blocking dye can be used with fluorescein if desired.

It is believed that the release or fixation of the indicator material is due to the formation of secondary valence forces between the Ag, the Ab, and the indicator material. The indicator material is released or its release is inhibited, i.e., it becomes fixed, by the relative strength of the binding forces of the indicator material with respect to the Ag or the Ab. Among these secondary valence forces are Van der Waals forces, coulombic forces, polar forces, hydrogen bonding, and hydrophobic bonding.

## PREFERRED EMBODIMENT OF THE INVENTION

An immunological reagent capable of detecting the antibody to an unfractionated myoglobin sample is made up in the following manner. Particles of p-aminobenzyl cellulose in a fluid suspension are diazotized by reacting them with nitrous acid ($HNO_2$) and the thus diazotized particles are then reacted with a myoglobin containing sample. Fluorescein is used as an indicator material by adding it to the fluid suspension of the treated particles.

The prepared reagent is then separated from the treatment fluid and washed several times, after which a drop of a concentrated suspension of the particles is placed onto a strip of filter paper and allowed to dry in order to provide a dry reagent area. One end of the paper strip is then contacted with an unfractionated antiserum sample which is known to contain myoglobin antibodies. As the sample moves along the filter paper by capillary action the fluorescein indicator is released and is transported with the moving chromatographic fluid front.

This immunological reaction provides a definite visual effect which can be easily observed.

In order to show a control for this reaction another strip of paper is prepared with the dried immunological reagent and is contacted with a serum sample which does not contain the myoglobin antibodies. The sample moves along the paper by capillary action and no dye movement is observed as the fluid front moves beyond the reagent area.

While it is not essential that a control be run with the normal serum samples, it is preferred to run a control so that the realease of the indicator material can be positively correlated with presence of the antibody in the test sample.

The movement of the fluorescein indicator material can be more easily viewed under an ultraviolet light which, while not essential, is preferred.

## EXAMPLE 1

An immunological reagent for detecting the antibody to human myoglobin was prepared and placed onto a strip of filter paper in the form of a suspension and allowed to dry prior to its use for testing an unfractionated rabbit antiserum sample containing the antibody.

One (1) g. of paraminobenzyl cellulose particles was suspended in 25 ml. of 2 N HCl and cooled to 0° C. Five (5) ml. of a 14% solution of sodium nitrite in water was added dropwise to form nitrous acid and the mixture was allowed to stand at 0° C. for one hour. The treated cellulose was then vacuum filtered and washed with 50 ml. of 5% sodium acetate in water, 50 ml. of 5% urea in water and 50 ml. of distilled water in succession. The diazotized cellulose was then suspended in 10 ml. of water containing 178 mg. of previously purified human myoglobin. The resulting suspension was stirred for two (2) hours and then collected by filtration. Thereafter 1 g. of beta-naphthol dissolved in 2 N sodium hydroxide was diluted to 1 liter and adjusted to pH 8. This blocking dye solution was reacted with the diazotized and sensitized immunological reagent and then the resulting particles were filtered off and suspended in 20 ml. of water. Five (5) ml. of this suspension was then reacted with a 1% by weight fluorescein solution in distilled water. The sensitized immunological reagent was collected by suction filtration and then a drop of a concentrated suspension thereof was placed onto a strip of filter paper and dried.

The testing was carried out by contacting an edge of the thus prepared strip with the rabbit anti-serum sample containing antibody to human myoglobin with the result that the fluorescein dye moved with the advancing liquid front. In a control test, wherein another strip of filter paper prepared with the same immunological reagent was contacted with a NRS sample, no movement of the fluorescein dye with the advancing liquid front was observed. The tests were conducted under both ultraviolet light and polychromatic light.

## EXAMPLE 2

An immunological reagent for detecting the antibody to human hemoglobin was prepared in the same manner as for Example 1 by using a solution of 11 mg. of human hemoglobin in 10 ml. of water in place of the myoglobin in that example.

The prepared immunological reagent was placed onto filter paper strips in the same manner as above, and the fluorescein indicator material was observed to move with the advancing chromatographic fluid front when the strips were contacted with a rabbit antiserum fraction containing hemoglobin antibodies. No indicator material was released when a NRS control sample was employed.

## EXAMPLE 3

A reagent was made up to directly detect the myoglobin in an unfractionated serum sample. The reagent was placed onto filter paper strips and tested in the manner above set out.

In making the reagent, diazotized p-aminobenzyl cellulose was prepared as in Example 1 and suspended in 20 ml. of a pH 7 phosphate buffer. To this suspension 4 to 12 ml. of an antiserum to myoglobin were added, after which the mixture was allowed to react for twelve (12) hours. A blocking dye, beta-naphthol, was then used as in Example 1. A 1% water solution of hematoxylin was mixed with 5 ml. of the prepared suspension, and then 2 ml. of a 2% aqueous ferrous sulfate solution was added. The prepared reagent was then collected by filtration and washed.



Upon being dried onto filter paper strips and contacted wth a human urine sample containing myoglobin the hematoxylin dye was observed to move with the advancing with myoglobin-free human urine sample.

## EXAMPLE 4

An immunological reagent was prepared in the same manner as for Example 3 except that for the indicator material a 1% by weight solution of fluorescein in distilled water was employed. A release of the fluorescein dye was observed when the prepared paper strips were contacted with a human urine sample containing myoglobin, and no release of the indicator material was observed when such paper strips were contacted with a myoglobin-free human urine-sample.

## EXAMPLE 5

Example 3 was repeated using an antiserum to HCG, rather than the antiserum to myoglobin, and employing a 1% solution of titan yellow in distilled water as the indicator material. When paper strips prepared with the resulting immunological reagent were contacted with a solution containing HCG, the titan yellow was seen to move with the advancing fluid front, whereas upon contact of such strips with a HCG-free solution, no release of the indicator material was observed.

## EXAMPLE 6

Diazotized p-aminobenzyl cellulose was prepared as in Example 1 and then a solution containing 125 mg. of HCG (2500 I.U./mg.) dissolved in 7 ml. of saline solution which had been previously adjusted to pH 7 were added thereto. The suspension was stirred for one (1) hour at 0° C. and the unreacted diazonium groups were blocked with beta-naphthol, after which the particles were reacted with fluorescein.

When paper strips prepared with this indicator were contacted with the rabbit antibody to HCG the fluorescein indicator material was released and moved with the fluid front. In the case of contact with a NRS sample no release of the fluorescein occurred.

## EXAMPLE 7

An immunological reagent was prepared to detect the presence of human hemoglobin in test solutions or in serum samples. Upon placing the indicator material on a strip of filter paper, drying, and contacting it with a test solution no movement of the indicator material with the advancing fluid front was observed. However, when another identically prepared paper strip was contacted with a saline control solution, advancement of the indicator material with the fluid front was observed. The indication from this observation is that when the human hemoglobin from the serum sample contacts the indicator material, the indicator material becomes fixed and does not move, whereas in the case where there is no human hemoglobin present the dye is free to move with the chromatographic advancing fluid front.

The immunological reagent of this example is prepared by making up a suspension of a diazotized p-aminobenzyl cellulose in the manner set out in Example 1 above. Twenty (20) ml. of the cellulose particles suspended in a pH 7 phosphate buffer were reacted with rabbit antiserum to hemoglobin by contacting the suspension with from 4 to 12 ml. of the antiserum. The unreacted diazonium groups were blocked with beta-naphthol as in Example 1. Five (5) ml. of the cellulose suspension were then mixed with 3 ml. of a 1% water solution of hematoxylin and 2 ml. of a 2% water solution of ferrous sulphate. The thus prepared immunological indicator particles were recovered by vacuum filtration and washed thoroughly with water, after which they were placed onto strips of filter paper as above set out.

Upon contacting the strips with a serum sample containing hemoglobin no advancement of the indicator material with the moving liquid front was observed. However, when the strips were contacted with a hemoglobin-free serum sample movement of the hematoxylin was observed.

## EXAMPLE 8

An immunological indicator was made up following the procedure of Example 7 except that the antiserum to HCG was employed rather than the antiserum to human hemoglobin. Identical results were obtained when the strips prepared therefrom were contacted with a solution containing HCG.

## EXAMPLE 9

An immunogolical reagent was prepared following Example 8 with the substitution of gamma-globulin from a rabbit antiserum to HCG as the sensitizing material. When paper strips prepared therefrom were contacted with a solution containing the rabbit antibody to the gamma-globulin, no movement of the hematoxylin indicator material, was observed. However, when the paper strips were contacted with a NRS control, movement of the indicator material with the chromatographic fluid was observed.

## EXAMPLE 10

Following Example 7, an immunological indicator was made employing the specific gamma-globulin fraction from mule serum containing antibodies to HCG in the place of the antiserum to hemoglobin of that example.

In this instance beta-naphthol was additionally employed in order to assure that all unreacted diazonium groups were blocked. Known pregnancy urine was used as a test fluid.

The immunological reagent prepared was placed on a filter paper strip, dried and then contacted with the test fluid. No release of the hematoxylin indicator material was observed. However, when the reagents were tested against known non-pregnancy urine samples, release of the indicator material and movement with the advancement of the chromatographic fluid front was observed.



What is claimed is:

**1.** An immunological reagent comprising paraaminobenzylcellulose particles covalently bound to a substance selected from the group consisting of human myoglobin, human hemoglobin, human chorionic gonadotropin and modified gamma globulin fraction of an antiserum to which is physically adsorbed thereon a dye selected from the group consisting of fluorescein, hematoxylin and titan yellow.

**2.** A reagent as in claim **1** comprising paraaminobenzylcellulose particles covalently bound to human myoglobin on which fluorescein is physically adsorbed.

**3.** A reagent as in claim **1** comprising paraaminobenzylcellulose particles covalently bound to human hemoglobin on which fluorescein is physically adsorbed.

**4.** A reagent as in claim **1** comprising paraaminobenzylcellulose particles covalently bound to human myoglobin on which hematoxylin is physically adsorbed.

**5.** A reagent as in claim **1** comprising paraaminobenzylcellulose particles covalently bound to human chorionic gonadotropin on which titan yellow is physically adsorbed.

**6.** A reagent as in claim **1** comprising paraaminobenzylcellulose particles covalently bound to the gamma globulin fraction of rabbit antiserum on which hematoxylin is physically adsorbed.

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# UNITED STATES PATENT OFFICE
# CERTIFICATE OF CORRECTION

Patent No. 3,641,235 Dated Feb. 8, 1972

Inventor(s) Margaret Rozman Weiss

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

| | |
|---|---|
| Column 1 - line 38 | Ninth word should read -- these -- rather than "The" |
| Column 2 - line 36 | First word should read --fluorescein-- rather than "flourescent" |
| Column 2 - line 48 | Immediately following the first word insert -- ) -- |
| Column 2 - line 62 | Second word should read -- leutinizing -- rather than "leutininzing" |
| Column 3 - line 18 | Insert the word -- which -- between the words "material" and "is" |
| Column 4 - line 13 | Insert the word -- powdered -- between the words "purified" and "cellulose" |
| Column 6 - line 5 | First word should read -- with -- rather than "wth" |
| Column 6 - line 7 | Between the words "ing" and "with" insert -- fluid front. No release was observed in the case of contact -- |

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# CERTIFICATE OF CORRECTION

Patent No. 3,641,235 Dated February 8, 1972

Inventor(s) Margaret Rozman Weiss

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

| | |
|---|---|
| Column 6, line 7 | Also, between the words "with" and "myoglobin-free" insert the word -- a -- |

Signed and sealed this 19th day of March 1974.

(SEAL)
Attest:

EDWARD M.FLETCHER,JR.
Attesting Officer

C. MARSHALL DANN
Commissioner of Patents

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