Competitive immunosorbent assays for the model antigen biotin were performed using both unilamellar vesicles with covalently attached biotin and horseradish peroxidase (HBVs) and commercially available biotin-labeled horseradish peroxidase (B-HRP) as the enzyme-labeled antigen. The assays were performed using anti-biotin antibody (ABA) surface densities ranging from one-tenth to full monolayer coverage. It was found that assays using HBVs strongly depended on the antibody surface density, while assays using B-HRP were relatively insensitive to the antibody surface density. The HBV assay dependence on the ABA surface density was most likely due to multiple point attachment of vesicles to the surface. The lowest detectable antigen concentration (least detectable dose) for vesicles (~10^{-9} M) was an order of magnitude lower than the value found for B-HRP (~10^{-8} M). The sensitivity (slope of the response vs biotin concentration curve) of assays with B-HRP was comparable to the sensitivity of assays with HBVs at low antibody surface density, probably due to less extensive multipoint attachment. It was also found that assays could be performed with vesicles at antibody surface densities that were at least 5 times lower, in terms of the bulk antibody concentrations used to coat the wells, than antibody surface densities at which B-HRP gave comparable signals (~1 \Delta A/min).

Introduction

In previous work, small unilamellar phospholipid vesicles (~1000 Å diameter) were prepared with both an enzyme label (horseradish peroxidase, HRP) and a small ligand (biotin) covalently attached to their outer surface (Jones et al., 1993). It was demonstrated that these bifunctional vesicles bound specifically to anti-biotin antibody (ABA) on microtiter plates with an equilibrium association constant of approximately 10^9 M^{-1} and could be used in a competitive assay to detect biotin. Because of the large number of HRP molecules that can be bound to the surface of a vesicle of this size, it was postulated that a higher signal could be generated in competitive and noncompetitive assays using enzyme- and antigen-conjugated vesicles (HBVs) as the marker rather than a conventional HRP-ligand or HRP-antibody conjugate (Jones et al., 1993). Figure 1 illustrates how a bifunctional vesicle with both antigen and enzyme label covalently attached to the outer surface competes with free antigen for antibody-binding sites on the surface of a microtiter well.

In this work, HBVs and B-HRP are used as marker-labeled antigens in competitive assays for the model antigen biotin at antibody surface densities ranging from one-tenth to full monolayer coverage. The isotherm of B-HRP adsorbing to ABA-coated wells was measured to determine the association constant (K) and to establish the practical range of B-HRP concentrations to be used in competitive assays. Radiolabeled HBVs and radiolabeled B-HRP were prepared and used to determine the difference between signals measured from the radiolabel and those from the enzyme label and to determine association constants. In addition, the adsorption of [14C]-biotin to ABA-coated wells was examined to determine the antibody specific activity and the association constant of biotin to adsorbed antibody.

It was found that the least detectable dose for biotin in a competitive assay using HRP-conjugated vesicles was approximately 10 times lower than that obtained with the conventional conjugate B-HRP. Because the binding strength of labeled vesicles to the surface was high, this assay was successful at antibody surface concentrations on the microtiter plates that were considerably lower than those required in conventional assays. The results obtained with this model system indicate that bifunctional...
vesicles may offer a suitable alternative for enhancing the
detection limits of enzyme-linked immunosorbent assays.

Materials and Methods

Materials. Horseradish peroxidase (HRP) type VI-A,
biotin-conjugated horseradish peroxidase (B-HRP, with
a 1:1 molar ratio of biotin to enzyme) type VI-A, goat
affinity purified polyclonal anti-biotin antibody (ABA),
bovine serum albumin (BSA), 2,2'-azinobis(3-ethylbenz-
thiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide
(H2O2), biotin, cholesterol (Chol), dimyristoylphosphati-
dyethanolamine (DMPE), and distearoylphosphatidy-
choline (DSPC) were obtained from Sigma Chemical
Company (St. Louis, MO). The nonionic
detergent limits of enzyme-linked immunosorbent assays.

Biological extinction coefficient of 1.4 L/g.cm at 280 nm

activity of ABA when adsorbed to plates were determined
based on Polystyrene Wells.

A high-protein-binding microtiter plates were obtained from
Pierce Chemical Company (Rockford, IL). The nonionic
surfactant pentaethylene glycol mono-n-dodecyl ether
was Cytoscint from ICN Biomedicals,


t-medium, pH 7.4. Casein (technical grade, from Sigma)
was a mixture of 74% α-, 23% β-, and 3% γ-casein.
Biotinylimidohexanoic acid N-hydroxysuccinimide ester,
or long chain biotin (biotin-LC-NHS), was obtained from
Pierce Chemical Company (Rockford, IL). The nonionic
surfactant pentaethylene glycol mono-n-dodecyl ether
(C12E5) was obtained from Nikko Chemical Co. (Tokyo,
Japan).

The scintillation cocktail used in counting
radioactive samples was CY-Scint from ICN Biomedicals,
Inc. (Irvine, CA). [14C]Biotin and [14C]cholesterol were
obtained from Amersham (Arlington Heights, IL). All
other chemicals were from Fisher Scientific and were
reagent grade or better. Polystyrene 96-well, flat-bottom,
high-protein-binding microtiter plates were obtained from
Costar Corporation (Cambridge, MA).

Methods. Total ABA and Active ABA per Unit
Area on Polystyrene Wells. Competitive assays for
biotin, as will be described later, were performed at various
antibody surface loadings in polystyrene 96-well flat-
bottom microtiter plates. The surface density of ABA
adsorbed to wells was determined using radiolabeled ABA,
which was prepared by reductive alkylation (Jentoft
and Dearborn, 1983) as described previously (Jones et
al., 1993). Antibody concentrations were determined using a mass-
based extinction coefficient of 1.4 L/g.cm at 280 nm
(Goding, 1986). The specific radioactivity of the labeled
antibody was determined by placing a sample of known
radiolabeled antibody concentration in 20 mL of scintil-
ant cocktail and counting on a Packard 1500 liquid
scintillation counter.

The ABA adsorption isotherm was determined at 4 °C
in an American Scientific refrigerator with shaking using
an IKA-Labortechnik MTS-4 microtiter plate shaker at a
setting of 600 cycles/min. At each ABA concentration
tested, between four and eight wells were incubated with
200 µL of ABA solution per well at antibody concentrations
ranging from 0.05 to 20 µg/mL in PBS for at least 15 h
with shaking. After they were rinsed three times, the wells
were immersed in 20 mL of scintillation cocktail and
counted as above. The radioactivity of the adsorbed
antibody was determined by subtracting the blank counts
(i.e., scintillation cocktail only) from the total counts. The
total mass of ABA adsorbed per unit area was calculated
to the antibody specific radioactivity and an area of
1.76 cm² per well when the well is filled with 200 µL of
liquid (Jones, 1993). The ABA adsorption isotherm
experiments were performed three times, and average
values were obtained. The area per ABA molecule was
calculated from the surface density assuming a molecular
weight of 160 000 (Goding, 1986).

The association constant of biotin for ABA and the
activity of ABA when adsorbed to plates were determined
using [14C]biotin. Microtiter plates were coated with
antibody at 4 °C, with shaking for at least 15 h and using
200-µL aliquots of antibody solutions at 10 µg/mL, and
washed two times. Wells were then blocked by incubating 200
µL of 1 wt % casein in PBS for 1 h at room temperature
and washed three times. Additional wells that were simply
blocked with casein were treated as background wells and
used to determine nonspecific adsorption. The isotherm
measurements for [14C]biotin adsorbing to ABA at 37 °C
were performed three times and average values were
obtained. Sample incubations were done for 1 h. The
number of moles of [14C]biotin bound specifically to the
surface per unit area was calculated from the radioactivity
of adsorbed [14C]biotin and the [14C]biotin specific
radioactivity (50 µCi/µmol), using a well area of 1.15 cm²
when the well is filled with 100 µL of liquid (Jones, 1993).
The activity of the adsorbed antibody was estimated from
the moles of adsorbed biotin at high bulk biotin concentra-
tions, with the assumption that 2 mol of biotin binds
to 1 mol of active antibody. Therefore, the percent active
antibody was calculated by dividing the moles of specifically
bound [14C]biotin by twice the total ABA surface
density and multiplying by 100. The association constant
for biotin adsorbing to ABA was determined from the
Langmuir adsorption isotherm and the Sips equation,
as will be described later.

Vesicle Preparation and Characterization. Small
unilamellar vesicles with composition DSPC/Chol/DMPE
DMPE–LC–biotin (42.4:3.5:20:0.0 mol %) and radiola-
beled vesicles with composition DSPC/Chol/[14C]Chol
DMPE/DMPE–LC–biotin (42.4:33.3:9.3:20:0.1 mol %)
were prepared by sonication (Jones et al., 1993). Bioti-
nylated phospholipid (DMPE–LC–biotin) was synthesized
by coupling DMPE with biotin-LC-NHS using the method
of Bayer and Wilcheck (1984), as described previously
(Jones et al., 1993).

Mesoporous peroxidase was covalently linked to the
outer surface of vesicles to obtain HRP-conjugated bi-
oitinylated vesicles (HBVs) using the periodate method
(Nakane and Kawaoi, 1974; Heath et al., 1980; Jones et
al., 1993). Free enzyme and HBVs were separated by gel
permeation chromatography. Non-radiolabeled enzyme-
vesicle conjugates are called 0.1 mol % HBVs, while
radiolabeled vesicles (also containing 0.1 mol % biotinyl-
ated phospholipid) are called [14C]HBVs. Concentrations
of phospholipid in non-radiolabeled vesicle samples were
determined by phosphate assay using the method of Chen
et al. (1956). For [14C]HBVs, the radiolabel was used to
determine the lipid concentration. The immobilized
enzyme concentration was determined from the absorb-
bance at 405 nm after subtraction of the absorbance due
to light scattering by vesicles. The HRP to lipid ratio
(µg/µmol) was calculated by dividing the immobilized HRP
concentration (µg/mL) by the lipid concentration (mmol/
µL) in solution. The mean HRP vesicle diameter was
determined by quasi-elastic light scattering. The number
of HRP molecules immobilized per vesicle was estimated
from the mean HRP vesicle diameter and the HRP to
lipid ratio. The vesicle concentration was estimated from
the concentration of total lipid, vesicle diameter, and values
for the area per lipid molecule as described previously
(Jones et al., 1993). The 0.1 mol % HBVs were used in
the competitive assays, as is described in the following
sections. The [14C]HBVs were used to determine the
adsorption isotherms and the maximum surface coverage
by vesicles and in competitive assays as described in the
following sections. Vesicles were stored at 4 °C.

Enzyme activity measurements to determine kinetic
parameters for [14C]HBVs were performed in 50 mM
citrate buffer (pH 6.0) at 25 °C, using a fixed H2O2 concentration of 2.75 mM and ABTS concentrations ranging from 0.0156 to 2.0 mM. After 3.2 mL of substrate solution was pipetted into the reference and sample cuvettes, 10 μL of enzyme solution was added to the sample cuvette and agitated. This was then zeroed against the reference cuvette, and the change in absorbance was recorded at 410 nm with a Shimadzu Model 160UV-visible spectrophotometer. The immobilized HRP concentration in the cuvettes was 8.7 × 10−10 M. Kinetic parameters (Michaelis constant, K, and maximum reaction rate, V max) were determined from the Michaelis–Menten equation by nonlinear least-squares regression analysis. The enzyme specific activity, kₚ, was determined by dividing V max by the enzyme concentration in the cuvettes. In experiments to be described later, 1 wt % C₁₂E₆ was used to solubilize radiolabeled vesicles that had been adsorbed to an antibody-coated surface. Thus, the effect on the enzyme activity of including 1 wt % C₁₂E₆ in the substrate solution was examined. Enzyme activity was determined at pH 4.0 with 2.75 mM H₂O₂ and 2.0 mM ABTS, using the procedure detailed above, with substrate solution containing 1 wt % C₁₂E₆ and with substrate solution containing no surfactant.

**Preparation and Characterization of Radiolabeled B-HRP.** Radiolabeled B-HRP ([¹⁴C]BHRP), used in experiments to be described later to determine the adsorption isotherm and maximum surface coverage, was prepared by reductive methylation (Jentoft and Dearborn, 1983). The concentration of [¹⁴C]BHRP was determined using a mass-based extinction coefficient of 1.85 L/g-cm at 403 nm (Jones et al., 1993). The specific radioactivity of the labeled HRP was determined by placing a sample of known radiolabeled enzyme concentration in 20 mL of scintillation cocktail and counted on a Packard 1500 liquid scintillation counter. It was assumed that the biotin functional group on B-HRP was unaffected by the radiolabeling procedure, so that the biotin to enzyme molar ratio remained 1.

Enzyme activity measurements to determine kinetic parameters for [¹⁴C]BHRP were performed as described above, but at pH 4.0. This has been found to be the optimal pH for B-HRP activity (Jones et al., 1993). Kinetic parameters (Michaelis constant, K, and maximum reaction rate, V max) were determined from the Michaelis–Menten equation by nonlinear least-squares regression analysis. The enzyme concentration used in the cuvettes was 1.2 × 10−10 M. The enzyme specific activity, kₚ, was determined by dividing V max by the enzyme concentration in the cuvettes. In experiments to be described later, 1 wt % C₁₂E₆ was used to solubilize [¹⁴C]BHRP that had been adsorbed to an antibody-coated surface. Thus, the effect on the enzyme activity of including 1 wt % C₁₂E₆ in the substrate solution was examined. Enzyme activity was determined at pH 4.0 with 2.75 mM H₂O₂ and 1.0 mM ABTS, using the procedure detailed earlier, with substrate solution containing 1 wt % C₁₂E₆ and with substrate solution containing no surfactant.

**Adsorption of B-HRP, [¹⁴C]BHRP, and [¹⁴C]HBVs to ABA-Coated Wells.** Three sets of experiments were performed to quantify the adsorption of enzyme-labeled biotin to ABA-coated polystyrene wells. First, the adsorption isotherms of B-HRP, [¹⁴C]BHRP, and [¹⁴C]HBVs to antibody-coated wells were determined by measuring the signals generated from the adsorbed enzyme label. The adsorption isotherms of [¹⁴C]BHRP and [¹⁴C]HBVs to antibody-coated wells were also determined from the adsorbed radiolabel. In addition, the nonionic surfactant C₁₂E₆ was used to solubilize adsorbed [¹⁴C]BHRP and [¹⁴C]HBVs before the addition of substrate to determine the effect of adsorption on enzyme activity.

The first two sets of experiments were designed to determine the association constants and the maximum surface coverage of adsorbed enzyme label to ABA-coated wells. Microtiter plates were coated with antibody at 4 °C with shaking for 16 h using 200-μL aliquots of antibody solutions in PBS and rinsed twice with PBS. For B-HRP and [¹⁴C]BHRP, 10 μg/mL antibody was used to coat the plates. For [¹⁴C]HBVs, 1 and 10 μg/mL antibody were used to coat the plates. The wells were blocked by incubating 200 μL of 1 wt % casein in PBS for 1 h at room temperature and rinsed three times. Additional wells that were simply blocked with casein were treated as background wells. At each concentration tested, between two and eight wells were incubated with 100-μL aliquots of B-HRP, [¹⁴C]BHRP, or [¹⁴C]HBVs ranging from approximately 0.05 to 50 nM for B-HRP, from 0.05 to 500 nM for [¹⁴C]BHRP, and from 0.004 to 7 nM for [¹⁴C]HBVs. The diluent for B-HRP and [¹⁴C]BHRP was 0.5 wt % BSA in PBS, while the diluent for [¹⁴C]HBVs was PBS only. B-HRP and [¹⁴C]BHRP were incubated for 1 h, while the [¹⁴C]HBVs were incubated for 2 h at 37 °C. Incubations at 37 °C were performed in a Precision Scientific Model 4EM mechanical convection incubator. Wells containing B-HRP and [¹⁴C]BHRP were rinsed three times while wells containing [¹⁴C]HBVs were rinsed four times with PBS. The enzyme signals (ΔA/min) were measured on a Biotek EL 340 plate reader at 410 nm after 100 μL of substrate solution was added to the wells. For B-HRP and [¹⁴C]BHRP, the substrate contained 1.0 mM ABTS with 2.75 mM H₂O₂ in 50 mM citrate buffer (pH 4.0), which are the optimum substrate conditions for free enzyme (Jones et al., 1993). For [¹⁴C]HBVs, the substrate contained 2.0 mM ABTS with 2.75 mM H₂O₂ in 50 mM citrate buffer (pH 6.0). The pH of the substrate solution used for the vesicles was chosen to be 6.0 rather than the optimum of 4.0, because the reaction rate was so fast when pH 4.0 buffer was used that accurate ΔA/min values could not be obtained at high vesicle concentrations.

The adsorption of [¹⁴C]BHRP and [¹⁴C]HBVs to ABA-coated wells was determined from the radiolabel using the procedures outlined above, but instead of adding substrate to the wells, the wells were immersed in 20 mL of scintillation cocktail and counted on a Packard 1500 liquid scintillation counter. The specific signals (S) were calculated by subtracting the signal obtained in the casein-coated wells (S max) from the signal obtained in the ABA-coated wells (SABA) for both enzyme label and radiolabel. The maximum specific signal (S max) was used to calculate the maximum experimental surface density (I max,exp) as will be described in the following section. The isotherm determinations for B-HRP and [¹⁴C]BHRP were performed at 290 ng/cm² ABA surface density, while the isotherm determinations for [¹⁴C]HBVs were performed at 290 and 79 ng/cm² ABA surface density. The association constants for B-HRP, [¹⁴C]BHRP, and [¹⁴C]HBVs adsorbing to ABA were determined from the Langmuir adsorption isotherm, as described in the following section. Vesicles are likely to adsorb to antibody-coated wells through multiple point attachments, and at high antibody loadings, surface exclusion effects could reduce the probability of additional vesicles adsorbing to the surface. As a result, the adsorption data for [¹⁴C]HBVs were also fit to a large ligand model that accounts for the large relative size of the vesicles, as described in the following section. In the third set of experiments to quantify the adsorption...
of enzyme-labeled biotin to ABA-coated wells, the effect of
saturating the adsorbed enzyme label from the well
surface by the addition of surfactant immediately before
the addition of substrate was investigated. Microtiter
plates were coated with 10 μg/mL ABA and blocked with
1 wt % casein, as described above. Aliquots (100 μL) of
[14C]BHRP or [14C]HBVs were added to the wells at
concentrations ranging from 0.16 to 5 mM for [14C]BHRP
and from 1.6 to 50 pM for [14C]HBVs. The diluent for
[14C]BHRP was 0.5 wt % BSA in PBS, while the diluent
for [14C]HBVs was PBS only. After incubation and rinsing
as above, 50 μL of 1 wt % C12E6 in 50 mM citrate buffer
was added to wells to solubilize adsorbed enzyme label,
while 50 μL of citrate buffer without surfactant was added
to identical wells. Triplicate wells at each conjugate
were added to wells to solubilize adsorbed enzyme label,
while 50 μL of citrate buffer without surfactant was added
to identical wells. Triplicate wells at each conjugate
concentration were prepared with and without surfactant.
The plates were agitated with an IKA-Labortechnik
MTS-4 microtiter plate shaker at a setting of
600 cycles/min for 5 min to ensure solubilization. Next, 50 μL of
substrate solution was added to each well, so that the ABTS
concentrations in the wells were 1.0 and 2.0 mM for [14C]-
BHRP and [14C]HBVs, respectively, with 2.75 mM H2O2
in 50 mM citrate buffer (pH 4.0). The enzyme signals
(ΔA/Δmin) were measured on a Biotek EL 340 plate reader
at 410 nm and averaged.

**Modeling of Adsorption Data.** The adsorption of four
labeled ligands ([14C]biotin, B-HRP, [14C]BHRP, and
[14C]HBVs) to ABA-coated microtiter plates was examined
as discussed above. The Langmuir model (Langmuir,
1918) was used to describe the adsorption of ligands to the surface:

\[ \Gamma = \frac{\Gamma_{\text{max,exp}} K(L)}{1 + K(L)} \]  

(1)

where \( \Gamma \) is the ligand surface density (number/cm²), \( K \)
is the equilibrium association constant, and \( L \) is the
equilibrium bulk concentration of the adsorbing species.
When the enzyme label was used to determine the surface
density of the ligand, it was assumed that the specific signal
measured in the plate reader was directly proportional
to the amount of adsorbed ligand and was calculated
as described previously (Jones et al., 1993) using

\[ \Gamma = \frac{S(K_m + [C]) v N_a}{k_s [C] n F M A_w} \]  

(2)

where \( S \) is the specific signal measured in the wells, \( K_m \)
is the Michaelis constant, \( [C] \) is the substrate concentration,
\( v \) is the volume (100 μL), \( N_a \) is Avogadro's number,
\( k_s \) is the enzyme specific activity, \( n \) is the number of HRP
molecules per conjugate (260 for [14C]HBVs; 1 for B-HRP
and [14C]BHRP), \( F \) is a factor that accounts for the
difference in path lengths of the kinetics in experiments
to determine kinetic parameters and adsorption
experiments (0.31), \( M \) is the molecular weight of HRP
(40 000; Keilin and Hartree, 1951; Maehley, 1955), and \( A_w \)
is the well area exposed to solution (1.15 cm²). When the
radioactivity was used to determine the surface density of
the ligand, the radioactivity of the adsorbed ligand (specific
radioactivity values reported in Results and Discussion)
measured per well was divided by the specific radioactivity
of the ligand and the area per well (1.15 cm²). The
maximum experimental surface density of adsorbed ligand
\( \Gamma_{\text{max,exp}} \) was determined from the maximum
specific signal \( (S_{\text{max}}) \), measured at high ligand concentrations for both
enzyme label and radioactivity. The association constants
for [14C]biotin, B-HRP, [14C]BHRP, and [14C]HBVs
adsorbing to ABA were determined by a weighted non-
linear least-squares fit to the Langmuir adsorption isotherm.
In addition, the maximum theoretical ligand surface density, \( \Gamma_{\text{max,tho}} \), was calculated and compared to
the experimental value. For B-HRP and [14C]BHRP, the
maximum theoretical surface coverage was taken to be
equal to the active antibody-binding site surface density
as determined from adsorbing [14C]biotin to the antibody-
coated surface. On the other hand, the surface coverage
by [14C]HBVs was estimated by assuming that the vesicles
adsorb as disks (in cross-section only) in a close-packed
configuration. The maximum theoretical vesicle surface
density was calculated as the reciprocal of the vesicle
cross-sectional area multiplied by a packing factor of 0.91.
The percent coverages were calculated by dividing \( \Gamma_{\text{max,tho}} \),
the corresponding \( \Gamma_{\text{max,tho}} \) value and multiplying by 100.

As mentioned above, the adsorption of vesicles to an
antibody-coated surface could occur through multiple
point attachment, depending on the antibody surface
density. The adsorption of a finite number of vesicles per
unit area can significantly reduce the probability that other
vesicles will find additional area with dimensions large
enough to accommodate their size. This phenomenon has
been called the large ligand effect, and some statistical
mechanical theories have been developed to estimate its
importance (Schaaf and Talbot, 1989; Stankowski, 1983;
Andrews, 1976). An equation describing the fractional
surface coverage of vesicles, \( \theta \), as a function of the ligand
concentration, \( [L] \), is (Schaaf and Talbot, 1989; Andrews,
1976)

\[ \theta = K_{\text{eff}} [L] P(\theta) \]  

(3)

where \( K_{\text{eff}} \) is the effective association constant and \( P(\theta) \)
is a term describing the probability of finding an area
large enough to accommodate a finite sphere. Schaaf and
Talbot (1989) obtained an expression for the probability
term as a function of the fractional surface coverage in the form

\[ P(\theta) = \left\{ 2 - \frac{2}{(1 - \theta)^3} + \frac{7\theta}{8(1 - \theta)^3} + \frac{7}{8} \ln(1 - \theta) \right\} \]  

(4)

The fractional coverage for vesicles, \( \theta \), is equal to the ligand
surface density (\( \Gamma \)) multiplied by area per vesicle (\( a \)). The
can be written as [14C]HBVs adsorption data to determine \( K_{\text{eff}} \) and \( a \) values by weighted
nonlinear least-squares from experimental plots of \( \Gamma \) vs
[14C]HBVs. The theoretical area per vesicle, \( a_{\text{theo}} \), was calculated
from the vesicle cross-sectional area and compared to
the area per vesicle from the large ligand model.

The Langmuir equation is strictly valid when each site
binds only one molecule, all of the binding sites have the
same affinity, there is no interaction between adsorbed
molecules, and the adsorbed molecules form at most a
monolayer. Since polyclonal antibodies often have a
distribution of association constants (Goding, 1985), p
46), the use of the Langmuir equation may be inappropriate
for characterizing the binding with antigen. Researchers
have recognized for some time that polyclonal antibodies
are heterogeneous and have used the Gaussian distribution
to model binding affinity (Pauling et al., 1944; Karush,
1956). Another method describes the association of
antibody with antigen in terms of an average association
constant and a heterogeneity index (Nisonoff and
Pressman, 1957; Watt and Voss, 1978) using a method outlined
by Sips (1948). This method has the advantage that
adsorption isotherm data can be fit to an equation
that accounts for binding site heterogeneity directly. The Sips
equation, in terms of $\Gamma$ and $\Gamma_{\text{max,exp}}$ defined earlier, is

$$\Gamma = \frac{\Gamma_{\text{max,exp}}(K_a[L])^h}{1 + (K_a[L])^h} \quad \text{(5)}$$

where $K_a$ is the average association constant and $h$ is the heterogeneity index, which varies between 1 and 0. When the antibody is homogeneous, $h$ is 1 and the Sips equation is the same as the Langmuir isotherm (eq 1). As the antibody heterogeneity increases, the value for $h$ decreases.

The Sips model (eq 5) was fit to adsorption data for [14C]-biotin, B-HRP, [14C]BHRP, and [14C]HBVs to determine average association constants and indices of heterogeneity using weighted nonlinear least-squares analysis.

**Competitive Assays Using HBVs and B-HRP.**

Competitive assays to detect biotin were performed using 0.1 mol % HBVs, B-HRP, and [14C]HBVs. Microtiter plates were coated with antibody and blocked with 1 wt % casein, as described in the previous section, using ABA solutions ranging from 0.5 to 10 $\mu$g/mL. Aliquots (100 $\mu$L) of HBVs in PBS (or B-HRP in 0.5 wt % BSA in PBS) containing biotin at concentrations ranging from 1 pM to 100 $\mu$M (1 pM to 10 $\mu$M) for [14C]HBVs were added to the wells and incubated at 37 °C. In addition, a blank solution containing vesicles (or B-HRP) without biotin was included. The concentrations of 0.1 wt % HBVs were 20, 31, and 61 $\mu$L, the concentrations of B-HRP were 1, 5, and 10 nM, and the concentrations of [14C]HBVs were 56 and 113 pM. Preliminary investigations indicated that binding was complete after incubation times of 2 h for vesicles and 1 h for B-HRP. As a result, these incubation times were used in the assays. After the HBVs were rinsed four times with PBS (B-HRP wells were rinsed three times), 100 $\mu$L of substrate solution in 50 mM citrate buffer (pH 4.0) was added. With HBVs, the substrate contained 2 mM ABTS and 2.75 mM H$_2$O$_2$, while with B-HRP the substrate contained 1 mM ABTS and 2.75 mM H$_2$O$_2$ which are the optimal substrate conditions found from earlier work (Jones et al., 1993). At each biotin concentration, the signals ($\Delta A$/min) from three ABA-coated wells (S$_{ABA}$) and from three casein-blocked wells (S$_{case}$) were measured with the Biotek EL 340 plate reader at 410 nm and averaged. The analysis of competitive assays is discussed in the next section.

**Analysis of Competitive Assays.** In competitive immunosorbent assays, a fixed concentration of labeled antigen competes with unlabeled antigen for surface-adsorbed antibody-binding sites. At low unlabeled antigen concentration, the amount of labeled antigen bound to the plate is high, so that the signal measured is high. As the concentration of unlabeled antigen increases, the amount of labeled antigen bound to the plate decreases, as does the signal. The specific signal ($S$) can be normalized by the average signal at low competing antigen concentrations ($S_a$), so that the response ($S/S_a$) ranges from 0.0 to 1.0. A plot of $S/S_a$ against the logarithm of the antigen concentration results in a sigmoidal curve for small labeled antigens (Campfield, 1983). The performance of competitive assays was assessed by determining three parameters from plots of $S/S_a$ vs the antigen concentration, the least detectable dose (LDD), the slope of the response (SR), and the half-maximal dose (HMD) (Campfield, 1983), in addition to the magnitude of the $S_a$ value. The LDD is the lowest concentration of antigen that can be determined from the response curve. The SR is an indication of the sensitivity of the assay ($\Delta$signal/$\Delta$antigen concentration). A large negative slope is desired. The HMD is the concentration of antigen that displaces the response ($S/S_a$) to 0.5. The $S_a$ value can be used to estimate the useful range of antibody surface densities and labeled antigen concentrations for designing competitive assays. An $S_a$ value of 1 $\Delta$A/min is an appropriate benchmark value to use in competitive assays. At this target $S_a$ value, the measured range of enzyme activity is broad (0.0–1 $\Delta$A/min), while at $S_a$ values higher than 2 $\Delta$A/min, the signal becomes difficult to measure accurately due to the optical limitations of the plate reader.

These performance criteria were determined from competitive assay data. The specific signal ($S$) from the adsorbed enzyme label (HBVs or B-HRP) was calculated by subtracting the signal obtained in casein-coated (S$_{case}$) wells from the signal in ABA-coated (S$_{ABA}$) wells. The average maximum specific signal ($S_a$) was calculated from the specific signals at biotin concentrations approaching zero. The response ($S/S_a$) was plotted against the logarithm of the biotin concentration. The data points that were less than 2$\sigma$ from $S/S_a = 1$ were fit by logarithmic regression analysis to determine the SR. The LDD, defined as the antigen concentration that results in a displacement of the maximum response equal to twice the standard deviation about the $S/S_a = 1$ value (1 – 2$\sigma$) (Campfield, 1983), was determined from the logarithmic curve fit. The HMD was calculated from the logarithmic curve fit at an $S/S_a$ value of 0.5.

**Results and Discussion**

**Adsorption of ABA and ABA Activity on Polystyrene Wells.** Figure 2 shows the ABA adsorption isotherm at 4 °C, in which antibody concentrations ranging from 0.05 to 20 $\mu$g/mL resulted in antibody surface concentrations ranging from 5 to 310 ng/cm$^2$. Kinetic measurements.
of ABA adsorption indicate that the total antibody adsorbed remains constant between 6 and 24 h under these conditions (Jones, 1993). The maximum surface concentration of adsorbed ABA (310 ± 10 ng/cm² at 20 μg/mL bulk ABA concentration) is slightly lower than the binding capacity of 400 ng/cm² reported for these plates by Costar. The maximum surface concentration in Figure 2a (310 ng/cm²) represents an area per antibody molecule of 5600 Å² or 1.17 × 10⁻¹² mol/cm². If we assume that the antibody molecules adsorb to the surface as close-packed spheres, this surface coverage gives an antibody diameter of approximately 100 Å. This size is in the range of determinations by electron microscopy measurements of human and rabbit immunoglobulins, which showed that the antibody diameters were between 80 and 120 Å (Feinstein and Rowe, 1965). As a result, it was assumed that the ABA formed a monolayer at 310 ng/cm². The antibody surface density increased linearly at bulk ABA concentrations between 0.05 and 2.0 pg/mL, with antibody concentrations greater than 20 pg/mL. The reason that the ABA formed a monolayer at 310 ng/cm² is slightly heterogeneous. The association constant for antibody adsorbing to ABA is approximately 100 times and 10 times smaller than the association constants reported for HBVs and B-HRP, respectively (Jones et al., 1993), indicating that biotinylated vesicles adsorb most strongly to ABA, followed by B-HRP and free biotin. The reason for the increase in the association constants could be due to additional attractive forces (electrostatic, hydrogen bonding, van der Waals, or hydrophobic) between the antibody and the adsorbed species. Adsorbed enzyme could interact more extensively through such forces than biotin alone, while the additional interaction between vesicles and the surface would be even greater than that of enzyme.

The activity of adsorbed ABA was estimated from the number of moles of specifically bound [¹⁴C]biotin at concentrations greater than 2 μM [¹⁴C]biotin (see Figure 3). The maximum surface density of specifically adsorbed [¹⁴C]biotin was found to be 4.3 × 10¹¹ molecules/cm² or 7.2 × 10⁻¹² mol/cm². From Figure 2a, the total antibody adsorbed to the surface at 10 μg/mL bulk ABA concentration is 290 ng/cm² or 1.8 × 10⁻¹² mol/cm². If we assume two binding sites per active ABA molecule, the percent active ABA is 20% at 290 ng/cm². This percent activity of adsorbed antibody is in reasonable agreement with the value of 10% for anti-theophylline adsorbed to a polystyrene surface (Plant et al., 1991). The percent activity of adsorbed ABA could depend on the antibody surface density. At high antibody surface densities, the well surface area occupied per antibody molecule is smaller than it is at low antibody surface densities. This situation could lead to more extensive hydrophobic interactions of the antibody with the polystyrene plate at low antibody surface densities, which could result in lower activity of adsorbed antibody. Since experiments to determine the activity of adsorbed ABA were conducted only at 290 ng/cm², the activity is known only at this particular ABA surface density. The surface density of active antibody (or, equivalently, surface density of binding sites) on microtiter wells is important to the adsorption of HBVs, because vesicles are much larger than an individual antibody molecule and are likely to attach to the surface through multiple point attachment.

Results of HRP Immobilization to Vesicles. Table 1 summarizes the results of immobilizing HRP to biotinylated vesicles in terms of the micrograms of HRP immobilized per micromole of total lipid, average hydrodynamic diameter, number of HRP molecules immobilized per vesicle, and the number of biotin molecules on the outer vesicle surface. Methods for computing these quantities were described in the Materials and Methods section in Jones et al. (1993). Table 1 shows that 91 μg/μmol were immobilized to 0.1 mol % HBVs and 140 μg/μL to [¹⁴C]HBVs. These HRP to lipid ratios are similar to the ratios obtained in previous work (Jones et al., 1993). It was found from quasi-elastic light-scattering measurements that the 0.1 mol % HBVs had a mean diameter of 1460 ± 115 Å and [¹⁴C]HBVs had a mean diameter of 890 ± 130 Å. The slightly larger size and the lower immobilized enzyme concentration of 0.1 mol % HBVs in comparison to [¹⁴C]HBVs could result from a small amount of vesicle cross-linking through HRP molecules during enzyme immobilization.

Table 1 also lists the number of HRP molecules immobilized per vesicle and the number of outer biotin molecules per vesicle for the two preparations. The number of HRP molecules immobilized per vesicle for 0.1 mol % HBVs was calculated to be 550, which is a result of the larger average size of the HRP-vesicle conjugates. For [¹⁴C]HBVs, the number of HRP molecules immobi-

**Figure 3.** Surface density of [¹⁴C]biotin on microplate wells coated with 290 ng/cm² ABA as a function of the [¹⁴C]biotin concentration with the Langmuir equation (---), \( K = 7.25 \times 10^6 \) M⁻¹, and with the Sips equation (--), \( K_a = 3.71 \times 10^7 \) M⁻¹, \( h = 0.82 \). The \( \Gamma_{max} \) value was determined to be 4.33 × 10⁻¹⁰ [¹⁴C]biotin/cm².

**Table 1.** Results of HRP Conjugated to Biotinylated Vesicles

<table>
<thead>
<tr>
<th>HRP/lipid (μg/μmol)</th>
<th>diameter (Å)</th>
<th>HRP per vesicle</th>
<th>outer biotin per vesicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mol % HBVs</td>
<td>91</td>
<td>1480 ± 115</td>
<td>550</td>
</tr>
<tr>
<td>[¹⁴C]HBVs</td>
<td>140</td>
<td>890 ± 130</td>
<td>260</td>
</tr>
</tbody>
</table>
Figure 4. Surface density of B-HRP on microplate wells coated as measured by enzymatic assay) with 290 ng/cm² ABA as a function of the B-HRP concentration with the Langmuir equation (- - -), K = 2.72 × 10⁹ M⁻¹, and with the Sips equation (---), K₀ = 2.93 × 10⁶ M⁻¹, h = 1.0. The I_max value was 6 × 10⁶ B-HRP/cm².

HBVs or P4C₁BHRP. As will be discussed later, enzyme activity of P4C₁HBVs and P4C₁BHRP was also determined at pH 6.0 (Jones et al., 1993). These results indicated that 1

Enzyme Activity and Specific Radioactivity of [¹⁴C]HBVs and [¹⁴C]BHRP. The kinetic parameters (Kₘ, V_max, and kₚ) for HRP immobilized to radiolabeled vesicles at pH 6.0 and for [¹⁴C]BHRP at pH 4.0 were determined. For vesicles, it was found that Kₘ, V_max, and kₚ were 0.463 mM, 0.192 ΔA/min, and 5500 (ΔA/min)/(mg/mL), respectively, using an enzyme concentration of 3.49 × 10⁻⁵ mg/mL. For [¹⁴C]BHRP, it was found that Kₘ, V_max, and kₚ were 0.164 mM, 0.60 ΔA/min, and 1.24 × 10⁻⁴ (ΔA/min)/(mg/mL), respectively, using an enzyme concentration of 4.84 × 10⁻⁶ mg/mL. Earlier measurements of Kₘ and kₚ were found to be 0.162 mM and 9.3 × 10⁻⁴ (ΔA/min)/(mg/mL), respectively, for B-HRP (Jones et al., 1993). These values are in reasonable agreement with values found for [¹⁴C]BHRP, indicating that the radiolabeling procedure had little or no effect on the enzyme activity. The kₚ value for [¹⁴C]HBVs measured at pH 6.0 is much lower than the kₚ value for [¹⁴C]BHRP measured at pH 4.0, because the pH optimum for HRP activity is 4.0 (Jones et al., 1993). The specific radioactivity in terms of the disintegrations per minute (dpm) for vesicles was found to be 5.56 × 10⁷ dpm/vesicle, and the enzyme specific radioactivity was found to be 2.28 × 10⁻⁸ dpm/enzyme molecule. The vesicle and enzyme specific radioactivity values and the values for Kₘ and kₚ noted above were used to estimate the maximum surface coverage of [¹⁴C]HBVs and [¹⁴C]BHRP adsorbing to ABA-coated wells. The results of which will be discussed later. The enzyme activity of [¹⁴C]HBVs and [¹⁴C]BHRP was also determined at pH 4.0, with and without 1 wt % C₁₂E₅ as the substrate solution. For [¹⁴C]HBVs, the activity values were found to be 1.01 ± 0.05 ΔA/min without surfactant and 1.094 ± 0.05 ΔA/min with surfactant. For [¹⁴C]BHRP, the activity values were found to be 0.495 ± 0.025 ΔA/min with surfactant and 0.506 ± 0.025 ΔA/min without surfactant. These results indicated that 1 wt % C₁₂E₅ did not significantly affect the enzyme activity of either [¹⁴C]HBVs or [¹⁴C]BHRP. As will be discussed later, enzyme labels adsorbed to ABA-coated wells were solubilized by adding 1 wt % C₁₂E₅ and the effect of surfactant on the enzyme activity must be known.

B-HRP, [¹⁴C]BHRP, and [¹⁴C]HBV Adsorption to ABA-Coated Wells. Figure 4 shows the adsorption of B-HRP to ABA-coated polystyrene wells, as measured by HRP activity, at an antibody surface density of 290 ng/cm² (10 μg/mL bulk ABA) at equilibrium concentrations ranging from 0.05 to 50 nM. The B-HRP surface density levels out at a maximum surface density of 6 × 10⁶ molecules/cm², with an S_max value of 1.5 AA/min, near a B-HRP concentration of 10 nM. The background signal was less than 1% of the total signal generated from the ABA-coated wells throughout the range of B-HRP concentrations used. The association constant, K, determined from the Langmuir equation was 2.7 × 10⁸ M⁻¹. The average association constant, K₀, and the heterogeneity index, h, determined from the Sips equation were 2.9 × 10⁸ M⁻¹ and 1.0, respectively. The two models were indistinguishable, indicating that the antibody heterogeneity was negligible when interacting with B-HRP. The absence of antibody heterogeneity when binding with B-HRP could result from the biotin moiety being covalently attached to the enzyme and therefore restricted in its possible binding orientations. Free biotin, on the other hand, has no such restrictions. The B-HRP S_max value, association constant, and percent nonspecific signal values are comparable to previous results (Jones et al., 1993). This experiment established the practical B-HRP concentration range for use in competitive assays. At B-HRP concentrations ranging from 1 to 10 nM, the specific signal ranged from 0.2 to 1.5 AA/min. When these B-HRP concentrations are used in competitive assays, the S_max value should likewise range from 1.5 to 0.2 AA/min when the ABA surface density is 290 ng/cm².

The adsorption isotherms for [¹⁴C]BHRP to ABA-coated polystyrene wells at an antibody surface density of 290 ng/cm² (10 μg/mL bulk ABA), as determined by the enzyme label and by the radiolabel at equilibrium concentrations ranging from 0.05 nM to 0.5 μM [¹⁴C]BHRP, are shown in Figure 5. The Langmuir model and Sips model fits are also shown. The [¹⁴C]BHRP surface density from the enzyme label levels out at 6 × 10⁶ molecules/cm², with an S_max value of 1.5 AA/min, near a concentration of 10 nM, which agrees with the non-radiolabeled case (see Figure 4). The association constant (K) determined from the enzyme label using the Langmuir model (eq 1).
There are two likely explanations for this effect. One is that there are limitations to the diffusion of substrate to and from the enzyme on the adsorbed vesicle surface, and the other is that enzyme molecules on the underside of the vesicle are occluded or denatured as the vesicle adsorbs to the ABA-coated surface. When 1 wt% C₁₂E₅ was added to wells to solubilize adsorbed [¹⁴C]BHVP and the enzyme activity was measured, no difference was observed when compared to wells that had no surfactant added, as seen in Figure 6a. The surfactant probably solubilized the enzyme, but did not disrupt the antibody-antigen bond. These experiments indicate that the enzyme activity is probably inhibited through binding to antibody on the well surface and that diffusion limitations of substrate to the enzyme are unlikely. A notable difference between the isotherms depicted in Figures 4 and 5 is that the apparent surface density determined from the enzyme label begins to decrease at enzyme label concentrations above 20 nM, but the surface density determined from the radiolabel remains constant. The drop in the enzyme signal is probably due to enzyme inhibition caused by surface crowding effects (Pesce et al., 1981).

As discussed earlier, the surface density of binding sites determined from the adsorption of [¹⁴C]biotin at an ABA surface density of 290 ng/cm² is 4.3 × 10¹¹ binding sites/cm². This value can be compared to the maximum surface density of [¹⁴C]BHVP as determined by the radiolabel (1.8 × 10¹¹ molecules/cm²) of 0.42 [¹⁴C]BHVP binding site, indicating that 42% of the binding sites are occupied by the biotinylated enzyme. This could be due to sites being inaccessible to the labeled enzyme through steric hindrance.

The adsorption isotherm for [¹⁴C]BHVs adsorbed to ABA-coated polystyrene wells at an antibody surface density of 79 ng/cm² (1 μg/mL bulk ABA), as determined by the enzyme label and the radiolabel at concentrations ranging from 4 pM to 7 nM [¹⁴C]BHVs, is shown in Figure 7. Figure 7a shows the model fits to the Langmuir model (eq 1). Figure 7b shows the model fits to the large ligand model (eq 3), and Figure 7c shows the model fits to the Sips model (eq 5). The Langmuir association constant determined from the enzyme label was 1.3 × 10⁸ M⁻¹, while the radiolabel was 1.7 × 10⁹ M⁻¹. The large ligand equation gave Kₐ and a values of 3.7 × 10⁸ M⁻¹ and 4 × 10⁻¹⁰ cm², respectively, using the enzyme label and Kₐ and a values of 4.9 × 10⁸ M⁻¹ and 3.2 × 10⁻¹⁰ cm², respectively, using the radiolabel. The Sips equation gave Kₛ and h values of 1.7 × 10⁹ M⁻¹ and 1.1, respectively, using the enzyme label and Kₛ and h values of 1.7 × 10⁹ M⁻¹ and 1.0, respectively, using the radiolabel. These results indicate that, for a given model, the two different labels did not give significantly different association constants. According to Sips (1948), the heterogeneity index varies between 1 and 0, and values greater than 1 are not physically realistic. The h value of 1 determined from the enzyme label is probably the result of experimental deviation and is not considered significant. Thus, the antibody heterogeneity is negligible when interacting with vesicles. As seen in Figure 7, the [¹⁴C]BHVP surface density from the enzyme label levels out at 8.6 × 10⁸ vesicles/cm², while the surface density determined from the radiolabel levels out at 1 × 10⁸ vesicles/cm² near a vesicle concentration of 4 nM, indicating that the maximum surface density determined from the enzyme label was about 35% lower than that determined from the radiolabel. There are two likely explanations for this effect. One is that there are limitations to the diffusion of substrate to and from the enzyme on the adsorbed vesicle surface, and the other is that enzyme molecules on the underside of the vesicle are occluded or denatured as the vesicle adsorbs to the ABA-coated surface. When 1 wt% C₁₂E₅ was added to wells to solubilize adsorbed [¹⁴C]BHVPs and the enzyme activity was measured, no difference was observed when compared to wells that had no surfactant added, as seen in Figure 6b. Since the concentration of

![Figure 6. Effect of solubilizing [¹⁴C]BHVP or [¹⁴C]BHVs adsorbed to microplate wells with 1 wt% C₁₂E₅ just before the addition of substrate. Wells were coated with 290 ng/cm² ABA. (a) Measured activity for [¹⁴C]BHVP with surfactant (O) and without surfactant (■). (b) Measured activity for [¹⁴C]BHVPs with surfactant (O) and without surfactant (■).](image-url)
Table 2 summarizes the results of adsorbing [14C]HBVs to wells coated with 79 and 290 ng/cm² ABA as determined by both the enzyme label and the radiolabel in terms of the maximum signal, Smax, maximum experimental vesicle surface density, \( \Gamma_{\text{max}, \text{exp}} \), percent surface coverage, the association constant from the Langmuir equation, \( K \), the average association constant, \( K_a \), and the heterogeneity index, \( h \), from the Sips model, the effective association constant, \( K_{\text{eff}} \), and the area per vesicle, \( a \), from the large ligand model, and the ratio of \( a \) to the theoretical area per vesicle, \( a_{\text{theo.}} \). The \( S_{\text{max}} \) values were used to determine the maximum surface density, \( \Gamma_{\text{max}, \text{exp}} \), as discussed above. Since the [14C]HBV diameter is 890 Å (see Table 1), the theoretical area per vesicle \( a_{\text{theo.}} \) is 6.22 × 10⁻¹⁰ cm², and the maximum theoretical vesicle surface coverage under close-packed conditions is 1.48 × 10¹⁰ vesicles/cm². Comparison of this value to the maximum surface density of vesicles listed in Table 2 gives experimental surface coverages of vesicles between 4.5 and 6.8% at 79 ng/cm² and between 27 and 44% at 290 ng/cm² ABA surface density. As discussed above, the difference between the values determined from the enzyme label and the radiolabel is probably due to denaturation or occlusion of enzyme molecules on the underside of adsorbed vesicles. The association constants from the Langmuir equation, the Sips equation, and the large ligand equation are listed in Table 2. The Langmuir association constants \( K \) were approximately 1.5 × 10⁶ and 2.0 × 10⁶ M⁻¹ at 79 and 290 ng/cm², respectively. The Sips association constants \( K_a \) were approximately 1.7 × 10⁹ M⁻¹ at 79 ng/cm² and between 2.3 × 10⁹ and 3.1 × 10⁹ M⁻¹ at 290 ng/cm². The large ligand association constants \( K_{\text{eff}} \) were between 3.7 × 10⁸ and 4.9 × 10⁸ M⁻¹ at 79 ng/cm² and between 2.9 × 10⁸ and 3.8 × 10⁸ M⁻¹ at 290 ng/cm². These results indicate that the strength of vesicle binding did not depend on the ABA surface density over the range studied. The heterogeneity index \( h \) was approximately 1 at 79 ng/cm² and 1.3 at 290 ng/cm². These results indicate that the Sips model predicted negligible antibody heterogeneity for vesicle binding at low antibody surface density, but gave anomalous heterogeneity at high antibody surface density. The reason for \( h \) values greater than 1 at high antibody surface density might be due to multiple point attachment of vesicles to adsorbed antibody, which would result in an isotherm that has a heterogeneity index larger than theoretically possible as predicted by the Sips equation. The ratio of \( a \) from the large ligand equation to \( a_{\text{theo.}} \) listed in Table 2 is between 6.4 and 6.1 at 79 ng/cm² and between 0.64 and 0.46 at 290 ng/cm². The overestimation of the area per vesicle by the large ligand model at low antibody surface density is probably due to low surface binding site density and not size exclusion effects. At high antibody surface coverage (290 ng/cm²), the slight underestimation of the area per vesicle at ABA surface density is not considered significant, since experimental nonidealities such as vesicle size polydispersity could lead to higher vesicle surface coverages. The Langmuir association constants determined for vesicles prepared with 0.1 mol % DMPE-LC-biotin are on the same order of magnitude (10⁶ M⁻¹) as those determined for vesicles prepared with 2.5 mol % DMPE-LC-biotin in earlier work (Jones et al., 1993). This indicates that the equilibrium binding affinity of biotin-conjugated vesicles to ABA-coated surfaces is not changed significantly when the number of biotin molecules per vesicle is changed from ~1500 to 30. As a result, association constants are based on vesicle molar concentrations rather than molar concentrations of biotin.

Figure 7. Surface density of [14C]HBVs on microplate wells coated with 79 ng/cm² ABA as a function of the [14C]HBV concentration. (a) Surface density as determined by the enzyme label (○) and the radiolabel (○) with the Langmuir equation (---) gave \( K = 1.3 \times 10⁸ \) M⁻¹ for the enzyme label and \( K = 1.7 \times 10⁸ \) M⁻¹ for the radiolabel. (b) Surface density as determined by the enzyme label (○) and the radiolabel (○) with the large ligand equation (---) gave \( K_{\text{eff}} = 3.7 \times 10⁸ \) M⁻¹ for the enzyme label and \( K_{\text{eff}} = 4.9 \times 10⁸ \) M⁻¹ for the radiolabel. (c) Surface density as determined by the enzyme label (○) and the radiolabel (○) with the Sips equation (---) gave \( K_a = 1.7 \times 10⁹ \) M⁻¹ and \( h = 1.1 \) for the enzyme label and \( K_a = 1.7 \times 10⁹ \) M⁻¹ and \( h = 1.0 \) for the radiolabel. The \( \Gamma_{\text{max}, \text{exp}} \) values were 6.6 × 10⁸ and 1 × 10⁹ [14C]-HBV/cm² for the enzyme label and the radiolabel, respectively.

1 wt % C₁₂E₅ (0.024 M) is far above the CMC of ~0.1 mM (Rosen et al., 1982), it is probably high enough to solubilize vesicles. Under these conditions, the surfactant and enzyme covalently attached to phospholipid could form molecular aggregates in which the active enzyme remains inaccessible to substrate. Another possibility is that after vesicle adsorption to the surface the enzyme molecules on the underside of the vesicle are denatured through hydrophobic interactions with proteins or the polystyrene surface.
Table 2. Summary of Binding Parameters for [$^{14}$C]HBVs Adsorbing to ABA-Coated Wells

<table>
<thead>
<tr>
<th>ABA surface density (ng/cm²)</th>
<th>label type</th>
<th>$S_{max}$ (vesicle/cm² × 10⁻⁹)</th>
<th>% surface coverage</th>
<th>Langmuir $K_a$ (nM⁻¹)</th>
<th>Sips $K_a$ (nM⁻¹)</th>
<th>large ligand $K_a$ (nM⁻¹)</th>
<th>$h$ (cm²/vesicle × 10⁰³)</th>
<th>$a$</th>
<th>$a/a_{ab}$</th>
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</thead>
<tbody>
<tr>
<td>79</td>
<td>enzyme</td>
<td>0.18 (ΔA/min)</td>
<td>0.66</td>
<td>4.5</td>
<td>1.3</td>
<td>1.7</td>
<td>1.1</td>
<td>0.37</td>
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<tr>
<td>79</td>
<td>radiolabel</td>
<td>615 (dpm/well)</td>
<td>1.0</td>
<td>6.8</td>
<td>1.7</td>
<td>1.7</td>
<td>1.0</td>
<td>0.49</td>
<td>3.2</td>
</tr>
<tr>
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<td>enzyme</td>
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<td>1.3</td>
<td>0.29</td>
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<td>44</td>
<td>2.0</td>
<td>2.3</td>
<td>1.3</td>
<td>0.38</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* $K_a$ was determined from the Langmuir equation. * $K_{0a}$ was determined from the Sips equation. * $K_{a}$ was determined from the large ligand equation.

Figure 8. Typical competitive assay results in which the normalized specific signal ($S/S_o$) is plotted as a function of the biotin in concentrations ranging from $10^{-12}$ to $10^{-4}$ M. The blank (zero biotin) signal is plotted at a biotin concentration of $10^{-15}$ M. (a) Assay using 61 pM 0.1 mol % HBVs. (b) Assay using 1 nM B-HRP. (c) Assay using 66 pM [$^{14}$C]HBVs.

Competitive Assay Results. Typical results from competitive assays are shown in Figure 8, in which the specific signal ($S$) has been normalized by the average maximum specific signal ($S_o$) and plotted vs the biotin concentration from $10^{-12}$ to $10^{-4}$ M. The blank (no biotin in solution) is plotted at a biotin concentration of $10^{-15}$ M.

Figure 9. Least detectable dose (LDD) values for B-HRP (●) and for vesicles (○) as a function of the bulk ABA concentration used to coat the wells.

Results using 0.1 mol % HBVs, B-HRP, and [$^{14}$C]HBVs are shown in Figure 9a–c, respectively. The ABA surface density in Figure 9a was 79 ng/cm² (1 μg/mL bulk ABA) and the 0.1 mol % HBV concentration was 61 pM, which resulted in an $S_o$ value of 1.2 ΔA/min. The ABA surface density in Figure 9b was 250 ng/cm² (5 μg/mL bulk ABA) and the B-HRP concentration was 1 nM, giving an $S_o$ value of 0.18 ΔA/min. The ABA surface density in Figure 9c was 50 ng/cm² (0.75 μg/mL bulk ABA) and the [$^{14}$C]-HBV concentration was 56 pM, which resulted in an $S_o$ value of 0.46 ΔA/min. The least detectable dose (LDD) values, slope of the response (SR), half-maximal dose (HMD) values, and $S_o$ values, determined for competitive assays as described earlier, are shown in Figures 9–12, with sketched-in lines depicting the approximate trends in the performance criteria. The LDD, SR, and HMD values were not significantly different for 0.1 mol % HBVs compared to the values for [$^{14}$C]HBVs, so the results are combined in Figures 9–11.

Figure 9 shows the least detectable dose (LDD) values for HBVs and B-HRP at bulk ABA concentrations ranging from 0.5 to 10 μg/mL. For HBVs, the LDD was approximately 1 nM, while for B-HRP the LDD was approximately 10 nM. This indicates that competitive assays employing HBVs are capable of detecting approximately an order of magnitude lower antigen concentration than assays using B-HRP.

Figure 10 shows the slope of the response (SR) at bulk ABA concentrations ranging from 0.5 to 10 μg/mL. For B-HRP, the slope ranged from −0.42 to −0.26 M⁻¹ and was insensitive to the ABA surface density. For HBVs, the slope ranged from −0.33 to −0.19 M⁻¹ when 0.5 to 1.0 μg/mL bulk ABA was used to coat the plates. At higher ABA concentrations, the slope increased and ranged from −0.18 to −0.07 M⁻¹ when 2 to 10 μg/mL ABA was used to coat the plates. The increase in slope with HBVs indicates that the assay becomes less sensitive as the antibody surface density increases. The decrease in sensitivity is probably due to multiple point attachment of vesicles to
concentrations of both HBVs and B-HRP. The practical range of B-HRP measurement from 0.5 to the HMD increases.

increased as the bulk ABA concentration increased for HBVs and B-HRP at bulk ABA concentrations ranging is shown in Figure 1. The maximum specific signals at 5 pg/mL ABA for HBVs increased monotonically from 290 ng/cm². 0.1 mol % HBVs cover approximately 44, 20, or 8 binding sites when wells are coated with 2, 1, or 0.5 μg/mL bulk ABA, respectively. Similarly, [14C]HBVs, with a diameter of 890 Å, would cover approximately 27, 16, 7, or 3 binding sites on wells coated with 290, 2, 1, or 0.5 μg/mL bulk ABA, respectively. As the number of binding sites covered per vesicle decreases, the likelihood of multiple attachment of vesicles to the surface decreases. As seen in Figure 10, the effect of multiple binding at low antibody surface density is small, since the slopes for vesicles are comparable to the slopes for B-HRP.

Figure 11 shows the half-maximal dose (HMD) values for HBVs and B-HRP at bulk ABA concentrations ranging from 0.5 to 10 μg/mL. The B-HRP HMD values are independent of the ABA surface density and range from 5 x 10^-8 to 1 x 10^-7 M. On the other hand, the HMD for HBVs increased monotonically from 10^-8 M at 0.5 μg/mL bulk ABA to 10^-7 M at 10 μg/mL bulk ABA. The increase in HMD for HBVs as the ABA surface density increases is probably due to multiple binding of vesicles to the surface, as discussed above. When multiple attachment of vesicles occurs, the slope of the response increases and the HMD increases.

The maximum specific signal (Sₕ) for HBVs and B-HRP is shown in Figure 12 at bulk ABA concentrations used to coat the wells ranging from 0.5 to 10 μg/mL. The Sₕ value increased as the bulk ABA concentration increased for both HBVs and B-HRP. The practical range of B-HRP concentrations established from adsorption isotherm measurement (1-10 nM) is represented in Figure 12. The maximum specific signals at 10 μg/mL ABA for B-HRP concentrations of 10 and 1 nM were 1.4 and 0.2 ΔA/min, respectively. These signals agree with Sₕ values obtained for B-HRP adsorption, indicating that the isotherm and competitive assay data were consistent. As discussed earlier, an appropriate Sₕ value for competitive assays is ~1 ΔA/min. At a bulk ABA concentration of 5 μg/mL, using a B-HRP concentration of 10 nM results in an Sₕ value of 1.1 ΔA/min, which is comparable to this target value. Therefore, a 5 μg/mL bulk antibody concentration may be thought of as representing the lower concentration limit when coating plates for assays using B-HRP. At a bulk ABA concentration of 1 μg/mL, the Sₕ value for 10 nM B-HRP is 0.1 ΔA/min, which is about 10 times less than the Sₕ value of 1.2 ΔA/min found using 61 pM 0.1 wt % HBVs. This demonstrates that adequate signals (~1 ΔA/min) can be obtained using HBVs at antibody concentrations that are at least 5 times below the antibody concentrations that result in a comparable signal using B-HRP. The Sₕ values for the [14C]HBVs were about half as large as the Sₕ values for 0.1 mol % HBVs at comparable bulk vesicle concentrations. For example, as seen in Figure 12, the Sₕ values using 113 pM [14C]HBVs were comparable to Sₕ values using 61 pM 0.1 mol % HBVs. Similarly, the Sₕ values using 56 pM [14C]HBVs were comparable to Sₕ values using 31 pM 0.1 mol % HBVs. This is because 0.1 mol % HBVs had 650 HRPs/vesicle, while [14C]HBVs had 260 HRPs/vesicle (see Table 1).

Conclusions

Horseradish peroxidase (HRP) was immobilized to unilamellar vesicles prepared with 0.1 mol % biotinylated phospholipid to obtain HRP conjugated to biotinylated
vesicles (0.1 mol % HBVs and [14C]BV). In addition, radiolabeled B-HRP ([14C]BHRP) was prepared. The adsorption isotherms of [14C]BHRP and [14C]BV were determined using both enzyme and radiolabel. For [14C]-BHRP it was found that the association constants were independent of label type (approximately 1 x 10^5 M^-1). The maximum [14C]BHRP surface density value determined from the enzyme label was a factor of 3 lower than that determined from the radiolabel, probably due to enzyme inhibition by binding with antibody. For [14C]-HBVs it was found that the association constants were independent of the antibody surface density and label type (approximately 1 x 10^5 M^-1). The maximum [14C]-HBV surface density determined from the enzyme label was about 35% lower than that determined from the radiolabel, probably due to enzyme denaturation or active site occlusion by adsorbed vesicles. The activity of the adsorbed antibody was found to be 20% using radiolabeled antigen and slightly heterogeneous. Competitive assays were performed for the model antigen biotin using either 0.1 mol % HBVs, [14C]HBVs or B-HRP as the marker-labeled antigen, and the results were analyzed in terms of the least detectable dose (LDD), the slope of the response curve (SR), the half-maximal dose (HMD), and the maximum specific signal at low biotin concentrations (S₀). It was found that the assays performed with vesicles depended strongly on the ABA surface density, while assays with B-HRP were relatively insensitive to the antibody surface density. This is most likely due to multiple point attachment of vesicles to the surface. The LDD for vesicles at low antibody surface density was approximately 10^-9 M, while the LDD for B-HRP was approximately 10^-8 M. This indicates that HBVs are capable of detecting antigen concentrations 10 times below those that could be detected with B-HRP. It was also found that the B-HRP slopes and HMD values were independent of the ABA surface density, while the HBV slopes and HMD values strongly depended on the ABA surface density. At low bulk ABA concentrations (∼1 μg/mL), the HBV slopes and HMD values were comparable to the B-HRP slopes and HMD values. As the amount of antibody used to coat wells increased, the HBV slopes became less negative and the HMD values increased. Finally, the S₀ values indicated that adequate signals (∼1 ΔA/min) can be achieved with HBVs at bulk ABA concentrations at least 5 times lower than the bulk antibody concentrations that result in comparable signals using B-HRP.

**Notation**

0.1 mol % HBVs | HBVs prepared with 0.1 mol % biotinylated phospholipid
---|---
[14C]HBVs | HBVs prepared with 0.1 mol % biotinylated phospholipid and radiolabeled cholesterol
[14C]BHRP | radiolabeled B-HRP
α | area per vesicle from the large ligand equation
ABA | anti-biotin antibody
ABTS | 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)
\(a_{\text{theo}}\) | theoretical area per vesicle under close-packed conditions
\(A_w\) | area of microtiter plate well exposed to solution
B-HRP | biotinylated horseradish peroxidase
biotin-LC-NHS | biotinylaminomidoheptanoic acid N-hydroxysuccinimide ester

**Greek Letters**

\(\Gamma\) | surface density of HBVs or B-HRP
\(\Gamma_{\text{max,exp}}\) | experimentally determined surface density of HBVs or B-HRP at high concentration
\(\Gamma_{\text{max,tho}}\) | theoretical maximum surface density of HBVs or B-HRP at high concentration
\(\theta\) | fractional surface coverage of vesicles
\(\sigma\) | standard deviation

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