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[54] **MACROMOLECULAR ENVIRONMENT CONTROL IN SPECIFIC RECEPTOR ASSAYS**

[75] Inventors: **David J. Litman**, Palo Alto; **Zvi Harel**, Stanford; **Edwin F. Ullman**, Atherton, all of Calif.

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

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[58] **Field of Search** .... 23/230 B; 424/1, 15, 424/8, 12; 435/5, 7, 810, 177, 178

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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 protein binding assays involving a homologous pair consisting of ligand and receptor for the ligand. The method employs a label conjugated to a member of said homologous pair and a uniformly dispersed discontinuous phase of discrete particles in a continuous aqueous phase, where the discrete particles create microenvironments which allow for discrimination between the label associated with the particle—in a discontinuous phase—and the label in the continuous phase.

Various conjugates and particles are provided which find use in the subject method.

46 Claims, No Drawings

Mylan v. Genentech

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MACROMOLECULAR ENVIRONMENT CONTROL IN SPECIFIC RECEPTOR ASSAYS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The measurement of trace amounts of a wide variety of organic compounds has become essential in medicine, ecology, quality control, and the like. One class of methods commonly referred to as immunoassays is dependent upon the use of a compound or receptor which specifically binds to another compound having a particular spatial and polar organization. The compound and its receptor form a homologous pair, referred to as ligand and receptor, where the receptor is normally antibody. One of the members of the homologous pair is bound to a label which is capable of providing a detectible signal.

The category of immunoassays may be further broken down into what is referred to as heterogeneous and homogeneous. The heterogeneous techniques are dependent upon separating associations or complexes of the homologous pair from members of the pair which are not associated. Since the complexes will substantially differ in molecular weight from the dissociated members, techniques such as centrifugation can be used to separate the associated from the dissociated members. One can then measure the label either in the phase containing the dissociated members or the phase containing the associated members. For the most part the labels which have found use in the heterogeneous methods are radiolabels, enzymes, and fluorescent molecules.

An alternative to physical separation is to bind one of the members of the homologous pair to a solid support, which may or may not absorb the aqueous medium. The solid support can then provide for the separation since the complexed or associated ligand and receptor is bound to the solid support. This allows for relatively easy separation between the aqueous assay medium and the solid support.

The homogeneous methods rely on the formation of complexes to modulate the signal obtained from the label. The dissociated conjugated label provides for a different level of signal from the associated conjugated label with its receptor. For example, where the ligand is conjugated to a stable free radical, the association of the conjugate with its homologous receptor results in a substantial flattening of the esr peaks. With enzymes as labels to which ligands have been conjugated, the binding of receptor to the ligands can result in steric inhibition of the approach of substrate to the active site of the enzyme or allosteric modification of enzyme activity. The presence of ligand in the assay medium reduces the amount of available receptor for binding to the label conjugate and thus affects the amount of the label conjugate which becomes associated with receptor. Therefore, by measurement of the signal from the label, one can relate the level of signal to the amount of ligand in the assay medium.

An alternative to employing the receptor to directly affect the signal by its bulk is the opportunity to bring together two labels which interact. Where a ligand is polyepitopic or a polyepitopic ligand is formed from monoepitopic ligands, the opportunity exists to allow for receptors which are labeled differently to be brought together when bound to the ligand or to have ligand with one label and receptor with a different label, which when the ligand and receptor are associated

bring the labels into close spatial proximity. Where the different labels interact to affect the amount of signal observed, the associated ligand and receptor will provide for a different signal level from the dissociated labeled receptor.

This technique has been employed with chromophores which are related by one of the chromophores fluorescing at a wavelength of an energy which is accepted by the other chromophore, which acts as a quencher. Also, by employing two different enzymes, where the product of one enzyme is the substrate of the other enzyme, one can observe an enhanced turnover in the complex, as compared to the dissociated label.

The focus of effort in the homogeneous immunoassay area has been directed to either employ the properties of the complex to modulate the signal or to provide for the complex to bring together in close spatial proximity different labels which are related and provide for different degrees of interaction in relation to their distance from each other.

In developing immunoassays, there are many considerations, not the least of which is sensitivity. For measuring extremely small amounts of a ligand, it is either necessary to have a label which is detected at very low levels with high accuracy or to provide for a plurality of events associated with an individual ligand. Another consideration is interference by the foreign materials present and the degree to which the interference can be minimized or removed.

Another problem associated with immunoassays is labeling, particularly where the ligand or receptor is impure. The background resulting from conjugation of the label to compounds other than those of the homologous pair must be maintained at a minimum in order to obtain a satisfactorily sensitive assay. Other considerations include simplicity of protocol, ease of measurement, reproducibility, sensitivity to extraneous factors and the like.

2. Description of the Prior Art

Engasser and Horvath, Applied Biochem. Bioengineering, Vol. 1, 127 (1976) Academic Press, report the kinetic and diffusion effects on the immobilization of enzymes. 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. Copending application Ser. No. 893,650, filed Apr. 5, 1978, now U.S. Pat. No. 4,233,402, describes a technique employing a plurality of enzymes, where the substrate of one enzyme is the product of the other enzyme. Copending application Ser. No. 815,636, U.S. Pat. No. 4,160,645, filed July 14, 1977, **now U.S. Pat. No.** 4,160,645, describes a homogeneous immunoassay employing a non-enzymatic catalyst as a label. Co-pending application Ser. No. 906,514, now U.S. Pat. No. 4,193,983, filed May 16, 1978, describes a labeled liquid discontinuous phase for use in immunoassays. Application Ser. No. 667,996, abandoned, filed Mar. 18, 1976, describes a homogeneous immunoassay employing as a label an enzyme substrate. See also U.S. Pat. No. 3,853,987, which discloses particles to which are conjugated radioactive and fluorescent labels and antibodies. See also U.S. Pat. No. 4,001,400.

SUMMARY OF THE INVENTION

Methods and compositions are provided for the determination of an analyte which is a member of a spe-

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cific binding pair—ligand and homologous receptor—-where no separation or segregation is required for the determination. The method does not rely on a bulk effect where one observes the sum of the signal from the labels of associated members, but rather relies on an enhancement or diminution of the signal as a result of association. The method employs a substantially uniformly dispersed discontinuous phase of discrete solid (includes solvent swelled) particles (beads) in an aqueous assay medium. The particles are labeled with one of the members of the specific binding pair.

The particles create a physical or chemical environment distinctively different from the continuous aqueous phase. A signal producing system is provided which produces a substantially different level of detectible signal depending upon whether the signal producing system operates in the solid or liquid aqueous phase. By causing the distribution between the solid and liquid phase of the signal producing system to be related to the amount of analyte in the assay medium, the observed signal will be a function of the amount of analyte in the assay medium.

Conjugates to particles are provided for use in the method, as well as reagent compositions and kits. Also, specific compounds are provided as special substrates.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

A method is provided for determining low concentrations of organic compounds in a wide variety of media, particularly having physiological activity, either being naturally present in physiological fluids, or administered to vertebrates. The method employs as an assay medium a continuous liquid aqueous phase and a discontinous solid phase comprised of discrete small particles having relatively slow settling rates and being capable of providing an environment different from the environment of the continuous phase.

The particles are large discrete solid beads providing an environment for a label which may be distinguished from the environment of the bulk solution, preferably porous, providing channels or surface indentations of substantial depth where the liquid environment in the channel or indentation is significantly affected by the substantially encompassing solid phase. A signal producing system is provided, where the signal producing system, in whole or in part, is partitioned between the two phases in relation to the amount of analyte present in the assay medium. Since the observed signal will be substantially different depending upon the degree to which the signal producing system is partitioned between the liquid and the solid phase, the measured signal will reflect the amount of analyte in the assay medium.

The analyte will be a member of a specific binding pair consisting of the ligand and its homologous receptor. The solid phase particles or beads will be bound, directly or indirectly, covalently or non-covalently to one of the members of the specific binding pair. There is an exception where a specific type of receptor to a specific ligand is the analyte, three specific binding components are required, viz receptor, antireceptor or ligand, which may be bound to the particle, and ligand or antireceptor respectively, employed for other labeling. Thus receptor as an analyte allows for a number of alternative conjugates. In addition, one of the members of the signal producing system will be bound or become bound to the reciprocal member of the specific binding pair. By appropriate choice of specific binding pair conjugates, the amount of signal producing member bound to the particle can be related to the amount of analyte in the assay medium.

In carrying out the method, one combines the analyte containing sample, the labeled particles, the labeled specific binding pair member, as well as any additional reagents and determines the signal from the assay medium. By comparing the observed signal with a signal obtained from an assay medium having a known amount of analyte, one can qualitatively or quantitatively determine the analyte of interest. One can use the properties of the discrete particles in a number of different ways. Arbitrarily will be divided into two categories: (1) diffusion; and (2) physical effects;

By appropriate choice of porous particles, one can affect the rate at which a molecule or molecular assembly moves through the volume of the liquid phase adjacent to the solid particle surface. The effect of the steric bulk and narrow channels of the particles is to reduce the rate of migration of a molecule or molecular assembly toward and away from the particle surfaces, as compared to the rate of migration in the bulk solution, by virtue of physical constraint, and the like. Thus, one can create a substantial concentration gradient between the bulk liquid aqueous phase and the liquid portion adjacent the solid phase surface. A signal producing system which is sensitive to the concentration of a species will give substantially different signal levels in the bulk liquid phase as compared to the solid phase.

By having two members of the signal producing system which cooperate, that is, one member provides a compound which interacts with the second member, one can greatly enhance the localized concentration of the compound in the solid phase as compared to the bulk liquid phase. In these situations, the particle would not only be labeled with a member of the specific binding pair, but also a member of the signal producing system.

The second effect is a physical effect as a result of the chemical nature of the particle. The physical effect can be observed as pH, spectroscopic properties, and the like. In effect, the environment created by the particle surfaces, particularly in the channels or pores, for a molecule is substantially different from the environment in the bulk solution. Where the signal producing member is sensitive to its environment, there will be a substantially different signal depending upon whether the signal producing member is in the solid phase or in the bulk solution. For example, the activity of an enzyme is pH dependent. By appropriate choice of buffer and an ion exchange resin, the pH at the surface of the solid phase can be distinctively different from the pH in the bulk solution. The enzymatic activity would therefore vary depending upon the partitioning of the enzyme between the two phases.

The polarity between the particle and the bulk solution can be greatly varied by employing a hydrophobic particle. The hydrophobic character could activate or deactivate an enzyme or chromogen e.g. fluorescer.

The spectroscopic effect can be exemplified by employing opaque, transparent or partially transparent (within a limited wavelength range) particles. One could therefore control the light that entered or exited from the particle. Alternatively, phosphorescent labeled (includes embedded) particles could be employed or particles having labels capable of energy transfer to a chromogen.

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In performing the subject method, there will be at least two reagents: the particle conjugate; and the specific binding pair member conjugate. These conjugates will vary depending upon the nature of the analyte, the nature of the signal producing system, and the nature of the particle. In addition, by covalently bonding molecules, particularly enzymes to the particle, one can create a concentration gradient, where the bulk solution has a relatively low concentration of the particular compound or enzyme product. These molecules can be part of the signal producing system or merely provide an environment which affects the signal producing system.

DEFINITIONS

Analyte—the compound or composition to be measured, which may be a ligand, which is mono- or polyepitopic, antigenic or haptenic, a single or plurality of compounds which share at least one common epitopic site or a receptor.

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 (antiligand)—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.

Ligand Analog—a modified ligand which can compete with the analogous ligand for a receptor, the modification providing means to join a ligand analog to another molecule. The ligand analog will normally differ from the ligand by more than replacement of a hydrogen with a bond which links the ligand analog to a hub or label.

Poly(ligand-analog)—a plurality of ligands or ligand analogs joined together covalently, normally to a hub nucleus. The hub nucleus is a polyfunctional material, normally polymeric, usually having a plurality of functional groups e.g. hydroxy, amino, mercapto, ethylenic, etc. as sites for linking. The hub nucleus may be water soluble or insoluble, preferably water soluble, and will normally be at least about 35,000 molecular weight and may be 10 million or more molecular weight, but usually under 600,000, more usually under 300,000. Illustrative hub nucleii include polysaccharides, polypeptides, including proteins, nucleic acids, ion exchange resins and the like. Water insoluble hub nucleii can be the same as those indicated for the particle.

Particle (solid phase)—the particle is a discrete solid particle, which may be swelled or remain unswelled by the liquid phase, and composed of a wide variety of both hydrophobic and hydrophilic materials. The particles will be solid, hollow or porous, having a substantially smooth or irregular surface, having a primarily concave or convex surface, preferably being porous and having channels or indentations, which can be widely varied as to the size of molecule or assembly which is excluded, defining an environment different from the medium in which the particles are dispersed. The particles will be readily dispersible in an aqueous medium, and either polyfunctionalized or capable of polyfunctionalization for linking to other molecules. Depending on the signal producing system, the particles may be substantially transparent to light in a substantial wavelength range between 300 and 800 nm, preferably through the range or be opaque over the entire ultraviolet and visible range.

Signal producing system—the signal producing system may have one or more components, at least one component being conjugated to a specific binding pair member. The signal producing system produces a measurable signal which is detectible by external means, usually the measurement of electromagnetic radiation, and depending on the system employed, the level of signal will vary to the extent the signal producing system is in the environment of the solid phase particles. For the most part, the signal producing system will involve enzymes and chromophores, where chromophores include dyes which absorb light in the ultraviolet or visible region, phosphors, fluorescers, 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 changes, and the like may also find application.

Label—the label may be any molecule conjugated to another molecule and is arbitrarily chosen as to which molecule is the label. In the subject invention, the labels will be the specific binding pair molecule that is conjugated to the particle or a molecule which is part of the signal producing system that is conjugated to a member of the specific binding pair or to a particle.

Particle conjugate—the particle to which is bound, directly or indirectly a member of the specific binding pair, and, as appropriate one or more members of the signal producing system. A substantial proportion of the labels conjugated to the particle will be influenced by the particle surface, usually within the channels and pores of the particle when these are present, so that where the signal producing member is bound to the particle, there is a property of the conjugate which differentiates the signal obtained from the particle as compared to the signal obtained from the bulk solution.

Binding pair label—a member of the specific binding pair employed for binding its homologous member to the particle directly bonded to the particle.

Signal label—a member of the signal producing system which is directly or indirectly (through the binding of a specific binding pair) bonded to a binding pair member or to the particle.

Binding pair member conjugate or signal label conjugate—the conjugate of the binding pair member with a member of the signal producing system (signal label).

Labeled ligand—the conjugate of the ligand member of the specific binding pair with a member of the signal producing system, either covalently or noncovalently bound, when covalently joined, either joined by a bond, linking group, or hub nucleus. The labeled ligand may have one or more ligands (includes ligand analogs) or one or more labels or a plurality of both, the latter being referred to as poly(ligand analog)-polylabel.

Labeled receptor—the conjugate of receptor with a member of the signal producing system, where the two are bound either covalently or non-covalently, usually covalently by a linking group, where there may be one or more receptors bound to the label, but usually one or more labels bound to the receptor.

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Macromolecular reagent—a reagent capable of interacting with a member of the signal producing system to modulate the signal and at least in part sterically excluded from interacting with a member of the signal producing system in the environment of the particle conjugate through steric constraints or reduced rates of diffusion. The reagent will usually have a minimum molecular weight of at least about 20,000, more usually at least about 40,000 and preferably at least about 100,000. The reagent may naturally have such molecular weight or the active compound linked to a hub nucleus to provide the desired molecular weight.

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 particle conjugate, the binding pair member conjugate, all of the materials required for the signal producing system for producing a detectible signal, as well as members of the specific binding pair or their analogs, as required.

The presence of ligand or its homologous receptor (antiligand) in the unknown will affect the partition of the signal producing system between the particle or solid phase and the bulk solution in the assay medium.

In carrying out the assay, an aqueous medium will normally be employed. Other polar solvents may also be included, usually oxygenated organic solvents of from 1-6, more usually from 1-4 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 4-11, more usually in the range of about 5-10, 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 particle 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°-50° C., more usually from about 15°-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, semi-qualitative 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 and 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 0.3-10 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 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 conjugates can be varied quite widely. For example, one could have very large excesses of the binding pair label in the particle conjugate, by first allowing the binding pair member conjugate to react with the unknown, followed by combining with the particle conjugate. Where a competition mode was employed, in that the particle conjugate and the binding pair member conjugate are added to the unknown simultaneously, large excesses of the binding pair label might reduce the sensitivity of the assay. Therefore, as indicated previously, by employing various concentrations of the various reagents with analyte at concentrations in the range of interest, one would obtain ratios which would optimize the assay response.

The order of addition of the various reagents may vary widely, depending upon the particular labels, the compound to which the label is conjugated, the nature of the conjugates, the nature of the analyte, and the relative concentrations of the analyte and reagents. Also affecting the order of addition is whether an equilibrium mode or rate mode is employed in the determination.

Since with many receptors, the association of the specific binding pair members is almost irreversible during the time period of the assay, one will normally avoid combining the particle conjugate with the signal label conjugate, prior to the addition of the analyte, where the two conjugates are reciprocal members of the specific binding pair. By contrast, where the two conjugates have the same member of the specific binding pair, one could combine them prior to introduction of the unknown sample into the assay medium. Regardless of the nature of the analyte, all the reagents can be added simultaneously and either a rate or equilibrium determination made.

One or more incubation steps may be involved in preparing the assay medium. For example, it may be desirable to incubate an antigen analyte with labeled receptor. In addition, it may be desirable to have a second incubation after addition of the particle conjugate. 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 receptor to the ligand. 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, particu-

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