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(54) Titre : ELEMENT ET PROCEDE ANALYTIQUE DE DETERMINATION D'UN LIGAND SPECIFIQUE DE LIAISON
UTILISANT LA BROMOPEROXYDASE DE VANADIUM COMME ENZYME GENERATRICE DE SIGNAUX
(54) Title: ANALYTICAL ELEMENT AND METHOD FOR THE DETERMINATION OF A SPECIFIC BINDING LIGAND
USING A VANADIUM BROMOPEROXIDASE AS A SIGNAL-GENERATING ENZYME

(57) **Abrégé/Abstract:**

An analytical element can be used to sensitively and rapidly detect a wide variety of specific binding ligands in either a competitive or sandwich assay format. The assays are carried out using a vanadium bromoperoxidase-labeled immunoreactant and a chemiluminescent signal-providing wash composition which comprises a 2,3-dihydro- 1,4-phthalazinedione derivative; a halogen, pseudohalogen, halogen-providing source or pseudohalogen-providing source; and a peroxide or a peroxide-generating reagent composition.



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(57) Abstract

An analytical element can be used to sensitively and rapidly detect a wide variety of specific binding ligands in either a competitive or sandwich assay format. The assays are carried out using a vanadium bromoperoxidase-labeled immunoreactant and a chemiluminescent signal-providing wash composition which comprises a 2,3-dihydro-1,4-phthalazinedione derivative; a halogen, pseudohalogen, halogen-providing source or pseudohalogen-providing source; and a peroxide or a peroxide-generating reagent composition.

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ANALYTICAL ELEMENT AND METHOD FOR THE DETERMINATION
OF A SPECIFIC BINDING LIGAND USING A VANADIUM
BROMOPEROXIDASE AS A SIGNAL-GENERATING ENZYME

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Field of the Invention

The present invention relates to analytical elements and methods for using the same to detect specific binding ligands in fluid samples. More particularly, the present invention relates to the use of a vanadium bromoperoxidase as a signal-generating enzyme in such analytical elements.

10 Background of the Invention

There is a continuing need in medical practice and research, and in analytical and diagnostic procedures for rapid and accurate determinations of chemical and biological substances which are present in various fluids, such as biological fluids. For example, the presence of proteins, hormones, drugs, viruses, microorganisms, narcotics and steroids must be determined rapidly and accurately for effective research, diagnosis and treatment.

20 A wide variety of analytical methods have been developed in recent decades to detect the noted substances. The methods have become highly reliable and in some instances, suitable for automation, as well as suitable for use in kit form. Most of such methods rely on what are known in the art as "specific binding reactions" between a substance to be detected (identified herein as a "specific binding ligand" or "ligand") and a corresponding "receptor" which recognizes and reacts with the ligand specifically.

30 Most known specific binding reactions are between immunoreactants (in "immunoassays"), such as antibodies

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with antigens or antibodies with haptens, but others are
1 also known such as avidin with biotin.

In general, immunoassays can provide a
qualitative and/or quantitative determination of the
presence or absence (or quantity) of a specific antigen,
5 antibody or antigen-antibody complex. In one form of
immunoassay, known as a "competitive binding
immunoassay", a labeled analog of the ligand to be
determined is placed in competition with a fixed amount
of an appropriate antibody which can react with both the
10 ligand and the ligand analog. The label on the analog
can be appropriately detected in its "free" or complexed
(that is, reacted) form. Such detection will then tell
the user how much ligand is in the sample being tested.

In an alternative immunoassay format known as
15 a "sandwich" immunoassay or immunometric assay, the
ligand is contacted with two or more receptor molecules
which bind to the ligand at different epitopic sites.
One receptor is appropriately labeled and the other is
either immobilized on a solid substrate, or is capable
20 of being immobilized thereon. The amount of ligand is
directly proportional to the amount of bound complex
among the ligand and the two receptors.

Immunoassays have been traditionally carried
out in solution, or in test devices where fluids are
25 removed in some fashion from the reagents participating
in the assay. Although solution techniques have enjoyed
broad acceptance in this area, they typically require
analyzer equipment often having intricate solution
handling and transport capabilities. Moreover, the
30 analytical equipment used in such assays can involve
complex liquid handling, and may require skilled

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1 personnel, both for operation and the precise cleaning
that may be needed to avoid sample to sample
contamination.

5 An alternative to solution chemistry is the
use of dry analytical elements. It should be understood
that not all aqueous solutions can be employed in dry
analytical elements because of interference from coating
agents, binders, and other reagents necessary to
maintain structural integrity in said elements. Also, a
multiplicity of scientific disciplines are often
10 required in successful element construction. Moreover,
dry analytical elements must use compartmentalization to
segregate incompatible components; such is not the case
in solution chemistry where separate liquid storage and
successive liquid additions can be employed.

15 Dry analytical elements and their use for
immunoassays are described in numerous publications,
including U.S. Patent No. 4,258,001 to Pierce et al.,
U.S. Patent No. 4,670,381 to Frickey et al., WO 82/2601
(published August 5, 1982), European Patent Application
20 No. 051 183 (published May 12, 1982) and European Patent
Application No. 066 648 (published December 15, 1982).

Improved dry analytical elements and their use
in immunoassays are described in
coassigned U.S. Patent 5,958,339, September 28,
25 1999 to Belly et al. in which enzyme labels
are utilized for detection. Horseradish peroxidase is
the preferred enzyme label disclosed in the application
of Belly et al. Such elements allow for the detection
of analytes present in very low concentrations using a
30 particular washing technique to separate unbound
reactants from bound (or complexed) immunoreactants.

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In the immunoassays carried out in the dry
1 analytical elements using peroxidase as the label, the
stability of the peroxidase is highly important since
any change in its concentration critically affects assay
sensitivity. In the assays described in U.S. Patent No.
5 5,372,932 to Friedman et al., 4'-hydroxy or 4'-
alkoxyarylacetamides are used as agents to enhance the
stability of the enzyme or enzyme label in a dry
analytical element. Although these agents have resulted
in improved stability, it has been observed that the
10 stability of the peroxidase label is still less than
desirable in dry analytical elements.

Recently, Butler and Walker have described a
family of vanadium bromoperoxidases (VBrPO) extracted
from aquatic and marine algae and some from terrestrial
15 lichens and fungi that, in the presence of hydrogen
peroxide and bromide anion, catalytically produce
activated bromine species which are potent oxidants.
(See, Butler et al., Chem. Revs., 93, pp. 1937-1944,
1993 and references cited therein).

20 One of the problems with most haloperoxidases
is that their pH optima are generally in the lower pH
range, i.e., 3-5. This presents a problem with prior
art analytical elements which depend upon a peroxy
anion; peroxides typically have a pKa of about 11.5.
25 The vanadium bromoperoxidases work effectively in a pH
range of about 6-10.

Although the analytical elements and methods
disclosed in the prior art show enhanced assay
sensitivity and stability, there is still a need for
30 further improvements in this field. For example, there
is still a need for providing a simpler analytical

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1 element which does not require the use of stabilizing
agents as described in U.S. Patent No. 5,372,932 to
Friedman et al. Furthermore, there still is a need to
provide an element which has improved enzyme stability
and more sensitivity than the known prior art systems.

5

Summary of the Invention

The problems identified above in regard to
prior art dry analytical elements and immunoassays have
been overcome by the present invention by utilizing an
10 analytical element which comprises, in at least one of
its zones, a vanadium bromoperoxidase-labeled
immunoreactant.

More specifically, the present invention
relates to an analytical element useful for the
15 determination of a specific binding ligand comprising:

- (i) a porous spreading zone,
- (ii) one or more additional zones which are in
fluid contact with the porous spreading zone, and
- (iii) optionally, an absorbent material in
20 fluid contact with the above zones,

wherein said element, in at least one of the
zones, contains a vanadium bromoperoxidase-labeled
immunoreactant which is capable of specifically reacting
with either a specific binding ligand of interest or its
25 receptor.

The present invention also provides a method
for the determination of a specific binding ligand using
the above immunoassay analytical element and comprising:

- (A) contacting a fluid sample suspected of
30 containing the specific binding ligand with said
analytical element;

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(B) contacting said analytical element with a
1 wash composition comprising:

(a) a chemiluminescent signal-generating
reagent which provides a signal in response to the
catalytic activity of a vanadium bromoperoxidase, said
5 signal-generating reagent is a 2,3-dihydro-1,4-
phthalazinedione derivative;

(b) a halogen, pseudohalogen, halogen-
providing source or pseudohalogen-providing source; and

(c) a peroxide or peroxide-generating
10 reagent composition; and

(C) detecting either the unreacted or reacted
form of the vanadium bromoperoxidase-labeled
immunoreactant as a measure of the specific binding
ligand of interest.

15 The present invention provides a highly
sensitive and stable composition for use in dry
analytical elements. The element of the instant
invention is useful for determining a wide variety of
specific binding ligands, but it is particularly useful
20 for the determination of ligands at low concentrations.
The advantages of enzyme stability and enhanced
detection are achieved by incorporating a vanadium
bromoperoxidase-labeled immunoreactant into the element.

25 Brief Description of the Drawings

Fig. 1 is a graphical representation of the
chemiluminescent signal obtained using an element of the
present invention which contains a vanadium
bromoperoxidase signal-generating enzyme as one of its
30 components and a control element which contains
horseradish peroxidase (HRP) as the signal-generating

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enzyme, and 3'-chloro-4'-hydroxyacetanilide as an
1 enhancer contained in the wash composition as described
in Example 1 of U.S. Patent No. 5,372,931 to Friedman et
al.

Fig. 2 is a graphical representation of the
5 signal-to-noise response obtained using an element of
the present invention which contains a vanadium
bromoperoxidase signal-generating enzyme as one of its
components and a control element which contains
horseradish peroxidase (HRP) as the signal-generating
10 enzyme, and 3'-chloro-4'-hydroxyacetanilide as an
enhancer contained in the wash composition as described
in Example 1 of U.S. Patent No. 5,372,931 to Friedman
et al.

Fig. 3 is a graphical representation of the
15 stability of a spotted enzyme assay using an element of
the present invention which contains a vanadium
bromoperoxidase signal-generating enzyme and a control
element which contains horseradish peroxidase as the
signal-generating enzyme.

20 Figs. 4(a) and (b) are bar graphs depicting
the percent of chemiluminescent signal remaining after
room temperature storage at various times using an
element of the present invention which contains a
vanadium bromoperoxidase signal-generating enzyme and a
25 control element which contains horseradish peroxidase
(HRP) as the signal-generating enzyme.

Detailed Description of the Invention

The method of the present invention is a
30 specific binding assay, such as an immunoassay, which
can be either competitive binding or immunometric as

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those terms are known in the art. The analyte of
1 interest to be determined is a specific binding ligand,
a labeled ligand, a labeled ligand analog, or a receptor
thereof.

The present invention is used advantageously
5 to determine low concentrations of ligands in various
aqueous liquids, such as human and animal biological
fluids, foods, industrial or municipal effluents, and
other fluids commonly tested in this manner. Biological
fluids which can be tested include, but are not limited
10 to, whole blood, serum, plasma, urine, spinal fluid,
lacrimal fluid, synovial fluid, lymphatic fluid,
suspensions of tissues or plaque, gingival fluid,
vaginal fluid, cranial fluid and other fluids readily
apparent to one skilled in the art.

Ligands which can be determined include, but
15 are not limited to, peptides, polypeptides, proteins
(such as enzymes, antibodies, lipoproteins and
glycoproteins), drugs, narcotics, steroids, toxins and
saccharides (such as polysaccharides). Specific binding
20 ligands of particular interest include digoxin,
phenytoin, phenobarbital, theophylline, C-reactive
protein, human chorionic gonadotropin, thyroid
stimulating hormone, a thyronine derivative (such as
thyroxine and triiodothyronine), creatine kinase-MB,
25 carbamazepine, gentamicin, tobramycin or vancomycin.
The present invention is most useful in the
determination of digoxin, phenytoin, phenobarbital and
C-reactive protein.

The present invention is carried out using an
30 analytical element comprising a porous spreading zone,
usually a coated layer which has suitable porosity for

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accommodating a test sample (for example 1 to 50 μ l),
1 diluted or undiluted. The element of the present
invention is assembled using techniques that are well
known in the art. Preferably, the porous spreading zone
is isotropically porous, which property is provided by
5 interconnected spaces among the particles, fibers or
other physical components of the zone. By isotropically
porous is meant that fluids are uniformly spread
throughout the zone. Useful materials for such zones
are water-insoluble and maintain their structural
10 integrity during the assay. Conventional materials and
means for assembling the element are described, for
example, in U.S. Patent No. 3,992,158 to Przybylowicz et
al., U.S. Patent No. 4,258,001 to Pierce et al., U.S.
Patent No. 4,292,272 to Kitazima et al. and U.S. Patent
15 No. 4,430,436 to Koyama et al.

The preferred
porous spreading zones are prepared from organopolymeric
particles and a polymeric adhesive in a coherent, three-
dimensional structure, as described in U.S. Patent No.
20 4,258,001 to Pierce et al.

There are one or more additional zones in the
element, all of which are in fluid contact with the
porous spreading zone. It should be understood that the
term "fluid contact" is used herein to denote that
25 fluids can readily move from one zone to another. Such
additional zones, preferably coated polymer layers,
include subbing, reagent, and radiation blocking zones
and are composed of one or more hydrophilic binder
materials as are known in the art, such as gelatin,
30 acrylamide polymers and vinylpyrrolidone polymers. Some

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1 zones may be water-insoluble while others may be water-soluble.

5 The zones of the element of the present invention can be self-supporting, but preferably, these zones are disposed on a suitable dimensionally stable, chemically inert support. Preferably, the support is nonporous and transparent to electromagnetic radiation. A support of choice should be compatible with the intended mode of detection (for example, transmission or reflectance spectroscopy). Useful support materials include, but are not limited to, paper, metal foils, polystyrenes, polyesters, polycarbonates and cellulose esters.

15 Optionally, the element comprises an absorbent material in fluid contact with the zones of the element of the instant invention. Absorbent materials as used in this invention provide overall added liquid capacity to the element providing a reservoir for the wash fluid used in the method of this invention. Suitable absorbent materials comprise, but are not limited to, glass microfibers, papers, sponges, fabrics, plastics and the like, so long as the material is capable of absorbing a liquid.

20 In a preferred embodiment, an annular section of the absorbent material is placed on the spreading zone permitting access of the spreading zone to sample and wash.

25 In at least one of the zones of the element of this invention is a vanadium bromoperoxidase-labeled immunoreactant which is capable of specifically reacting with either the specific binding ligand of interest or its receptor. In competitive binding immunoassays, the

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1 labeled immunoreactant is usually a labeled analog of
the specific binding ligand. In sandwich assays, the
labeled immunoreactant can be a labeled receptor for the
ligand, or it can be a labeled molecule which
specifically binds to the receptor (such as a labeled
5 anti-antibody).

Such labeled immunoreactant can be prepared
using any of a number of known procedures including that
described by Yoshitake et al. Eur. J. Biochem., 101,
395, 1979 and in U.S. Patent No. 5,106,732 to Kondo et
10 al.

As used herein, "vanadium bromoperoxidase" is
meant to be any vanadium haloperoxidative substance
(enzymatic or otherwise) which catalyzes the oxidation
of a substance such as luminol to produce an appropriate
15 chemiluminescent signal. Specific examples of
proteinaceous vanadium bromoperoxidase sources that may
be employed in the present invention can be found in
Butler et al., Chem. Revs., 93, pp. 1937-1944, (1993)
and include, but are not limited to, those obtained from
20 *Ascophyllum nodosum*, *Ceramirum rubrum*, *Laminaria*
saccharina, *Fucus distichus*, *Corallina pilulifera*,
Corallina officinalis, *Macrocystis pyrifera* and the
like. Procedures for isolation of vanadium
bromoperoxidases may be found within the aforementioned
Butler et al. reference. Of these proteinaceous
25 vanadium bromoperoxidase sources, *Ascophyllum nodosum* is
particularly preferred.

As stated hereinabove, by incorporating a
vanadium bromoperoxidase into the dry analytic element,
30 the element of the instant invention quite unexpectedly
exhibits improved enzyme stability and enhanced

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1 detection without the need of adding enhancers, such as
described in U.S. Patent No. 5,372,931 to Friedman et
al., into the element.

5 The amount of vanadium-bromoperoxidase-labeled
immunoreactant can vary widely due to the amount of the
other components used in the reaction and the suspected
amount of analyte in the test sample. Generally, the
amount present in the element is at least about 0.01
 $\mu\text{g}/\text{m}^2$, with the preferred range being from about 0.1 to
about 100 $\mu\text{g}/\text{m}^2$.

10 The element of the present invention,
optionally, may contain an unlabeled immunoreactant
which is capable of specifically reacting with either
the specific binding ligand of interest or its receptor.
In competitive binding immunoassays, this immunoreactant
15 is generally a receptor (such as an antibody) to the
ligand. In sandwich immunoassays, the unlabeled
immunoreactant can be a receptor for the ligand, or a
binding molecule for the receptor. In preferred
embodiment, the immunoreactant is an antibody specific
20 to the ligand.

When present, the unlabeled immunoreactant is
located in any zone of the element, except it is
generally not located in the same zone as the vanadium
bromoperoxidase-labeled immunoreactant. Thus, those two
25 components of the element are kept separated in some
fashion until the assay has begun. These components may
be separated by locating them in different zones of the
element, or they may be in the same zone, but one is
encapsulated with a material that releases the
30 immunoreactant when the assay is begun. Preferably, the
immunoreactants are kept in separate zones or layers.

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It is also preferred that the unlabeled
1 immunoreactants are immobilized on suitable particles
that are dispersed throughout a zone of the element.
Such particles can be composed of any suitable material
including, but not limited to, glass, iron oxides,
5 ceramics, organic synthetic or naturally occurring
polymers, and have an average particle size of from
about 0.01 to about 10 μm . Preferably, the particles
are prepared from synthetic polymers and have suitable
surface groups for adsorption or covalent attachment of
10 the immunoreactant molecules. A wide variety of such
materials are known in the art such as those described
in U.S. Patent No. 4,828,978 to Warren et al., U.S.
Patent No. 4,997,772 to Sutton et al., U.S. Patent No.
5,147,777 to Sutton et al. or U.S. Patent No. 5,177,023
15 to Sutton et al., and references identified therein.

Particularly useful polymeric particles are
those prepared from ethylenically unsaturated
polymerizable monomers having reactive carboxy, 2-
substituted ethylsulfonyl or vinylsulfonyl groups,
20 particularly as described in the Sutton et al. patents
referenced in the preceding paragraph.

In a preferred embodiment of this invention, a
multilayer analytical element for the determination of a
specific binding ligand is provided. Specifically, the
25 multilayer element comprises a nonporous support having
thereon, in fluid contact:

a first reagent or buffer layer,
a subbing layer comprising an unlabeled
immunoreactant capable of reacting with either a
30 specific binding ligand of interest or its receptor,

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a porous spreading layer containing uniformly
1 distributed, a vanadium bromoperoxidase-labeled
immunoreactant which is capable of reacting with the
specific binding ligand of interest or its receptor.

The multilayer element of the present
5 invention may optionally contain an absorbent material
which is in fluid contact with one of the aforementioned
zones.

In a more preferred embodiment, the vanadium
bromoperoxidase-labeled immunoreactant is localized in
10 the top portion of the porous spreading layer by using
manufacturing techniques such as, but not limited to,
ink jet deposition, spray coat deposition, and gravure
deposition as is well known to those skilled in the art.

The elements of this invention can include a
15 variety of additives in appropriate zones as are known
in the art to aid in manufacture, fluid spreading,
absorbance of unwanted radiation and receptor stability.

The element of the present invention can be
prepared using conventional coating procedures and
20 equipment as are described in the prior art (including
gravure, curtain, hopper and other coating techniques).
The elements can be configured in a variety of forms,
including elongated tapes of any desired width, sheets,
slides or chips. Further, the method of this invention
25 can be manual or automated using appropriate analytical
equipment and procedures. Generally, the method
includes contacting the reagents in the element by
spotting a test sample (for example, 1 to 50 μ l) on the
porous spreading zone. The movement of fluid within the
30 element effectively mixes the reagents for the reactions
to take place.

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After sample application, the element is
1 exposed to any conditioning, such as incubation, heating
or other procedure, that may be desirable to quicken or
otherwise facilitate forming the appropriate specific
binding complexes in the various zones of the element.
5 While in some instances, a suitable signal can be
obtained without effective separation of the reacted and
unreacted forms of the vanadium bromoperoxidase-labeled
immunoreactant, it is preferred that the forms be
separated within a zone of the element, as is typical in
10 what are known as heterogeneous immunoassays. Thus, a
signal can be better read in a defined region of the
zone.

Applying a wash solution (from about 5 to
about 200 μ l) to the element is the preferred procedure
15 for effecting this separation. The wash solution can be
applied in any suitable manner known in the art, but
preferably, it is applied at a continuous metered rate,
for example of up to 10 μ l/sec and most preferably, at
about 1 μ l/sec. However, any rate and method of wash
20 solution application can be used as long as the porous
spreading zone readily absorbs the fluid during
application as is readily provided by using the
aforementioned absorbent material in the element.

The wash solution can comprise multiple fluid
25 compositions applied sequentially, but at least one of
the fluids must comprise the signal-generating
composition as described hereinbelow. Alternatively, a
surfactant can also be used in any of the wash
solutions.

30 The wash solution employed in the method of
the present invention is an analytical composition for

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providing a chemiluminescent signal having a pH of from
1 about 6.5 to about 10, said composition comprising:

- (a) a chemiluminescent signal-generating reagent which provides a signal in response to the catalytic activity of a vanadium bromoperoxidase, wherein the signal-generating reagent is a 2,3-dihydro-1,4-phthalazinedione derivative;
- (b) a halogen, pseudohalogen, halogen-providing source or pseudohalogen-providing source; and
- (c) a peroxide or peroxide-generating reagent
10 composition.

As indicated above, the signal-providing composition employed in the instant invention contains, as one of its components, a 2,3-dihydro-1,4-phthalazinedione derivative (identified herein as
15 "DPD"). Any free or conjugated DPD that can be converted to an excited state in a chemiluminescent reaction and subsequently returns to a non-excited state with the emission of light, is useful in the practice of this invention. A considerable number of such compounds
20 are known in the art, including those described in U.S. Patent No. 4,598,044 to Kricka et al. and in Grundermann et al., Chemiluminescence in Organic Chemistry, Springer-Verlag, Berlin, 1987, pages 204-207.

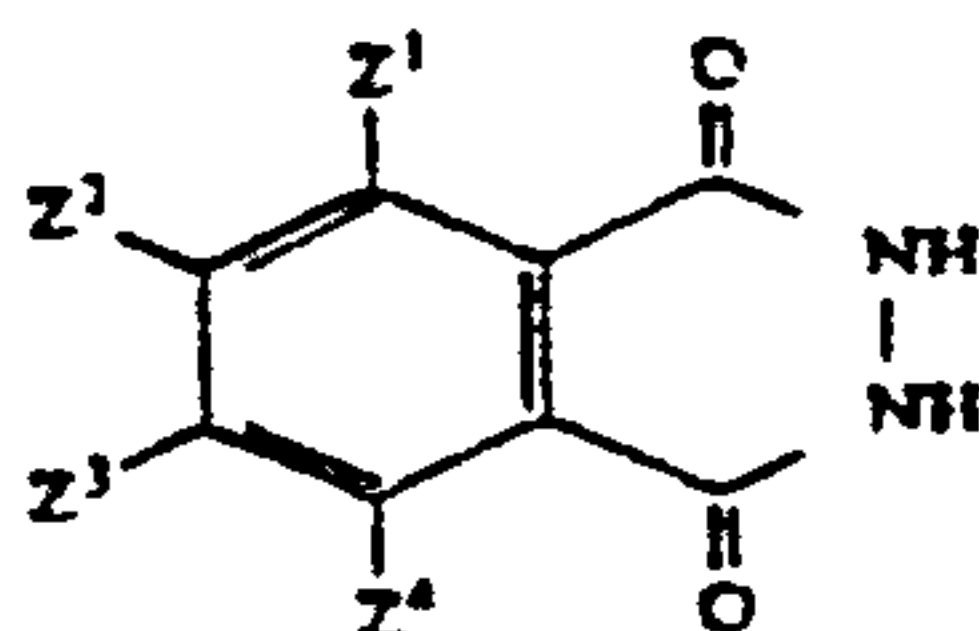
Such compounds are generally known as
25 "luminol-type hydrazides" and include phthalic hydrazides, naphthalene-1,2-dicarboxylic acid hydrazides, anthracene-2,3-dicarboxylic acid hydrazides, phenanthrene-1,2-dicarboxylic acid hydrazides, fluorene-1,2-dicarboxylic acid hydrazides, benzo[g,h,i]-perylene-1,2-dicarboxylic acid hydrazides, coronene-1,2-
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dicarboxylic acid hydrazides, and others readily
 1 apparent to one skilled in the art.

In particular, the DPD's that can be employed
 in the instant invention are defined by the following
 structural formula:

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wherein Z^1 , Z^2 , Z^3 , and Z^4 are independently hydrogen; an
 alkyl containing from about 1 to about 6 carbon atoms
 10 such as methyl, ethyl, isopropyl, t-butyl, sec-pentyl
 and hexyl; an alkenyl containing from about 2 to about 6
 carbon atoms such as ethenyl, 1-propenyl, isobutenyl, 2-
 (N,N-diisopropylamino)vinyl, 2-(N,N-
 diisobutylamino)vinyl, 2-(N,N-diisopentylamino)vinyl and
 15 2-hexenyl; a hydroxy; an alkoxy containing from about 1
 to about 6 carbon atoms such as methoxy, ethoxy,
 isopropoxy, t-butoxy and hexoxy; a carboxy; an amino
 including amino substituted with alkyl or alkanoyl, such
 as methylamino, ethylamino; amido (for example,
 20 acetamido and hexanamido); dimethylamino, diethylamino
 and diisobutylamino; a conjugated aminoalkenyl (for
 example, as defined below); or an aminoaryl including
 substituted aminoaryl, such as p-(N,N-
 dimethylamino)phenyl, p-(N,N-diethylamino)phenyl and 5-
 25 amino-2,3-dihydro-1,4-phthalazinedione-8-yl (also known
 as luminy).

At least one of Z^1 and Z^2 is an amino
 (including substituted amino, as defined above),
 conjugated aminoalkenyl (including substituted
 30 aminoalkenyl as described above) or an aminoaryl such as
 p-(N,N-dimethylamino)-phenyl, p-(N,N-diethylamino)phenyl

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and 5-amino-2,3-dihydro-1,4-phthalazinedione-8-yl. As
1 used herein, "conjugated aminoalkenyl" refers to a
monovalent group capable of electron resonance from the
amino group through the alkenyl group to the aromatic
ring of the phthalazinedione where it is substituted,
5 and includes for example, a dialkylaminovinyl group such
as 2-(N,N-diisopropylamino)vinyl, 2-(N,N-
diisobutylamino)vinyl and 2-(N,N-diisopentylamino)-
vinyl; and dialkylaminobutadienyl groups, such as 4-
(N,N-diethylamino)-1,3-butadiene-1-yl.

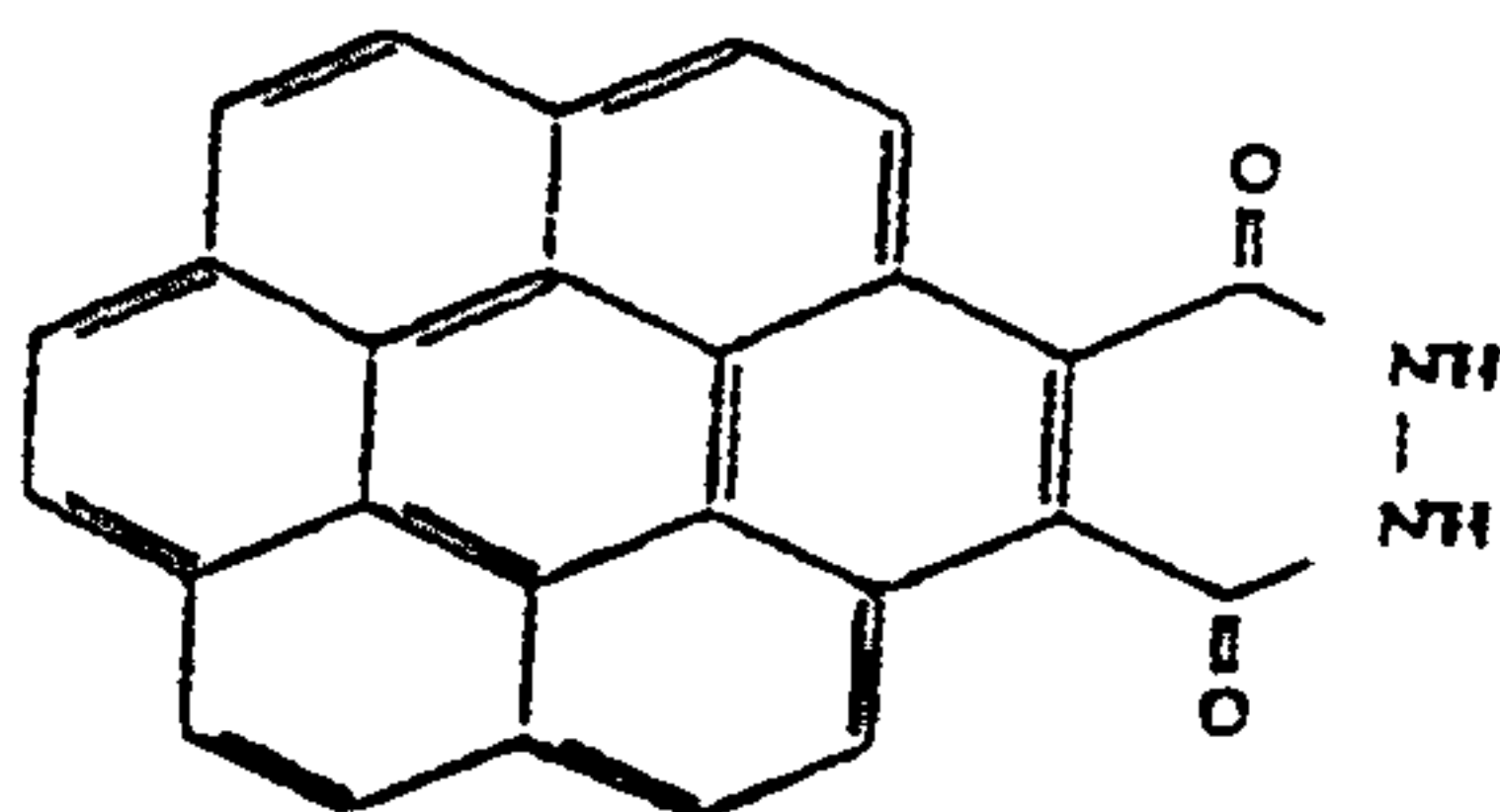
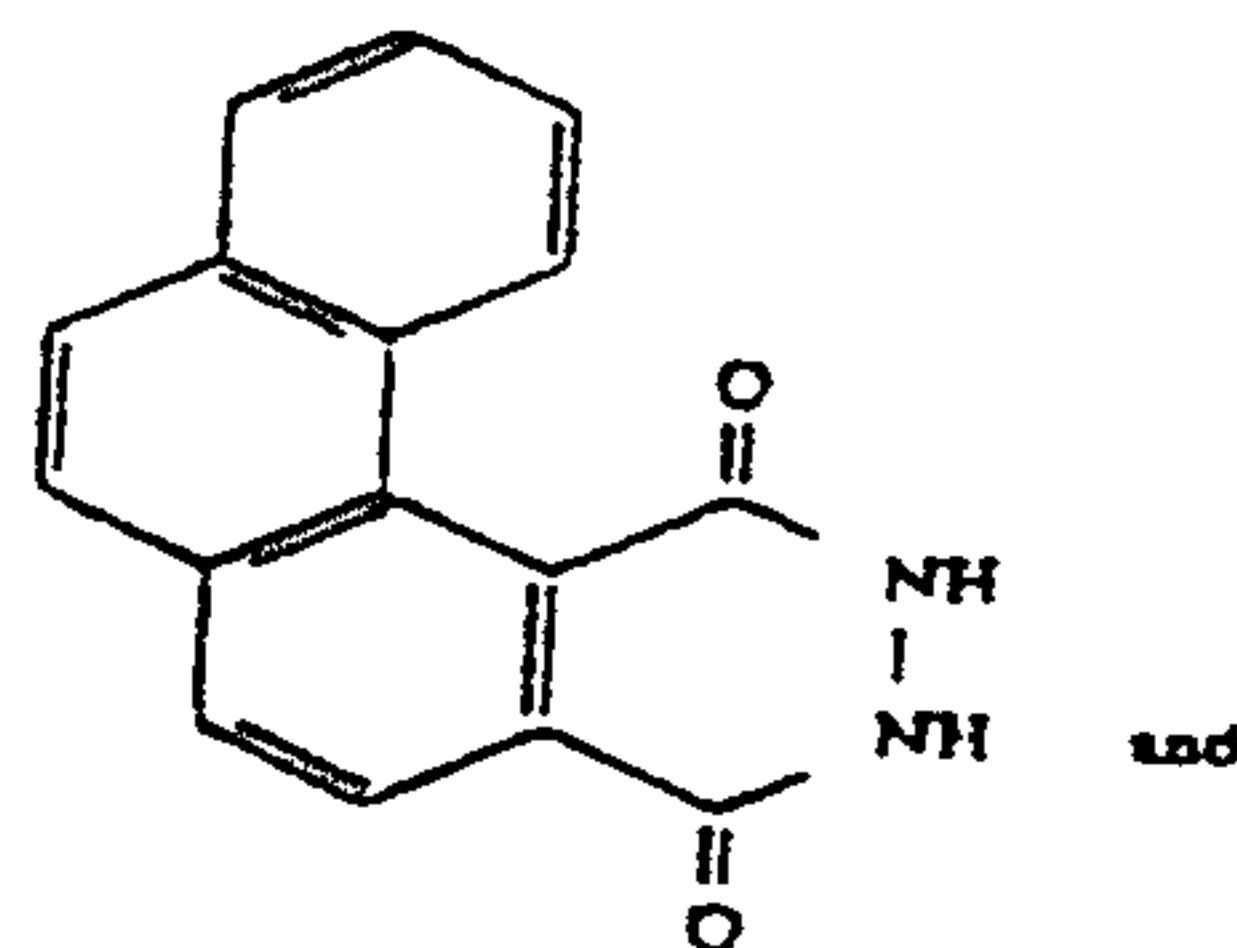
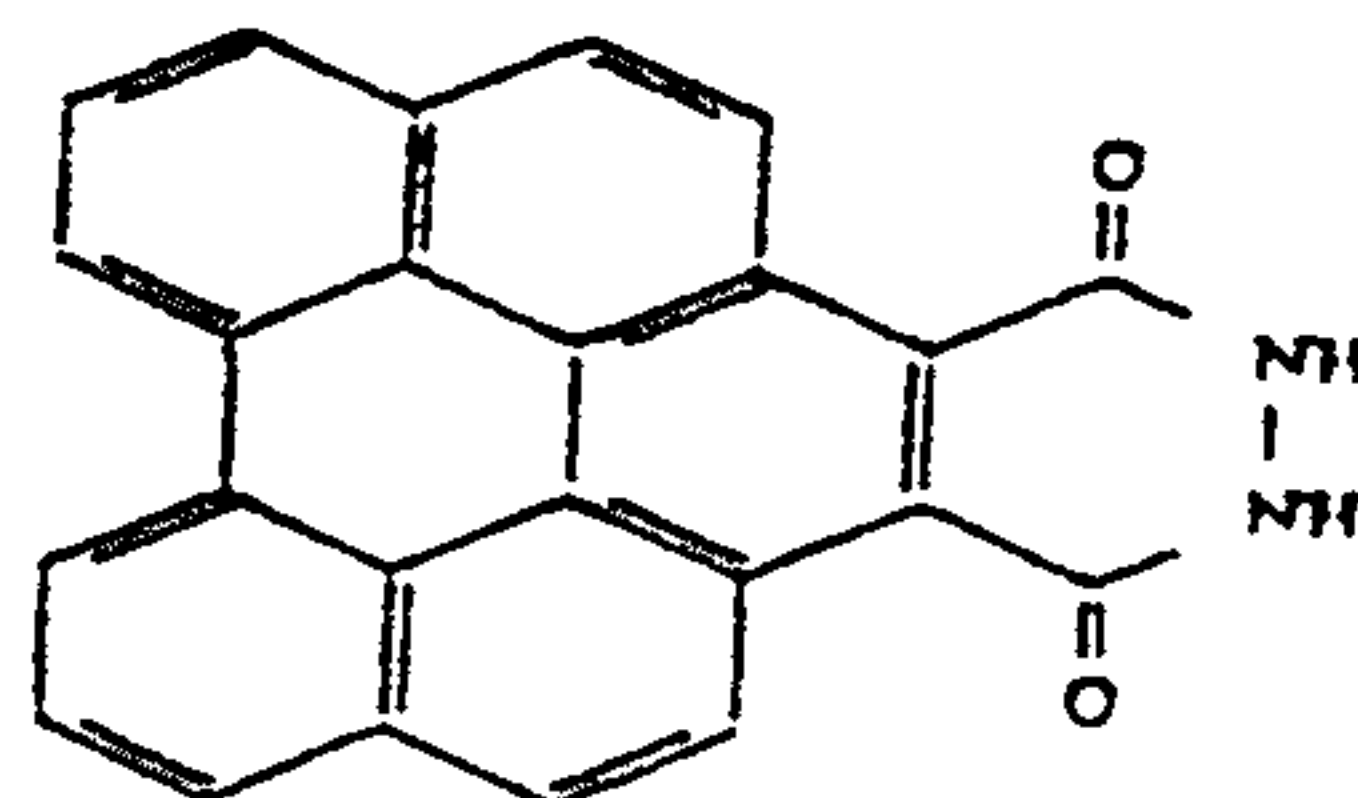
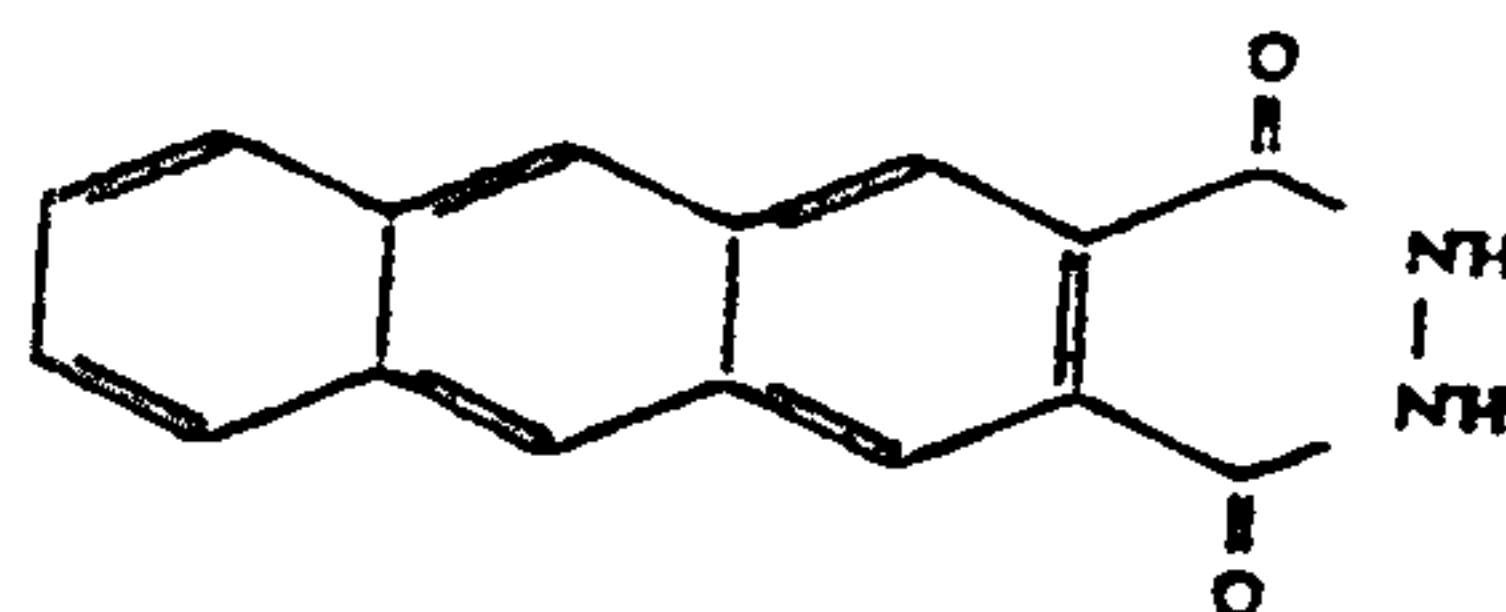
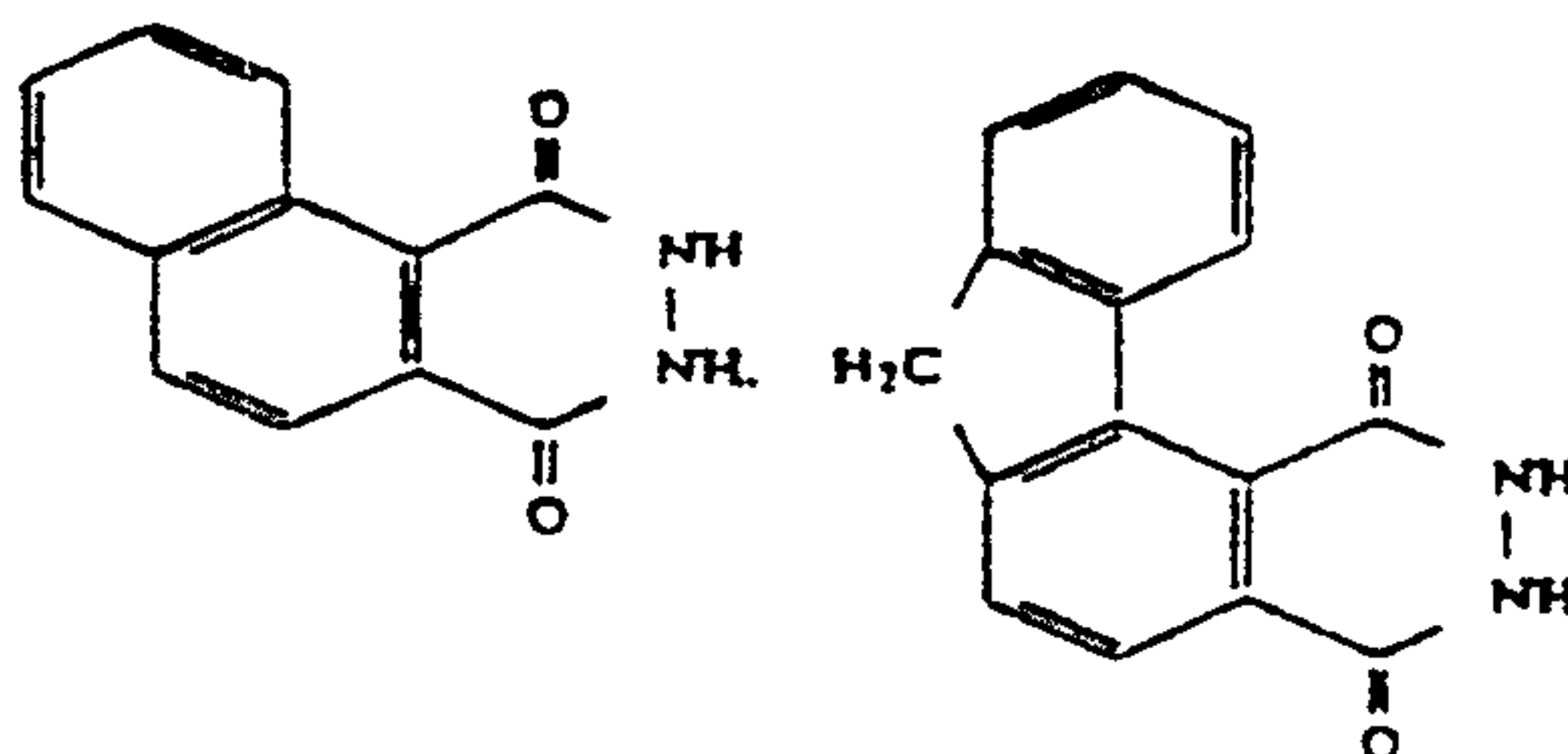
10 Alternatively, any adjacent two, adjacent
three or all of Z¹, Z², Z³ and Z⁴ (that is, combinations
of two or three adjacent groups, or all four groups) can
be taken together to form a fused ring system containing
one or more aromatic rings. Such fused rings can be
15 substituted with one or more hydroxy, amino (substituted
or unsubstituted as described above) or an alkoxy having
from about 1 to about 4 carbon atoms such as methoxy,
ethoxy and isopropoxy. Preferably, such fused rings are
substituted with one or more primary, secondary or
20 tertiary amines, hydroxy or alkoxy as described above.

Representative useful DPD compounds include,
but are not limited to, luminol, isoluminol, N-(4-
aminobutyl)-N-ethylisoluminol hemisuccinimide, N-(6-
aminoethyl)-N-ethylisoluminol, N-ethylisoluminol and 7-
25 dimethylaminonaphthalene-1,2-dicarboxylic acid
hydrazide. Luminol (5-amino-2,3-dihydro-1,4-
phthalazinedione) and isoluminol (6-amino-2,3-dihydro-
1,4-phthalazinedione) are preferred, and luminol is most
preferred.

30 Other useful classes of DPD compounds are
described in the Gundermann et al. publication cited

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above, and include substituted or unsubstituted phthalic
1 acid hydrazides, anthracene-2,3-dicarboxylic acid
hydrazides, phenanthrene dicarboxylic acid hydrazides,
fluorene-1,2-dicarboxylic acid hydrazides,
benzo[g,h,i]perylene-1,2-dicarboxylic acid hydrazides
5 and coronene-1,2-dicarboxylic acid hydrazides, such as
those illustrated by the following structures:



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Other phthalazinedione analogs recognized by
1 those skilled in the art as being useful in this
invention include those disclosed in European Patent
Application No. 91310875.9 to Masuya et al. and U.S.
Patent No. 5,324,835 to Yamaguchi.

5 The compounds identified above can be obtained
commercially, or be prepared using conventional starting
materials and procedures well known to those skilled in
the art.

10 The second component of the wash composition
employed in the instant invention is a halogen,
pseudohalogen, halogen-providing source or
pseudohalogen-providing source. The terms "halogen or
halogen-providing source" as used herein denote any
15 material which when added to the wash composition of the
instant invention provides a halogen anion, i.e. a
fluoride, chloride, bromide or iodide anion. This
includes the halogens like chloride, bromide and iodide.
The halogen providing source may comprise a metal or
20 non-metal halogen salt. Non-metal salts include, but
are not limited to, ammonium, alkylammonium, substituted
alkylammonium, arylammonium, substituted arylammonium,
hydrazinium, oxonium, tropylium, sulfonium and the like.
The halogen-providing source may also be a polymeric
25 material comprising a homopolymer or copolymer
containing any combination of the above cationic species
with a halogen as a counter-ion. An example of such a
material is NH_4Br . Typically, the halogen-providing
source is added to the composition as a Group IA metal
30 salt. Other metal salts besides Group IA-containing
metals are also contemplated herein. In a

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highly preferred embodiment of the instant invention,
1 the halogen-providing source is NaBr.

The terms "pseudohalogen or pseudohalogen-providing source" are used herein to denote any material which when added to the composition employed in the
5 instant invention provides anions which have a high electron affinity that is comparable to the halogens. It should be noted that the pseudohalogen-providing source is typically, but not limited to, a Group IA metal compound. The pseudohalogen or pseudohalogen-
10 providing source may be a metal or non-metal compound as described above for halogen-providing sources, as for example, ammonium, alkylammonium and etc. and polymers thereof. Suitable pseudohalogens that may be present in the analytical signal-providing composition include, but
15 are not limited to, CN^- , SCN^- , OCN^- , azide, isonitrile and the like. Of the pseudohalogens, SCN^- is particularly preferred. A preferred pseudohalogen-providing source employed in the instant invention is NaSCN.

20 The third component of the wash composition employed in the instant invention is a peroxide or a peroxide-generating reagent composition. The term 'peroxide' includes inorganic or organic peroxide compounds that are typically employed in the prior art.
25 By peroxide-generating reagent composition, it is meant to include any reagent composition which is capable of producing peroxide when added to the signal-providing composition employed in the instant invention. Such peroxide-generating reagent compositions are well known
30 to those skilled in this art and include, but are not limited to, perborates, peracetates, urea peroxide, and

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organic peroxides such as peracids, peroxybenzoic acid
1 and oxones as well as hydroperoxides.

The wash composition employed in the present
invention is generally buffered to a pH of from about
6.5 to about 10, more preferably from about 6.8 to about
5 8.0, using one or more suitable biological buffers
having a pKa of about 6.0 to about 8.0. These buffers
are well known in the art. Illustrative examples of
such buffers include, but are not limited to,
Tris(hydroxymethyl)aminomethane-HCl (Tris-HCl); 2-[N-
10 morpholino]ethanesulfonic acid (MES); Piperazine-N,N'-
bis[2-ethanesulfonic acid] (PIPES); 3-[N-Morpholino]-2-
hydroxypropanesulfonic acid (MOPSO), phosphate buffers
and the like. Of the biological buffers mentioned
above, Tris-HCl is particularly preferred.

15 In the wash composition employed in the
present invention, the amounts of each component may
vary depending upon its intended use, the particular
sensitivity of the reagents and other factors well
understood by one skilled in the art. Thus, the
20 following general ranges are meant to provide guidance
for the skilled worker, and not to limit the practice of
the invention.

Specifically, the amount of buffer employed in
the present invention is readily apparent to one skilled
25 in the art since it is well known how to determine the
quantity of any buffer needed to maintain a desired pH.
The amount of signal-generating compound, DPD, is
generally at least about 0.01 mMolar, with an amount in
the range of from about 0.1 to about 10 mMolar being
30 preferred.

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1 In regard to the second component, i.e. the
halogen, pseudohalogen, halogen-providing source or
pseudohalogen-providing source, it is normally present
in an amount of at least about 0.1 mMolar. More
preferably, the halogen, pseudohalogen, halogen-
5 providing source or pseudohalogen-providing source is
present in the signal-providing composition in an amount
of about 50 to about 100 mMolar.

Regarding the third component, i.e. the
peroxide or peroxide-generating reagent composition,
10 that component is present in the wash composition in an
amount of at least about 0.02 mMolar. More preferably,
the peroxide or peroxide-generating reagent composition
is present in an amount of from about 0.5 mMolar to
about 1.0 mMolar.

15 The element of the present invention can be
used in a variety of assay formats to provide
chemiluminescent signal in response to the reacted or
unreacted form of the vanadium bromoperoxidase-labeled
immunoreactant.

20 The following examples are given to illustrate
the scope of this invention. Because these examples are
given for illustrative purposes only, the invention
embodied therein should not be limited thereto.

25 Except where noted, all reagents and equipment
were obtained from Eastman Kodak Company or other
commercial sources.

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EXAMPLE 11 Preparation of an Aqueous Chemiluminescent Composition
and a Vanadium Bromoperoxidase Solution

5 An aqueous composition for providing a
chemiluminescent signal was prepared as follows:

A buffer solution (pH 8.0) was prepared
containing 100 μ M of diethylenetriaminepentaacetic acid
(DTPA), 0.75 M NaBr and 1% cetyltrimethylammonium
bromide (CTAB) in a 0.05 M Tris-HCl buffer.

10 The signal-providing composition was prepared
from a two-part mixture. The first part contained 1 mM
of Na luminol and the second part contained 1mM H_2O_2 ,
both of which were prepared in the above buffer. These
two parts were combined immediately before use.

15 A vanadium bromoperoxidase (from *Ascophyllum*
nodosum) stock solution stored at 4°C was serially
diluted in 0.05 M Tris-HCl, pH 8, and 0.01% bovine serum
albumin (BSA) to provide a known concentration of the
vanadium bromoperoxidase ranging from 10 to 50,000
20 attomoles.

A coated element was then prepared having the
following reagent matrix composition:

Bead Spread Layer:	0.1M Tris, pH 8.0
	Polymeric beads (VtE)
25	Binder (MaWnaMt)
	0.5 g/m ² TSH Ab beads
	DTPA (10 ⁻⁴ M)

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1	Gel Pad:	Gelatin 0.2M Tris pH 8.0 DTPA (10 ⁻⁴ M) TX-100 Hardener (BVSME)
5	Support:	7 mil Estar [*] (polyethylene terephthalate)

The following definitions apply to the above reagent matrix:

10 Tris: Tris(hydroxymethyl)amino-methane buffer.

Polymeric beads: Poly(vinyltoluene-co-methacrylic acid beads).

15 Binder: Poly(methyl acrylate-co-sodium 2-acrylamido-2-methyl-propanesulfonate-co-2-acetoacetoxyethyl methacrylate).

TSH Ab beads: poly(styrene-co-3-(p-vinylbenzylthio)propanoic acid) having covalently attached an antibody against thyroid stimulating hormone (TSH).

20 TX-100: Triton X-100^{*}, an octylphenoxy polyethoxy ethanol surfactant sold by Rohm and Haas Co.

BVSME: Bis(vinylsulfonyl)methyl ether.

25 MaWnaMt = poly(methyl acrylate-co-2-acrylamido-2-methyl propane sulfonic acid, sodium salt-co-acetoacetoxyethylmethacrylate) (90/4/6).

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* Trademark

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Evaluation of Vanadium Bromoperoxidase for1 Chemiluminescent Signal on a Sensitive Detection
Immunoassay Coating

5 The above prepared chemiluminescent signal-providing composition and vanadium bromoperoxidase buffered solutions were used as follows:

10 An element in the form of a slide was assembled by placing a 16 mm² section of the above prepared coating on an EKTACHEM (trademark) C-Slide base (as described in U.S. Patent 5,958,339 to Belly et al. earlier referenced). A Whatman GF/B absorbent microfiber glass material (Whatman* Specialty Products Division, Product No. PD 008-12A-100) with a 10 mm diameter centered hole to enable a sample application, was positioned over the coating. An impervious annular plastic top-piece was then placed on the absorbent layer and the entire assembly was sealed with tape (For complete assembly details see Figs. 1 and 2 of U.S. Patent 5,958,339 to Belly et al.).

20 The element was then washed with 100 µl of the above described chemiluminescent signal-providing composition at a rate of 1 µl/sec. Following the application of the wash solution, the appropriate dilution of vanadium bromoperoxidase described above in 5 µl of the chemiluminescent signal-providing composition was spotted onto the coating. The element was immediately disassembled and the chemiluminescent signal of the coating was measured using a TURNER (trademark) TD-20e luminometer at ambient temperature. The luminometer was modified to accommodate the disassembled 16 mm² coating by use of an adapter that

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35 * Trademark

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permitted read out of the central 10 mm diameter portion
1 of the coating.

Fig. 1 shows the chemiluminescent output (10
sec. integral at $t=4$ minutes after placement in the
modified luminometer) under the above conditions for
5 this assay. More specifically, Fig. 1 shows that the
element of the instant invention produces an absolute
signal which is easily detectable like that of the prior
art element.

In Fig. 1 the control element contains
10 horseradish peroxidase as a signal-generating enzyme,
and 3'-chloro-4'-hydroxyacetanilide as an enhancer in
the wash composition as described in U.S. Patent No.
5,372,931 to Friedman et al., wherein the wash
composition was modified as follows: 0.05 M Tris-HCl,
15 pH 8.0, 0.1% cetyltrimethyl ammonium chloride (CTAC),
150 μ M 3'-chloro-4'-hydroxyacetanilide in addition to
stated components therein.

Fig. 2 shows the signal-to-noise ratio, a
recognized measure of system sensitivity to those
20 skilled in the art. The data in Fig. 2 were obtained
using the element of the present invention and a control
element which contained horseradish peroxidase as a
signal-generating enzyme. The wash composition for the
HRP control included 3'-chloro-4'-hydroxyacetanilide as
25 an enhancer as described in U.S. Patent No. 5,372,931 to
Friedman et al., modified as follows: 0.05 M Tris-HCl,
pH 8.0, 0.1% cetyltrimethyl ammonium chloride (CTAC),
150 μ M 3'-chloro-4'-hydroxyacetanilide in addition to
stated components therein. This figure shows that the
30 element of the present invention provides greater
sensitivity than the control element.

EXAMPLE 21 Comparison of Element Stability

5 This example compares the stability of vanadium bromoperoxidase in a dry element to that of HRP in a dry element. A base coated element was prepared having the following reagent matrix composition coated on a 7 mil Estar (polyethylene terephthalate) support:

LAYER	COMPONENT	LAYDOWN (g/m ²)
10 Porous Spread Layer	Polymer Beads-VtE Latex Binder-MaWnaMt Buffer-TES	130 2.583 0.219
15 Subbing (Receptor)	Copolymer-IMnAg(85/5/10 Buffer-TES Surfactant-TX-100	0.50 0.10 0.02
15 First Reagent	Matrix-Gelatin Buffer-TES Surfactant-TX-100 Hardener-BVSME	10.0 4.58 0.02 0.150

20 KEY: VtE = poly(vinyltoluene-co-methacrylic acid)
 (98/2)

 MaWnaMt = poly(methyl acrylate-co-2-acrylamido-2-methyl propane sulfonic acid, sodium salt-co-acetoacetoxyethylmethacrylate) (90/4/6)

25 TES = N-[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl] taurine

 BVSME = Bis(vinylsulfonyl)methyl ether

30 IMnAg = poly(N-isopropylacrylamide-co-2-hydroxyethylmethacrylate-co-methylenebisacrylamide) (85/5/10).

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1 The same reagents as described in Example 1
for vanadium bromoperoxidase stock solution were
employed in this example except that the vanadium
bromoperoxidase and HRP stock solutions were diluted to
10⁻⁸ M in a 10 mM phosphate buffer, pH 7.0.

5 Both elements were assembled using the
procedure described in Example 1 except the absorbent
material was not included in the slide assembly. Then,
10 μ l of either the above vanadium bromoperoxidase or
the above HRP stock solutions were spotted on the
elements.

Each element was then aged in the dark at room
temperature for 30 minutes, 2, 4, 6, 24 and 48 hours.
After each interval, the element was cut to remove the
coating material which was then extracted in 1 ml of a
15 buffer containing phosphate (pH 7.0), 0.15 M NaCl and
0.1% BSA. The extracted material was then centrifuged
for about 2 minutes to separate the particulate and
solid materials.

The measurement of enzyme activity was
20 accomplished by the addition of 200 μ L of the
appropriate signal-generating solution to 10 μ L of the
extracted supernatant. For the vanadium
bromoperoxidase, the signal-providing wash composition
of the instant invention was used. For HRP, the reagent
25 composition disclosed in Example 1 of U.S. Patent No.
5,372,931 to Friedman et al. was used. The
chemiluminescent signal was then determined using a
DYNATECH (trademark) ML3000 luminometer at 37°C.

The results of these experiments are shown
30 graphically in Figure 3 (1.0 sec. integral, at t=5 min.
for each interval point). Figures 4(a) and (b) are

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plots of the data from Figure 3 depicting percent
1 remaining activity of (a) HRP and (b) vanadium
bromoperoxidase. Specifically, these figures clearly
show that the stability of the vanadium bromoperoxidase-
containing element remains essentially constant over a
5 long period of time (up to 48 hours). In contrast, a
dramatic decrease in the HRP-containing element is
clearly shown.

The above experiments and examples are given
to illustrate the scope and spirit of the present
10 invention. These embodiments and examples will make
apparent to those skilled in the art, other embodiments
and examples. These other embodiments and examples are
within the contemplation of the present invention;
therefor, the instant invention should be limited only
15 by the appended claims.

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WHAT IS CLAIMED IS:

1

1. An analytical element for the determination of a specific binding ligand, said element comprising:

5

(i) a porous spreading zone; and

(ii) at least one additional zone which is in fluid contact with said porous spreading zone,

10

wherein said element contains, in at least one of said zones, a vanadium bromoperoxidase-labeled immunoreactant which specifically binds to a receptor for the specific binding ligand of interest.

15

2. The element of Claim 1 wherein said vanadium bromoperoxidase is obtained from *Ascophyllum nodosum*, *Ceramirum rubrum*, *Laminaria saccharina*, *Fucus distichus*, *Corallina pilulifera*, *Corallina officinalis* or *Macrocystis pyrifera*.

3. The element of Claim 2 wherein said vanadium bromoperoxidase is obtained from *Ascophyllum nodosum*.

20

4. The element of Claim 1 further comprising (iii) an absorbent material in fluid contact with one or more of said zones.

25

5. A multilayer analytical element for the determination of a specific binding ligand, wherein said element comprises a non-porous support having thereon, in fluid contact:

(i) a first reagent or buffer layer;

30

(ii) a subbing layer comprising an unlabeled immunoreactant which specifically binds with the specific binding ligand of interest; and

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(iii) a porous spreading layer containing a
1 vanadium bromoperoxidase-labeled immunoreactant which
specifically reacts with the specific binding ligand of
interest or its receptor.

6. The multilayer element of Claim 5 wherein
5 said vanadium bromoperoxidase is obtained from
Ascophyllum nodosum, Ceramirum rubrum, Laminaria
saccharina, Fucus distichus, Corallina pilulifera,
Corallina officinalis or Macrocystis pyrifera.

10 7. The multilayer element of Claim 6 wherein
said vanadium bromoperoxidase is obtained from
Ascophyllum nodosum.

15 8. The multilayer element of Claim 5 wherein
said porous spreading layer is a beaded spreading layer.

9. The multilayer element of Claim 5 further
comprising (iv) an absorbent material in fluid contact
with one or more of said layers.

20 10. A method for the determination of a
specific binding ligand comprising:

(A) contacting a fluid sample suspected of
containing said specific binding ligand with an analytic
element comprising:

25 (i) a porous spreading zone, and
(ii) at least one additional zone which is in
fluid contact with said porous spreading zone, said
element containing in at least one of said zones a
vanadium bromoperoxidase-labeled immunoreactant which
30 specifically binds said ligand;

(B) applying a wash solution to said element
to bring about a separation of the unreacted form of said
vanadium bromoperoxidase-labeled immunoreactant from the
35 reacted form of said vanadium bromoperoxidase-labeled
immunoreactant, said wash solution comprising a

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composition having a pH of from about 6.5 to about 10,
1 wherein said composition comprises:

(a) a chemiluminescent signal-generating
reagent which provides a signal in response to the
catalytic activity of said vanadium bromoperoxidase,
5 wherein said signal-generating reagent is a 2,3-dihydro-
1,4-phthalazinedione derivative;

(b) a halogen, pseudohalogen, halogen-
providing source or pseudohalogen-providing source; and

(c) a peroxide or a peroxide-generating
10 reagent composition, and

(C) detecting either said unreacted or
reacted form of said vanadium bromoperoxidase as a
measure of said specific binding ligand.

11. The method of Claim 10 wherein said fluid
15 sample is of human or animal biological origin.

12. The method of Claim 10 wherein said
vanadium bromoperoxidase is obtained from *Ascophyllum*
nodosum, *Ceramirum rubrum*, *Laminaria saccharina*, *Fucus*
distichus, *Corallina pilulifera*, *Corallina officinalis* or
20 *Macrocyctis pyrifera*.

13. The method of Claim 12 wherein said
vanadium bromoperoxidase is obtained from *Ascophyllum*
nodosum.

14. The method of Claim 10 wherein said 2,3-
25 dihydro-1,4-phthalazinedione derivative is luminol,
isoluminol, N-(4-aminobutyl)-N-ethylisoluminol
hemisuccinimide, N-(6-amino-hexyl)-N-ethylisoluminol, N-
ethylisoluminol or 7-dimethylaminonaphthalene-1,2-
dicarboxylic acid.

15. The method of Claim 14 wherein said 2,3-
30 dihydro-1,4-phthalazinedione is luminol.

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16. The method of Claim 10 wherein said
1 halogen-providing source is a metal or a non-metal salt.

17. The method of Claim 16 wherein said
halogen-providing source is NaBr.

18. The method of Claim 10 wherein said
5 pseudohalogen-providing source is a metal or non-metal
compound containing as one of its ligands CN^- , SCN^- ,
 OCN^- , azide or isonitrile.

19. The method of Claim 18 wherein said
pseduohalogen is SCN^- .

20. The method of Claim 10 wherein said
10 peroxide-generating reagent is a perborate, peracetate,
urea peroxide, peroxybenzoic acid, peracid, oxone or a
hydroperoxide.

21. The method of Claim 10 wherein said
15 peroxide is hydrogen peroxide.

22. The method of Claim 10 further comprising
(iii) an absorbent material in fluid contact with one or
more of said zones.

23. The method of Claim 10 wherein said
20 vanadium bromoperoxidase is obtained from *Ascophyllum*
nodosum, said 2,3-dihydro-1,4-phthalazinedione
derivative is luminol, said halogen-providing source is
NaBr and said peroxide is hydrogen peroxide.

24. The method of Claim 10 wherein said
25 vanadium bromoperoxidase is obtained from *Ascophyllum*
nodosum, said 2,3-dihydro-1,4-phthalazinedione
derivative is luminol, said pseudohalogen-providing
source NaSCN and said peroxide is hydrogen peroxide.

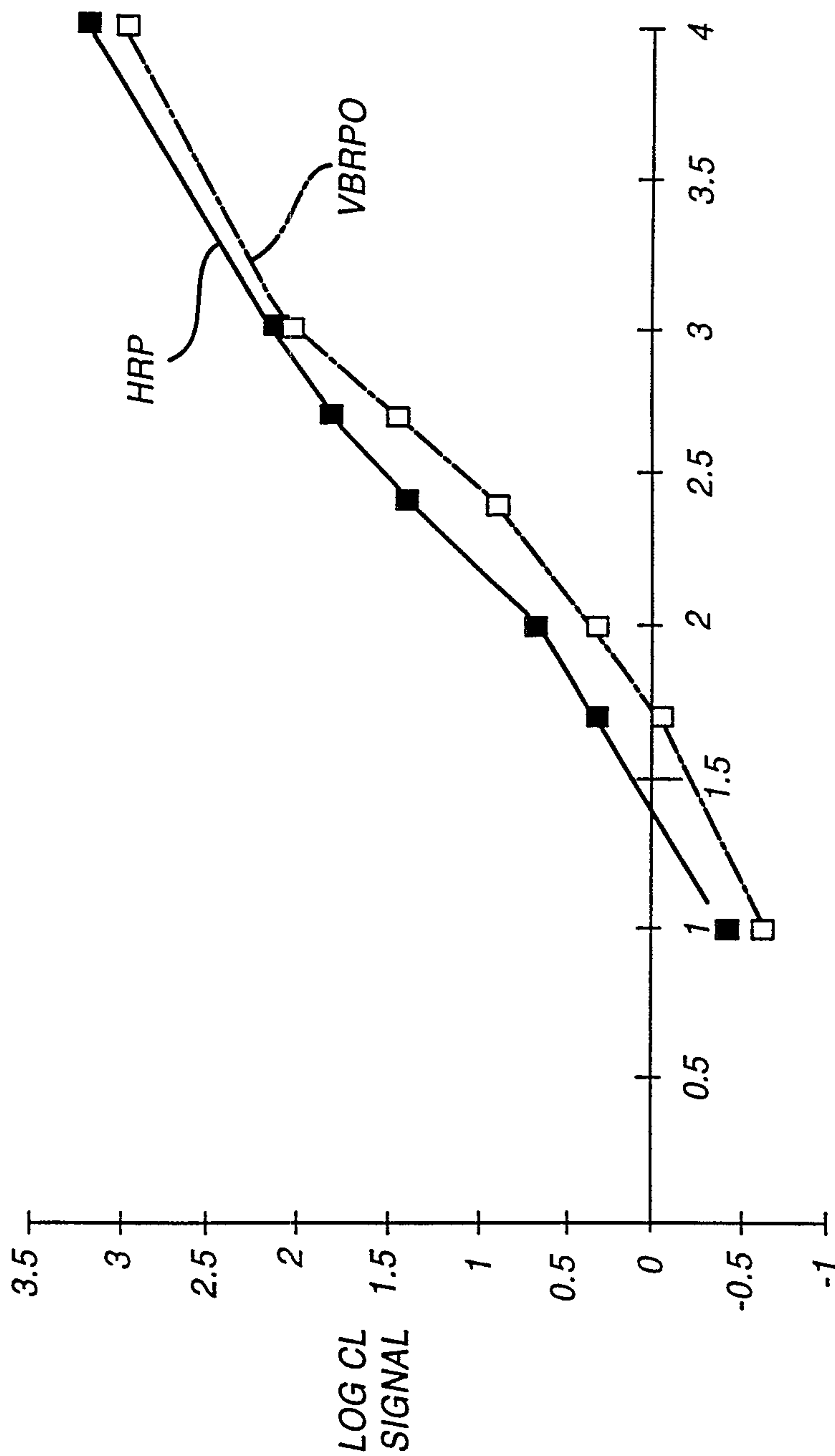
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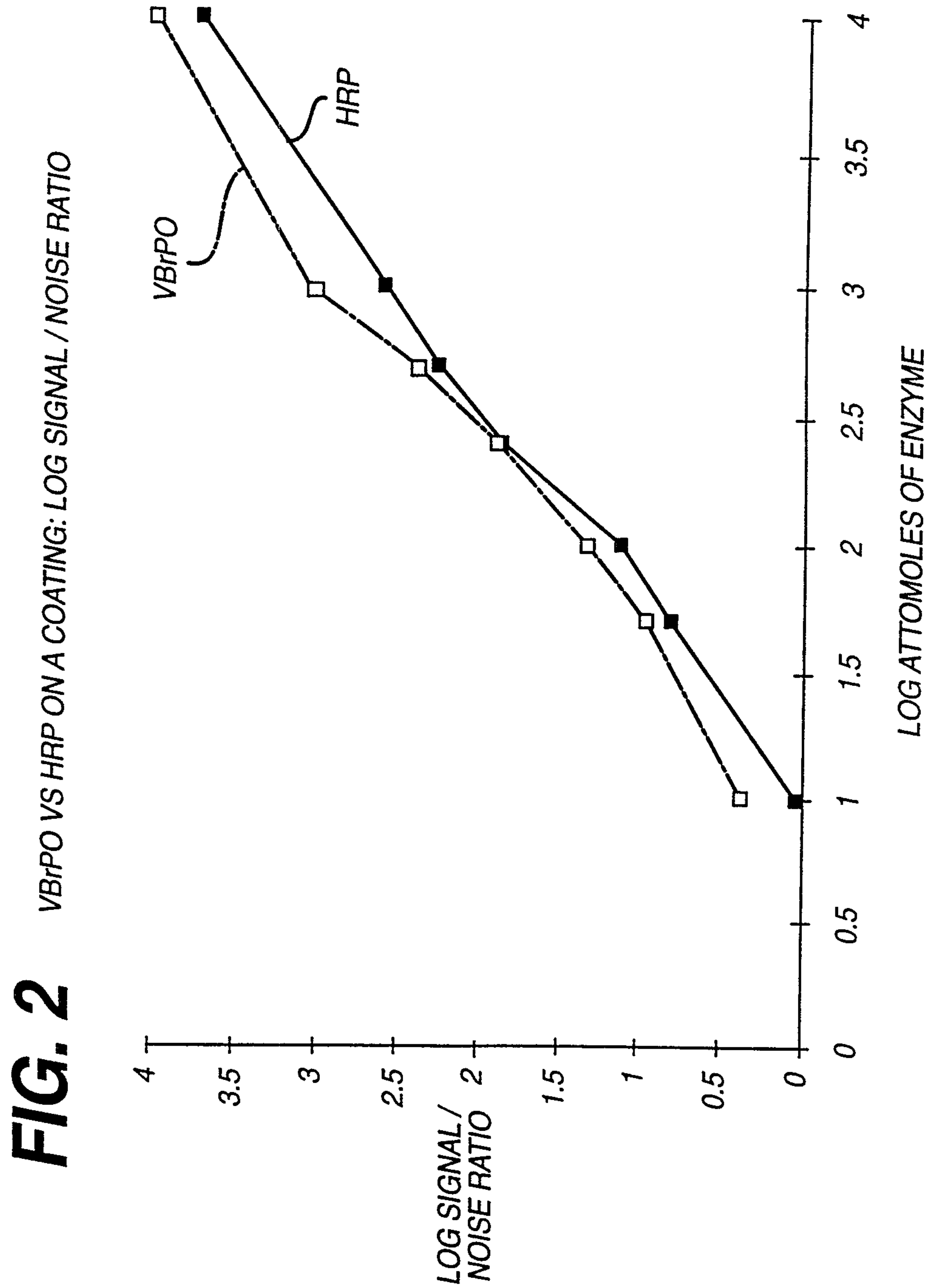
FIG. 1

VBRPO VS HRP ON A COATING: LOG CL SIGNAL

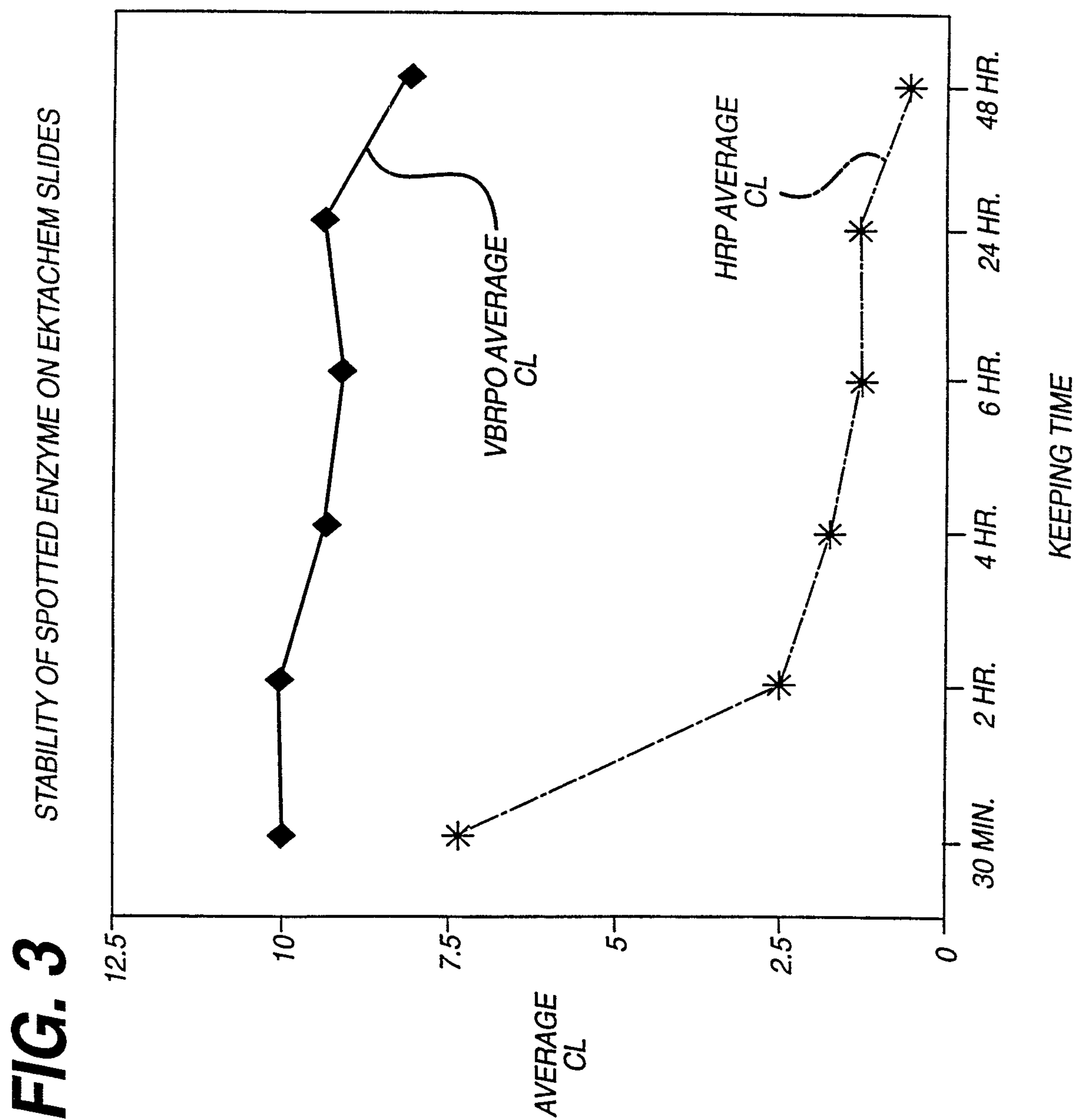


LOG ATOMMOLES ENZYME

2/5



3/5



4/5

FIG. 4(a)

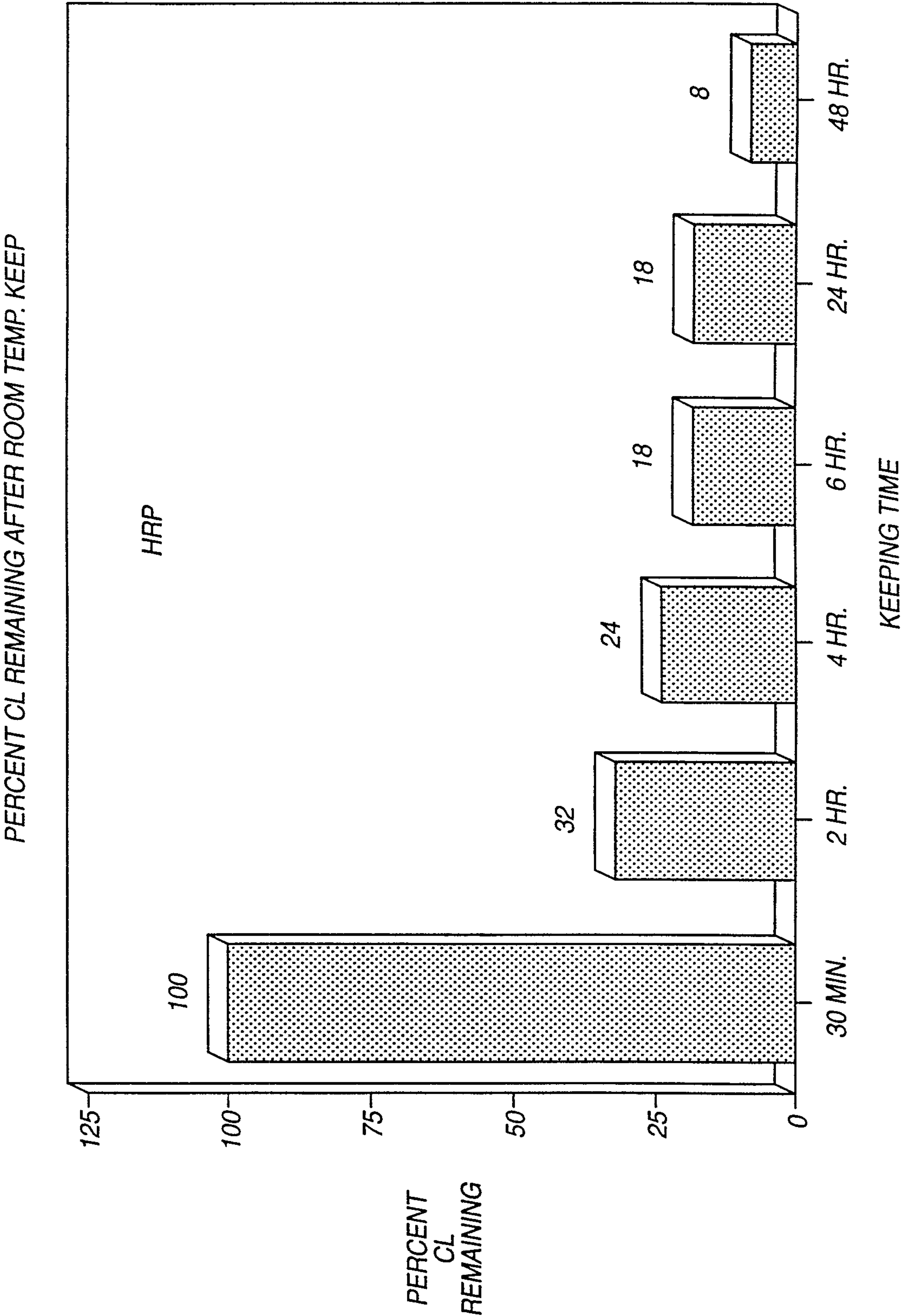


FIG. 4(b)

