# The Use Of Avidin And Biotin In Enzyme Based Immunoassays.

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# The Use of Avidin and Biotin in Enzyme Based Immunoassays

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# Non-Standard Abbreviations

AKP, Alkaline Phosphatase

DSPCEIA, Dual Solid Phase Competitive Enzyme Immunoassay

DSPDIA, Dual Solid Phase Dye Immunoassay

DSPEIA, Dual solid Phase Enzyme Immunoassay

EIA, Enzyme Immunoassay

EMIT, Enzyme Multiplied Immunoassay Technique

G6P, Glucose-6-phosphate

G6PDH, Glucose-6-phosphate Dehydrogenase

HABA, Hydroxyazobenzoic Acid

INT, p-Iodonitrotetrazolium Violet

MBS, m-Maleimidobenzyl-N-hydroxysuccinimide Ester

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# Abstract

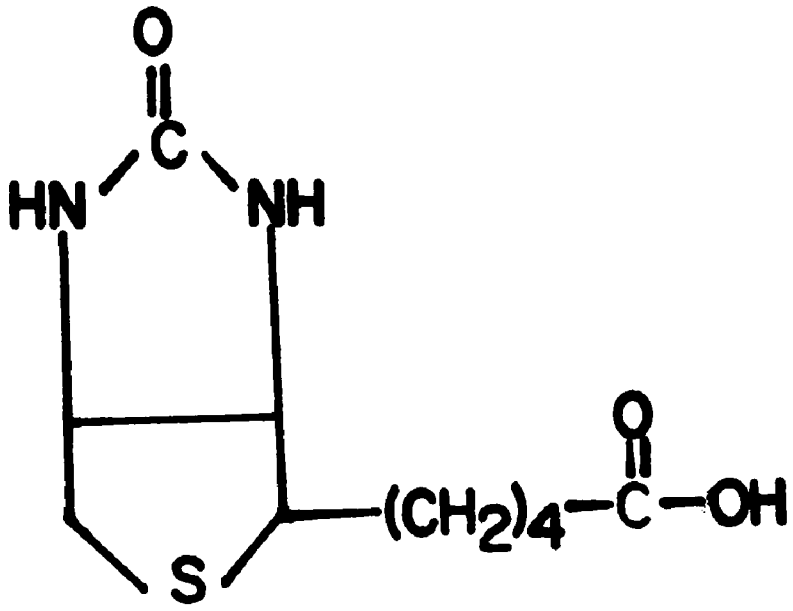
The use of avidin and biotin in several different immunoassay schemes is presented. Biotinylated glucose-6-phosphate dehydrogenase is used as the enzyme marker in most of the assay schemes. An improved biotinylation methodology for this enzyme is presented and subsequently used to significantly improve the enzymatic assay of biotin and avidin. Avidin and biotinyl-glucose-6-phosphate dehydrogenase were also used to develop a new homogeneous assay technique used for detection of small and large molecular weight analytes. This new homogeneous assay scheme relies upon a central enzyme conjugate partitioning between two solid phases. The enzyme is inactivated when bound to one solid phase and active when bound to the other. Using this assay design as little as 2.0ng/ml macromolecular analyte was detectable. Variations of the dual solid phase scheme included use of a dye, hydroxyazobenzoic acid, as a marker in the assay. In another variation the competition between two biotinylated proteins was used after the partitioning of the central conjugate. The two variations of the dual solid phase scheme did not attain the the high level of sensitivity of the original scheme. However, the use of biotin and avidin in the assays described demonstrate the construction of a homogeneous EIA for macromolecular analytes.

# Chapter 1
# General Introduction

Avidin and biotin because of their affinity for one another, $K_D 10^{-15}$M, have been adapted to numerous applications in biochemistry, microbiology, histology, and cytology. Biotin, vitamin H, was discovered around the turn of the century but not isolated until 1936 by Kogl and Tonnis(1). Several years later the chemical structure was determined(2)(fig. 1) followed by the synthesis of the compound(3). However, due to the small cellular requirement for biotin it was not until many years later that the physiological function in transcarboxylation reactions was elucidated(4,5). Clinical studies have shown that even with the minute daily requirment of biotin, its deficiency can cause drastic physiological changes(6).

Avidin, a glycoprotein, was first isolated to a high degree of purity by Fraenkel-Conrat, Snell and Ducay in 1952(7). Subsequently much of the characterization of avidin was performed by Green including its assay, purification, composition, and analysis of binding site(8). Avidin from egg white has a molecular weight of 67,000 and has been shown to contain four subunits, each able to bind one biotin, each having a molecular weight of 15,600. Each subunit contains 1.0 oligoscharide, 4-5 mannose, 3 glucosamines, and 4 tryptophans. No function has been defined for avidin, however it has been suggested that it acts as an antimicrobial agent because it aggressively binds biotin necessary for microbial growth.

It is odd that avidin should posses such a high affinity for biotin. Analysis of the interaction between biotin and avidin has shown that the intact ureido ring of biotin ad the tryptophan residues of avidin are required for




**The Structure of Biotin**

binding to avidin. Tryptophan generally participates in charge transfer or hydrophobic reactions and would not be expected to interact with the ureido ring of biotin but rather the more hydrophobic portions of the molecule(9). Recent X-ray crystallographic analysis of tetrameric avidin has shown it to exist as a symmetric dimer having 222 molecular symmetry. Thus the avidin molecule is heterogeneous, possibly in the carbohydrate portion of the molecule(10).

Although the precise function of avidin, the metabolic pathway of biotin, and the exact nature of the interaction of the two have not been defined, the tenacious binding of avidin to biotin has found many applications. In the field of histology avidin and biotin have been used for localization of tissue components while cytological applications include detection of cell surface analytes(11,12,13). Because of the ease of binding biotin and avidin to cell surface components or cytoplasmic proteins, avidin and biotin have been used to purify such proteins. The high affinity of avidin for biotin allows isolation of desired proteins and in some cases selective retrieval.(14-17).

More recently, avidin and biotin have found application to the field of genetics. The strong and specific interaction of avidin with biotinylated nucleotides provides probes for DNA and RNA studies. By labeling a nucleotide with biotin it is possible to detect insertions into genetic material with enzyme-labeled avidin providing a simple, rapid, non-isotopic tool(18-21)

Another area that has found wide application for avidin and biotin is immunoassays. Biotin, because it is available commercially in several reactive derivatives can be bound to proteins under mild conditions without compromising the biological activity of the protein. Biotinylated assay

components can be used along with labeled avidin to increase sensitivity of enzyme immunoassays. Biotin and avidin have been used in two different types of EIA, homogeneous and heterogeneous. For homogeneous EIA, requiring no separation step, biotin is used to label an enzyme component of the assay. When avidin binds the biotin labeled enzyme it inactivates the enzyme producing a change in enzymatic activity proportional to analyte concentration. Homogeneous EIA using avidin and biotin to modulate enzyme activity have produced superior assays compared to assays without avidin and biotin(22,23,24). A second type of EIA is a heterogeneous method which requires a separation step prior to measurement of enzyme activity. In this type of assay biotin labeled antibody will sandwich the antigen between itself and solid phase antibody. After a wash step an avidin-enzyme conjugate is added and binds to biotinylated antibody. The ensuing enzymatic reaction is measured and is proportional to analyte concentration. This technique has been widely used without avidin and biotin and is termed ELISA, enzyme linked immunosorbant assay, with outstanding results for detection of macromolecules(25). However when used with avidin and biotin the assays, termed BLA-S-ELISA, biotin labeled-sandwich-enzyme linked immunosorbant assay, have yielded significant improvements in sensitivity(26-29).

When applied to EIA, avidin and biotin can significantly improve sensitivity. Thus their application is advantageous if attempting to improve or develop EIA. In this thesis experimentation is reported using avidin and biotin to improve presently used EIA, and to develop new and novel strategies which incorporate avidin and biotin. Since heterogeneous EIA require a separation step and are useful largely for detection of macromolecules, a major goal was to

develop an assay which is homogeneous, requiring no separation step, yet is comparable to heterogeneous EIA in sensitivity. Several developments toward this goal have been tested and the results reported here.

# Chapter 2
# A Comparison of Heterogeneous Enzyme Immunoassays with and without Avidin and Biotin.

## 2.1 Abstract

In chapter 2 is presented a comparison of heterogeneous EIA, one with and one without the use of avidin and biotin. The assay consists of antibody bound to small polystryene latex spheres for which enzyme-labeled and unlabeled antigen compete. The difference between the two assays is the method by which the antibody is bound to solid phase latex. In the non-avidin/biotin assay, antibody is adsorbed directly onto latex while in the second assay system the antibody is bound via biotin and solid phase avidin. Analysis of the amount of antibody bound to the latex by the two methods showed a 21 molar increase in the amount of antibody bound by simple adsorption. Comparison of sensitivity using similar dilutions of antibody-latex and antibody-biotin-avidin-latex over a similar range of analyte concentrations showed the avidin/biotin system to be approximately 5 times more sensitive. The average between "run error" of the assay using avidin and biotin is 4.0% while that for the non-avidin/biotin assay scheme is 8.0%.

## 2.2 Introduction

Avidin possesses a high affinity for biotin which is reflected in a binding constant of $K_D=10^{-15}\underline{M}$. This attribute applied to histochemistry, immunoassays, cytochemistry, and molecular biology can aid detection of cell surface components, analytes in solution, or gene insertions. Use of biotin and avidin in such applications provides a label or probe which is non-isotopic, conjugated to proteins under mild conditions, and stable for long periods(26).

Avidinylation or biotinylation of proteins can occur under various conditions depending on the targeted moiety. Avidin, a glycoprotein molecular weight 68,000, has four active sites each containing various amino acids(8). Thus avidin may be conjugated via selected amino acids with at least one active site remaining. Biotin, a simple molecule of molecular weight 244, is composed of a uriedo ring and a short carbon sequence ending with a carboxylic acid group. The uriedo ring must be intact to retain biological activity, however the chain and carboxylic acid may be modified without affecting biological activity(27). Various derivatives have already been constructed and used in conjugation procedures. Further, many of these biotin derivatives are available commercially adding to the convenience of biotinylation.

The use of avidin and biotin has been shown to increase the sensitivity of enzyme immunoassays(25). The increase in sensitivity may be due to the high affinity of avidin for biotin or it may be due to the link that avidin and biotin provide between assay components which may reduce steric hindrance created by other conjugation procedures.

Presented is a comparison between two identical heterogeneous assay systems in which one employs the use of avidin and biotin while the other does

not. For the EIA using avidin/biotin, avidin has been adsorbed onto latex spheres with antibody bound via biotinylated immunoglobulin. In the second system antibody has been adsorbed directly onto the latex surface without the aid of avidin/biotin. In both cases the solid phase bound antibody is then competed for by unlabeled and enzyme-labeled antigen, the unbound label washed away, and the amount of enzyme activity determined.

## 2.3 Materials/Methods

Calf intestinal alkaline phosphatase(AKP) and phosphatase substrate, were obtained from the Sigma Chemical Co. Rabbit IgG was obtained from the Pel Freez Co. and sheep anti-rabbit IgG from Cooper Biomedical Corp. Polystyrene latex spheres, 4.56%, 0.17$\mu$, were a gift of Dr. Terry Michael of the Emulsion Polymer Institute, Lehigh University. All other reagents and materials were of the highest quality available. Biologicals were stored at 4°C lyophilyzed or reconstituted in PBS, pH 7.4, as was appropriate. For microfiltration 25mm, 0.1$\mu$M Membra-fil, membrane filters were obtained from the Nuclepore Corp.

Solid phase avidin and antibody were prepared by mixing 0.5-1.0 mg of protein with 2$\mu$l of 4.56% latex, 0.17$\mu$, in 1.5 ml of PBS, pH 7.4. The protein-latex solutions were incubated for 2 hours at 21°C, the unadsorbed protein separated by microfiltration, washed three times with 5.0 ml of buffer, and redissolved in 5.0 ml of PBS. The concentration of protein in the eluant was determined using commassie blue(28).

Antibody, affinity purified as described previously(29), was biotinylated by mixing 5.0$\mu$g of N-hydroxysuccinimido biotin with 1.2 mg of antibody, incubating 2.3 hours at 21°C, and separating using sephadex G-25. Once eluted, protein and biotin concentrations were determined in each fraction by methods described previously(30).

The enzyme conjugate, AKP-antigen, was prepared by mixing 5.0 mg of alkaline phosphatase, 1100 units/mg protein, with 2.24 mg of antigen, rabbit IgG, in a final concentration of 25% glutaraldehyde for 2 hours at 21°C, quenching with 0.5 mg of glycine, and dialyzing overnight in 4 liters of PBS at 4°C.

To prepare the latex-avidin-biotin-antibody complex, latex-avidin was prepared as described above. From the concentration of avidin in the eluent the amount of avidin bound to the latex was determined. To this latex-avidin enough biotinylated-antibody was added to yield a 1:1 molar ratio, it was incubated 2 hours at 21°C, and the unbound biotin-antibody removed by microfiltration.

To determine the amount of latex-protein to use in each assay, various dilutions of latex-antibody were mixed with 7.5$\mu$g of AKP-antigen, incubated 6 hours at 21°C, and the unbound enzyme conjugate separated by microfiltration. The enzyme activity remaining was then determined by diluting the latex-bound enzyme remaining in the separation vessel to 0.6 ml, adding 0.4 ml of phosphatase substrate mixture(1.0 mg/ml) and measuring the absorbance at 400nm.

For each assay, both with and without avidin/biotin, 7.5$\mu$g of AKP-antigen was mixed with various concentrations of unlabeled antigen. To this solution 50$\mu$l of the latex-protein was added, incubated 6.0 hours at 21°C, separated by microfiltration, and rediluted with 0.6 ml of PBS. The solution was removed from the separation flask, 0.4 ml of phosphatase substrate mixture added, and the absorbance at 400nm measured.

## 2.4 Results

The quantitiy of proteins adsorbed onto polymeric surfaces can vary depending on the nature of the protein adsorbed and the character of the surface(31). In this report, analysis of avidin and antibody adsorbed onto polystyrene latex spheres showed 10$\mu$g avidin per $cm^2$ bound and 1.73$\mu$g antibody bound per $cm^2$.

After preparation of latex-avidin, biotinylated antibody was added to form the latex-avidin-biotin-antibody complex. The biotinylated antibody was found to contain approximately 2.6 moles of biotin bound per mole of antibody. Adding equivalent molar amounts of antibody-biotin to latex-avidin showed 1.4 x $10^{-11}$ moles antibody bound to the latex-avidin, or 0.083$\mu g/cm^2$ antibody.

Simple adsorption of antibody directly onto the latex showed 3 x $10^{-10}$ moles of antibody bound. Compared to the 1.4 x $10^{-11}$ moles of antibody bound to latex-avidin, this is a 21-fold molar difference between simple adsorption and linkage via avidin/biotin.

Prior to performing the comparison of assay systems the dilution of latex-protein to use in each assay was determined. To accomplish this the effect of increasing dilution of latex-protein with a constant amount of enzyme conjugate was studied. Using 7.5$\mu$g/ml of the enzyme conjugate it was found that as dilution increased the amount of enzyme conjugate bound decreased(Fig. 1). Based on the data in figure 1 a dilution of 40 was chosen for each assay.

Comparison of the assays using the above parameter showed a limit of sensitivity of 125$\mu$g/ml for the avidin/biotin system and 600$\mu$g/ml without the use of avidin and biotin(Fig. 2). This difference shows the avidin/biotin assay system to be approximately 5 times more sensitive. Further, when both assays

were done in quadruplicate the avidin/biotin system had an average "between run" error of 4.0% while the non avidin/biotin system had an average error of 8.0%. The time to perform each assay was identical in both procedures.

**Figure 2-1:** Finding the dilution of latex-protien to use in each assay. To various dilutions of latex-antibody, 7.5$\mu$g of AKP-antigen was added, incubated 6 hours at 21°C, and the unbound enzyme conjugate removed by microfiltration. The latex was rediluted to 0.6ml, removed from the separation flask, 0.4ml of substrate added, and the absorbance measured at 400nm.

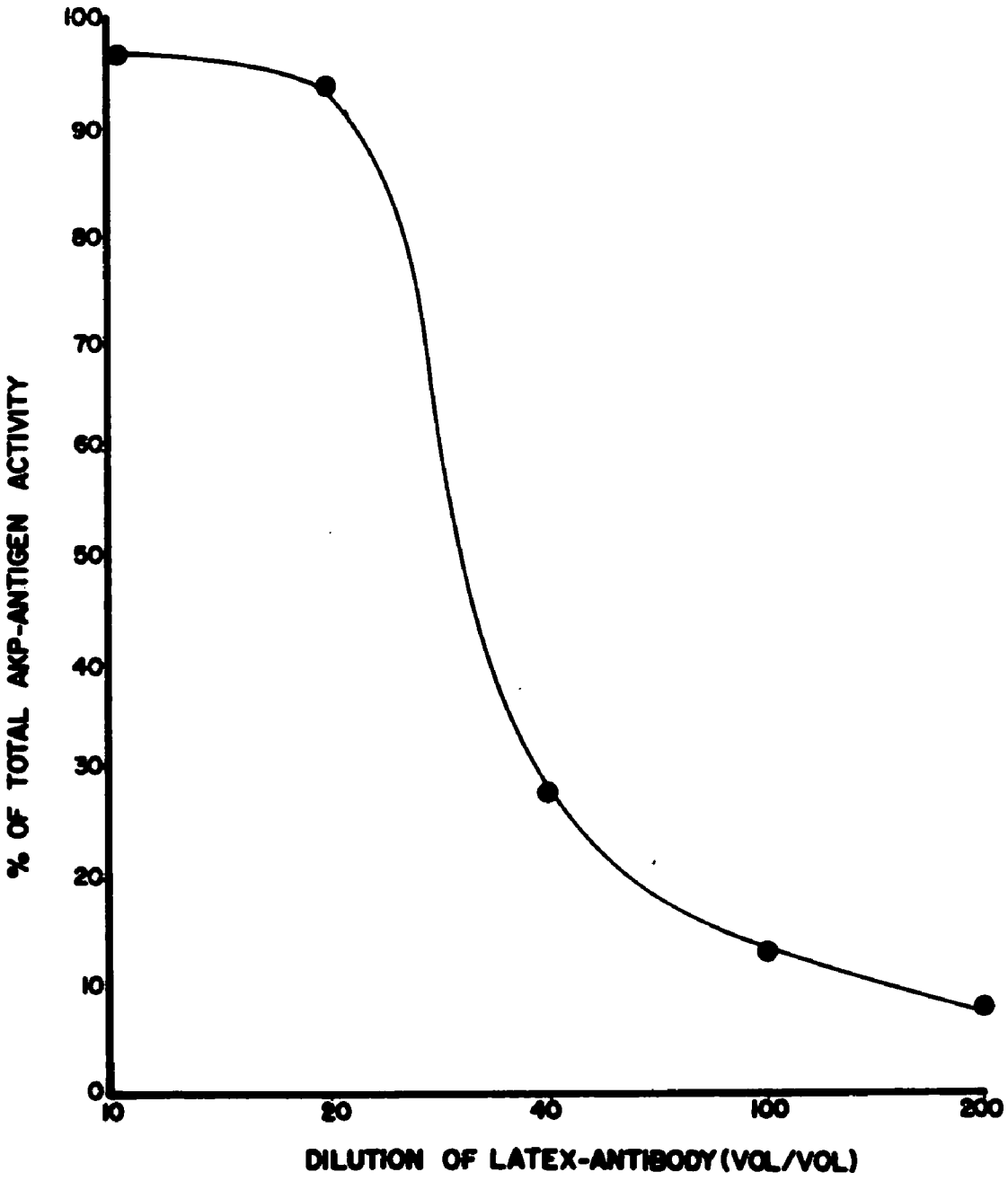
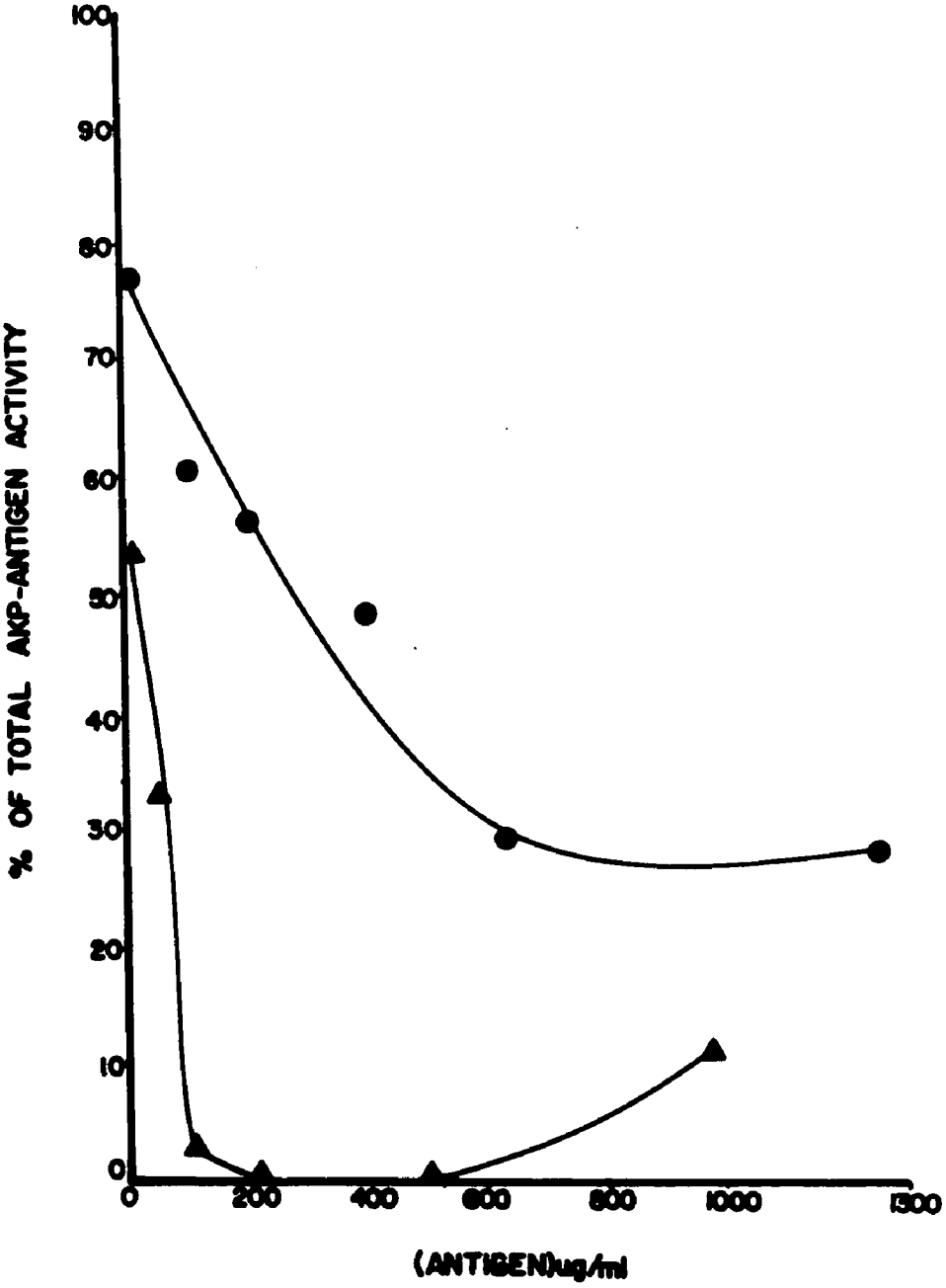

100
90
80
70
60
50
40
30
20
10
0
% OF TOTAL AKP-ANTIGEN ACTIVITY
10
20
40
100
200
DILUTION OF LATEX-ANTIBODY (VOL/VOL)

**Figure 2-2:** Comparison of EIAs with(▲) and without(●) avidin/biotin. To various concentrations of unlabeled antigen, 7.5$\mu$g of AKP-antigen was added. To this mixture 1.0ml of a 40 fold dilution of latex-protein was added, incubated 6 hours at 21°C, and the unbound enzyme conjugate separated by microfiltration. The latex remaining was rediluted to 0.6ml, removed from the separation flask, 0.4ml of substrate added, and the absorbance measured at 400nm.


% OF TOTAL AKP-ANTIGEN ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
1500
(ANTIGEN)ug/ml

## 2.5 Discussion

Biotinylation of antibody in this report resulted in 2.6 moles of biotin per antibody using a 4 molar excess of N-hydroxysuccinimido biotin. It was not desirable to fully biotinylate the antibody since only one avidin-biotin-antibody link would be sufficient to form the latex-avidin-biotin-antibody complex.

Adsorption of protein to artificial surfaces such as polystyrene is limited by a number of circumstances. The exact mechanism by which proteins adsorb onto latex is not certain, however it has been postulated that the proteins hydrophobic regions will orientate such that they will face the latex surface(31). This rearrangement of the adsorbed protein will result in a increase in entropy but may cause conformational changes in the protein that can cause a loss in biological activity. It has been shown that for some proteins the amount of protein adsorbed may increase near the pI, however for other proteins this has not been true(32). Such inconsistencies reflect the confusion as to the mechanism of the adsorbtion process. In this report avidin and antibody were allowed to adsorb onto latex at a pH of 7.4. At this pH there was 21 fold more antibody adsorbed onto equal amounts of latex. Bagchi and Birnbaum have shown the adsorption of rabbit IgG to be relatively constant onto polystyrene latex between pH 3 and 11(33). In both cases, latex-avidin and latex-antibody, a saturating amount of protein was present during the adsorption process giving the greatest chance of close packing the protein on the latex surface. Such close packing provides the best chance of the protein having biological activity after adsorption.

The comparison between two assay systems which rely on the maintainance of biological activity after adsorption onto latex depends not only

the characteristics of the adsorption process, but also on the resulting steric hindrance that may accompany adsorption. The close packing of proteins onto latex sufaces may provide the opportunity for the protein to retain its natural conformation, however it can cause the distortion of active regions of the molecule(34). In the comparison of avidin/biotin vs non avidin/biotin there was a 21 molar difference between the amount of protein on the latex surfaces. Yet the latex-avidin-biotin-antibody complex contained the lesser amount of antibody and provided a more sensitive assay. The thickness of IgG adsorbed onto latex has been found to be 54 angstroms(33), and avidin 55 angstroms(35). If avidin is added to IgG the resulting thickness can conservatively be estimated at 109 angstroms. This large increase in the distance of the IgG molecule from the latex surface using the avidin/biotin system suggests the increase in sensitivity to result from a decrease of steric hindrence. Finally the reduction in error between runs with avidin/biotin may reflect the tight binding of the antibody to the latex via avidin/biotin whereas in the non avidin/biotin assay the equilibrium between adsorption and desorption may introduce error in the assay.

Thus the avidin/biotin system has the specific advantages over the non-avidin/biotin system of sensitivity and precision. The use of avidin and biotin may also increase the distance of competing proteins from the solid phase thereby facilitating the above improvements.

# Chapter 3
# Improved Biotinylation of Glucose-6-phosphate Dehydrogenase Using Active-Site-Blocking Agents.

## 3.1 Abstract

In chapter 3 characteristics of the biotinylation of glucose-6-phosphate dehydrogenase are presented. The enzyme is inactivated in the presence of N-hydroxysuccinimido biotin but can be protected by an appropriate concentration of NADPH used as an active site blocker. A Ki of 1.6+1.0$\mu$M calculated for NADPH for this protection shows it to be an active-site phenomena. Enzyme inactivation is irreversible with consistent kinetic results requiring the presence of 10 mM EDTA. An improved methodology was developed for biotinylation allowing 100% protection of the enzyme with loading factors up to 30.8 mol of biotin per enzyme.

## 3.2 Introduction

N-Hydroxysuccinimido biotin(NHS-biotin) under mild experimental conditions has been bound to a variety of proteins including enzymes and immunoglobulins via their lysine residues(36,37,38). The usual purpose of such biotinylations is to make use of the biotin-avidin couple to bind otherwise nonassociable components(one biotin-labeled and one avidin-labeled) in cytochemistry, immunoassays, and enzymatic systems(9,26). Avidin, a glycoprotein of molecular weight 68,000, possesses an extremely high affinity for biotin, $K_D=10^{-15}$M, making the binding of the two components very effective. In some proteins these lysine residues are important for biological activity; thus biotinylation can severely affect such activites.

Glucose-6-phosphate dehydrogenase(G6PDH) has been used as a marker in the EMIT enzyme assay system for drug determinations(Enzyme Multiplied Immunoassay Technique, a trademark of the Syva Co.)while Ngo et al. prepared biotinylated G6PDH for use in determining biotin concentrations(21). However during biotinylation a 26-fold decrease in activity was reported with subsequent loss in the effectiveness of the assay.

The kinetics of the G6PDH inactivation and methods of blocking the loss of enzymatic activity are reported here. G6PDH and the biotin/avidin system are likely candidates for use in areas such as diagnostics because of their stability and long shelf life. The ability to improve biotinylation procedures without loss of significant enzyme activity has the possibility of improving sensitivity of these enzyme-based assays.

## 3.3 Materials and Methods

G6PDH(EC 1.1.1.49) from yeast with a specific activity of 270 units/mg protein, NHS-biotin, NADP, NADPH, and glucose-6-phosphate(G6P) were all obtained from the Sigma Chemical Company. G6PDH was assayed at 25°C using a Beckman Model 25 spectrophotometer by methods described elsewhere(39). All materials were reconstituted in 0.02M sodium phosphate buffer, pH 7.4, except G6PDH as indicated.

The effect of NHS-biotin on G6PDH was studied by combining various concentrations of NHS-biotin with 1 $\mu$g of enzyme having 0.3 units of activity in 0.5ml, incubating appropriately, and assaying for activity after 14 minutes. An identical procedure was used for the substrate protection experiments using various concentrations of NADPH. 20mM Tris/10mM EDTA buffer, pH 8.0, was used to ensure that the simplified kinetics of the dimeric form of the enzyme were studied.

The reversibility of the effect of NHS-biotin on the enzyme was studied by inactivating 1 $\mu$g of enzyme having 0.3 units of activity in two changes of 8 liters of 20mM Tris/10mM EDTA, pH 8.0, dialyzing overnight and assaying enzymatic activity. A control was run to test the denaturation of native enzyme under similar conditions.

A preparative-scale biotinylation procedure was developed. To a fixed concentration of NHS-biotin various amounts of G6PDH, NADPH, and G6P were added and incubated from 1 to 2 hours. Over the course of the biotinylation aliquots were removed and assayed for enzyme activity. The enzyme was then separated on a G-25 column, assayed for protein and enzyme activity, and dialyzed overnight. After dialysis the fractions were assayed for

enzymatic activity and biotin content as described elsewhere(30,39).

## 3.4 Results

Biotinylation of glucose-6-phosphate dehydrogenase with NHS-biotin results in successful biotinylation accompanied by virtually complete inactivation(21). Figure 1 shows that the enzyme inactivation exhibits a linear dependence on NHS-biotin concentration. Above 4.92mM NHS-biotin the inactivation was too rapid to measure.

A series of experiments, shown in fig. 2, using various concentrations of NADPH with fixed concentrations of NHS-biotin and 1 $\mu$g of G6PDH having 0.3 units of activity in 20mM Tris/10mM EDTA buffer, pH 8.0, showed the inactivation by NHS-biotin could be prevented using appropriate concentrations of NADPH. Reproducibility is good with a "between run" error of 7.75%. A similar effect was seen using G6P. For example, 1.1 $\mu$Mol of NADPH was sufficient to completely prevent the inactivation of 27 units of G6PDH by 100 $\mu$g of NHS-biotin. Using the data from similar protection experiments with fixed concentrations of NADPH and various concentrations of NHS-biotin a Ki for NADPH of approximately $1.6 \pm 1.0 \mu$M was determined using Dixon plots(40).

The inactivation of the enzyme was found to be irreversible. After the enzyme reaction with NHS-biotin it was assayed and dialyzed for 24 hours at 4°C. There was no return in enzyme activity after dialysis over this time. A control of active enzyme lost only 13% of enzymatic activity.

The protection by NADPH and G6P of enzyme inactivation was used to develop a preparative method for the biotinylation of G6PDH. An optimum experimental protocol uses 1.1 $\mu$mol of NADPH, 28.5 $\mu$mol of G6P, and 19.2 units of G6PDH in 1.0 ml of 20mM Tris/10mM EDTA, pH 8.0, and gives complete protection over 1.5 hours. This 382 molar excess of NHS-biotin during

biotinylation results in 30.8 mol of NHS-biotin bound/mol of enzyme after separation. The ratio of NHS-biotin and enzyme may be adjusted to yield different biotinylation levels.

**Figure 3-1:** Inactivation of G6PDH by NHS-biotin.
One microgram of G6PDH having 0.3units of activity was mixed with 0.82-4.92mM biotin in 0.5ml of 20mM Tris/10mM EDTA, pH 8.0, incubated 14min at 21°C, diluted fourfold, and assayed over a 5-min period.

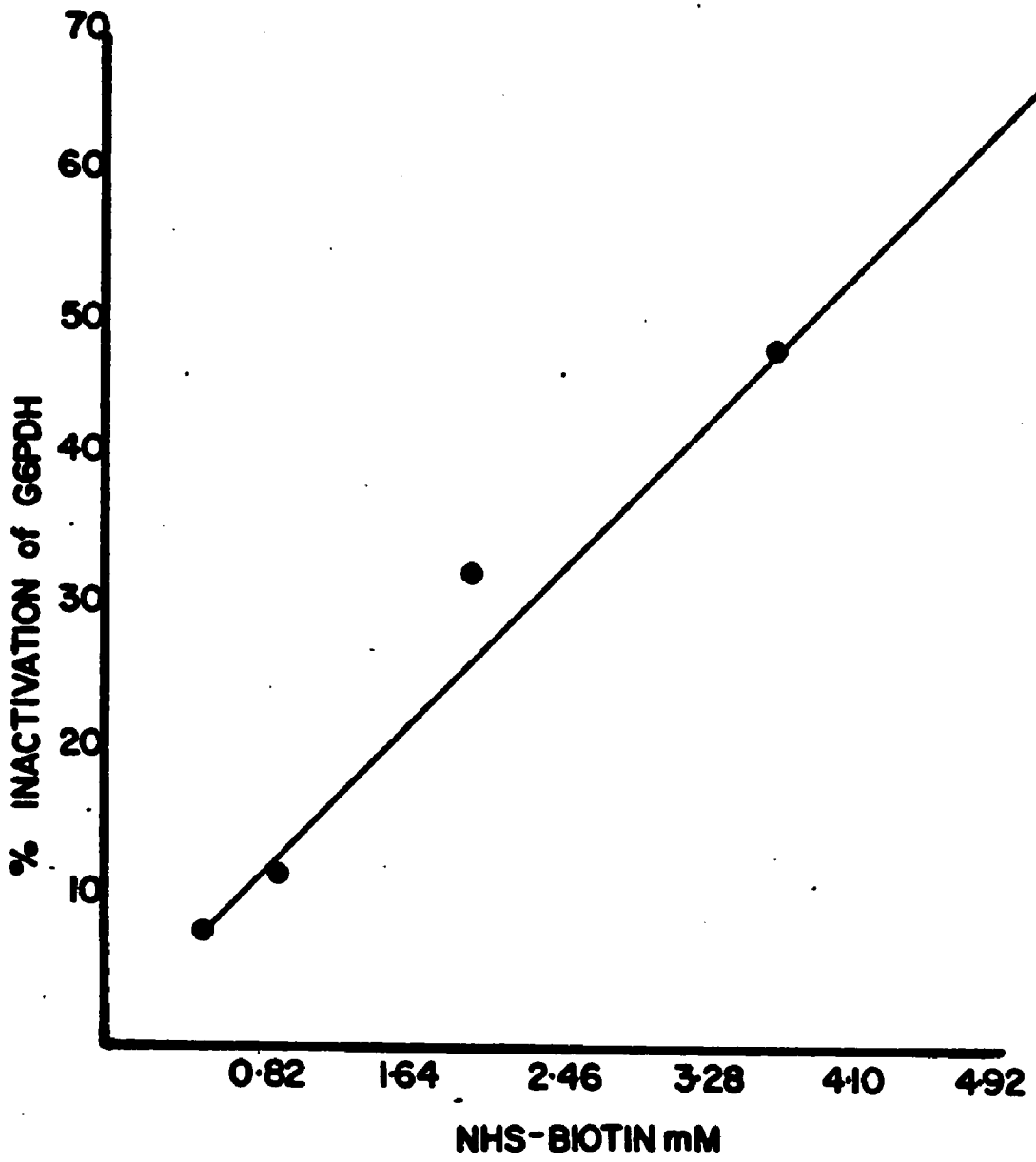
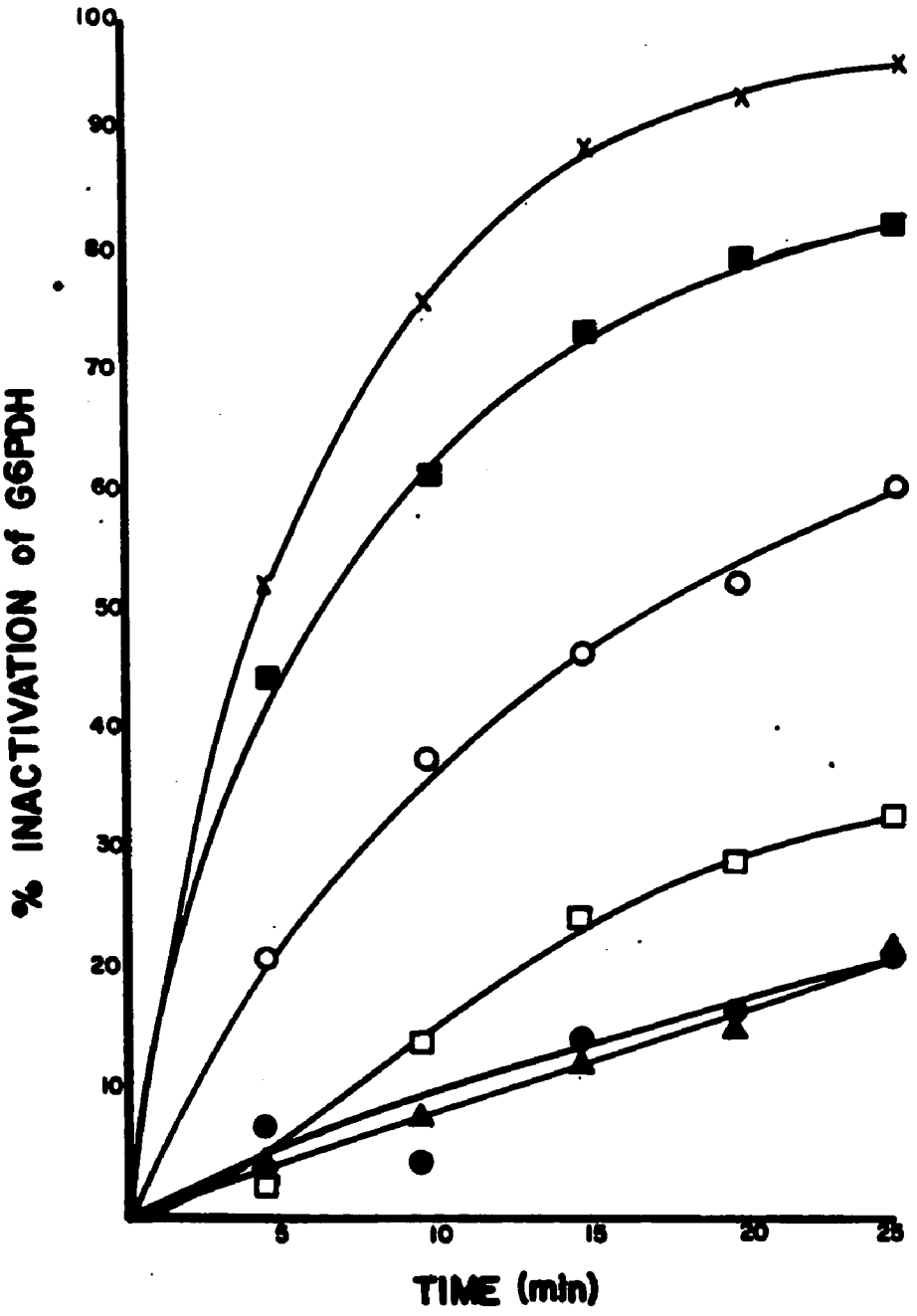

70
60
50
40
30
20
10
% INACTIVATION of G6PDH
0·82
1·64
2·46
3·28
4·10
4·92
NHS-BIOTIN mM

**Figure 3-2:** NADPH protection of G6PDH inactivation by NHS-biotin. One microgram of G6PDH having 0.3 units of activity was added to 0.82mM NHS-biotin in the presence of NADPH, 0.0mM(X),11$\mu$M(■),22$\mu$M(○),0.22mM(□),2.2mM(●),and a control containing enzyme alone . The solutions were incubated at 22°C for 25 min in 0.5ml of 20mM Tris/10mM EDTA, pH 8.0, with aliquots removed every 5min, diluted 70-fold, and assayed.


% INACTIVATION of G6PDH
100
90
80
70
60
50
40
30
20
10
5
10
15
20
25
TIME (min)

## 3.5 Discussion

Previously, numerous biologically active molecules such as antibodies, antigens, and enzymes have been biotinylated without appreciable loss of biological activity or specificity(30,41). However G6PDH biotinylation resulted in a 26 fold decrease in activity(21).

The results of this study suggest that the inactivation of G6PDH is related to the ability of NHS-biotin to bind to an essential group in the active site while reactions elsewhere on the molecule do not affect activity. Inactivation directly related to biotin concentration exhibited second-order kinetics. Substrate protection experiments carried out allowed 100% protection of the enzyme using NADPH and G6P as active-site-blocking agents. From this data the Ki for NADPH, using Dixon plots, was calculated to be approximately $1.6 \pm 1.0 \mu M$, which is similar to Ki values determined for NADPH as an inhibitor of enzyme activity(24). G6PDH from several sources have been shown to contain lysine in their active sites(43). NHS-biotin has been shown to react primarily with lysine residues, suggesting the mode of inactivation of G6PDH(9). These kinetics and protection studies exhibited consistent kinetics only in the presence of 10 mM EDTA. The use of EDTA has been demonstrated to prevent macromolecular association/dissociation reactions of G6PDH between dimeric and tetrameric forms, presumably by prevention of hydrophobic interactions and not chelation(44,45).

Protection of the enzyme from inactivation provided insight for developing methodologies for biotinylation. Previously, long incubation times of enzymes and NHS-biotin resulted in a significant decrease, approximately 96%, in enzyme activity(21). By using appropriate ratios of G6P and NADPH to NHS-biotin,

various concentrations of enzyme could be biotinylated for 1-2 hours with little activity loss. Levels of 30.8 NHS-biotin/mol of G6PDH have been achieved. The ability to biotinylate G6PDH without significant loss of enzyme activity should be of considerable use in efforts aimed at utilizing G6PDH as a marker enzyme.

# Chapter 4
# A Spectrophotometric Assay for Nanogram Quantities of Biotin and Avidin.

## 4.1 Abstract

In chapter 4 parameters and conditions for an enzyme based assay for biotin and avidin are presented. Biotinylated glucose-6-phosphate dehydrogenase when complexed with avidin becomes inactivated. Thus it was possible to construct a competitive assay system for biotin. The assay is sensitive between 100-500ng/ml and could detect as little as 10ng in 0.1ml with a between run error of 2.4%. It requires a 60 minute incubation at 21°C and 5 minutes to assay. The avidin assay, based on the degree of inactivation of biotinylated-glucose-6-phosphate dehydrogenase in relation to the concentration of avidin, could detect as little as 0.25ng in 0.1ml or 2.5ng/ml with an assay time of 10 minutes with a between run error of 3.9%. Both assays are rapid with significant improvements over other non-isotopic methods in sensitivity and comparable to radioisotopic methods in sensitivity with the added advantage of ease of method.

## 4.2 Introduction

The high affinity of avidin for biotin, $K_D = 10^{-15}$M, makes this couple useful for conjugation of non-associating components, cytologic identification of antigens, and amplification of analyte detection(11,22,24). The biotinylation and avidinylation of proteins is performed under mild conditions preserving full biological activity in many cases. Prepared avidin and biotin conjugates have a long shelf life and are stable under extreme experimental conditions(26). The use of avidin and biotin in detection of analytes can improve sensitivity by bridging components or inhibiting biological activity upon binding and is finding wide spread use(45,46).

Biotin and avidin are not only useful because of their high affinity for one another, but also function physiologically. Biotin, vitamin H, functions as a cofactor in carboxylase reactions and its deficiency can result in alopecia, dermatitis, metabolic acidosis, ketosis, and organic-aciduria(47). Although the physiologic role of avidin has not yet been defined one of its functions may be to act as an antibacterial agent. Thus assay methods for avidin and biotin are not only useful for quantifying biotinylated or avidinylated conjugates but also to the clinician for diagnosis.

Assay methods for avidin and biotin previously described have utililized isotopically labeled biotin, colorimetric determination using dyes, or competitive binding enzymatic systems. The most sensitive of these methods have been the radiolabeled and colorimetric systems(30,48). The competitive binding enzymatic system employed the use of biotinylated glucose-6-phosphate dehydrogenase. However, the sensitivity of the assay was limited since 96% of enzymatic activity was lost during biotinylation(21). Recently, active site blocking agents

have been used to improve biotinylation of G6PDH(49). Thus it has become possible to develop an assay superior to previous assays in either simplicity, sensitivity, or both.

## 4.3 Materials/Methods

The following materials were obtained from the Sigma Chemical Co.; avidin, biotin, N-hydroxysuccinimido biotin, glucose-6-phosphate dehydrogenase, G6PDH(E.C.1.1.1.49) having a specific activity of 270U/mg of protein, glucose-6-phosphate(G6P), NADPH, and NADP. All other materials and reagents were of the highest quality obtainable. Experiments were carried out in 10mM EDTA/20mM Tris buffer pH 8.0 unless stated otherwise.

The biotinylation of G6PDH was carried out by methods described elsewhere(49). Briefly, to 72U of enzyme enough G6P and NADPH was added to give final concentrations of 18mM and 0.44mM respectively. After incubation for 10 minutes at 21°C 40$\mu$g/ml of N-hydroxysuccinimido biotin was added and incubated for two hours at 21°C. During this period aliquots were removed and assayed for enzymatic activity. The solution was applied to a Sephadex G-25 column with eluted fractions containing protein dialyzed overnight at 4°C. These fractions were assayed for biotin content and enzymatic activity(30,43).

The competitive assay for biotin was set up as follows: To 0.5$\mu$g of avidin in 0.1ml of 10mM EDTA/20mM Tris buffer pH 8.0 various concentrations of biotin were added and incubated for 30 minutes at 21°C. 2.9$\mu$g/ml of biotinylated enzyme was then added and incubated for 30 minutes at which time the solution was assayed for enzymatic activity for 5 minutes. Avidin was assayed by taking 0.3$\mu$g of biotinylated G6PDH in 0.1ml 10mM EDTA/20mM Tris pH 8.0, adding various concentrations of avidin, incubating 10 minutes at 21°C, and assaying enzymatic activity for 5 minutes. Results from duplicate experiments were plotted to construct standard curves with the between run error calculated from the difference between numerical values for each point.

## 4.4 Results

The biotinylation of glucose-6-phosphate dehyrogenase(G6PDH) resulted in 100% of enzymatic activity conserved(49). Enzyme with two different biotinylation levels was used in the assay methods. The preparation used for the assay of biotin contained a ratio of biotin to enzyme of approximately 8:1 while the fraction used for the assay of avidin contained a ratio of approximately 5:1.

The basis for the assay procedures is the fact that biotinylated G6PDH is inactivated following complexation by avidin as originally shown by Ngo(21). The time dependence of this inactivation is shown in Fig. 1. The extent of inactivation for a given incubation time is dependent on the avidin concentration as shown in Fig. 3, and forms the basis for both assays. Fig. 3 constitutes a standard curve for assay of avidin. The assay procedure for avidin, requiring a 10 minute incubation at 21°C and 5 minutes to assay, was sensitive to 0.25ng in 0.1ml or 2.5ng/ml with a between run variation of 3.9%. The biotinylated G6PDH used in the avidin assay was inactivated ~60% in 10 minutes while that used in the biotin assay was inactivated ~70% in 60 minutes.

The avidin level selected for use in the assay of biotin is an optimal concentration providing a high degree of enzyme inactivation while allowing a desirable level of sensitivity to competing biotin. The assay of biotin is rapid and measured spectrophotometrically via enzyme activity for 5 minutes after a 60 minute incubation at 21°C. The entire assay procedure required 65 minutes without any special conditions other than 10mM EDTA, and was linear between 100-500ng/ml or could detect as little as 10ng in 0.1ml with a between run

error of 2.4%(Fig. 2).

**Figure 4-1:** Time dependence of avidin-mediated inactivation of biotinylated G6PDH. 2$\mu$g of biotinylated G6PDH was mixed with 10$\mu$g of avidin in 1ml of 10mM EDTA/20 mM pH 8.0 buffer, incubated at 21°C, with aliquots removed from 0 to 30 minutes, and assayed for enzymatic activity.

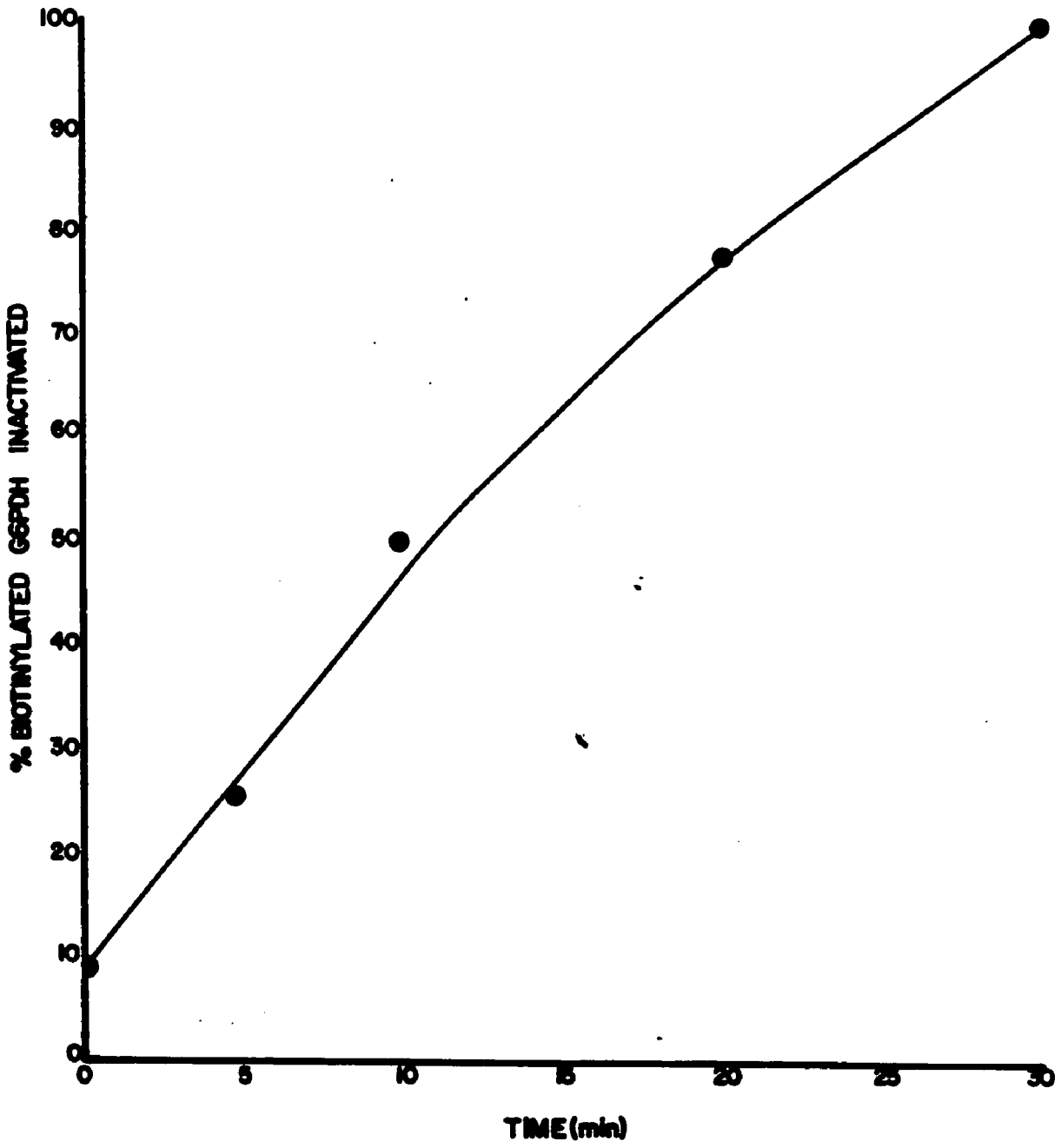

% BIOTINYLATED G6PDH INACTIVATED
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
TIME(min)

**Figure 4-2:** Parameters used for the assay of biotin. To 0.1ng to 25$\mu$g of biotin in 0.1ml 10mM EDTA 20mM Tris buffer pH 8.0, 0.5$\mu$g of avidin was added and incubated 30 minutes at 21°C. 0.29$\mu$g of biotinylated G6PDH was then added, incubated 30 minutes at 21°C, and assayed at 340nm for 5 minutes.

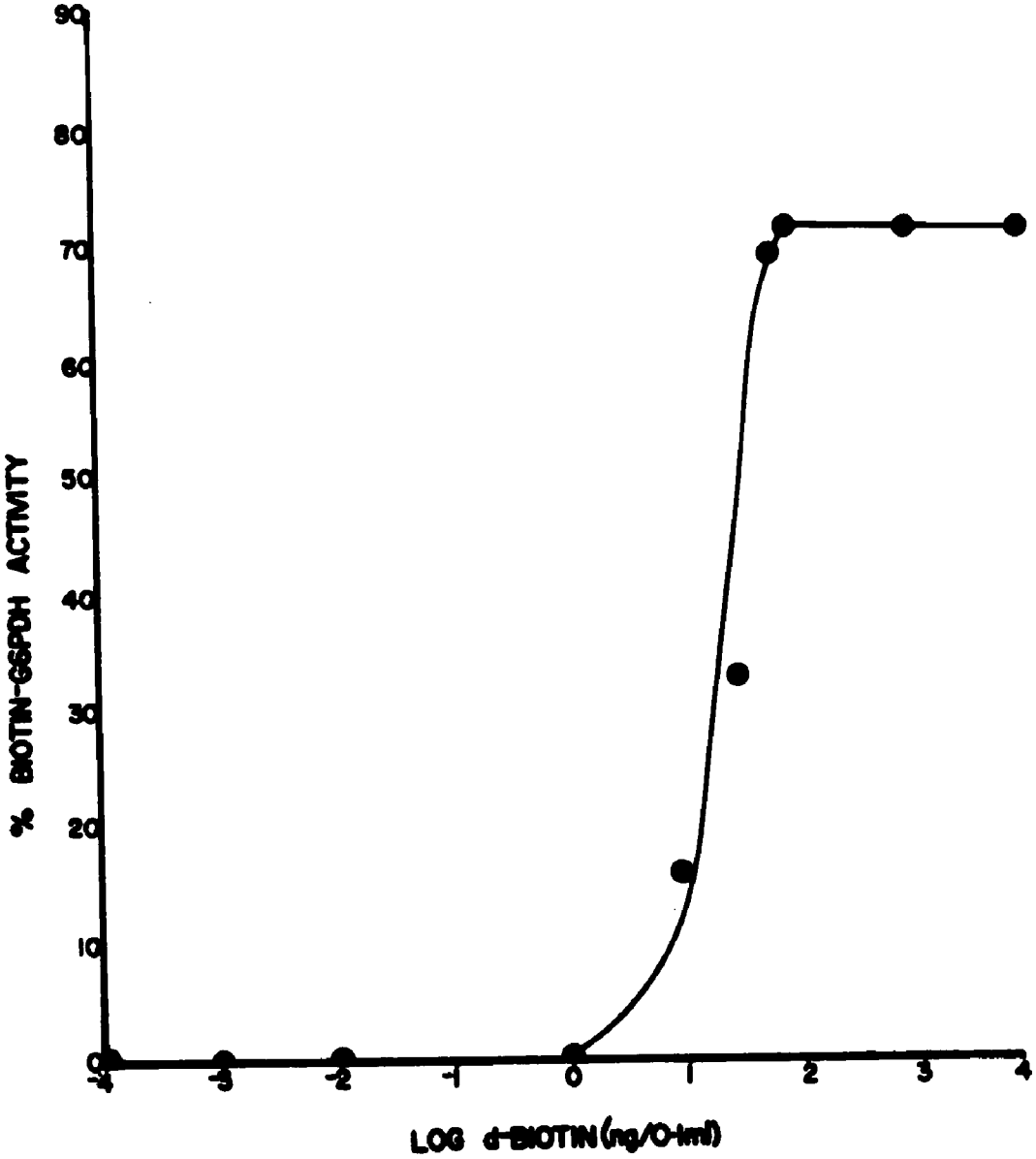
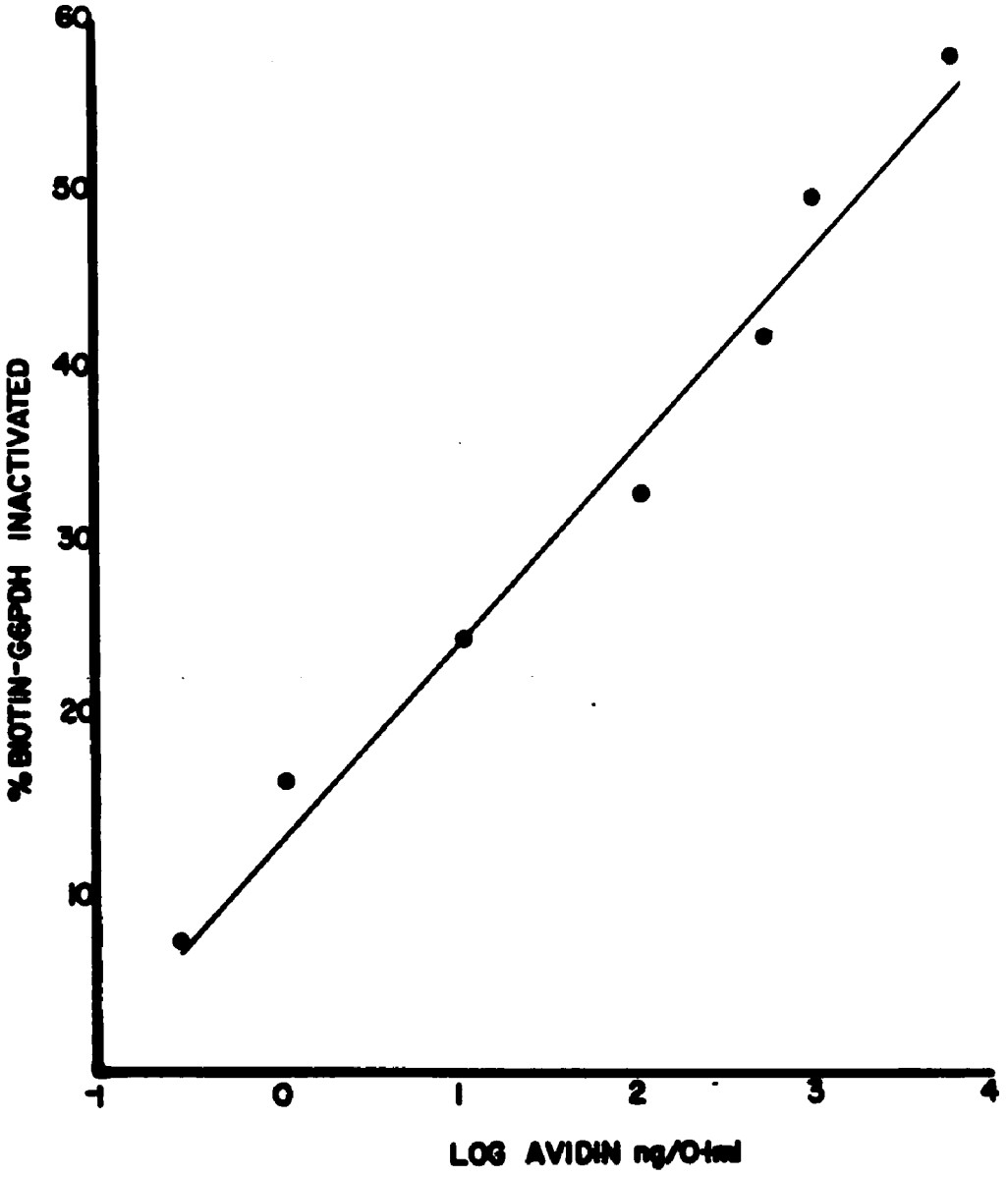

% BIOTIN-G6PDH ACTIVITY
90
80
70
60
50
40
30
20
10
0
-4
-3
-2
-1
0
1
2
3
4
LOG d-BIOTIN (ng/0·1ml)

**Figure 4-3:** Parameters used for the assay of avidin. To 0.25ng to 10$\mu$g of avidin in 0.1ml 10mM EDTA/20mM Tris buffer pH 8.0, 0.3$\mu$g of biotinylated G6PDH was added, incubated 10 minutes at 21°C, and assayed for 5 minutes at 340nm.


60
50
40
30
20
10
% BIOTIN-GAPDH INACTIVATED
-1
0
1
2
3
4
LOG AVIDIN ng/0·1ml

## 4.5 Discussion

The avidin biotin system has been used for numerous applications designed to amplify sensitivity and specificity of analyte detection. In creating such systems the concentration of biotin or avidin must be determined accurately in minimal time in order to determine such things as degree of biotinylation. Green pioneered much of the fundamental characterization of avidin and was the first to develop an assay for it using $^{14}$C-biotin as a marker for detection of 1$\mu$g avidin and 0.01$\mu$g of biotin(50). However, the assay involves the usual problems of radiolabeled components such as handling, disposal, shelf life, and commercial availability of labeled biotin. A similar method for assaying avidin has been used by O'Malley which involves the use of labeled biotin bound to avidin, precipitated after -interaction with avidin antibody and counted(51). Although more sensitive than the method of Green and linear between 0.1-0.5$\mu$g avidin, it still requires labeled biotin and considerable handling. A spectrophotometric assay of biotin and avidin has been used relying on the change in absorbance upon displacement of dye bound to avidin(30). This method which employs no radiolabeled component is facile and can detect 50$\mu$g/ml of avidin and 0.65$\mu$g/ml of biotin. However the dye binds non-specifically in the presence of albumin.

The enzymatic assay of biotin previously developed by Ngo et al. could detect 40-60mg/ml of biotin and 25-95$\mu$g/ml of avidin in two minutes(21). However this work employed the use of G6PDH which had a 26-fold decrease in enzymatic activity during biotinylation. The assay procedures for avidin and biotin presented here have utilized an improved biotinylation procedure. The improvement has increased enzyme detectability due to decreased inactivation of

the enzyme. This has allowed orders of magnitude improvement in sensitivity over those previously reported using this system(21). (Discrepancies in text and figure presentations in the first publication of the enzymatic method prevents statements of precise improvements in the work presented here.) Thus, this assay is considerably simpler than isotopic assays yet achieves similar detection levels and achieves much greater sensitivity than other non-isotopic assays.

# Chapter 5
# A Homogeneous Enzyme Immunoassay for Macromolecules: DSPEIA, The Dual Solid Phase Enzyme Immunoassay.

## 5.1 Abstract

In chapter 5 is presented the Dual Solid Phase Enzyme Immunoassay system,DSPEIA, which has characteristics of both hetero- and homogeneous EIA. DSPEIA relies on the partitioning of an enzyme conjugate of biotin-G6PDH-antibody polystyrene latex bound antigen. When enzyme conjugate binds to latex-antigen the enzyme activity is unaffected, while binding to latex-avidin inhibits enzymatic activity. The assay utilizes a competition between this conjugate and analyte antibody for the latex-antigen. As the concentration of competing unlabeled antibody is increased there is a concomitant decrease in enzymatic activity due to conjugate binding to the latex-avidin. Using DSPEIA as little as 2.0ng/ml antibody could be detected, 1.0 to 1000ng/0.5ml, with a "between run" error of 2.9% in 25 hours when reagents were added sequentially, or 20ng/ml, 10 to 10,000ng/0.5ml, with a "between run" error of 3.0% in 24 hours when reagents were added in one step. DSPEIA requires no separation step, is applicable to macromolecules, is easy to perform and thus combines the most advantageous characteristics of hetero- and homogeneous EIA into one assay system.

## 5.2 Introduction

Enzyme Immunoassays(EIA's) have proven to be extremely useful tools for sensitive, non-isotopic detection of macromolecular and small molecule analytes. EIA's may be heterogeneous, requiring a separation step, or homogeneous, requiring no separation step. The heterogeneous EIA is by far the more sensitive technique, and is applicable to the quantitation of macromolecules such as antigens and antibodies(52). However, the necessity of several wash steps greatly reduces the ease of performing heterogeneous EIA's. Competitive homogeneous EIA's, which require no separation step, rely on the competition between labeled analyte and unlabeled analyte for an antibody(53). The binding of enzyme-labeled analyte to antibody results in activation or inactivation of the enzyme. Thus the amount of enzyme activity is proportional to the concentration of unlabeled analyte. Unfortunately, homogeneous EIA's are unable to attain comparable sensitivity to heterogeneous EIA's and are inapplicable to macromolecules.

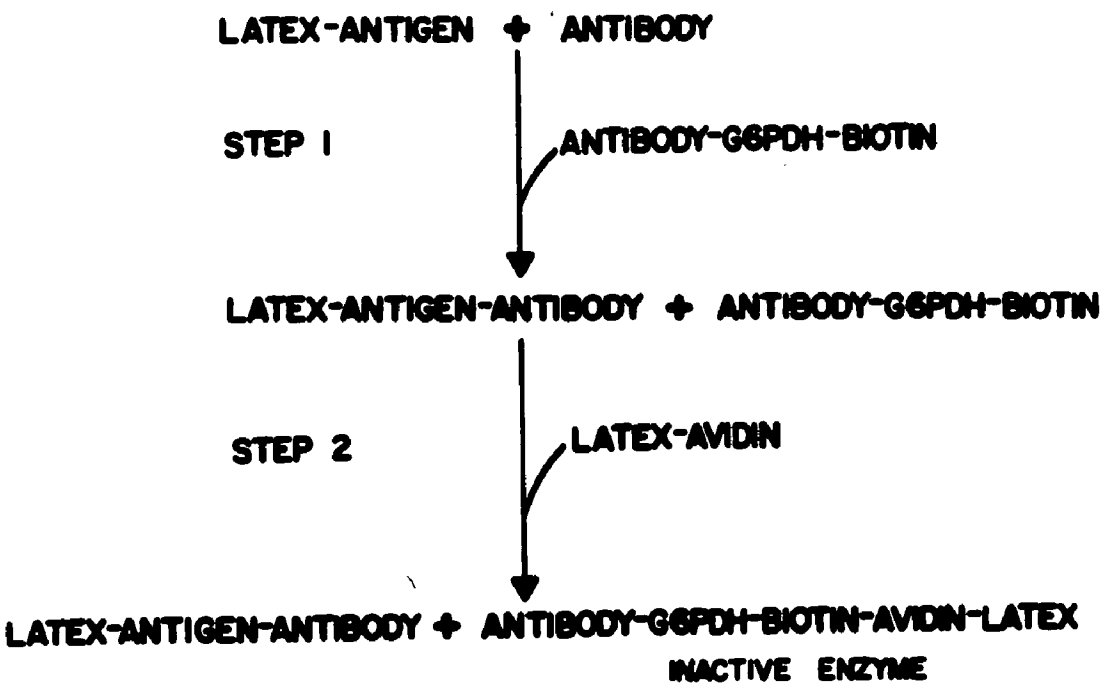
Compounds other than antibodies may be included in an EIA system to improve sensitivity. For example; avidin, a glycoprotein, and biotin, a vitamin, posess an extraordinary affinity for one another, $K_D=10^{-15}\underline{M}$, making them ideally suited for binding dissimilar compounds(8). Avidin and biotin can not only increase the sensitivity of an assay system, but also have the advantages of availability, stability, and are conjugated to proteins under mild condition by numerous methods depending on the targeted prosthetic group(26).

An EIA system has been devised which has the sensitivity of heterogeneous EIA's, yet the ease of method found in homogeneous EIA's. This was done using two solid phases composed of protein adsorbed onto polystyrene latex

spheres. One solid phase consists of avidin on latex while the other contains antigen on latex. The enzymatic component of the system consists of biotinylated glucose-6-phosphate dehydrogenase conjugated to antibody. When the concentration of analyte, macromolecular antigen or antibody was low, the enzyme conjugate binds to the latex-antigen solid phase and retains enzymatic activity. However, when the concentration of analyte increases the enzyme conjugate binds to the avidin-latex solid phase becoming inactive. The amount of enzyme activity is thus proportional to the amount of analyte(see scheme 1).

Scheme 1: Priciples of the Dual Solid Phase Enzyme Immunoassay(DSPEIA). The key to the assay is the fact that when biotin-G6PDH-antibody reacts with the latex-antigen solid phase the enzyme remains active. In step 1 as labeled antibody is added there is competition between the enzyme conjugate and free analyte antibody. When the second solid phase of latex-avidin is added, unbound biotin-G6PDH-antibody binds to the latex-avidin solid phase inactivating the enzyme.

SCHEME I


LATEX-ANTIGEN + ANTIBODY
STEP 1
ANTIBODY-G6PDH-BIOTIN
LATEX-ANTIGEN-ANTIBODY + ANTIBODY-G6PDH-BIOTIN
STEP 2
LATEX-AVIDIN
LATEX-ANTIGEN-ANTIBODY + ANTIBODY-G6PDH-BIOTIN-AVIDIN-LATEX
INACTIVE ENZYME

## 5.3 Materials/Methods

Glucose-6-phosphate dehydrogenase(G6PDH),(EC 1.1.1.4.9) from yeast, NADP, NADPH, N-Hydroxysuccinimido biotin(NHS-Biotin), avidin, glucose-6-phosphate(G6P), diaphorase from Cl. kluyveri, p-iodonitrotetrazolium violet(INT), m-maleimidobenzyl-N-hydroxysuccinimide ester(MBS), and 2-iminothiolane were all obtained from the Sigma Chemical Company. Sheep anti-rabbit IgG was obtained from Cooper Biomedical, purified as described previously(29), and stored at 0°C. Lyophylized rabbit IgG was obtained from Pel Freez and stored at 5°C. Polystyrene latex, 0.17$\mu$, 4.56% solid, was produced and donated by Dr. Terry Michael of the Emulsion Polymers Institute of Lehigh University.

G6PDH was assayed using diaphorase by methods described elsewhere(54). Briefly, 200$\mu$l of assay mixture composed of 100$\mu$g of G6P, 100$\mu$g of NADP, 2.0 units of diaphorase, and 0.05$\mu$g of INT in 20mM Tris/10mM EDTA pH 8.0 was added to G6PDH, allowed to react, quenched with 100$\mu$l of 1.0M HCl, and the absorbance at 492nm measured on a Beckman model 25 spectrophotometer. Once quenched the absorption was stable for at least one hour at 21°C.

Biotinylated G6PDH was prepared by methods described elsewhere(49). The preparation of the biotin-G6PDH-anti-IgG conjugate required the activation of biotinylated-G6PDH using 2-iminothiolane and of the anti-IgG using m-maleimidobenzyl-N-hydroxysuccinimide ester(MBS)(29). Biotinylated-G6PDH was activated by mixing biotinylated-G6PDH with 2.8mM NADPH, 42mM G6P in 20mM Tris/10mM EDTA, pH 8.0, incubating 5 minutes at 21°C, and adding 0.37mM 2-iminothiolane(29). While incubating 30 minutes at 21°C 5$\mu$l aliquots were removed every 10 minutes and assayed for enzymatic activity. Finally, the

activated biotin-G6PDH was passed through a Sephadex G-25 column using 20mM Tris/10mM EDTA pH 8.0 as eluent and assaying fractions for protein content and enzymatic activity.

Sheep(anti rabbit)IgG was activated by mixing 1.1$\mu$M antibody with 10mM MBS, incubating 30 minutes at 21°C, and separating on a Sephadex G-25 column measuring protein concentration by the absorbance at 280nm. When both biotin-G6PDH and anti-IgG were activated equimolar concentrations of each were mixed, incubated 1.5 hours at 21°C, and dialyzed for 48 hours at 4°C in 20mM Tris/10mM EDTA pH 8.0.

To test the effects of avidin and rabbit IgG on the biotin-G6PDH-anti-IgG conjugate various concentrations of each were mixed with 5.5$\mu$g of the biotin-G6PDH-anti-IgG, incubated 10 minutes at 21°C, and assayed for enzymatic activity.

Latex-protein solid phases were prepared by placing 2$\mu$l of latex in 1.5ml of 0.1M sodium phosphate buffer, pH 7.2, containing 0.5 to 1.0mg of protein and sonicated for 3 to 5 seconds at 200 watts. The solution was allowed to incubate for one hour at 21°C after which it was centrifuged for 30 minutes at 39,000 x g. The supernatant was removed, 1.0ml of the above buffer was added, and the sample sonicated as above. The dilution of each solid phase selected for use in the DSPEIA was determined by incubating various dilutions of each latex-protein solid phase with 0.5$\mu$g of biotin-G6PDH-anti-IgG in 0.5ml of 20mM Tris/10mM EDTA pH 8.0 for 30 minutes at 21°C and measuring enzymatic activity over 24 hours.

The procedure for the DSPEIA was constructed in two ways. For the first procedure each reagent was sequentially added and incubated as follows.

100$\mu$l of a 1:25 dilution of latex-IgG in 10x75mm glass tubes was followed by various concentrations of anti-IgG and incubated 30 minutes at 21°C. 0.5$\mu$g of biotin-G6PDH-anti-IgG was added and again incubated 30 minutes at 21°C. 100$\mu$l of a 1:3 dilution of latex-avidin was added and incubated 30 minutes at 21°C. Finally 200$\mu$l of assay mixture, previously described, was added, incubated 24 hours at 21°C, quenched with 100$\mu$l of 1.0M HCl and the absorbance at 492nm measured. In the second procedure for the DSPEIA reagent additions were made in the same order, however reagents were not incubated between additions but were rapidly added. After multiple runs of the sequential and one step procedures the error between samples was calculated and reported as the average between run error.

## 5.4 Results

The dual solid phase enzyme immunoassay(DSPEIA) as shown in Scheme 1 combines the solid phase characteristics of heterogeneous EIA with the inactivation of enzyme upon complexation found in homogeneous EIA. This requires two protein-solid phases and a conjugate which is partitioned between them. These were synthesized as follows.

ASSAY COMPONENTS

Avidin and rabbit IgG were bound to latex as described in methods. After protein was absorbed onto the latex the assay of the supernatent for protein from each preparation of latex-avidin and latex-IgG showed an average of 4.25$\mu$g/cm$^2$ and 3.81$\mu$g/cm$^2$ surface area bound, respectively. This latex-IgG is the latex-antigen of Scheme 1. Biotin-G6PDH was prepared as previously described(49) and conjugated to purified sheep anti-rabbit IgG as described in methods with G6PDH activity fully preserved during biotinylation and conjugation. When biotin-G6PDH-anti-IgG was mixed with various concentrations of free avidin the enzyme was inactivated as a function of avidin concentration as was seen with biotin-G6PDH previously(21,55). The presence of IgG minimaly affected enzymatic activity as was the case with IgG bound to latex.

ASSAY PARAMETERS

In order to provide an adequate degree of enzyme inactivation and an acceptably low backround absorbance due to latex, a 1:3 dilution of latex-avidin stock, as prepared in methods, was chosen for use in the assay. When various dilutions of latex-IgG were mixed with 0.5$\mu$g of biotin-G6PDH-anti-IgG in the presence of 100$\mu$l of a 1:3 dilution of latex-avidin the enzymatic activity

decreased as the dilution increased, as shown in Fig. 1. Based on the curve from Fig. 1 a 1:25 dilution of latex-IgG corresponding to 50% inactivation was chosen for use in the DSPEIA procedure.

ASSAY PROCEDURE

Choice of a 1:3 dilution of latex-avidin and a 1:25 dilution of latex-antigen provided parameters for the DSPEIA in which the smallest concentration of antibody would compete with the biotin-G6PDH-anti-IgG conjugate for latex-antigen. Enzyme conjugate that is not bound to latex-antigen binds to the latex-avidin solid phase becoming inactivated. Using the above parameters in the DSPEIA allowed detection of as little as 2.0ng/ml or 1.0 to 1000ng/0.5ml antibody with a "between run" error of 2.9% when the reagents were added sequentially, or 20ng/ml or 10 to 10,000ng/0.5ml antibody with a "between run" error of 3.0% when the reagents were added in one step(Fig. 2). Addition of reagents in a sequential fashion required 25 hours to perform the assay while one step addition of the reagents required 24 hours. The long assay time is required primarily for enzymatic generation of product.

**Figure 5-1:** Effect of various concentrations of latex-IgG on enzyme activity of biotin-G6PDH- antibody conjugate in the presence of latex-avidin. To 100$\mu$l of various dilutions of latex-IgG(vol/vol) 0.5$\mu$g of biotin-G6PDH-anti-IgG was added and incubated 30 minutes at 21°C. 100$\mu$l of a 1:3 dilution of latex-avidin was added and again incubated for 30 minutes at 21°C after which 200$\mu$l of assay mixture was added, incubated for 30 minutes at 21°C, and quenched with 100$\mu$l of 0.1M HCl. The absorbance was then measured at 492nm.

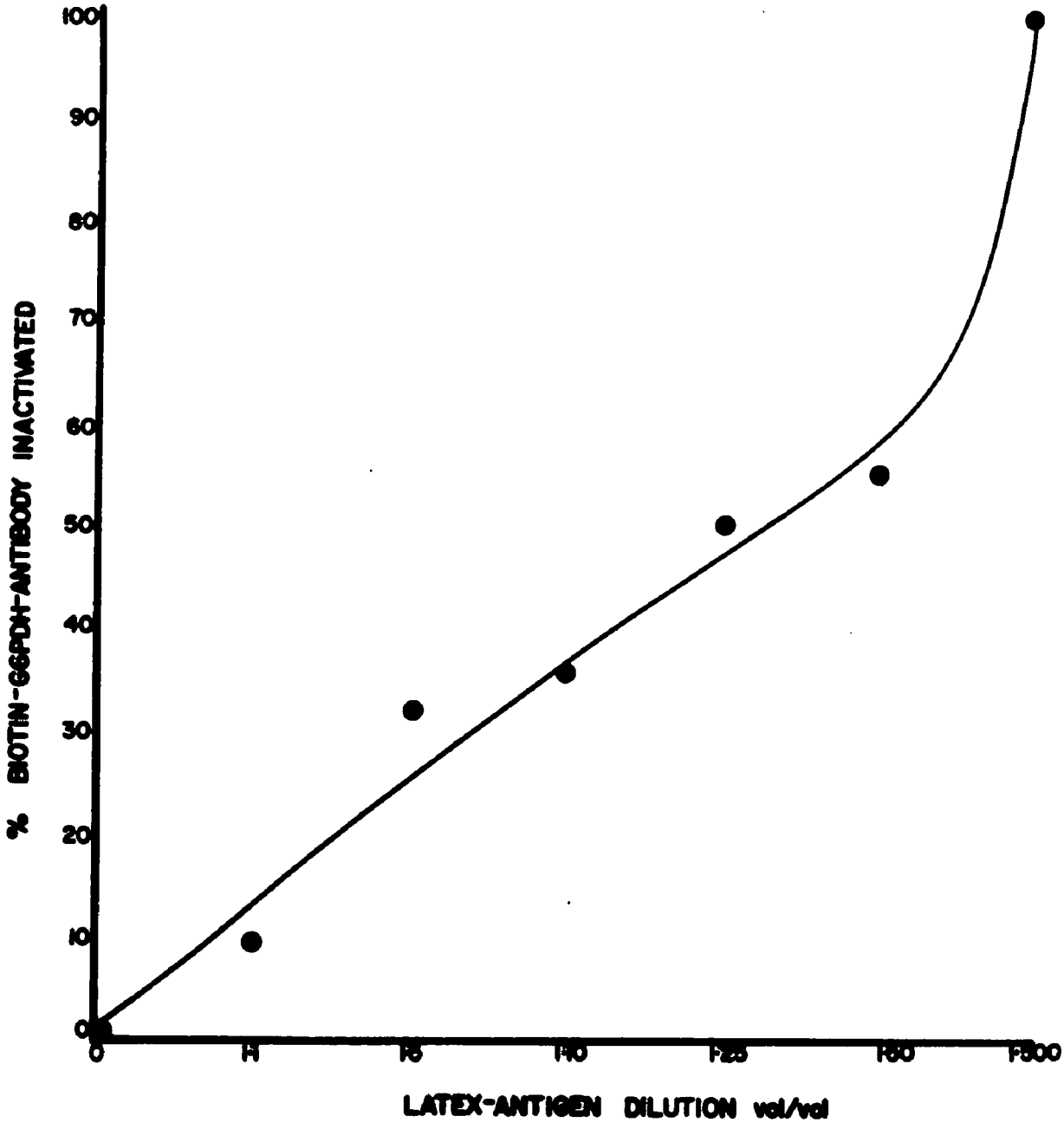
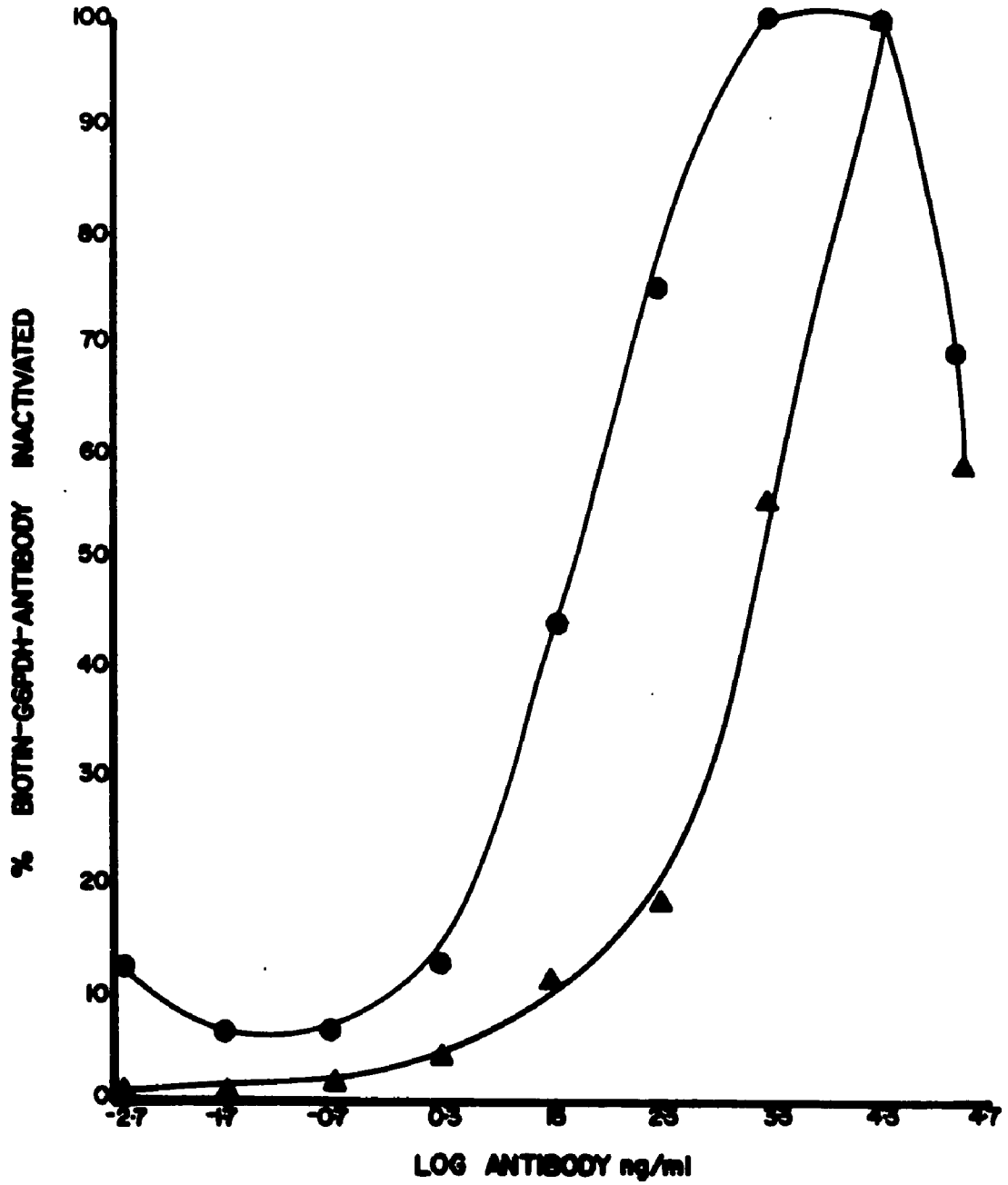

% BIOTIN-G6PDH-ANTIBODY INACTIVATED
100
90
80
70
60
50
40
30
20
10
0
0
F5
F10
F25
F50
F100
LATEX-ANTIGEN DILUTION vol/vol

**Figure 5-2:** Results of the Dual Solid Phase Enzyme Immunoassay, DSPEIA. When reagents were added in the sequential fashion(●), described in methods, there is a 10 fold increase in sensitivity,2.0ng/ml antibody detectable, compared to mixing all reagents together in one step(▲) 20.0ng/ml detectable. In the sequential procedure various amounts of antibody are added to 100μl of a 1:25 dilution of latex-antigen and allowed to incubate 30 minutes at 21°C. 0.5μg of biotin-G6PDH-antibody conjugate is then added and incubated 30 minutes at 21°C, after which 100μl of a 1:3 dilution of latex-avidin is added and incubated 30 minutes at 21°C. Finally 200μl of substrate is added, allowed to react 24 hours at 21°C, quenched with 100μl of 0.1M HCl, and the absorbance at 492nm measured.


% BIOTIN-GGPDH-ANTIBODY INACTIVATED
100
90
80
70
60
50
40
30
20
10
0
-2·7
-1·7
-0·7
0·3
1·3
2·3
3·3
4·3
4·7
LOG ANTIBODY ng/ml

## 5.5 Discussion

Heterogeneous EIA's have been shown since their conception to be useful methods of assaying macromolecular analytes(23). The basic advantage of heterogeneous EIA's over homogeneous EIA's is sensitivity. However the greatest disadvantage of heterogeneous EIA is the tedious manipulations involved in the procedure including the necessity for several wash steps. In opposition to the several separation steps of a heterogeneous EIA are simple homogeneous assay systems which require no separation step(53). However these homogeneous systems are generally less sensitive and inapplicable to macromolecules.

DSPEIA combines the elements of the two basic types of EIA's resulting in a homogeneous system with the sensitivity of a heterogeneous system for macromolecules. In the initial work by Engvall and Perlman(56) using the heterogeneous EIA system, they were able to detect 1.0 to 100ng/ml of antigen. Since this initial work, heterogeneous EIA's have been used to assay numerous macromolecular analytes but without a significant improvement in sensitivity(57,58). In the DSPEIA detection limits were 2.0ng/ml and 20ng/ml antibody when reagents were added sequentially or in one step, respectively. The DSPEIA not only rivaled heterogeneous EIA's in terms of sensitivity but also in terms of time required which was 24 to 25 hours for the DSPEIA and 4.5 to 21 hours for heterogeneous EIA's. The majority of the time required for the DSPEIA was for enzyme assay. Furthermore, DSPEIA requires no separation steps thereby increasing the ease of method.

The sensitivity of sequential addition of reagents was 10 fold better than one step reagent addition. Such differences in sensitivity allow choice as to which DSPEIA procedure is better suited to the desired macromolecule to be

assayed. For many macromolecules, especially in biological samples, 20ng/ml would be an adequate level of sensitivity. Thus DSPEIA could detect macromolecules in a homogeneous system using simple spectrophotometric equipment and also allow flexibility in procedure according to the level of desired sensitivity.

The model system for the DSPEIA presented in this paper should in no way suggest the system to be limited to the use of avidin/biotin and antigen/antibody. The principle of the DSPEIA assay system relies on the affinty of two different compounds for a conjugate of enzyme and analyte. The only limit to the assay design is that enzyme must be activated or inactivated upon binding to one of the solid phases while binding to the other solid phase does not affect enzymatic activity. Therefore it will be possible to apply DSPEIA to the assay of other macromolecules by varying the components of the system. For example; two antigen/antibody reactions such as anti-enzyme and anti-analyte on two different solid phases for an enzyme-analyte conjugate could satisfy the principles of the DSPEIA assay system.

# Chapter 6
# The Use of Hydroxyazobenzoic Acid As An Indicator In Immunoassays

## 6.1 Abstract

In chapter 6, a dye, hydroxazobenzoic acid binds specifically to the active site of avidin causing an increase in absorbance which decreases upon addition of biotin by displacement of the dye from avidin. This characteristic of the dye has been applied to the dual solid phase assay scheme for detection of digoxin and macromolecular antibody. Three different assays are presented, each having a different conjugate partitioning between two solid phases and a different solid phase protein to which the conjugate binds. For assay scheme 1 using an avidin-digoxin conjugate, solid phase biotin-BSA, and solid phase anti-digoxin, a dose response curve showed the assay to be sensitive from 0.2pg/ml to 2.0$\mu$g/ml, with an average "between run" error of 1.3% and 60 minutes to perform the assay. For scheme 2 which used a biotin-digoxin conjugate, solid phase avidin and solid phase anti-digoxin, no dose response curve was produced. For scheme 3 which used an avidin-anti-IgG conjugate, solid phase biotin, and solid phase antigen, a dose response curve showed the assay sensitive between 0.2 to 20$\mu$g/ml with a between run error of 1.0% and 60 minutes to perform the assay. Using the biotin-digoxin conjugate from scheme 2, anti-digoxin was purified from whole sera by affinity chromatography. Avidin-Sepharose bound to biotin-digoxin-anti-digoxin as whole sera passed through the column. The anti-digoxin was selectively retrieved by elution with 0.1M NaCl, pH 2.5.

## 6.2 Introduction

Perhaps the greatest advantage for using enzymes in immunoassays is their ability to amplify signal. One active enzyme can turn over many molecules of substrate to product which produces an easily measured spectrophotometric change(59). Enzymes are inactivated or activated during these assays, their level of activity being proportional to analyte concentration. The use of enzymes in enzyme immunoassays(EIA) has other advantages such as stability, reproducibility, long shelf life, and they are conjugated to other molecules usually with little loss of biological activity to either portion of a conjugate.

In most EIA's the antigen-antibody interaction, which is diffusion controlled, limits the rate at which the assay procedure can be carried out. Once formation of the antigen-antibody complex is complete, substrate is added and incubated until adequate product is formed to detect spectrophotometrically. It is this production or deficiency of product that one wishes to make as rapid as possible since it is the marker for determining an unknown concentration of analyte.

Other than enzymes any marker that provides a rapid signal can be used in an assay procedure. The oldest and still one of the most sensitive methods for detection of analytes involves the use of radioisotopes the principles of which are explained elsewhere(60). Although highly sensitive the use of radiolabels has inherent problems and is therefore not a label of choice. More recently fluorescent labels have been used for quantitation of analytes(61). While detection is rapid, there are also problems of backround fluorescence, particularly in sera. Finally, dyes have been used which change absorbance characteristics when complexed with proteins. Many of these proteins are non-specific and also

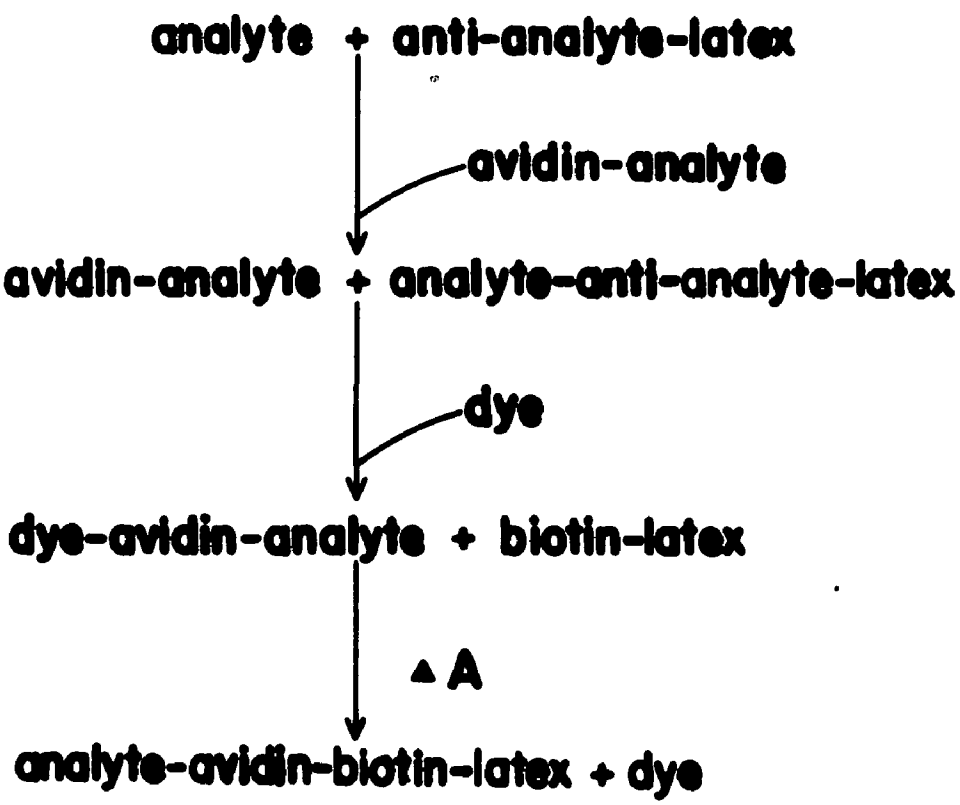
behave less than desirable in sera(62).

In this chapter are presented applications of a dye as an indicator in an immunoassay detection system to increase the speed of detecting analyte while maintaining desirable sensitivity. HABA, hydroxyazobenzoic acid, binds specifically and stoichiometrically to avidin. The binding of the dye to avidin results in a spectral shift which decreases when biotin is added by displacment of the dye from avidin. This distinctive property is ideal for an assay system and has been applied to the dual solid phase assay system discussed previously. HABA has already been used for detection of avidin and biotin(30) but not for other analytes. In this report we have attempted to use the HABA-avidin/biotin system to detect digoxin, an important cardiac drug, and IgG, a large macromolecule. Several variations of the Dual Solid Phase Dye Immunoassay(DSPDIA) were attempted to maximize HABA as a marker. The basic scheme, see fig 1, involves the competition between labeled and unlabeled analyte for solid phase antibody followed by addition of HABA. The dye binds to available avidin with a concomitant increase in absorbance. In the last step a biotinylated component is added and unbound avidin-dye binds tenaciously to biotin displacing the dye and causing a decrease in absorbance. The change in absorbance from the first to second measurement is calculated and proportional to analyte concentration.

**Figure 6-1:** General scheme for the Dual Solid Phase Dye Immunoassay. To solid phase antibody various concentrations of unlabeled analyte are added and incubated as appropriate. Labeled analyte is added, incubated, HABA dye added and the absorbance at 500nm measured($A_1$). As a final step a second solid phase is added and the absorbance measured a second time at 500nm($A_2$). Latex backround is subtracted from $A_1$ and $A_2$ followed by subtraction of $A_2$ from $A_1$ to give the change in absorbance. The change in absorbance is plotted vs analyte concentration.

# DSPDIA

## GENERAL SCHEME



## 6.3 Materials/Methods

The following materials were obtained from the Sigma Chemical Co.; N-hydroxysuccinimido biotin(NHS-biotin), biotin hydrazide, bovine serum albumin(BSA), 2-iminothiolane, m-maleimidobenzoyl-N-hydroxysuccinimido(MBS), digoxin, Dowex 50w cation exchange resin, CNBr-activated sepharose 4B and d-biotin. Anti-digoxin was obtained from PolySciences Co, Malvern PA, sheep anti rabbit IgG from Cappel laboratories, Malvern PA., Miles Laboratories, and New England Nuclear, Aquacide III from Calbiochem-Behring Corp, La Jolla CA, and rabbit IgG from Pel Freez Biologicals, Rogers, AR.

Scheme 1

Several variations of the DSPDIA scheme were attempted in order to maximize the HABA-avidin/biotin system. Scheme 1 involves use of solid phase biotin via biotinylated BSA prepared by mixing a 65 molar excess of NHS-biotin with 10mg BSA in 20mM Tris-10mM EDTA pH 8.5 for 2 hours at 21°C and dialyzing against several changes of buffer for 48 hours at 4°C. To 1.0mg of biotinylated-BSA, 2$\mu$l of 4.56% polystyrene latex was added, sonicated 2-4 seconds at 200 watts, and incubated 30 minutes at 21°C. The solution was centrifuged for 30 minutes at 39,000xg, the supernatent removed, and the absorbance at 280nm measured. The pellet was redissolved in 1.0ml of 2mM phosphate buffer, pH 7.4, sonicated 2-4 seconds at 200 watts, and diluted as appropriate.

Digoxin was conjugated to avidin by the method described previously(6). Briefly, to 1.0mg of digoxin dissolved in 46$\mu$l dioxane with 180$\mu$l ETOH, 140 $\mu$l 0.1M $NaIO_4$, and incubated 30 minutes at 21°C, 10$\mu$l of 1.0M ethylene glycol was added followed immediately by 1.0mg of avidin dissolved in 1.0ml of 2mM

tris/10mM EDTA pH 9.5, and the pH maintained between 9.0-9.5 with NaOH. After incubation for 60 minutes at 21°C 1.2mg of $NaBH_4$ was added and the mixture allowed to stand overnight at 21°C. The solution was chromatographed on a Sephadex G-25 column, measuring the absorbance at 280nm, pooling fractions containing protein, and reducing the volume with Aquacide III. The concentration of avidin and digoxin was determined by methods described elsewhere(30,63).

After preparation of avidin-digoxin, the activity of avidin was demonstrated by mixing 3.5μg of the conjugate with 25μug of HABA, measuring the absorbance at 500nm, adding various amounts of d-biotin and measuring the absorbance again at 500nm.

Setting the parameters for scheme 1, the dilution of latex-BSA-biotin to use with avidin-digoxin was determined. In 0.4ml of buffer 25μg of HABA was mixed with 3.5μg of avidin-digoxin and the absorbance measured at 500nm. To this 100μl of latex-BSA-biotin was added and the absorbance again measured at 500nm. The effect of latex was subtracted and the change in absorbance calculated.

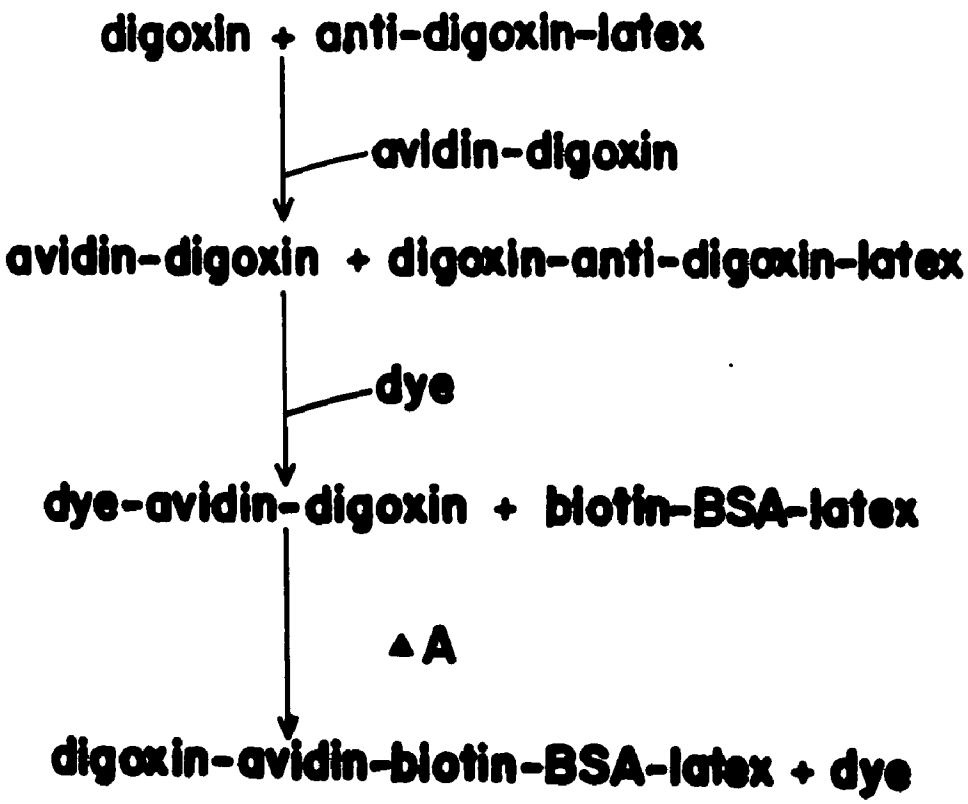
The second solid phase, latex-anti-digoxin, was prepared by incubating 0.5mg of antibody with 2μl of 4.56% latex, sonicated for 2-4 seconds at 200 watts, and incubated for 1.0 hour at 21°C. The solution was centrifuged for 30 minutes at 4°C, the supernatent removed and the pellet redissolved in 1.0ml of 20mM Tris-10mM EDTA, pH 8.5, and sonicated for 2-4 seconds at 200 watts. To determine the dilution of latex-anti-digoxin to use in the assay various dilutions of latex-anti-digoxin were prepared. To 100μl of each dilution 2.0μg of avidin-digoxin was added, incubated 30 minutes at 21°C, 25μg of HABA added,

and the absorbance at 500nm measured. 100$\mu$l of a 1:1 dilution of latex-BSA-biotin was added and the absorbance measured again at 500nm.

Assay of digoxin consisted of mixing various concentrations of unlabeled digoxin with 100$\mu$l of a 1:2 dilution of latex-anti digoxin prepared as described above. After 30 minutes 2$\mu$g of avidin-digoxin was added and the solution incubated again for 30 minutes at 21°C. 25$\mu$g of HABA was added and the absorbance at 500nm measured, 100$\mu$l of a 1:3 dilution of latex-BSA-biotin was added and the absorbance at 500nm measured a second time. The change in absorbance was calculated after subtraction of background latex.

Scheme 1: To solid phase anti-digoxin various dilutions of unlabeled digoxin were added and incubated 30 minutes at 21°C. To this solution 3.5$\mu$g of avidin-digoxin conjugate was added, incubated 30 minutes at 21°C, 25$\mu$g of HABA added, and the absorbance at 500nm measured($A_1$). 100$\mu$l of undiluted latex-BSA-biotin was added, the absorbance at 500nm measured($A_2$), and the change in absorbance calculated after subtraction of latex backround.

## SCHEME I


digoxin + anti-digoxin-latex
avidin-digoxin
avidin-digoxin + digoxin-anti-digoxin-latex
dye
dye-avidin-digoxin + biotin-BSA-latex
▲A
digoxin-avidin-biotin-BSA-latex + dye

Scheme 2

The second design of the DSPDIA involved the use of a digoxin-biotin conjugate as the portion of the assay partitioning between two solid phases,see scheme 2. It was prepared by oxidation of 30mg of digoxin in 5.5ml ETOH, 1.4ml dioxane, 6.2ml $NaIO_4$ and incubating 3 hours at 21°C. A 10 molar excess of ethylene glycol was added, followed immediately by 20.2 mg of biotin-hydrazide maintaining a pH of 6.0 at 21°C. After 24 hours 15mg of $NaBH_4$ was added to reduce the schiff's base and the solution incubated for 3 hours at 21°C. To remove unbound biotin-hydrazide the solution was applied to a Dowex-50w cation exchange column and the digoxin-biotin conjugate eluted with 0.35M sodium citrate buffer, pH 4.0. The concentration of digoxin and biotin were determined by methods described elsewhere(30,63).

Prior to determining the dilution of latex-anti-digoxin to use in the assay the antibody was purified. To accomplish this an avidin affinity column was prepared by mixing 9.1mg of avidin in 20ml of sodium bicarbonate buffer pH 8.0 with 5.0g of activated CNBr-Sepharose 4B, stirring slowly for 24 hours at 4°C, 50ml of 1.0M ethanolamine pH 8.0 was added, and the slurry stirred again for 24 hours at 4°C. The sepharose was washed with 500ml of buffer, the concentration of protein in the eluent determined, and the sepharose stored at 4°C with a few added crystals of $NaN_3$. For purification of anti-digoxin, digoxin-biotin was mixed with whole sera containing anti digoxin and incubated at 4°C for 24 hours stirring occasionally. The mixture was added to the avidin-sepharose, shaken gently for 24 hours at 4°C and washed with 0.1M carbonate buffer until no protein was detected in the washings. The solution was poured into a column and the antibody eluted with 0.1M NaCl pH 2.5

collecting fractions in 2.5ml of buffer to neutralize. The protein in each fraction was measured(64), the pooled fractions lyophylised and stored desiccated at 4°C.

To test the effect of the biotin-digoxin conjugate on avidin-latex, 2μl of 4.56% polystyrene latex 0.17μ, was mixed with 0.5-1.0mg of avidin in 1.0M sodium bicarbonate buffer pH 8.0 and incubated 1.0 hour at 21°C. The solution was centrifuged for 30 minutes at 39,000 x g, the supernatant removed, the pellet was redissolved in 1.0ml of PBS and sonicated 2-4 seconds at 200 watts. This solution was diluted 1:3 with buffer, 100μl removed and added to 25μg of HABA, the absorbance measured at 500nm, various amounts of the digoxin-biotin conjugate added, final volume 0.5ml, and the the absorbance measured again at 500nm.

After demonstrating that the digoxin-biotin conjugate was able to displace HABA from solid phase avidin, the dilution of latex-avidin to use in scheme 2 was determined. Latex-avidin prepared as descibed above was diluted, 25μg of HABA added, and the absorbance at 500nm measured. To this solution 25μl of digoxin-biotin was added and the absorbance measured again at 500nm.

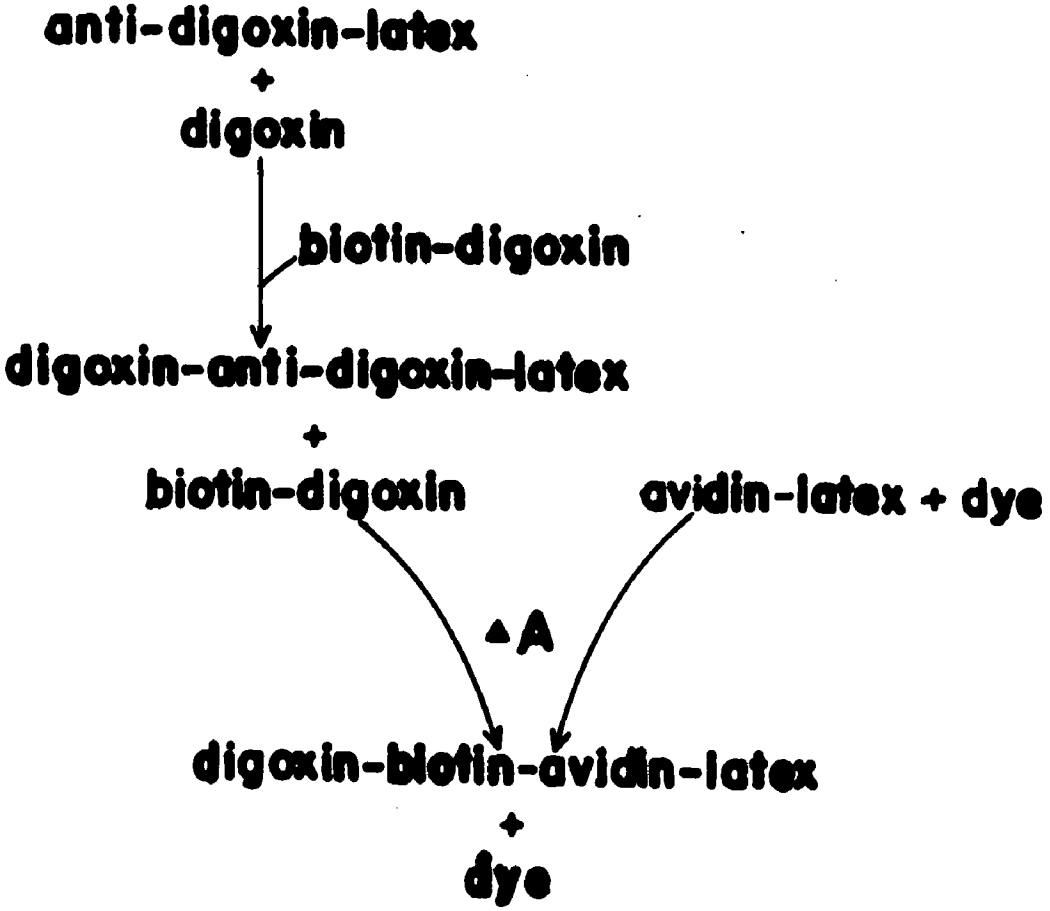
The final parameter, the dilution of latex-anti-digoxin, was determined by mixing 1.0μl of latex with 42μg of anti-digoxin, incubating 1.0 hour at 21°C and centrifuging at 39,000 x g for 30 minutes at 4°C. The supernatant was removed, the pellet redissolved in 1.0ml of 0.1M sodium bicarbonate buffer pH 8.0, and sonicated 2-4 seconds at 200 watts. Various dilutions of the latex-anti digoxin were mixed with 25μl of biotin-digoxin, and incubated 30 minutes at 21°C. To this solution 25μg of HABA, previously mixed with latex-avidin in which the absorbance was measured, was added and the absorbance at 500nm measured followed by 100μl of latex-avidin, prepared as above, and the

absorbance measured again at 500nm. Appropriate controls were used to subtract backround latex absorbance for the first and second measurements at 500nm.

Using the results from above, scheme 2 was performed by mixing various concentrations of unlabeled digoxin with 100$\mu$l of a 1:4 dilution of latex-anti digoxin. After incubating 30 minutes at 21°C, 50$\mu$l of biotin-digoxin solution containing 3.5$\mu$g of digoxin was added and incubated again for 30 minutes at 21°C. Undiluted latex-avidin, 100$\mu$l, was mixed with 25$\mu$g of HABA and the absorbance measured at 500nm. The HABA-avidin-latex solution was added to the biotin-digoxin solution and the absorbance measured again at 500nm.

Scheme 2: To solid phase anti-digoxin various concentrations of unlabeled digoxin were added, incubated 30 minutes at 21°C followed by addition of 50 $\mu$l of biotin-digoxin containing 3.5$\mu$g of digoxin and 13.5$\mu$g biotin, and again incubated 30 minutes at 21°C. Concurrently 100$\mu$l of a 1:3 dilution of latex-avidin was mixed with 25$\mu$g HABA and the absorbance at 500nm measured($A_1$). This solution was mixed with the latex-anti-digoxin solution, the absorbance at 500nm measured($A_2$), and the change in absorbance calculated after subtraction of backround absorbance by latex.

# SCHEME 2


anti-digoxin-latex
+
digoxin
biotin-digoxin
digoxin-anti-digoxin-latex
+
biotin-digoxin
avidin-latex + dye
ΔA
digoxin-biotin-avidin-latex
+
dye

Scheme 3

A third version of DSPDIA, scheme 3, employed avidin conjugated to IgG to partition between two solid phases(see scheme 3). The conjugate was prepared by reacting 2-iminothiolane with avidin and MBS with the antibody as described elsewhere(65). Briefly to 5.0mg of avidin enough 2 iminothiolane was added to yield a final concentration of 378$\mu$M, incubated 30 minutes at 21°C and the unbound 2 iminothiolane separated by gel filtration. To 2.6mg of purified sheep anti-rabbit IgG enough MBS was added to give a final concentration of 10mM, incubated 1.0 hour at 21°C, and unbound MBS separated by gel filtration. The absorbance at 280 of the eluent was monitored after which fractions containing protein were mixed, stirred for 2.0 hours at 21°C, and dialyzed at 4°C against several liters of PBS pH 7.4 at 4°C.

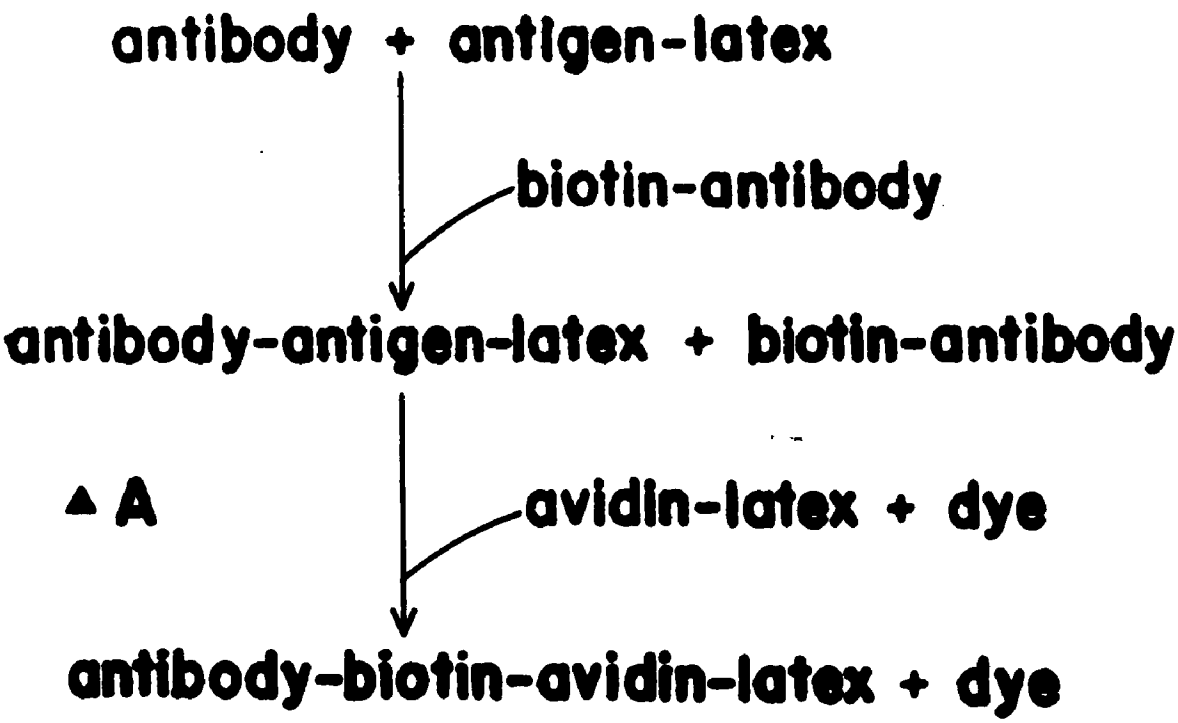
Solid phase biotin was prepared by mixing 1.0mg of d-biotin with 10$\mu$l of 4.56% latex, 0.17$\mu$, sonicating 2-4 seconds at 200 watts, and incubating 1.0 hour at 21°C. The solution was centrifuged for 30 minutes at 39,000 x g, the supernatent removed, and the pellet redissolved in 1.0ml of PBS and sonicated as before. To test the activity of the avidin-anti-digoxin conjugate with latex-biotin various concentrations of avidin-anti-IgG were mixed with 100$\mu$g of HABA and the absorbance at 500nm measured. To this solution 50$\mu$l of latex-biotin was added and the absorbance measured again at 500nm. The change in absorbance from the first to second measurement was calculated after subtracting the absorbance due to latex.

The assay procedure for scheme 3 was performed by mixing 100$\mu$l of a 1:20 dilution of latex-antigen, prepared as described, with various concentrations of unlabeled antibody and incubated 30 minutes at 21°C. Avidin-antibody,

3.13$\mu$g, was added, incubated 30 minutes at 21°C, 100$\mu$g of HABA added, and the absorbance at 500nm measured. To this solution 100$\mu$l of undiluted latex-biotin was added and the absorbance measured a second time at 500nm. The change from the first to second measurment was calculated after subtracting the effects of the latex.

Scheme 3: To 100$\mu$l of a 1:20 dilution of latex-antigen various concentrations of unlabeled antibody were added and incubated 30 minutes at 21°C. 3.13$\mu$g of avidin-antibody conjugate was added, incubated 30 minutes at 21°C, 25$\mu$g HABA added and the absorbance at 500nm measured($A_1$). 100$\mu$l of undiluted latex-biotin was added, the absorbance measured again at 500nm($A_2$), and the change in absorbance calculated after subtraction of background latex.

# SCHEME 3


antibody + antigen-latex
biotin-antibody
antibody-antigen-latex + biotin-antibody
▲ A
avidin-latex + dye
antibody-biotin-avidin-latex + dye

## 6.4 Results

Scheme I

Following preparation of biotinylated-BSA and avidin-digoxin each component was tested for activity. Avidin-digoxin in the presence of HABA and subsequently added d-biotin showed an increase in the change in absorbance as the concentration of avidin-digoxin increased. When biotin-BSA or latex-BSA-biotin was added instead of unlabeled biotin a similar change in absorbance was seen, see Fig. 2. Various dilutions of latex-BSA-biotin in the presence of HABA and avidin-digoxin conjugate showed the absorbance to decrease with increasing dilution of latex-BSA-biotin.

Determining the dilution of latex-anti-digoxin to use in scheme 1 showed the change in absorbance to increase with increasing dilution of latex-anti digoxin. This occurred with a fixed amount of latex-BSA-biotin, avidin-digoxin, and HABA demonstrating the partitioning between the two solid phases.

Using the procedure described in methods for scheme 1 a dose curve was found using concentrations of unlabeled digoxin from 0.2pg/ml to 2.0$\mu$g/ml, see Fig. 3. A change in absorbance of 0.011 was seen in the presence of as little as 0.2pg/ml while a change in absorbance as much as 0.025 for 2.0$\mu$g/ml digoxin compared to a control. For nine concentrations of analyte the average error between runs was 1.34% although the change in absorbance for 2.0pg/ml and 0.2ng/ml were far from the curve. The total time for the assay procedure was 60 minutes with the least amount of time for color change which was instantaneous.

Scheme 2

For scheme 2 the biotin-digoxin conjugate was purified by ion exchange

chromatography. After reduction with $NaBH_4$ and elution of product, analysis of each fraction showed a single large peak containing biotin to elute at fraction 10, see fig 4, a similar pattern was also found for digoxin, however the elution pattern was not as well-defined, see Fig. 4. The molar ratio of biotin to digoxin in the fraction showing the highest concentration of digoxin was 11.5 biotins/digoxin, an excess of 9.5 biotins.

Subsequently the biotin-digoxin conjugate was used to purify anti digoxin from whole sera. Analysis of eluted fractions for protein resulted in one peak containing approximately 430$\mu$g/ml of protein(fig 5).

After preparation biotin-digoxin the ability of the conjugate to displace HABA from avidin was shown since increasing concentrations of conjugate resulted in a concomitant increase in the change in absorbance. As little as 50$\mu$l of conjugate containing 3.5$\mu$g of digoxin and 13$\mu$g of biotin caused a change in absorbance of 0.152.

Once biological activity of biotin-digoxin was established parameters for scheme 2 were determined first by finding the dilution of solid phase avidin to use in the assay. It was found that with increasing dilution of latex-avidin the change in absorbance decreased. When avidin-latex was undiluted it produced the highest change in absorbance and therefore was chosen for use in the assay (Fig. 6).

Determining the dilution of latex-anti digoxin to use in scheme 2 was less conclusive. Various dilutions of solid phase antibody in the presence of biotin-digoxin conjugate, HABA, and avidin-latex did not show a change in absorbance consistent with changing dilutions of latex-anti digoxin. Thus the dilution of this solid phase to use in the assay could not be determined experimentally, and

was therefore chosen at 1:4 based on previous results from other dual solid phase schemes.

Using the above parameters no change in absorbance was seen for scheme 2 when a dose response curve was run using various concentrations of unlabeled digoxin.

Scheme 3

For scheme 3 adsorption of biotin onto latex was accomplished similarly to protein adsorption. Analysis of the supernatant after centrifugation to separate unbound biotin from latex showed 0.648mg of biotin bound to the latex, or $4.3\mu g/cm^2$. Addition of various concentrations of avidin-IgG showed an absorbance change proportional to the amount of conjugate added with fixed amounts of latex-biotin and HABA. p Since the first dual solid phase enzyme immunoassay worked best with a 1:20 dilution of latex-antigen the same was chosen for use in scheme 3. The change in absorbance was relatively constant from 0.0001 to $0.01\mu g/0.5ml$, however there was an increase of 0.067 in absorbance from 0.2 to $20\mu g/1.0ml$, see Fig. 7. The average "between run" error was 1.0% with approximately 60 minutes required to perform the assay.

**Figure 6-2:** Determining the dilution of Latex-BSA-biotin to use in scheme 1. To 3.5$\mu$g of avidin-digoxin in 0.9ml of 20mM $NaH_2PO_4$, pH 7.4, 25$\mu$g of HABA was added and the absorbance at 500nm measured($A_1$). 100$\mu$l of various dilutions of latex-BSA-biotin was added and the absorbance measured again at 500nm($A_2$). The change in absorbance from $A_1$ to $A_2$ was calculated after subtraction of background latex in $A_2$.

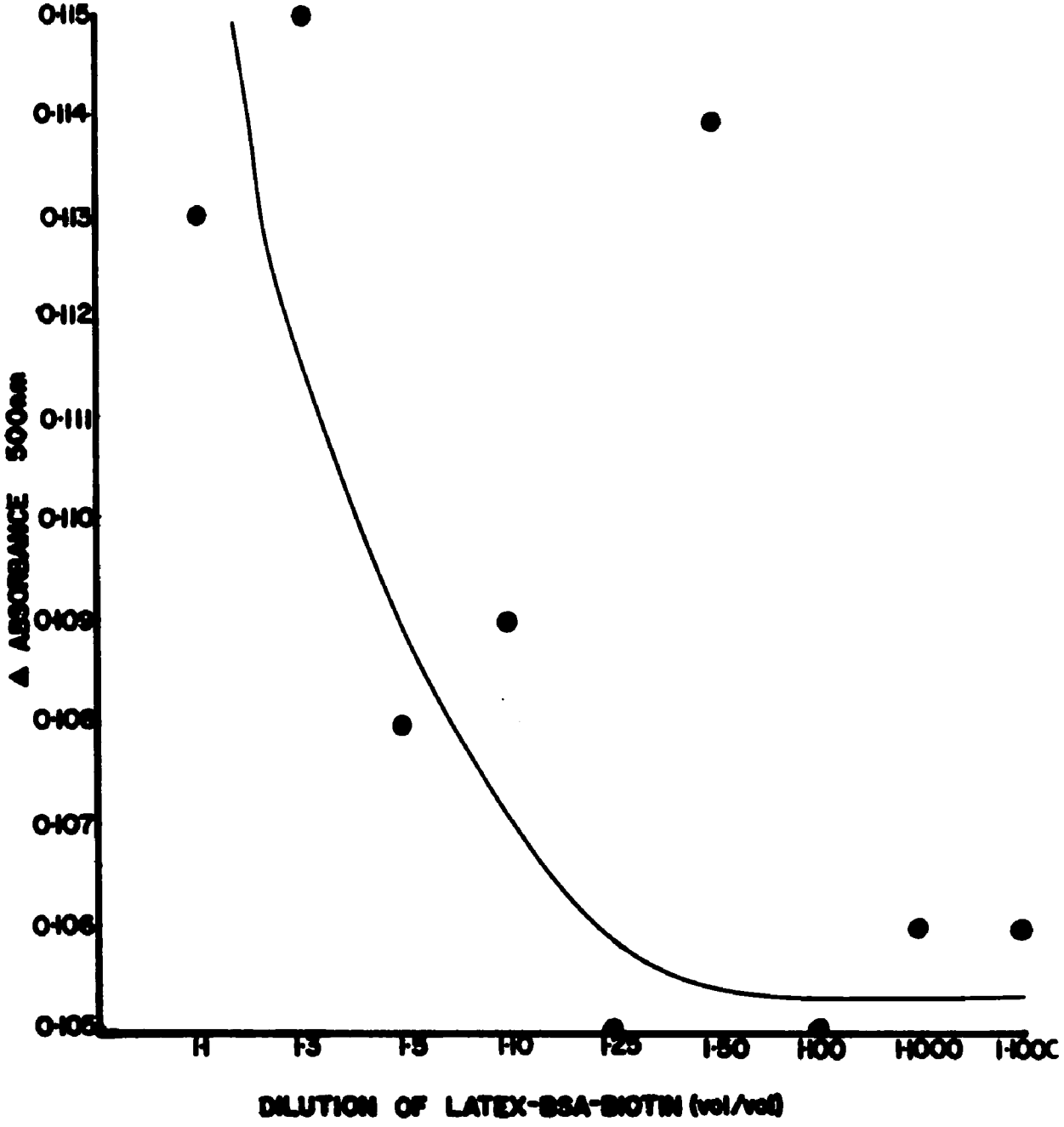

0·115
0·114
0·113
0·112
0·111
0·110
0·109
0·108
0·107
0·106
0·105
Δ ABSORBANCE 500nm
1
1·3
1·5
1·10
1·25
1·50
1·100
1·1000
1·1000
DILUTION OF LATEX-BSA-BIOTIN (vol/vol)

**Figure 6-3:** Dose response curve for scheme 1. To 100$\mu$l of a 1:3 dilution of latex-anti-digoxin various concentrations of unlabeled digoxin was added, incubated 30minutes at 21°C, 3.5$\mu$g of avidin- digoxin added and incubated 30minutes at 21°C. 25$\mu$g of HABA dye was added the absorbance at 500nm measured($A_1$), followed by addition of 100$\mu$l of undiluted latex-BSA-biotin and measurement of the absorbance again at 500nm($A_2$). The backround absorbance of latex was subtracted, the change in absorbance calculated and plotted vs the concentration of analyte.

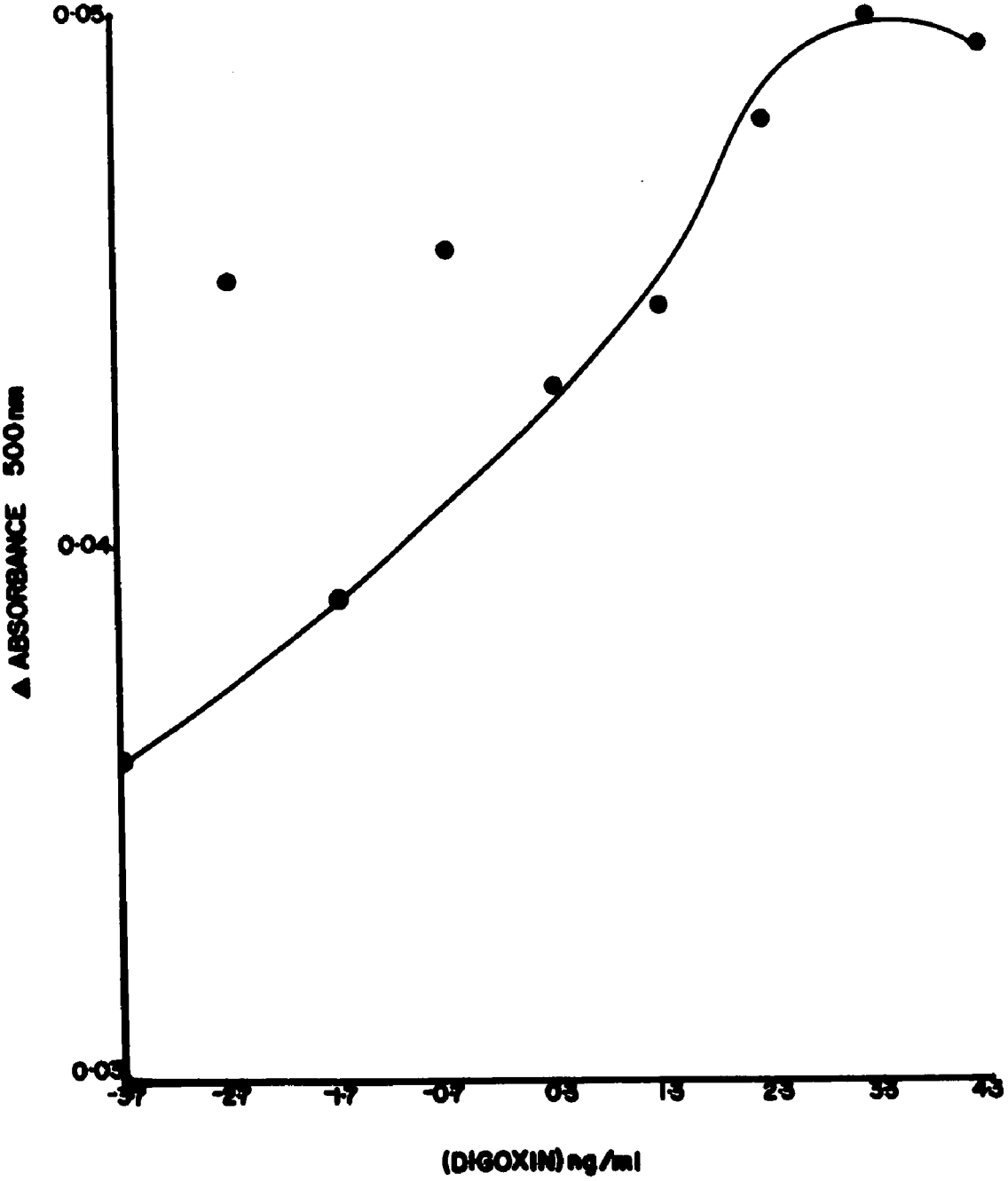

0·05
0·04
0·03
▲ ABSORBANCE 500nm
-3·1
-2·7
-1·7
-0·7
0·3
1·3
2·3
3·3
4·3
(DIGOXIN) ng/ml

**Figure 6-4:** Purification of biotin-digoxin by ion exchange chromatography. Oxidized digoxin was reacted with biotin-hydrazide, reduced with $NaBH_4$, applied to the ion exchange column and eluted with 0.35M sodium citrate buffer, pH 4.0. The concentration of biotin and digoxin was determined in each fraction with fraction 9, which contained the highest concentration of digoxin, having 11.5 moles of biotin/digoxin, an excess of 9.5 biotins.

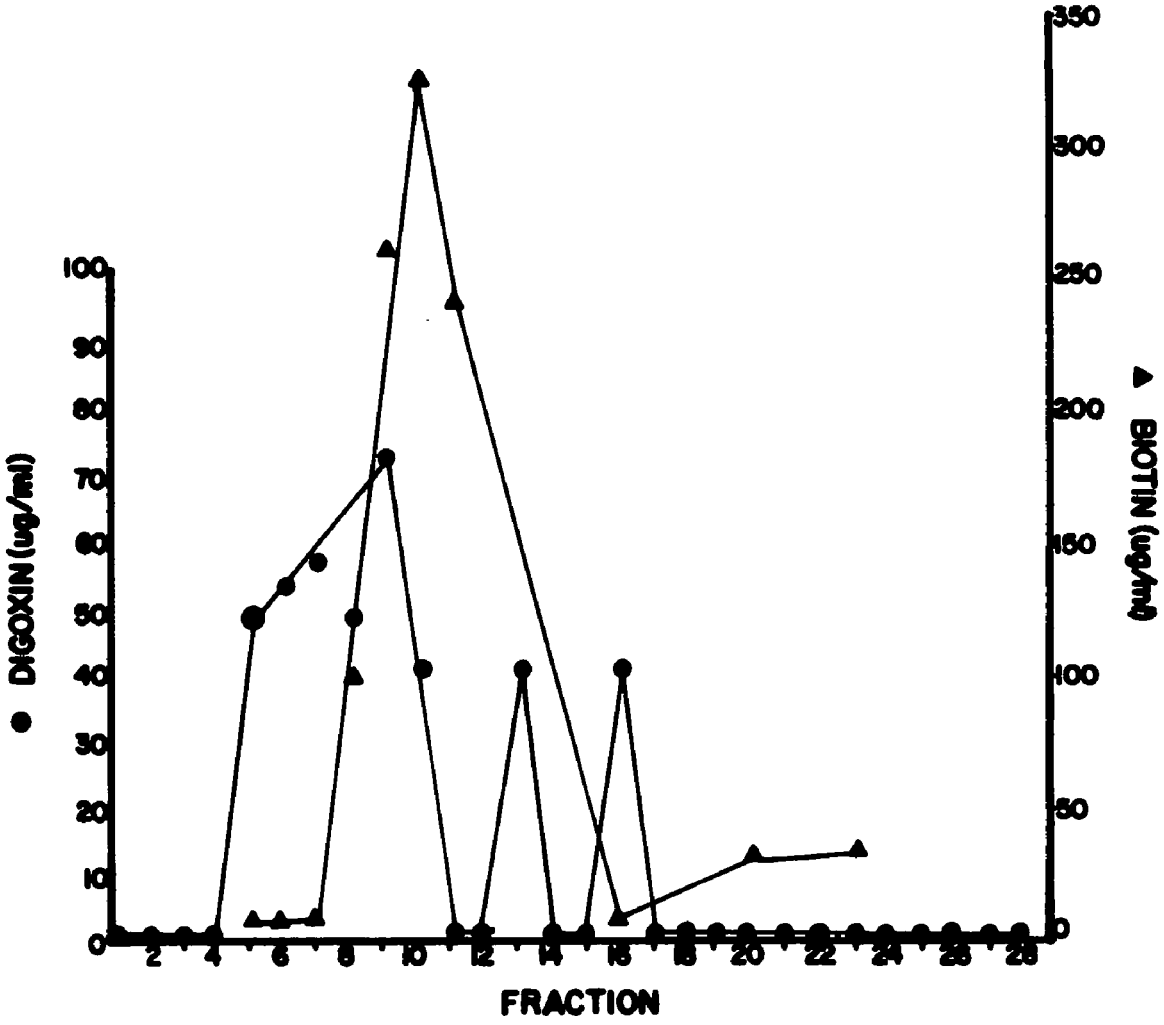

● DIGOXIN (ug/ml)
▲ BIOTIN (ng/ml)
FRACTION
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
250
300
350
2
4
6
8
10
12
14
16
18
20
22
24
26
28

**Figure 6-5:** Purification of anti-digoxin by affinity chromatography. A 3000 molar excess of digoxin-biotin was added to anti-digoxin in whole sera and incubated 24 hours at 4°C. The solution was mixed with avidin-Sepharose, and shaken gently for 24 hours at 4°C. The mixture was poured into a column and washed with 0.1M carbonate buffer until no protein was detected in the eluent. Anti-digoxin was eluted with 0.1M NaCl, pH 2.5, and the protein in each fraction determined.

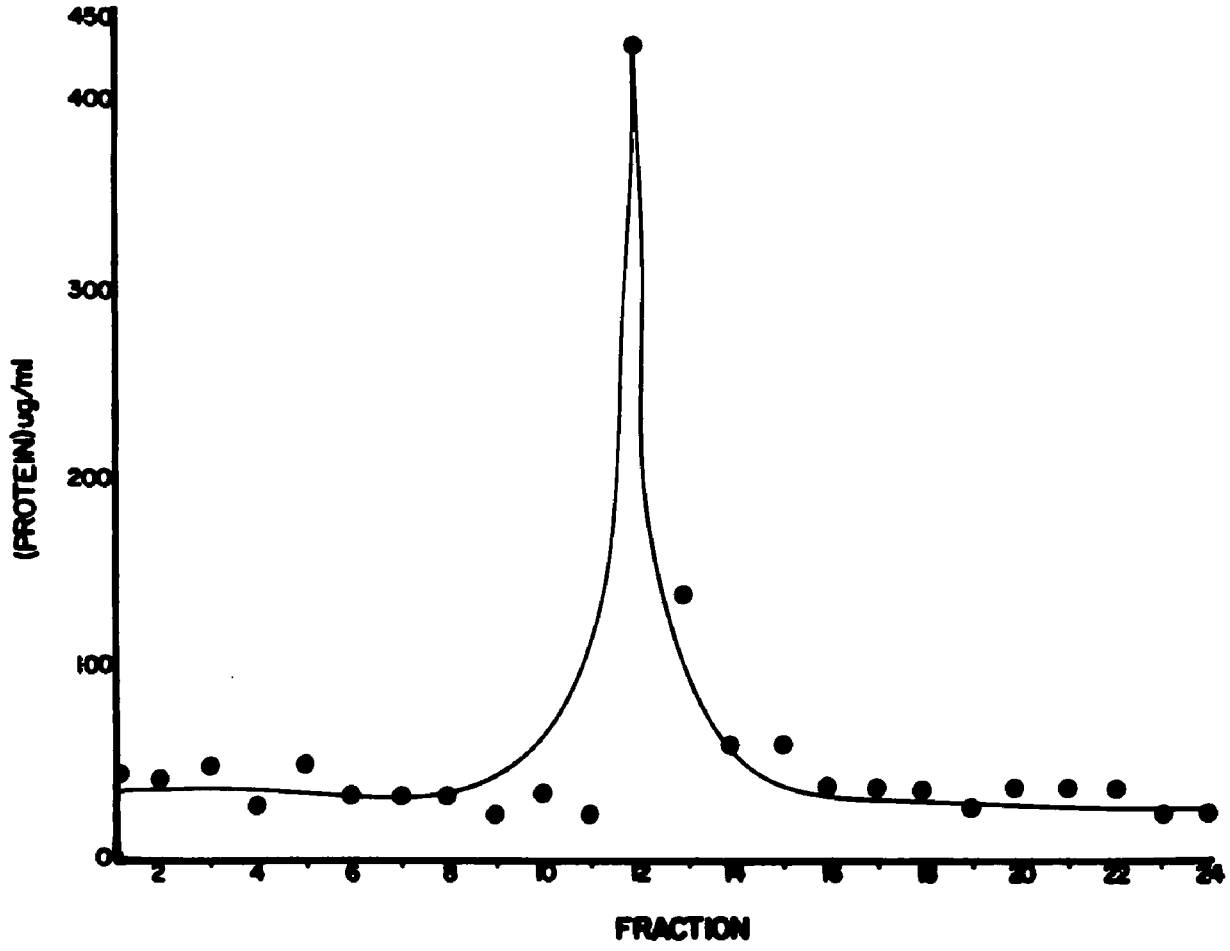

(PROTEIN)ug/ml
450
400
300
200
100
0
2
4
6
8
10
12
14
16
18
20
22
24
FRACTION

**Figure 6-6:** Determination of the dilution of latex-avidin to use in scheme 2. To 100$\mu$l of various dilutions of latex-avidin in 0.4ml of 0.1M $NaH_2PO_4$ pH 8.0 25$\mu$g of HABA dye was added and the absorbance at 500nm measured ($A_1$). To this solution 25$\mu$l of biotin-digoxin conjugate containing 1.8$\mu$g of digoxin and 6.5$\mu$g of biotin was added and the absorbance measured again at 500nm($A_2$). The change in absorbance from $A_1$ to $A_2$ was calculated and plotted after subtraction of backround latex in $A_2$.

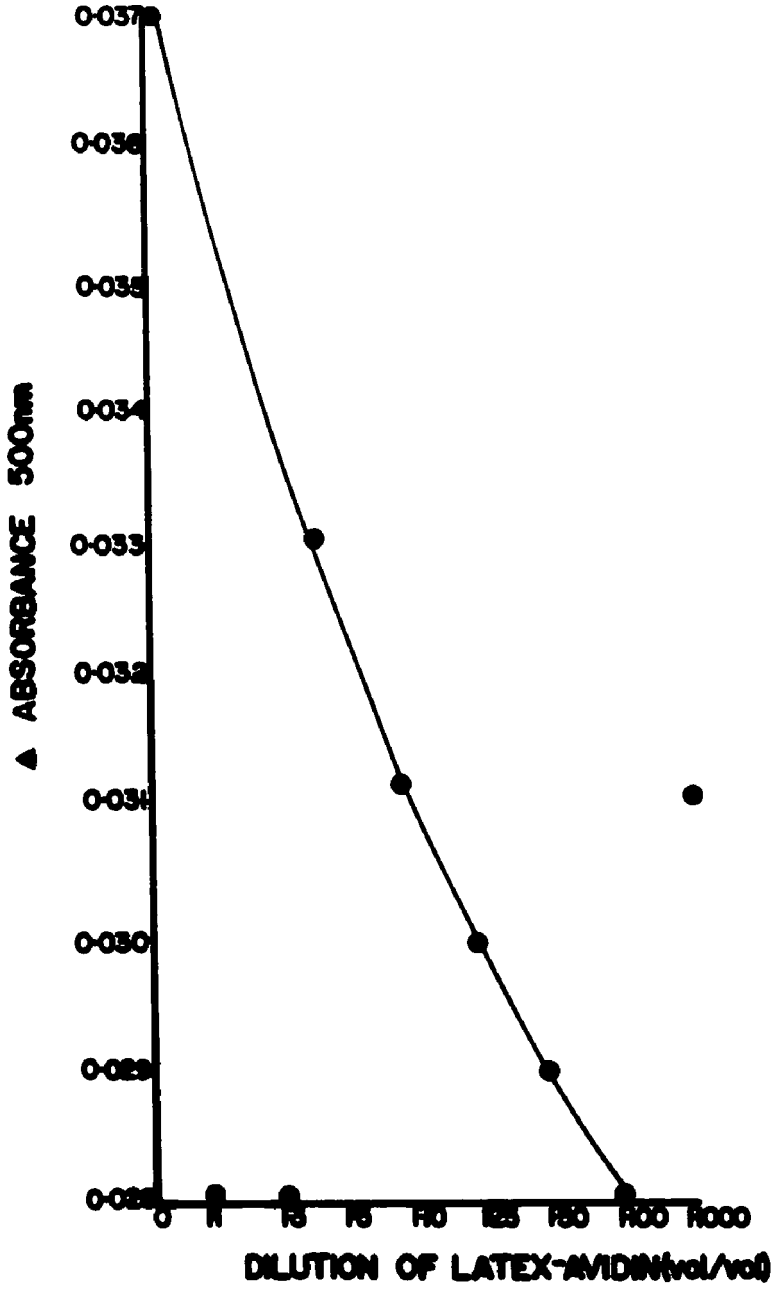
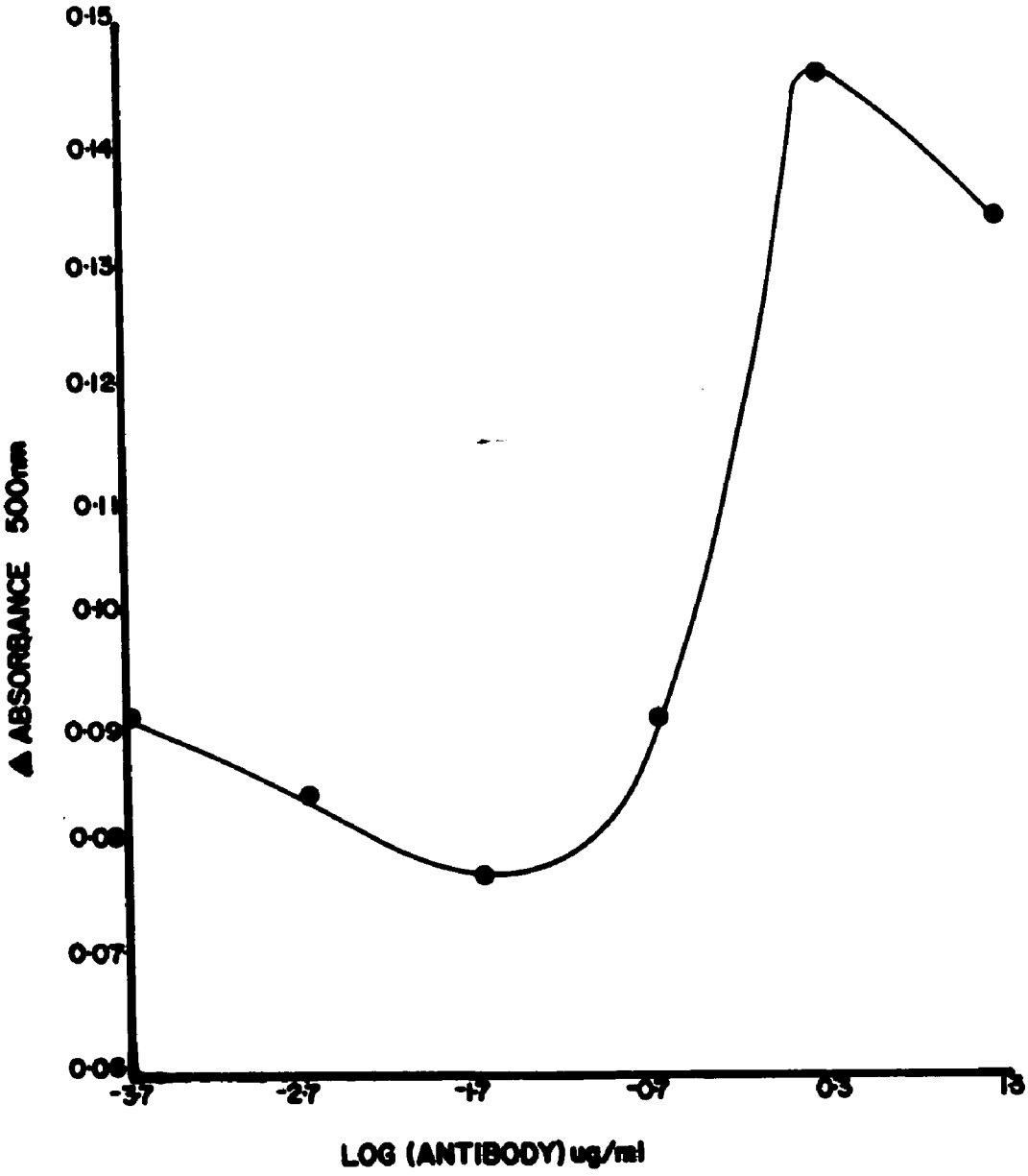

ABSORBANCE 500nm
0·036
0·035
0·034
0·033
0·032
0·031
0·030
0·029
0
DILUTION OF LATEX-AVIDIN(vol/vol)

**Figure 6-7:** Dose response curve for scheme 3. To 100$\mu$l of a 1:20 dilution of latex-antigen various concentrations of unlabeled antibody was added and incubated 30 minutes at 21°C. To this solution 3.13$\mu$g of avidin-antibody was added, incubated 30 minutes at 21°C, 25$\mu$g of HABA dye added, the absorbance at 500nm measured($A_2$), backround absorbance of latex subtracted, and the change in absorbance calculated and plotted.


0·15
0·14
0·13
0·12
0·11
0·10
0·09
0·08
0·07
0·06
▲ ABSORBANCE 500nm
-3·7
-2·7
-1·7
-0·7
0·3
1·3
LOG (ANTIBODY) ug/ml

## 6.5 Discussion

Although three DSPDIA assay schemes were attempted, only scheme 1 and 3 were able to produce dose response curves. Using scheme 1 and 3 it was possible to use the dual solid phase scheme to demonstrate detection of small and large molecular weight analytes. Previously such analytes were detected in competitive assay systems using enzymatic, isotopic, or fluorometric labels as the marker, or in assay schemes requiring separation steps. Here a dye producing a color change detected spectrophotometrically used with avidin and biotin could detect as little as 0.2pg/ml of digoxin and 0.1$\mu$g/ml macromolecular antibody.

Although scheme 1 and 3 were able to produce dose curves there were several problems with these assay schemes. For scheme 1 sensitivity was good, however the change in absorbance was small over several orders of magnitude change in analyte concentration. At 0.2pg/ml the change in absorbance was 0.011 while at 2.0$\mu$g/ml 0.025. This small change in absorbance over a wide range of analyte concentrations is unacceptable. In scheme 3 it was found that a dose response curve was produced using a 1:20 dilution of solid phase antigen similar to the dilution used in the first DSPEIA, however the sensitivity was 50 fold less in scheme 3. In the original DSPEIA scheme, see chapter 5, 2.0ng/ml was detectable while using scheme 3, 0.1$\mu$g/ml macromolecular antibody was detectable. This decrease in sensitivity results from the inadequacy of the dye to detect small changes in biotinylated-analyte concentration while the amplification effect of enzymes in the original DSPEIA allowed an increase in detectability.

For scheme 2 several explanations for its inability to produce a dose response curve are plausible. A large excess of biotin, 9.5moles/digoxin, could

have disrupted partitioning between the two solid phases. Excess biotin would have bound to solid phase avidin thus blocking binding sites for biotinylated assay components. Such an occurence would not have been detected since the binding of biotin to avidin would have been too rapid to measure. A second possibility for failure of scheme 2 may have been solid phase anti-digoxin. Purified by affinity chromatography, the antibody was not tested for activity after elution nor after it was adsorbed onto latex.

Purification of anti-digoxin using a digoxin-biotin conjugate was a unique application of the affinity of avidin for biotin. Conjugation of digoxin to biotin was simple and purified by ion exchange chromatography. When excess digoxin-biotin conjugate was mixed with antibody in whole sera the conjugate acted as a link to avidin immobilised onto sepharose. The binding of biotin to avidin is extremely tight and not easily broken(26), however a change in pH could disrupt the association between digoxin and anti-digoxin. Thus it was possible to use avidin and a biotin conjugate to isolate a desired serum component and recover it selectively. Elution of the anti-digoxin from the affinity column resulted in a single peak containing 430$\mu$g/ml of protein. However the biological activity of the product could not be determined.

The use of HABA coupled with the high affinity of avidin for biotin is a unique characteristic ideal for detecting the binding of biotin to avidin. Binding of biotin to avidin in the presence of HABA produces an instantaneous change in absorbance measured spectrophotometrically. Because avidin and biotin can be coupled to proteins and other analytes it was intriguing to see if HABA could be employed as a marker in an assay system. Although several assay schemes were tested, application of the HABA system to the dual solid phase

system was less than adequate. Perhaps if the spectrophotometric change due to HABA displacement from avidin could be amplified the detection method would prove more viable.

# Chapter 7
# The Dual Solid Phase Competitive Enzyme Immunoassay. DSPCEIA

## 7.1 Abstract

In chapter 7 a final variation of the dual solid phase assay is presented. Biotin-antibody binds to solid phase avidin, biotin-enzyme is then added and binds to unoccupied avidin inactivating the enzyme. The amount of enzymatic activity is proportional to analyte concentration. Using this assay scheme for detection of macromolecular antibody, the assay is sensitive between 1.0 and 100$\mu$g/ml with an absorbance reading of 0.082 at 1.0$\mu$g/ml and 0.106 at 100$\mu$g/ml. The assay requires 2 hours to perform with an additional 3 hours for substrate color development. When dose response curves were run 4 days apart in triplicate using the same concentrations of analyte the average "between run" error was 2.3%.

## 7.2 Introduction

Following closely the development of the first heterogeneous EIA by Engvall and Perlman(23), Rubenstein et al. introduced the first homogeneous EIA for morphine(53). Based on the competition between enzyme labeled analyte and unlabeled analyte for a common binding site, the amount of enzyme activity was proportional to enzyme concentration. This homogeneous technique has proven extremely useful for small molecular weight analytes yet is limited for sensitive detection of large macromolecules compared to heterogeneous EIA. In the area of diagnostics homogeneous EIA has been used for detection of numerous therapeutic drugs since the sensitivity of the assays is easily within clinically therapeutic serum concentrations and have a high degree of accuracy and reproducibility. Various enzyme labels have been used in these assays, the key being the inhibition of enzyme label upon binding to anti-analyte, common in the EMIT assay system(66,67).

The common binding site for labeled and unlabeled analyte is sometimes composed of anti-analyte bound to a solid phase. A variety of materials are available for use as a solid phase such as glass, polystyrene latex, nitrocellulose, charcoal, agarose, and sepharose. Each of these possess characteristics desirable under certain assay conditions, however a solid phase used in some heterogeneous EIA is polystyrene latex(68,69). Polystyrene is available in various forms from tubes to spheres and can be prepared containing various functional groups if covalent binding is necessary. Polystyrene prepared without available surface functional groups may have protein adsorbed onto the surface resulting in easily prepared solid phases of biologically active protein. The mechanism of the adsorption process has not yet been determined(70), however

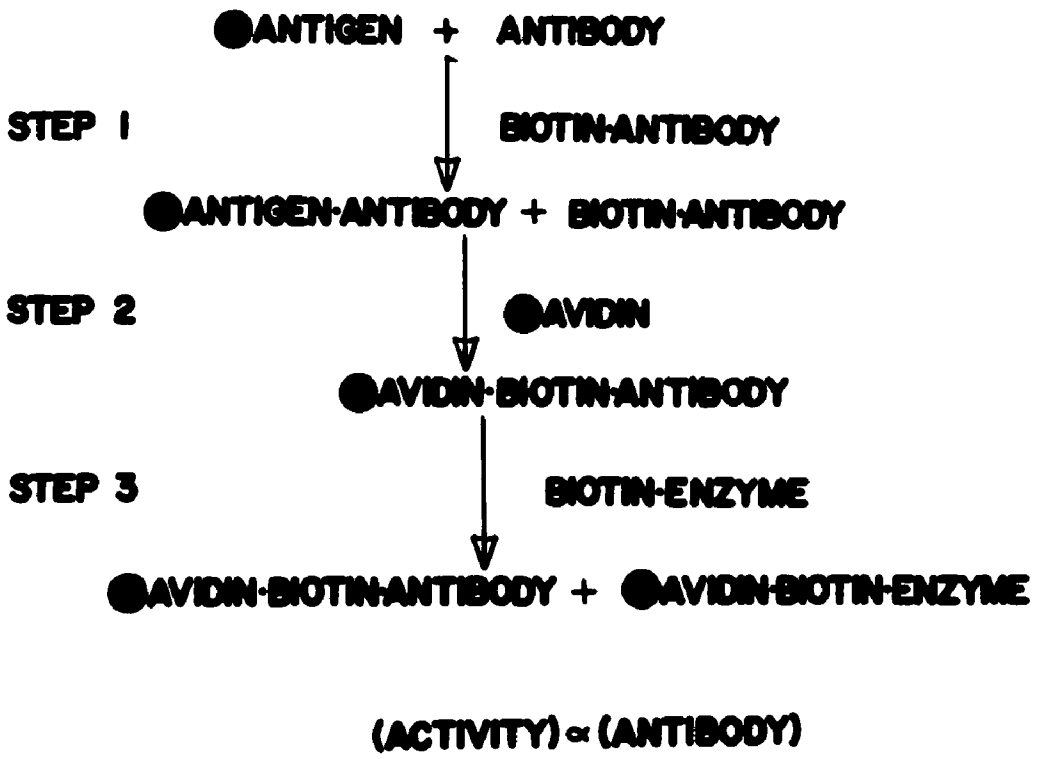
it is known that using polystyrene latex, hydrophobic regions of protein molecules will orient toward the latex surface leaving hydrophilic regions facing aqueous solution, and at or close to the pI of many proteins adsorption will occur with the highest efficiency(71). These prepared solid phases are stable and, if using spheres, have the added advantage of mobility by simply pipetting.

Since homogeneous EIA has predominantly been used for assay of small molecules it was of interest to examine a competitive homogeneous assay for macromolecules, and since the dual solid phase system is "homogeneous" it was a likely candidate for the assay scheme. In this assay scheme biotin labeled antibody and unlabeled antibody compete for solid phase antigen. Remaining biotin labeled antibody binds to solid phase avidin thus partitioning between the two solid phases. Biotinylated enzyme is added binding to unoccupied avidin on the latex becoming inactivated. Competition between biotinylated antibody and biotinylated enzyme followed by measurement of enzyme activity is proportional to analyte concentration(see scheme 1). Using this scheme, termed the Dual Solid Phase Competitive Enzyme Immunoassay(DSPCEIA), biotin labeled assay components compete for solid phase avidin, the analyte is a macromolecular antibody, and analyte concentration is proportional to enzyme activity similar to previous competitive homogeneous EIA.

Scheme 1: Principles of the Dual Solid Phase Competitive Enzyme Immunoassay. In step 1 solid phase antigen was added to various concentrations of unlabeled antibody, incubated, and biotinylated antibody was added and incubated. In step 2 avidinylated latex was added to the solution and incubated. In step 3 biotinylated G6PDH was added, incubated, substrate solution added, and the enzymatic activity measured and plotted against analyte concentration.

# DSPCEIA

## THE DUAL SOLID PHASE COMPETITIVE ENZYME IMMUNOASSAY




(ACTIVITY) ∝ (ANTIBODY)

## 7.3 Materials/Methods

Glucose-6-phosphate dehydrogenase(G6PDH)(E.C. 1.1.1.49) from yeast having 330 units/mg solid, N-hydroxysuccinimido biotin(NHS-biotin), iodonitrotetrazolium violet(INT), diaphorase from C. kluyveri(E.C. 1.6.4.3) containing 5.9 units/mg solid, glucose-6-phosphate, NADP, and NADPH were obtained from the Sigma Chemical Co.. All materials were stored at 0°C or 4°C as appropriate and reconstituted in 20mM Tris/10mM EDTA pH 8.0 unless described otherwise. G6PDH was assayed using a solution containing 50$\mu$g INT, 2 units diaphorase, 100$\mu$g G6P, 100$\mu$g NADP, reacted, quenched with 100$\mu$l of 1.0M HCl, and the absorbance at 492nm measured.

Glucose-6-phosphate dehydrogenase was biotinylated by methods described elsewhere(49). To a solution of 35mM G6P and 1.8mM NADPH, 1.04$\mu$M G6PDH was added, incubated 10 minutes at 4°C, 40$\mu$g of NHS-biotin was added and the solution was incubated 2 hours at 21°C. Aliquotes were removed every 30 minutes and assayed for activity. After 2 hours the solution was applied to a Sephadex G-25 column, eluted, the protein and enzymatic activity in each fraction was determined(64), fractions containing enzymatic activity were dialyzed overnight at 4°C. After dialysis biotin concentrations and enzymatic activity were determined(30).

Sheep anti-rabbit IgG, affinity purified as described previously(65), was biotinylated by mixing a four molar excess of NHS-biotin, incubating 2 hours at 21°C, and dialyzing overnight against several changes of buffer at 4°C.

To test the effect of avidin-latex on biotinylated G6PDH and to determine the dilution of solid phase avidin to use in the assay 0.5-1.0mg of avidin was mixed with 2$\mu$l of 4.56% polystyrene latex, 0.17$\mu$, sonicated 2-4 seconds at 200

watts, incubated 1.0 hour at 21°C, and centrifuged at 39000 x g for 30 minutes. The supernatant was removed, the pellet was redissolved in 1.0ml of buffer, and the solution was sonicated as before. Various dilutions of latex-avidin were prepared, 100$\mu$l of each dilution added to 0.6ml of buffer, 0.05$\mu$g of biotinylated enzyme was added, incubated 10 minutes at 21°C, 200$\mu$l of diaphorase assay mixture was added, incubated 10 minutes at 21°C, the reaction was quenched and the absorbance at 492nm was measured. After measurement of enzymatic activity the percent inactivation of the enzyme was calculated using a control containing no avidin-latex as 100% of activity.

To determine the concentration of biotinylated-antibody to partition between the two solid phases various concentrations were mixed with 100$\mu$l of a 1:1 dilution of avidin-latex, incubated 30 minutes at 21°C, 0.05$\mu$g of biotinylated G6PDH was added and incubated for 30 minutes at 21°C. To this solution 200$\mu$l of substrate mix was added incubated 30 minutes at 21°C, quenched with 100$\mu$l of 1.0M HCl, the absorbance at 492nm measured, and the % inactivation calculated by comparison to a control containing enzyme alone.

The last parameter of the assay to be determined was the dilution of latex-antigen. To 0.5-1.0mg of rabbit IgG, 2$\mu$l of 4.56% polystyrene latex, 0.17$\mu$, was added, sonicated 2-4 seconds at 200 watts, incubated 60 minutes at 21°C, centrifuged for 30 minutes at 39000 x g, the supernatant was removed, the pellet was redissolved in 1.0ml of buffer and sonicated 2-4 seconds at 200 watts. After sonication 100$\mu$l of various dilutions of latex-avidin was added to 0.5ml of buffer containing 3.0$\mu$g of biotinylated antibody, incubated 30 minutes at 21°C, 100$\mu$l of a 1:1 dilution of latex-avidin was added and incubated 30 minutes at 21°C. To this solution 0.05$\mu$g of biotinylated G6PDH was added,

incubated 30 minutes at 21°C, 200μl of assay mixture was added, incubated 3 hours at 21°C, the reaction was quenched with 100μl of 1.0M HCl, the absorbance at 492nm was measured and the % inactivation was calculated by comparison to a control containing enzyme alone.

Using the above results the DSPCEIA was performed by mixing various concentrations of unlabeled antibody with 100μl of a 1:10 dilution of latex-antigen, incubating 30 minutes at 21°C, adding approximately 3.0μg of biotinylated antibody, and incubating 30 minutes at 21°C. To this solution 100μl of a 1:1 dilution of latex-avidin was added, incubated 30 minutes at 21°C, 0.05μg of biotinylated G6PDH was added, and incubated again for 30 minutes. 200μl of substrate mixture was added, incubated 3 hours at 21°C, the reaction was quenched with 100μl of 1.0M HCl, and the absorbance at 492nm was measured. The background absorbance due to latex was subtracted and the percent inactivation of the enzyme calculated by comparison to a control consisting of enzyme alone.

## 7.4 Results

The key to the DSPCEIA was the ability of avidinylated latex to bind and inactivate biotinylated G6PDH. Analysis of biotin-G6PDH prepared by the method used in this chapter have been found to contain 30.8 biotin/mole of enzyme(49). When various concentrations of biotinylated G6PDH were mixed with avidinylated latex having 4.25$\mu$g/cm$^2$ avidin the enzyme was inactivated as dilution of avidin-latex decreased. Therefore a dilution of 1:1 avidin-latex was chosen for use in the assay because it provided the highest degree of enzyme inactivation with the lowest possible amount of latex backround absorbance. Further, when various concentrations of biotinylated-antibody were mixed with avidinylated latex followed 30 minutes later by 0.05ug of biotinylated G6PDH, enzyme activity increased with increasing concentration of biotin-antibody(see fig. 1).

Once the competition between the two biotinylated components was shown the correct dilution of solid phase antigen was determined to demonstrate the partitioning of biotin-antibody between the solid phases. Increasing dilution of latex-antigen in the presence of 3.0ug of biotin-antibody showed the enzymatic activity to decrease with increasing dilution of solid phase antigen(see fig. 2). Thus as less antigen was available to bind, the antibody-biotin competed with biotin-G6PDH for avidin reflected by an increase in enzymatic activity.

Parameters for the assay were as follows: 100$\mu$l of a 1:10 dilution of latex-antigen, 3.0$\mu$g of biotinylated-antibody, 100$\mu$l of a 1:1 dilution of latex-avidin, and 0.05$\mu$g of biotinylated G6PDH. Using the above parameters and procedure described in materials and methods the DSPCEIA was performed in 2.0 hours with an additional 3.0 hours for the enzymatic reaction. The assay was

sensitive between 1.0 and 100$\mu$g/ml with an absorbance reading of 0.082 at 1.0$\mu$g/ml and 0.106 at 100$\mu$g/ml. Running dose response curves in triplicate four days apart using the same concentrations of analyte produced similar curves with an average between run error of 2.3%, see fig. 3.

**Figure 7-1:** Competition between biotin-G6PDH and biotin-antibody for solid phase avidin. To 100$\mu$l of a 1:1 dilution of latex-avidin in 0.5ml of 20mM Tris/10mM EDTA pH 8.0, various concentrations of biotin-antibody was added and incubated. After 30 minutes 0.05$\mu$g of biotinylated-G6PDH was added, incubated 30 minutes at 21°C, 0.2ml of substrate mix added, incubated 30 minutes at 21°C, quenched with 1.0M HCl and the absorbance at 492nm measured.

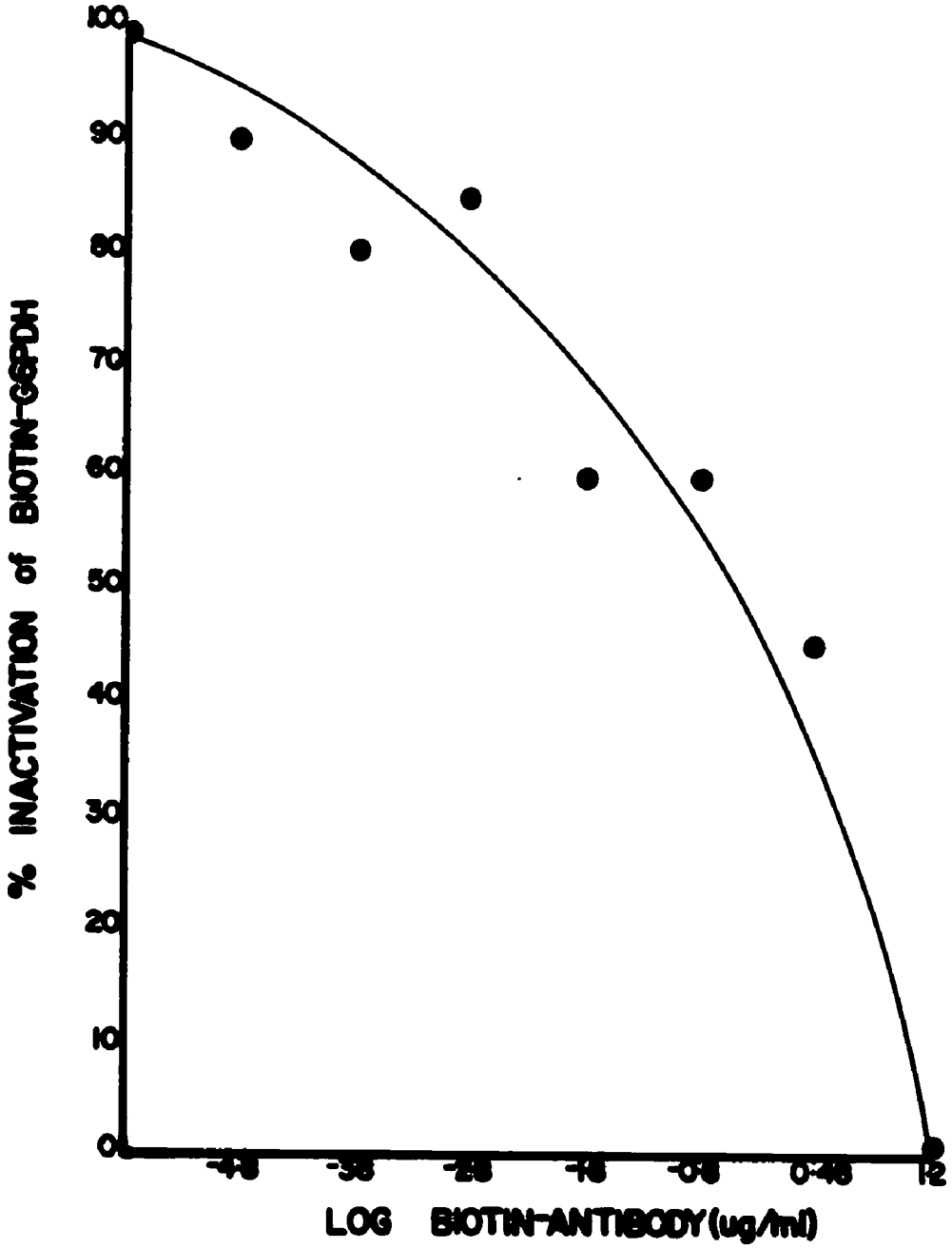

% INACTIVATION of BIOTIN-GSPDH
100
90
80
70
60
50
40
30
20
10
0
-1.2
LOG BIOTIN-ANTIBODY (ug/ml)

**Figure 7-2:** Effect of latex-antigen on biotin-G6PDH activity. To 3.0$\mu$g biotin-antibody in 0.48ml 20mM Tris/10mM EDTA pH 8.0, 0.1ml of various dilutions of latex-antigen were added and incubated 30 minutes at 21°C. To this solution 0.1ml of a 1:1 dilution of latex-avidin was added, incubated 30 minutes at 21°C, 0.05$\mu$g of biotin-G6PDH added, and incubated 30minutes at 21°C. Assay mixture was added, incubated 3 hours at 21°C, quenched with 1.0M HCl, the absorbance at 492nm measured and the % of biotin-G6PDH inhibition calculated.

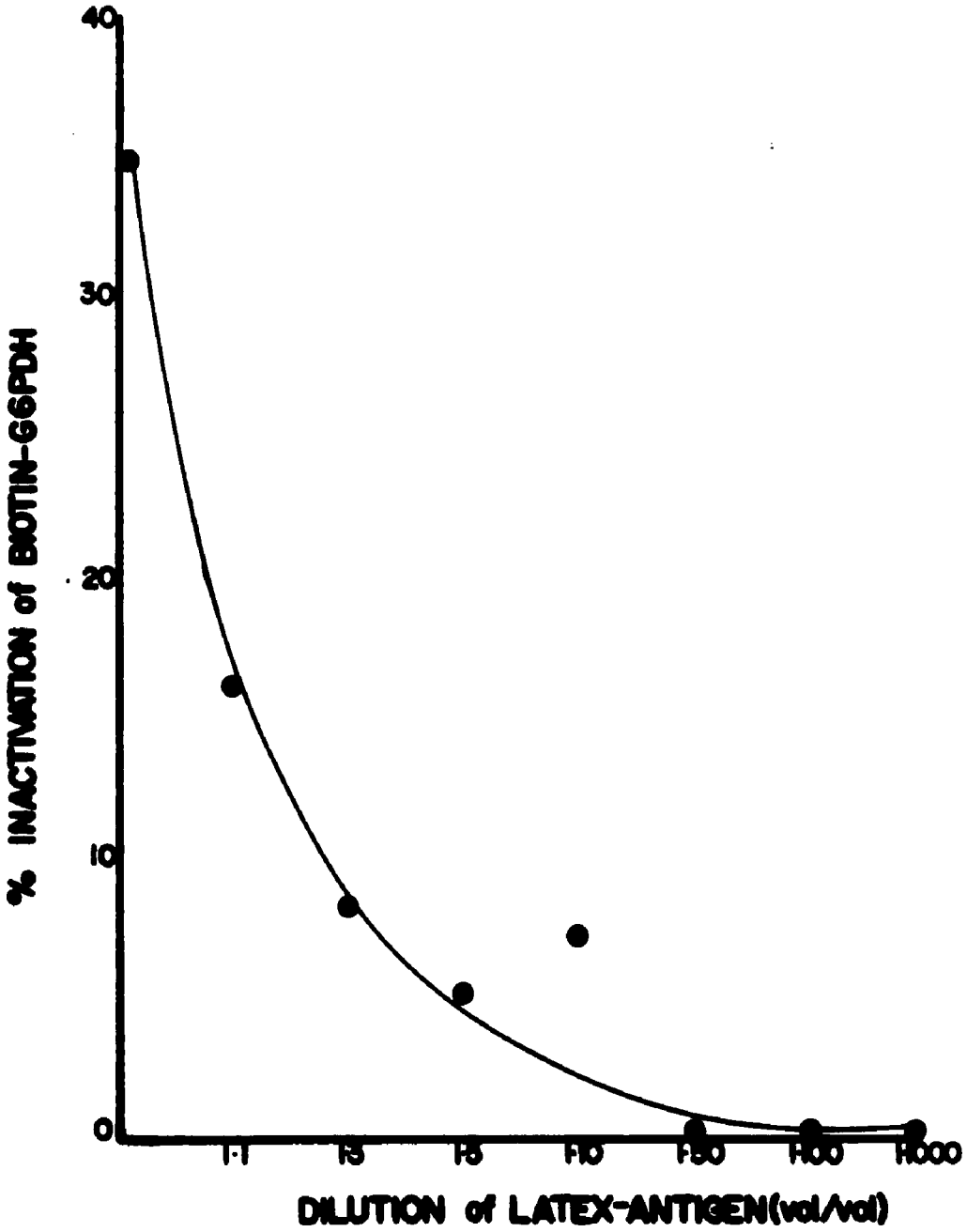
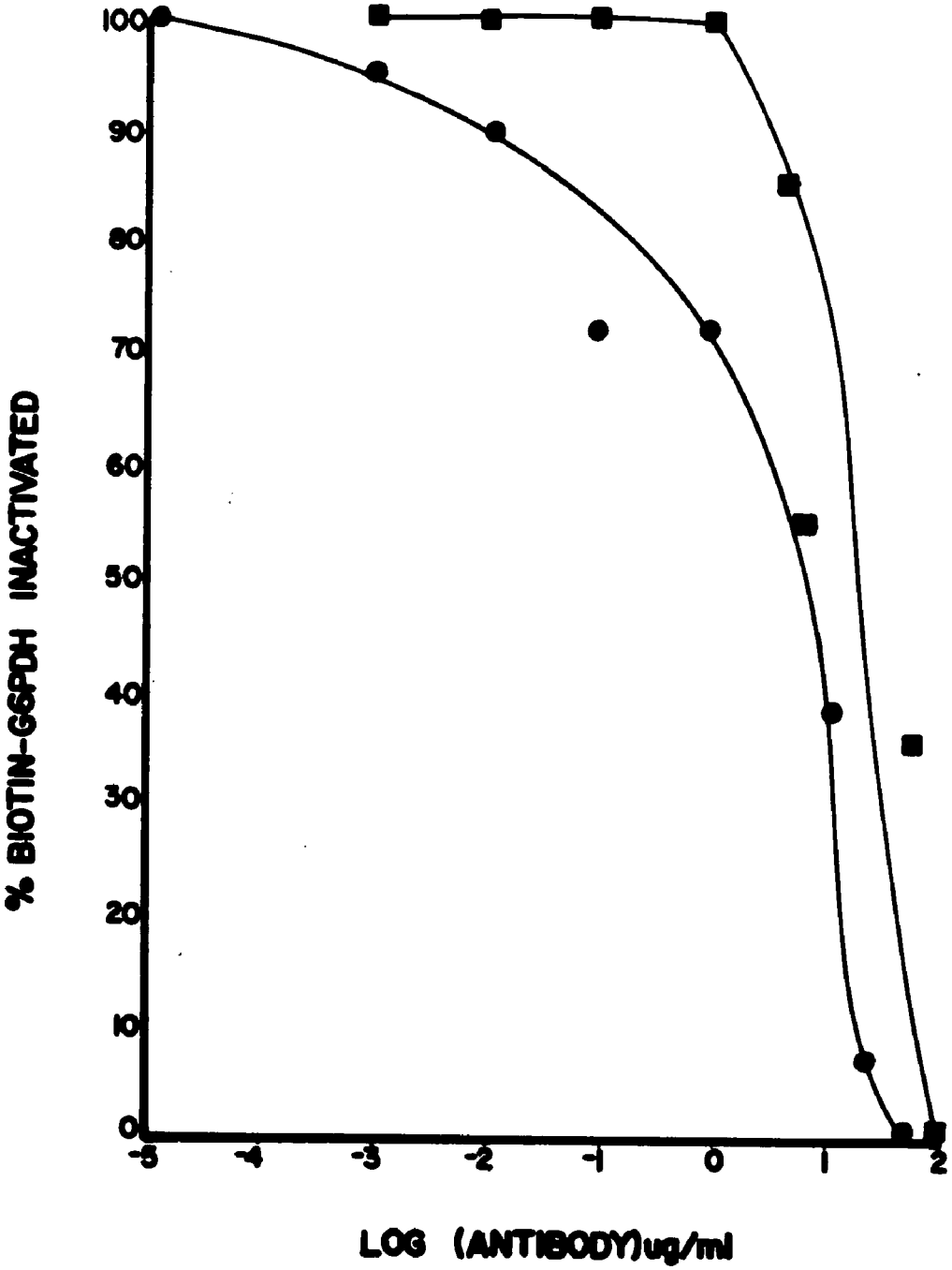

40
30
20
10
0
% INACTIVATION of BIOTIN-G6PDH
1:1
1:5
1:5
1:10
1:50
1:100
1:1000
DILUTION of LATEX-ANTIGEN(vol/vol)

**Figure 7-3:** Results of the Dual Solid Phase Competitive Assay. Performed on day 1(●) and day 4(■) latex-antigen was added to various concentrations of unlabeled antibody, incubated 30 minutes at 21°C, 3.0μg of biotin-antibody added and incubated 30 minutes. To this solution latex-avidin was added, incubated 30 minutes at 21°C, 0.05μg of biotin-G6PDH added, and incubated again for 30 minutes. Substrate was added, incubated 30 minutes at 21°C, quenched with 1.0M HCl, and the absorbance at 492nm measured and compared to a control to give the % of enzyme inactivated.


% BIOTIN-G6PDH INACTIVATED
100
90
80
70
60
50
40
30
20
10
0
-5
-4
-3
-2
-1
0
1
2
LOG (ANTIBODY)ug/ml

## 7.5 Discussion

Generally accepted, homogeneous EIA's are used for assay of small molecular weight analytes. Sensitivities of these assays vary but have been reported ranging from 0.5ng/ml for small molecular weight drug analytes(MW<300) to 40$\mu$g/ml for antimicrobial drugs requiring minutes to 1 hour to perform(72). The utility of such assays depends on the concentration of the analyte, especially if used clinically which many times requires less sensitivity and more speed. The DSPCEIA having a sensitivity of 1.0$\mu$g/ml was comparable to other homogeneous EIA for small molecular weight analytes. However 5 hours were required to perform the assay which is extremely long compared to other homogeneous assays. The time required for performance of the DSPCEIA was more closely comparable to heterogeneous EIA, which require a separation step and a similar amount of time.

Competitive enzyme immunoassays rely upon the affinity of antibody for labeled and unlabeled analyte and the affinity of antibody for antigen generally has a Ka of approximately $10^9$. In the DSPCEIA the use of avidin and biotin, $K_D=10^{-15}\underline{M}$, did not produce an assay of superior sensitivity. Sensitivty was 1.0ug/ml for macromolecular antibody influenced by the first step of the assay which involved competition between biotinylated antibody and unlabeled antibody. Other available methods for detection of macromolecules using avidin and biotin are a 1000 fold more sensitive, the only disadvantage being the necessity of separation steps. A second influence on the sensitivity of the DSPCEIA may have been the affinity of antibody for antigen. The biotinylation of the antibody may have compromised the biological activity of the protein although such effects have been found absent in other studies(73).

Thus using the DSPCEIA scheme no significant increase in sensitivity over presently available methods for macromolecules was found.

Although the DSPCEIA scheme tested in this report did not attain the sensitivity of heterogeneous EIA for macromolecules nor the speed of homogeneous EIA for small molecular weight analytes it did demonstrate the use of competition between biotinylated assay components. Previously avidin and biotin have been used for capture or delivery of assay components(37,74). Here biotin was bound to several different components presenting a unique application of the avidin/biotin system.

It was hoped the DSPCEIA could improve sensitive detection of macromolecules in a homogeneous assay scheme. However the final result was an assay which compared in sensitivity to presently available homogeneous EIA for small molecular weight analytes while the time required to perform the assay was similar to assay schemes presently available for macromolecules. Thus the DSPCEIA is most applicable to detection of macromolecules present in concentrations greater than 1.0$\mu$g/ml for which a simple non-labor-intensive assay procedure requiring no separation step is desirable.

## 7.6 General Conclusion

The major accomplishments of this work were as follows. Improved biotinylation procedures were developed and applied to an assay for biotin and avidin significantly more sensitive than previous assays. These methodologies were used to create several new enzyme immunoassay procedures. The focus of these new procedures was the creation of a single incubation, sensitive assay for macromolecules. This was accomplished in several assays the central feature of which is the use of two solid phases.

The dual solid phase enzyme immunoassay system was able to produce an increase in sensitivity for macromolecules yet was done by a homogeneous method. In the dual solid phase assay system, avidin or biotin was bound to solid phase polystyrene latex spheres. A central conjugate then partitioned between this solid phase and another, usually composed of solid phase anti-analyte. However when the dual solid phase competitive enzyme immunoassay and the dual solid phase dye immunoassays were tested, they did not yield detection levels similar to the original method in chapter 5. For the dual solid phase competitive assay the competition of two biotinylated compounds for solid phase avidin shortened the assay time to one eighth of the original DSPEIA, however the assay was 50 times less sensitive. It is plausable that the ability of two large biotinylated macromolecules to block one another from avidin does not occur in a consistent manner, thus the assay was not able to reach a high level of sensitivity. It is also possible that the affinity of the biotinylated proteins may have been different. For the dual solid phase dye immunoassay, the dye, hydroxyazobenzoic acid, was unable to produce a large enough shift in absorbance over a several fold change in analyte concentration. If a method for

amplifying the absorbance change was available, the use of the dye as an indicator in immunoassays may prove worthwhile.

Aside from the above uses of enzymes and dyes in the dual solid phase system, there are other indicators or markers which would be applicable. In chapters 5 and 7 the quantitation of analyte was dependant on the inactivation of G6PDH when it complexed with avidin. It is possible to use the activation of enzymes, rather than their inactivation, as an indicator of analyte concentration. For example, Ngo et al.(75) have prepared the apoenzyme of glucose oxidase. If the required enzyme cofactor, FAD, were bound to a solid phase and the apoenzyme partitioned between two solid phases, enzyme activity would be absent when the conjugate binds to one solid phase and present when bound to solid phase cofactor. Such an indicator would provide a simple spectrophotometric means of measuring analyte concentration.

In conclusion the use of avidin and biotin in immunoassay development is highly encouraged. Both compound are easy to work with and are easily conjugated to proteins. They are highly specific and can be used to develop assays not possible with ordinary antibody-antigen reactions as demonstrated in this thesis. Avidin and biotin are available commercially, and as the number of reactive derivatives grows, so will their common use in the area of immunoassays.

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# Vita

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