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## [54] DOUBLE ANTIBODY FOR ENHANCED SENSITIVITY IN IMMUNOASSAY


[75] Inventors: **Robert F. Zuk**, San Francisco; **Ian Gibbons**, Menlo Park; **Gerald L. Rowley**, Cupertino; **Edwin F. Ullman**, Atherton, all of Calif.

[73] Assignee: **Syva Company**, Palo Alto, Calif.



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[58] **Field of Search** ..... 424/8, 12; 23/230 B; 435/5, 7, 184, 188, 810



[56] **References Cited**

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*Primary Examiner*—Thomas G. Wiseman
*Attorney, Agent, or Firm*—Bertram I. Rowland



[57] **ABSTRACT**

Method and compositions are provided for performing homogeneous immunoassays. The method involves having a signal producing system, which provides a detectable signal, which system includes a macromolecular member. The determination of the analyte, which is a member of a specific binding pair consisting of a ligand and its homologous receptor, is performed by creating an extensive matrix in the assay medium by having in the assay medium in addition to the analyte, ligand labeled with one of the members of the signal producing system, antiligand either present as the analyte or added, a polyvalent receptor for antiligand, the macromolecular member of the signal producing system, and any additional members of the signal producing system. The labeled ligand, antiligand, and polyvalent receptor for the antiligand create a matrix which modulates, e.g. inhibits, the approach of the macromolecular member of the signal producing system to the labeled ligand. The extent and degree of formation of the matrix is dependent upon the concentration of the analyte in the medium. By comparing the signal from an assay medium having an unknown amount of analyte, with a signal obtained from an assay medium having a known amount of analyte, the amount of analyte in the unknown sample may be determined qualitatively or quantitatively.

Kits are provided having predetermined amounts of the various reagents to allow for enhanced sensitivity of the method.


**17 Claims, No Drawings**

# DOUBLE ANTIBODY FOR ENHANCED SENSITIVITY IN IMMUNOASSAY

## BACKGROUND OF THE INVENTION

1. Field of the Invention

There is a continuing and increasing need for accurate, sensitive techniques for measuring trace amounts of organic materials in a wide variety of samples. This need includes the measurement of drugs, naturally occurring physiologically active compounds or nutrients in physiological fluids, the presence of trace amounts of contaminants or toxic materials in foods, water or other fluids, and the like, as well as monitoring materials for trace contamination introduced during chemical processing.

Among the various techniques which have found increasing exploration are techniques involving receptors which recognize or bind to a specific polar and spatial organization of one or more molecules. For the most part, the receptors are antibodies and the techniques are referred to as immunoassays. These techniques conventionally employ a labeled ligand where the binding to the receptor allows for distinguishing between a bound labeled ligand and an unbound labeled ligand. Certain techniques, generally referred to as heterogeneous, rely on segregating the bound from the unbound labeled ligand. Other techniques, generally referred to as homogeneous, rely on the bound labeled ligand providing a signal level different from unbound labeled ligand.

In many of the homogeneous techniques, the label must interact with another substance in order to differentiate the signal. For example, in one technique, the label is an enzyme and when receptor is bound to the ligand the enzymatic activity is inhibited. This requires that the enzyme ligand combination be such that when receptor is bound to the enzyme ligand conjugate, either substrate is inhibited from entering the active site or the enzyme is allosterically modified, so that its turnover rate is substantially reduced. In another technique, a fluorescent label is employed in conjunction with a receptor for the fluorescer. The binding of the receptor to the fluorescer substantially diminishes the fluorescence when the fluorescer is irradiated with light which normally excites the fluorescer. When the fluorescer is conjugated to ligand, and antiligand is bound to the conjugate, the antifluorescer is inhibited from binding to the fluorescer.

While these techniques have found great use or show great promise, there is still an interest in enhancing the sensitivity of techniques which do not require separation. As lower and lower concentrations of analytes are encountered, improvements in available techniques are required to allow for accurate determination of the presence of extremely small amounts of the analyte. Therefore, there has been an ongoing effort to find new and improved ways to measure extremely small amounts of organic molecules in a wide variety of environments.

2. Description of the Prior Art

U.S. Pat. No. 3,817,837 describes a homogeneous enzyme immunoassay. U.S. Pat. No. 3,996,345 describes a homogeneous immunoassay employing two chromophores related by being a fluorescer and a quencher. Co-pending application Ser. No. 893,650, filed Apr. 5, 1978 describes a technique employing a plurality of enzymes, where the substrate of one enzyme is the product of the other enzyme. U.S. Pat. No. 3,935,074 describes an immunoassay involving steric hindrance between two antibodies. Co-pending application Ser. No. 815,487, describes an enzyme immunoassay, employing antienzyme as an inhibitor.

## SUMMARY OF THE INVENTION

Method and compositions are provided for enhancing the sensitivity of immunoassays requiring the proximity of two reagents for modulating a signal related to the amount of analyte in the assay medium. The subject method employs a second receptor, which is polyvalent, and binds to a receptor for ligand to affect the degree of interaction between the two reagents, particularly inhibiting interaction. The role of the second receptor is manifold depending upon the nature of the two reagents involved in signal production. The second receptor is employed in forming or extending matrices of ligand and ligand receptor which results in modulation of entry or exit from the matrix. Depending upon the role of the second receptor, different protocols will be employed.

A kit is provided having predetermined amounts of the various reagents, as well as ancillary reagents for optimizing the sensitivity of the immunoassay.

## DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Method and composition are provided for determining small amounts of organic compounds in a wide variety of media by employing an organic receptor which recognizes a specific spatial and polar organization of a molecule, either the organic compound or its receptor being the analyte of interest. In the subject method, a signal producing system is employed having at least two members which provides a detectible signal which may be modulated in accordance with the concentration of the analyte in the assay medium. The modulation of the signal is as a result of two members of the signal producing system being brought into proximity, which results in either an enhancement or reduction in the signal level. Two receptors are involved in the assay: the first receptor, which is added when the organic compound or ligand is the analyte, or is inherently present when the first receptor is the analyte; and a second receptor, which is polyvalent and specifically binds to the first receptor. The presence of the second receptor enhances the differentiation in signal level as a result of the degree of proximity of the two reagents of the signal producing system.

In the broadest sense in performing the assay, the analyte, which is a member of a specific binding pair—ligand and its homologous receptor—is introduced into an assay medium in combination with: (1) antiligand, when ligand is the analyte; (2) ligand conjugated to a label, where the label is a member of a signal producing system; (3) a second member of the signal producing system which interacts with the first member to modulate the signal depending on the proximity of the second member of the signal producing system to the first member of the signal producing system; (4) a polyvalent receptor for the antiligand; (5) and any ancillary reagents necessary for the signal producing system. Depending upon the particular signal producing system, various protocols will be employed.

By modulation is intended to create, destroy, modify, affect or change the signal, so as to allow a detectable

difference by virtue of the interaction of the label and macromolecular reagent.

## Definitions

Analyte—the compound or composition to be measured, which may be a ligand, which is mono- or polyepitopic (antigenic determinants) or haptenic, a single or plurality of compounds which share at least one common epitopic site, or a receptor capable of binding to a specific polar or spatial organization.

Specific binding pair—two different molecules, where one of the molecules has an area on the surface or in a cavity which specifically binds to a particular spatial and polar organization of the other molecule. The members of the specific binding pair are referred to as ligand and receptor (antiligand).

Ligand—any organic compound for which a receptor naturally exists or can be prepared.

Receptor—any compound or composition capable of recognizing a particular spatial and polar organization of a molecule i.e. epitopic site. Illustrative receptors include naturally occurring receptors, e.g. thyroxine binding globulin, antibodies, enzymes, Fab fragments, lectins, and the like.

In the subject invention, a polyvalent receptor, normally an antibody, for the ligand receptor anti(antiligand) will be employed. The anti(antiligand) is prepared by employing the antiligand as an immunogen in a vertebrate different from the source of the antiligand.

Signal Producing System—the signal producing system will have at least two components, at least one component being conjugated to ligand and another component which will be a macromolecular reagent. The signal producing system produces a measurable signal which is detectible by external means, usually the measurement of electromagnetic radiation. For the most part, the signal producing system will involve enzymes, antibodies, and chromophores, where chromophores include dyes which absorb light in the ultraviolet or visible region, fluorescers, phosphors and chemiluminescers. While for the most part, the signal is conveniently the absorption or emission of electromagnetic radiation, usually in the ultraviolet or visible range, electrochemical changes, thermal changes, nephelometric, and the like may also find application. The subject signal producing system requires that the macromolecular reagent interact with or react with, directly or indirectly, the member of the signal producing system (label) bound to the ligand, resulting in the production, enhancement or diminution of the signal observed for the label.

Macromolecular reagent—the macromolecular reagent is a member of the signal producing system, which reacts or interacts with, directly or indirectly, the member of the signal producing system bound to the ligand, so as to modulate the signal produced by the label. The macromolecular reagent will be at least $1\times10^4$, usually at least $5\times10^4$ molecular weight, and preferably greater than $1\times10^5$ molecular weight, and may be 10 million molecular weight or more. The macromolecular reagent may be a single compound, a group of compounds, covalently or noncovalently linked together, or one or a plurality, usually a plurality, of molecules bound to a hub nucleus. The hub nucleus may be water soluble or insoluble, and is a polyfunctional material, normally polymeric, having a plurality of functional groups, such as hydroxy, amino, mercapto, ethylenic, etc., as sites for linking. The hub nucleus will generally have a molecular weight equal to or greater than 10,000, usually 50,000 molecular weight. Illustrative hub nucleii include polysaccharides, polypeptides, including proteins, nucleic acids, ion exchange resins, vinyl polymers, such as polyacrylamides and vinyl alcohols, polyethers, polyesters, and the like. The significant factor of the macromolecular reagent is that its approach to the label bonded to the ligand will be affected, when a large immunological matrix is formed by polyvalent receptors about the labeled ligand associated with antiligand.

Ligand-receptor matrix—the matrix is a matrix formed from a plurality of ligands, ligand receptors, and antireceptors, where at least the antireceptors are polyvalent, and normally all the members of the matrix are polyvalent. What is intended is that a plurality of ligands, which are bound to ligand receptors, are connected by a plurality of bridges formed by antireceptors, which results in relatively large reticulated microstructures, capable of modulating, usually reducing, the rate of diffusion of a molecule in the environment of the matrix up to and including steric exclusion.

Labeled Ligand—the conjugate of the ligand member of the specific binding pair covalently bonded to a member of the signal producing system, either joined by a bond, linking group or hub nucleus. The labeled ligand may have one or more ligands or one or more labels or a plurality of both. Where conjugation of the label and ligand are required, the ligand will normally be modified to provide for a site for linking. The modified ligand is referred to as ligand analog, and the ligand analog may differ from the ligand by replacement of a hydrogen or more usually by the introduction of or modification of a functional group.

Poly(ligand analog)—a plurality of haptenic ligand analogs bonded to a water soluble hub nucleus of at least about 10,000 molecular weight, usually 30,000 to 600,000, e.g. proteins, polysaccharides, nucleic acids, synthetic polymers, etc.

## Method

The subject assay is carried out in an aqueous zone at a moderate pH, generally close to optimum assay sensitivity, without separation of the assay components or products. The assay zone for the determination of analyte is prepared by employing an appropriate aqueous medium, normally buffered, the unknown sample which may have been subject to prior treatment, the labeled ligand, ligand receptor (antiligand), the macromolecular reagent member of the signal producing system, and antireceptor as well as any additional materials required for the signal producing system for producing a detectible signal. In the event that antiligand is the analyte, antiligand need not be added.

The presence of ligand or its homologous receptor (antiligand) in the unknown will affect the extent to which the macromolecular reagent interacts with the labeled ligand.

In carrying out the assay, an aqueous medium will normally be employed. Other polar solvents may also be included, usually oxygenated organic solvents from one to six, more usually from one to four carbon atoms, including alcohols, ethers and the like. Usually these cosolvents will be present in less than about 40 weight percent, more usually in less than about 20 weight percent.

The pH for the medium will usually be in the range of about 4 to 11, more usually in the range of about five to ten, and preferably in the range of about 6.5–9.5. The

pH is chosen so as to maintain a significant level of specific binding by the receptor while optimizing signal producing efficiency. In some instances, a compromise will be made between these two considerations. Various buffers may be used to achieve the desired pH and maintain the pH during the determination. Illustrative buffers include borate, phosphate, carbonate, tris, barbital, and the like. The particular buffer employed is not critical to this invention, but in individual assays one buffer may be preferred over another.

Moderate temperatures are normally employed for carrying out the assay and usually constant temperatures during the period of the measurement, particularly for rate determinations. The temperatures for the determination will generally range from about 10° to 50° C., more usually from about 15° to 40° C.

The concentration of analyte which may be assayed will generally vary from about $10^{-4}$ to $10^{-15}$M, more usually from about $10^{-6}$ to $10^{-13}$M. Considerations such as whether the assay is qualitative, semiquantitative or quantitative, the particular detection technique and the concentration of the analyte of interest will normally determine the concentration of the other reagents.

While the concentrations of the various reagents in the assay medium will generally be determined by the concentration range of interest of the analyte, the final concentration of each of the reagents will normally be determined empirically to optimize the sensitivity of the assay over the range of interest. The total binding sites of the members of the specific binding pair which are reciprocal to the analyte will be not less than about 0.1 times the minimum concentration of interest based on binding sites of analyte and usually not more than about 1,000 times the maximum concentration of interest based on analyte binding sites, usually about 0.1 to 100 times, more usually about 0.3 to 10 times the maximum concentration of interest. By concentration is intended the available concentration, that is, the concentration at saturation and not necessarily the actual concentration where all members of the specific binding pair may not be equally available for binding.

Depending upon the particular signal producing system, as well as the manner in which the specific binding pair members are employed, the amount of the various members of the signal producing system can be varied relatively widely. That is, relatively large excesses of the macromolecular reagent may be employed, where either the rate of formation of the matrix is high as compared to the interaction between the label and the macromolecular reagent or the matrix is allowed to form initially, followed by introduction of the macromolecular reagent. As suggested above, the order of addition of the various reagents may vary widely, depending on the particular label and signal producing system, the nature of the analyte, the relative concentrations of analyte and reagents, the mode of determination, and the sensitivity desired.

When ligand is the analyte, the addition of ligand will normally diminish complex formation involving labeled ligand, so that the interaction between the labeled ligand and the macromolecular reagent will be enhanced with increasing amounts of ligand being present in the assay medium. By contrast, when ligand receptor is the analyte, there can be no complex formation in the absence of ligand.

For ligand analyte, conveniently the ligand may be combined with antiligand, so that available binding sites of the antiligand become filled by the available ligand. To the extent that ligand is present, the amount of antiligand available for binding labeled ligand will be reduced. Alternative ways would be to combine both the ligand and labeled ligand simultaneously with the antiligand, but one would normally not combine the labeled ligand with the antiligand prior to addition of the ligand analyte. This is due to the fact that with many receptors, the association of the specific binding pair members is almost irreversible during the time period of an assay.

While for the most part, ligand analytes will be antigens, which have a plurality of determinant (epitopic) sites, haptenic (mono-epitopic) compounds can also be employed by having a plurality of haptens conjugated to a large label (often greater than 10,000, usually greater than 30,000 molecular weight) or a plurality of haptens and labels bonded to a water soluble hub nucleus.

One or more incubation steps may be involved in preparing the assay medium. For example, it will usually be desirable to incubate an antigen analyte with the antiligand. In addition, it may be desirable to have a second incubation after addition of the antireceptor. Whether to employ an incubation period and the length of the incubation period will depend to a substantial degree on the mode of determination—rate or equilibrium—and the rate of binding of the receptors to their homologous members. Usually, incubation steps will vary from about 0.5 min to 6 hrs, more usually from about 5 min to 1 hr. Incubation temperatures will generally range from about 4° to 50° C., more usually from about 15° to 37° C.

After the reagents are combined the signal will then be determined. The method of determination may be the observation of electromagnetic radiation, particularly ultraviolet and visible light, either absorption or emission, calorimetric, electrochemical, nephelometric, or the like. Desirably, the signal will be read as electromagnetic radiation in the ultraviolet or visible region, particularly from about 250 to 750 nm, usually from about 350 to 650 nm.

The temperature at which the signal is observed will generally range from about 10° to 50° C., more usually from about 15° to 40° C.

Standard assay media can be prepared which have known amounts of the analyte. The observed signal for the standard assay media may then be plotted, so as to relate concentration to signal level. Once a standard curve has been established, a signal level may be directly related to the concentration of the analyte.

The time for measuring the signal will vary depending on whether a rate or equilibrium mode is used, the sensitivity required, the nature of the signal producing system and the like. For rate modes, the times between readings will generally range from about 5 sec to 6 hrs, usually about 10 sec to 1 hr. For the equilibrium mode, after a steady state is achieved, a single reading may be sufficient or two readings over any convenient time interval may suffice.

For the most part, the labels bound to the ligand will be catalysts, particularly enzymes or oxidation-reduction catalysts, chromogens, which absorb or emit light in the ultraviolet or visible region, such as dyes, fluorescers, chemiluminescers, donor-acceptor fluorescer combinations, phosphorescers, or enzyme binding substances e.g. chromogenic substrates, cofactors and inhibitors, and the like.

## Materials

The components employed in the assay will be the labeled ligand, antiligand when ligand is the analyte, macromolecular reagent, and receptor for the ligand receptor, anti(antiligand), as well as any additional members of the signal producing system.

## Analyte

The ligand analytes of this invention are characterized by being monoepitopic or polyepitopic. The polyepitopic ligand analytes will normally be poly(amino acids) i.e. polypeptides and proteins, polysaccharides, nucleic acids, and combinations thereof. Such combinations of assemblages include bacteria, viruses, chromosomes, genes, mitochondria, nuclei, cell membranes, and the like.

For the most part, the polyepitopic ligand analytes employed in the subject invention will have a molecular weight of at least about 5,000, more usually at least about 10,000. In the poly(amino acid) category, the poly(amino acids) of interest will generally be from about 5,000 to 5,000,000 molecular weight, more usually from about 20,000 to 1,000,000 molecular weight; among the hormones of interest, the molecular weights will usually range from about 5,000 to 60,000 molecular weight.

The wide variety of proteins may be considered as to the family of proteins having similar structural features, proteins having particular biological functions, proteins related to specific microorganisms, particularly disease causing microorganisms, etc.

The following are classes of proteins related by structure:

protamines
histones
albumins
globulins
scleroproteins
phosphoproteins
mucoproteins
chromoproteins
lipoproteins
nucleoproteins
glycoproteins
proteoglycans
unclassified proteins, e.g. somatotropin, prolactin, insulin, pepsin

A number of proteins found in the human plasma are important clinically and include:

Prealbumin
Albumin
$\alpha_1$-Lipoprotein
$\alpha_1$-Acid glycoprotein
$\alpha_1$-Antitrypsin
$\alpha_1$-Glycoprotein
Transcortin
4.6S-Postalbumin
Tryptophan-poor
$\alpha_1$-glycoprotein
$\alpha_1\chi$-Glycoprotein
Thyroxin-binding globulin
Inter-$\alpha$-trypsin-inhibitor
Gc-globulin
(Gc 1-1)
(Gc 2-1)
(Gc2-4 2)
Haptoglobin
(Hp 1-1)
(Hp 2-1)
(Hp 2-4 2)
Ceruloplasmin
Cholinesterase
$\alpha_2$-Lipoprotein(s)
Myoglobin
C-Reactive Protein
$\alpha_2$-Macroglobulin
$\alpha_2$-HS-glycoprotein
Zn-$\alpha_2$-glycoprotein
$\alpha_2$-Neuramino-glycoprotein
Erythropoietin
$\beta$-lipoprotein
Transferrin
Hemopexin
Fibrinogen
Plasminogen
$\beta_2$-glycoprotein I
$\beta_2$-glycoprotein II
Immunoglobulin G
(IgG) or $\gamma$G-globulin
Mol. formula:
$\gamma_2\kappa_2$ or $\gamma_2\lambda_2$
Immunoglobulin A (IgA) or $\gamma$A-globulin
Mol. formula:
$(\alpha_2\kappa_2)^n$ or $(\alpha_2\lambda_2)^n$
Immunoglobulin M
(IgM) or $\gamma$M-globulin
Mol. formula:
$(\mu_2\kappa_2)^5$ or $(\mu_2\lambda_2)^5$
Immunoglobulin D(IgD) or $\gamma$D-Globulin ($\gamma$D)
Mol. formula:
$(\delta_2\kappa_2)$ or $\delta_2\lambda_2)$
Immunoglobulin E (IgE) or $\gamma$E-Globulin ($\gamma$E)
Mol. formula:
$(\epsilon_2\kappa_2)$ or $(\epsilon_2\lambda_2)$
Free $\kappa$ and $\lambda$ light chains
Complement factors:
C'1
C'1q
C'1r
C'1s
C'2
C'3
$\beta_1$A $\alpha_2$D
C'4
C'5
C'6
C'7
C'8
C'9

Important blood clotting factors include:

BLOOD CLOTTING FACTORS

| International designation | Name |
|---|---|
| I | Fibrinogen |
| II | Prothrombin |
| IIa | Thrombin |
| III | Tissue thromboplastin |
| V and VI | Proaccelerin, accelerator globulin |
| VII | Proconvertin |
| VIII | Antihemophilic globulin (AHG) |
| IX | Christmas factor, plasma thromboplastin component (PTC) |
| X | Stuart-Prower factor, autoprothrombin III |

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