Enzyme-Enhancement Immunoassay: A Homogeneous Assay for Polyvalent Ligands and Antibodies

Ian Gibbons, Teresa M. Hanlon, Carl N. Skold, Mary E. Russell, and Edwin F. Ullman

A homogeneous enzyme immunoassay for proteins has been developed that avoids the need for a labeled antigen. The technique involves antibody labeled with β-galactosidase (EC 3.2.1.23), succinylated antibody, and a macromolecular o-nitrophenyl-β-galactoside substrate. The enzyme-labeled antibody and the succinylated antibody form an immune complex in the presence of sample antigen. An enzyme within this negatively charged microenvironment produces a product that forms a second light-scattering phase, whereas the product produced by free enzyme remains soluble. Thus the antigen modulates the rate of increase in light scattering. The technique has been applied to assays for human immunoglobulin G and C-reactive protein as well as for specific antibodies.

Additional Keyphrases: C-reactive protein - enzyme immunoassay - β-galactosidase - turbidimetry - proteins - immunoglobulins

Labeled antibody as a reagent was first described by Coons et al., for tissue staining (1). It was later used by Miles and Hales (2) in a related quantitative immunoradiometric technique. In a modification of the latter method, sample antigen binds to surface-immobilized antibodies and is then permitted to react with excess labeled antibody (3). This method is particularly attractive when preparation of a labeled antigen is precluded by limited supply or poor stability of the purified antigen. A major advantage of immunometric assays is that a large excess of antibody can be used, both on the solid phase and as liquid reagent, to accelerate the slow, diffusion-limited binding processes.

Although homogeneous immunoassay methods (4) offer significant advantages in terms of assay protocol, attempts to combine these advantages with those of labeled antibodies have met with limited success. Previously described homogeneous immunoassay methods with labeled antibody include enzyme-channeling immunoassay (5), antibody-labeled enzyme-inhibition immunoassay (6), and fluorescence excitation transfer immunoassay (7). Labeling the antibody in the last two methods offers little advantage because the antibodies must be highly purified and cannot be used in excess. Moreover, the fluorescence technique gives an undesirable biphasic response. Although less purification is required in enzyme-channeling immunoassay, the procedure requires the use of a bead suspension, which can adversely affect its reproducibility.

We describe a new enzyme immunoassay that combines certain advantages of labeled antibody reagents with the simplicity and convenience of homogeneous methodology. In this procedure β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is the label and a synthetic macromolecular dextran-linked o-nitrophenyl-β-galactoside is used as substrate. This enzyme-substrate combination was previously used in an antigen-labeled homogeneous enzyme-inhibition immunoassay for serum proteins (8).

The present assay is based on an increase in the observable signal when an enzyme-catalyzed reaction occurs within a microenvironment having a localized, highly negative charge. A limited amount of enzyme-labeled antibody combines with a polyvalent antigenic analyte, and then an excess of negatively charged antibody is added. The increased local charge about the enzyme directly affects the rate of formation of the product, which manifests itself by forming a second light-scattering liquid phase.

The second-phase product separates from the bulk solution phase as small droplets, which remain uniformly suspended for as long as an hour. Thus, the concentration of the second-phase product can be determined by turbidimetry. A complete description of the enzymology and physical chemistry of the formation of the second-phase product will be given elsewhere.

Materials and Methods

Chemicals. o-Nitrophenyl-β-D-galactoside,1 1-ethyl-3-diethylaminopropylcarboxydiimide hydrochloride, porcine pepsin, and rabbit serum albumin were obtained from Sigma Chemical Co., St. Louis, MO 63178; N,N-dimethyl-enediamine and succinic anhydride from Aldrich Chemical Co., Milwaukee, WI 53233; and N-hydroxysuccinimide ester of m-maleimidobenzoic acid from Pierce Chemical Co., Rockford, IL 61016. [1,4-14C]Succinic anhydride was from New England Nuclear, Boston, MA 02118. β-Galactosidase from Escherichia coli was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN 46250, and was purified by gel filtration on BioGel A-5m (Bio-Rad Laboratories, Richmond, CA 94804). Human IgG was purchased from Miles Laboratories, Elkhart, IN 46515. 125I-labeled human IgG, prepared by the Enzymobead method (Bio-Rad Laboratories), and human C-reactive protein were gifts of Dr. C.-J. Hau.

Buffers. The sodium phosphate buffer contained 50 mmol/L sodium phosphate, pH 7.0. Buffer A contained, per liter, 10 mmol of sodium phosphate, pH 7.0, 150 mmol of sodium chloride, 5 mmol of sodium azide, and 1 mmol of magnesium acetate; buffer B had 15% higher concentrations of these salts. When used in the assays, the buffers contained 1 g of rabbit serum albumin per liter.

Chromatography media. Biogel A-5m and A-15m (200-400 mesh) were the products of Bio-Rad Laboratories. DEAE-Sephadex A-50 was obtained from Pharmacia, Piscataway, NJ 08854.

Antibodies. Rabbit anti-sheep IgG was the product of Miles Laboratories, and sheep anti-rabbit γ-globulin was from Antibodies Inc., Davis, CA 95616. Antisera against human IgG (γ-chain) and human C-reactive protein were raised in sheep against protein immunogens purified by modifications of published procedures (9, 10). The immunization protocol included monthly injections, first plus complete Freund's adjuvant and later plus incomplete adjuvant. The antisera

1 Nonstandard abbreviations: ONPG, o-nitrophenyl-β-D-galactoside; EDCI, 1-ethyl-3-diethylaminopropylcarboxydiimide hydrochloride; MBSE, N-hydroxysuccinimide ester of m-maleimidobenzoic acid; IgG, immunoglobulin G.
were dialyzed overnight against 50% saturated ammonium sulfate at 4°C, and the precipitate was dissolved in 10 mmol/L sodium phosphate, pH 6.5, and dialyzed against the same buffer to remove ammonium sulfate. Chromatography on DEAE-Sephadex A-50 equilibrated with the same buffer allowed us to resolve two electrophoretically distinct fractions of pure IgG: an unadsorbed fraction 1 and a fraction 2 that eluted with buffer containing NaCl, 0.15 mol/L.

Antibody concentrations were calculated from data provided by the supplier, or by labeling with fluorescein isothiocyanate (7) and determining the fluorescence intensity of the label that bound to antigen-labeled Sepharose® (Pharmacia). Fluorescein labeling was assumed not to destroy antibody-binding sites. The anti-human IgG was 17% specific for human IgG. Possible reduction in titer as a result of labeling with enzymes or charged groups was not determined.

Fab' fragments of sheep anti-human IgG. Sheep anti-human IgG (fraction 1) was dissolved at 40 g/L in 0.1 mol/L sodium acetate, pH 4.5, containing 0.1 mol/mL EDTA and 1 mmol of sodium azide per liter. To 10 mL of the solution we added 0.4 mL of a solution of pepsin (100 g/L) and incubated for 27 h at 37°C. The supernatant solution obtained after centrifugation was adjusted to pH 8.1 with solid Tris base and treated with 25 mL of 250 g/L sodium sulfate. Under these conditions sheep IgG was essentially fully digested to [Fab']2. No Fc fragments remained after the sodium sulfate precipitation of the [Fab']2 as judged by cellulose acetate strip electrophoresis (Beckman Microzone®; Beckman Instruments, Fullerton, CA 92634). The [Fab']2 was dissolved in 80 mL of Tris-HCl, pH 8.4, containing, per liter, 0.12 mol of NaCl, 16 mmol of sodium acetate, 37 mmol of 2-mercaptoethanol, and 1.6 mmol of EDTA. After incubation overnight at 4°C, the solution was then dialyzed against oxygen-free 20 mmol/L sodium phosphate, pH 5.0, containing NaCl, 0.15 mol/L. Approximately 90% of the [Fab']2 was converted to Fab', as judged by chromatography on Biogel A-5m.

Succinylation of antibodies. Purified IgG (fraction 2) was dissolved in 0.1 mol/L Na2HPO4 at 20 g/L. To 1 mL of the stirred solution we added 28.9 µL of a 1 mol/L solution of succinic anhydride in dry dimethylformamide, followed immediately by 43 µL of 1 mol/L NaOH. After 30 min at room temperature, 0.1 mL of 1 mol/L hydroxyamine hydrochloride, adjusted to pH 8.0 with NaOH, was added to destroy unused reagent and any protein-bound esters that had formed. The product was dialyzed exhaustively against buffer A. When [1,4,4,5-14C]succinic anhydride (20 mCi/mol) was used to measure the extent of the reaction, we found 45 ± 2 succinyl groups per molecule of IgG.

Modification of sheep anti-human IgG with N,N-dimethylthelylenediamine. To 2 mL of 0.46 mol/L N,N-dimethylthelylenediamine-HCl, pH 5.0, containing 40 mg of sheep anti-human IgG (fraction 1), we added 25 µL of 0.52 mol/L EDCI. The reaction mixture was incubated for 2 h at room temperature and then dialyzed against the sodium phosphate buffer. This derivative was more positively charged than the native IgG, as shown by cellulose acetate strip electrophoresis.

Enzyme-labeled antibody. Following the method of Kitagawa and Aikawa (11), we dissolved (final concentration 25 g/L) either N,N-dimethylthelylenediamine-modified antibody or unmodified antibody (fraction 2) in the sodium phosphate buffer. MBSE (10 g/L, in dry dimethylformamide) was added to give a final concentration of 1.2 mmol/L. The mixture was incubated at 23°C for 30 min and then dialyzed against 20 mmol/L sodium acetate, pH 5.0, containing 150 mmol of NaCl per liter. To an aliquot containing 75 mg of sheep IgG adjusted to pH 7.0 with 0.5 mol/L sodium phosphate we added 5 mg of β-galactosidase in the sodium phosphate buffer containing magnesium acetate, 1 mmol/L; the final volume was 7.5 mL. After overnight incubation the solution was treated with 0.5 mL of 10 mmol/L cysteine·HCl for 30 min at room temperature to block any remaining maleimido groups. The product was chromatographed on a Biogel A-5m column (75 × 2.6 cm) equilibrated with buffer A. Those fractions containing most of the enzyme activity were pooled. Because the enzyme activity, as determined with ONPG as substrate, did not change during conjugation, the quantity of enzyme in the conjugate was determined directly from its activity. We calculated the ratio of sheep IgG to enzyme from the absorbance at 280 nm (1 cm path length), using ε = 2.24 × 10⁴ for IgG and ε = 9.3 × 10⁴ for β-galactosidase (12) and assuming relative molecular masses (Mr) of 160 000 and 465 000 (13), respectively.

Enzyme-labeled Fab' fragments. β-Galactosidase (1 g/L) was treated overnight at room temperature with 0.2 mmol/L N-ethylmaleimide in buffer A without Na2S, followed by dialysis against buffer A. The resulting derivative retained full enzyme activity with both ONPG and dextran-linked ONPG. Titration with 5,5'-dithiobis(2-nitrobenzoic acid) (14) showed that no rapidly reacting thiol groups remained, whereas eight to 10 groups per molecule were observed with native β-galactosidase. By titrating the derivatives in the presence of 8 mol/L urea, we found that approximately 19 out of a total of 64 thiol groups per enzyme molecule (13) had been blocked.

To a solution of 1.4 mg of this derivative in 1.5 mL of the sodium phosphate buffer, containing 1 mmol/L magnesium acetate, we added 0.9 mg of MBSE in 90 µL of dry dimethylformamide. After 20 min at room temperature the solution was dialyzed against the same buffer to remove unreacted MBSE. The dialysate was then incubated for 17 h at room temperature with 0.15 mL of 0.5 mol/L sodium phosphate, pH 7.0, and 1.5 mL of a solution containing 25 mg of Fab' fragments of sheep anti-human IgG. After addition of 0.2 mL of 10 mmol/L cysteine and further incubation for 20 min, the resulting enzyme conjugate was separated from unconjugated Fab' by chromatography on Biogel A-5m with buffer A. Full enzyme activity with ONPG was retained.

Complex formation between impure human IgG and enzyme-labeled anti-human IgG. Pure human IgG (4.3 mg, 125I-labeled, 2 × 10⁶ cpm/g) in 4.5 mL of sheep serum was dialyzed against buffer A. This was added with constant stirring over 8 h to 5 mL of the same buffer containing 0.61 mg of β-galactosidase conjugated to sheep anti-human IgG at a molar ratio of 1:1 (specific antibody/enzyme). After standing overnight and centrifugation, the supernatant solution was chromatographed on Biogel A-5m. A broad peak with a ratio of about 2 mol of human IgG per mole of enzyme, and containing most of the enzyme activity, emerged before the main peaks of radioactivity and protein.

Macromolecular substrate. A preliminary description of dextran-linked ONPG substrates has been given by Gibbons et al. (8). The substrate was prepared from carboxymethyl-dextran (M, 40 000) and had about 40 ONPG residues attached by 1,4-bis(3-aminopropyl)piperazine-linking groups and about six residual free carboxymethyl groups. Concentrations of the dextran-linked ONPG are given in terms of ONPG groups.

Enzyme assays. Enzyme activity was usually measured in 1.0 mL of buffer A containing 2 mmol of ONPG or 0.4 mmol of dextran-linked ONPG per liter. Immediately after adding the final reagent, we aspirated the sample into the temperature-regulated 1-cm-pathlength flow cell of a Stasar spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH 44074). After a few seconds delay to permit the sample to reach the 37°C cell temperature, the change in absorbance was monitored. In the immunoaassays, rates were usually followed for 30 s at 355 nm. For measurements with ONPG as substrate, rates were measured at 420 nm.
Results

In preparing conjugates of β-galactosidase with intact sheep antibody, we took advantage of the free sulfhydryl groups on the enzyme (13), which react readily with maleimide-substituted IgG antibodies. Because several maleimide groups were introduced per IgG, oligomeric conjugates were formed. Typically, the molar ratio of total sheep IgG to enzyme was about five to ten. The chromatographic data suggest the $M_r$ of the conjugate was about $2 \times 10^6$ to $5 \times 10^6$. Because the enzyme $M_r$ is 465 000 (13), we conclude that an average of two to four molecules of enzyme were present in each conjugate.

Conjugates of sheep Fab' fragments were prepared by reaction of maleimide-substituted enzyme with the Fab' sulfhydryl groups. For this purpose, the free sulfhydryl groups of the enzyme were first capped with excess N-ethylmaleimide. On the basis of an average Fab' conjugate $M_r$ of $10^6$, we conclude that the conjugates mostly contained one enzyme molecule, to which were attached about 10 Fab' fragments.

The specific activity of the conjugated enzyme toward ONPG was unchanged from that of the native enzyme. On the other hand, about 30% greater activities were observed with the dextran-linked substrate. When increasing amounts of human IgG (antigen) were added to enzyme-labeled sheep anti-human IgG, there was a slight further increase in activity with the dextran-linked substrate. This reached a maximum near the equivalence point and then diminished at higher antigen concentration (Figure 1). When succinylated antibody was added to the reaction mixture at the same time as or just before the substrate, the antigen effected a much larger increase in rate and the maximum rate shifted to higher antigen concentration (Figure 1). The antigen concentration at which the rate was highest was approximately 20% of the molar concentration of succinylated antibody, over a 16-fold concentration range.

When the added antibodies were not succinylated, no additional rate enhancement was observed; moreover, as many as 24 succinyl groups per IgG molecule produced negligible effects. However, enhancement increased sharply at higher degree of succinylation and had not reached a maximum even at the 45 succinyl groups per antibody used in the present study. These effects were highly dependent on the ionic strength of the buffer: an 80% smaller antigen-induced rate enhancement was observed on increasing the NaCl concentration from 0.18 to 0.23 mol/L. Antibody modified with other reagents (citric acid, mellitic acid) to introduce negative charge also produced strong enhancement (J. Olson, unpublished).

The effect of antigen on the catalytic activity of the antibody conjugates in the presence of succinylated antibody was to increase visibly the rate of formation of a light-scattering product. Some of this product was also produced by conjugate alone, but was minimal if the antibodies were prelabeled with $N,N$-dimethylatedamine before conjugation. Using such a conjugate, we measured the apparent rate of increase in absorbance in the presence of excess succinylated antibodies as a function of wavelength. This provided the difference spectra between product and unhydrolyzed substrate shown in Figure 2. In the absence of antigen (human IgG) the difference spectrum closely resembled that caused by hydrolysis of ONPG. By contrast, product formed in the presence of antigen transmitted less light at all wavelengths. When the spectrum obtained without antigen was subtracted from the spectrum with antigen, the difference showed a nearly structureless spectrum, as would be expected if the primary difference in the absorbance of the product in the two experiments was due to the formation of a light-scattering material. This interpretation is consistent with the observation of apparent absorbance at 550 nm, which could be removed by centrifugation. The o-nitrophenol chromophore does not absorb at this wavelength.

To confirm that the light scatter was due to enzymic hydrolysis of the dextran-linked substrate and was not an artifact associated with the immune reaction, we also performed the following control experiments. Essentially no measurable rate of light-scatter production was observed when ONPG was used in place of dextran-linked substrate, or when either enzyme-labeled antibody or substrate was omitted. In addition, the light scattering produced when native enzyme was substituted for enzyme-labeled antibody was not modulated by added antigen.

To determine the chemical changes that accompanied the production of turbidity, we needed to find conditions under which the enzyme reaction could be stopped and the second phase dissolved. Addition of Tris base or sodium hydroxide sufficient to raise the pH above 8.5 accomplished both objectives. The degree of hydrolysis of dextran-linked ONPG could therefore be determined by increasing the pH of a re-
action mixture and measuring the absorbance of the resulting single-phase solution at 420 nm, the absorbance maximum of dextran-linked o-nitrophenol. The formation of the second-phase product was measured independently by recording the increase of apparent absorbance at 355 nm, the isosbestic point for the hydrolysis of ONPG. Using this method, we found that antigen increased the rate of hydrolysis of dextran-linked ONPG by only 8%, whereas there was a threefold increase in apparent absorbance related to changes in turbidity. Thus the primary effect of the antigen was on the chemical nature of the product and its solubility.

The assay sensitivity depended on maximizing the ratio of the assay responses when antigen was present to the response in the absence of antigen. One method was to measure only the light-scattering component of the signal at 355 nm. To differentiate the rates further, we increased the positive charge on the enzyme conjugate by labeling with N,N-dimethyl-ethylenediamine. This suppressed second-phase formation in the absence of antigen, with less reduction in the signal when antigen was present. For example, with one preparation the background signal was reduced by 83%, whereas the antigen-modulated signal was decreased by only 43%. A third method of increasing the rate ratio was to use an enzyme conjugate prepared with Fab' fragments in place of intact IgG. This selectively reduced the rate in the absence of antigen to about one-half without much effect on the rate with antigen present. Finally, including lactose, a nonchroomogenic β-galactosidase substrate, at about 3 mmol/L almost completely inhibited the formation of light-scattering product in the absence of antigen while suppressing this product by only about 35% when antigen was present (Figure 3).

Optimization of the assay reagents has not yet been systematically approached. Clearly, however, an important factor controlling background rates is the ratio of specific antibody to enzyme in the conjugate. Any enzyme to which no antibody is attached will contribute to background but not to the modulated signal. The ratio of specific antibody to total sheep IgG in the sheep antibody–enzyme conjugate was in the range 4–18%, and the ratio of specific antibody to enzyme therefore averaged about 0.2 to 1. Adequate responses were obtained even with the lowest ratio. Therefore, it is probably safe to assume that IgG containing as little as 2% specific antibody could be used.

**Assay Protocols**

For the antigen assay we usually incubated the sample for 0.5 to 2 h with a limited amount of enzyme-labeled antibody. Succinylated antibody and substrate were then added, and the mixture was immediately aspirated into a flow cell where the change in absorbance was monitored over 30–60 s. An alternative protocol omitted the incubation step.

Useful assays for C-reactive protein were set up by either protocol (Figure 4), although the latter procedure required two- to threefold greater concentrations of the analyte to evoke a similar response. Using the incubation protocol in which the final assay mixture contained serum sample at 3 mL/L, we obtained results (y) that compared well with radial immunodiffusion results (x) on 17 patients' samples containing from 15 to 100 mg of C-reactive protein per liter: y = 1.04x − 2.1, r = 0.96, SEE = 7.1 mg/L. We determined the effect of serum-to-serum variation on the assay precision after adding C-reactive protein at 0.5 mg/L to 20 sera that had negligible concentrations of this protein. The coefficient of variation of the C-reaction protein concentration measured by the method of Figure 4 (incubation protocol) was only 5%.

To explore the effect of assay conditions on the detectability limits of the method, we decreased the concentration of the enzyme conjugate and increased the incubation times. In a model human IgG assay (Figure 5) as little as 10 ng/L (60 attomoles) in the assay was detectable. Unexpectedly, the standard curve displayed two distinct ascending zones separated by a plateau near 1 μg/L.

The method was also applicable to the assay of antibodies in two ways. Figure 6 shows a standard curve for a direct assay for rabbit anti-sheep IgG in which the sample was first allowed to bind to an enzyme conjugate of the antigen (sheep IgG Fab' fragments) and then substrate and excess succinylated antibody specific for rabbit IgG were added. The more analyte antibody from the sample that bound to the conjugate, the more succinylated anti-analyte antibody was brought into
proximity to the enzyme. Thus, increasing amounts of analyte antibody increased the signal from light scattering.

Alternatively, we determined antibody by using an enzyme conjugate of the antibody (sheep anti-human IgG). By forming an immune complex with human IgG, the conjugate could in effect be converted to an enzyme conjugate of the antigen. With this technique an antigen might be available in very impure form and still permit ready isolation of the complex. When antibody from the sample was allowed to bind to this complex, binding of subsequently added succinylated anti-

Fig. 5. Standard curve for a high-sensitivity enzyme-enhancement immunocoupsay for human IgG.
Antihuman IgG Fab' fragments labeled with 5 ng of β-galactosidase were incubated overnight with human IgG in 0.4 mL of buffer B at 23°C. The enzyme reaction was initiated by adding a mixture of 0.17 mg of succinylated anti-human IgG and 0.8 μmol of substrate in 0.8 mL of buffer B. After incubation at 37°C for 1 h, we determined the difference between the increase in absorbance at 355 nm and that of a control containing no antigen. Human IgG concentrations are those in the final assay solution.

body was blocked. Thus, increasing amounts of antibody reduced the rate enhancement (Figure 7).

Discussion

Binding of antibodies to antigen-β-galactosidase conjugates substantially inhibits the rate of hydrolysis of polymeric substrates (8). The inhibition phenomenon is associated with steric exclusion of the polymeric substrate from the immune complex and is not observed with monomeric ONPG.

A corresponding inhibition of antibody-β-galactosidase conjugates by antigen does not occur (Figure 1), possibly because of the presence of only two binding sites located at distal points on the antibodies, as opposed to multiple closely spaced determinants on the antigens. Instead, increases in rates were observed with added antigen. The cause of the increased rates can be inferred from the observation that the rates were strongly augmented when the resulting immune complexes were treated with excess succinylated antibody. As with the antigen conjugates, modulation occurred only with polymeric substrate. Because the activation effect was suppressed by increasing ionic strength or decreasing negative charge on the succinylated antibody, activation appears to be associated with coulombic attraction of the positively charged substrate polymer with a negatively charged enzyme complex.

On the basis of the coulombic attraction hypothesis, increases in the size or charge density of either the substrate or the complex might be expected to produce further rate acceleration. Thus, the small rate increase observed with the added antigen in the absence of succinylated antibody (Figure 1) is probably associated with aggregation of the enzyme, which is normally negatively charged, in the immune complex. Indeed, increased rates have been observed even with increasing degrees of aggregation of native enzyme preparations (Gibbons, unpublished). The native enzyme has also been found to act more rapidly with increasing molecular mass of the substrate (8).

Although accumulation of negative charge in the enzyme-labeled immune complex produces strong increases in light scattering, there are only slight increases in absorbance from the hydrolyzed ONPG groups (Figure 2, dotted curve). The light-scattering product consists of polymers on which most of the ONPG groups have been hydrolyzed and which are formed even though other substrate polymers remain nearly unchanged; by contrast, less negatively charged enzyme derivatives randomly hydrolyze groups on all polymer chains (Gibbons, unpublished). This suggests that the effect of the
coulombic interaction between the enzyme complex and the substrate is to reduce the rate of dissociation of the enzyme-substrate complex, thus permitting multiple catalytic events to occur before dissociation. A decrease of charge on either component promotes more rapid dissociation and random hydrolysis.

The enzyme-enhancement immunoassay requires a polyvalent analyte to bring together the enzyme-labeled antibody and the charged antibody within a single complex. However, excess antigen blocks the formation of these complexes and results in a biphasic response typified by Figure 1. Fortunately, this does not introduce a major problem in quantitation. The concentration of the enzyme-labeled antibody is usually minimized to avoid excess background rate in the absence of antigen. For most antigen concentrations to be measured the antibody-binding sites on the conjugate will be largely saturated by antigen. Nevertheless, the downward limb of the response curve occurs at antigen concentrations well above this saturating value. The response in the assay increases continuously and apparently stops only when the binding capacity of the succinylated antibody is also surpassed. There being no limit, in principle, to the amount of this reagent that can be added, one can avoid the biphasic response for any given concentration of antigen. Although in practice too much succinylated antibody can coprecipitate with the positively charged substrate, this is not a problem at the maximum concentration expected for most analytes.

The explanation for this relationship between the maximum response and the succinylated antibody concentration is not obvious (Figures 1 and 5). At low antigen concentrations most of the antigen-binding sites are bound to only a fraction of the total enzyme conjugate. Succinylated antibody apparently binds to the remaining antigenic sites, which are not accessible to the bulky conjugate. Added antigen increases recruitment of the conjugate. Complexation of an increased number of enzyme molecules by negatively charged antibody leads to increased rates of second-phase product formation. At antigen concentrations that are high relative to the concentration of the conjugate, each antibody binding site in the conjugate should be associated with one antigen molecule, and the remaining antigenic sites should be occupied by succinylated antibody. Additional antigen would not be able to bind directly to the fully saturated conjugate and thus might be expected to have little effect on the enzymic activity of these complexes. Because we have found large rate increases in this concentration range, we speculate that the combined excess antigen and succinylated antibody form oligomeric aggregates that incorporate several enzyme conjugates into large, highly negative complexes. This interpretation is supported by the observation that succinylated monovalent Fab fragments produce very weak rate enhancements. When succinylated Fab fragments were substituted for the succinylated antibody, the maximum rate enhancement was only 16% as great as with succinylated antibody, and was shifted to lower antigen concentration.

The complex standard curve obtained in the high-sensitivity assay illustrated in Figure 5 is consistent with this model. The first plateau region of the curve occurs at near equivalence of antigen and conjugate binding sites. In this region added antigen binds to additional succinylated antibody but simultaneously decreases the size of the aggregates of antigen and enzyme conjugate. The net charge surrounding each enzyme may therefore not be dramatically changed. Only after the conjugate is fully saturated and antigen is in excess will added succinylated antibody cause large increases in the degree of polymerization of the complexes; in fact, it is in this region of the standard curve that the signal increases most rapidly. A corresponding plateau region in the lower sensitivity assay (Figure 1) is observed only as a weak shoulder, probably because of the lower ratio of succinylated antibody to conjugate used.

Potential of Enzyme-Enhancement Immunoassay

Enzyme-enhancement immunoassay is expected to have broad applicability for antigens. In addition to the determinations of IgG and C-reactive protein described here, assays for serum ferritin and albumin have been demonstrated (J. Olson and Gibbons, unpublished). No affinity purifications of the antibodies were required.

The limitations of the method have been only partly explored. One obvious but not serious limitation is the requirement that the antigen have multiple determinant sites, to accommodate simultaneous binding of the enzyme-labeled antibody and the succinylated antibody. A major consideration regarding sensitivity is the amount of serum that can be tolerated in the assay mixture. In the study with C-reactive protein 10 μL of serum in the 1.2 mL assay volume did not cause significant interference. We could measure 10 ng of human IgG per liter in the assay medium; hence, the current practical limit of detectability in serum appears to be no better than 1 μg/L. However, at the high sensitivity limit of the method, the shape of the standard curve (Figure 5) may add unwanted complexity.

The techniques developed to improve discrimination of rates in the presence and absence of antigen may improve sensitivity of the method. N,N-Dimethylthlyenediamine substitution on the enzyme conjugate increases the positive charge on the conjugate molecules. Lactose, a natural substrate for the enzyme, acts as a competitive inhibitor of enzyme conjugate molecules that are not bound tightly by coulombic attraction to dextran-linked substrates. Thus, both techniques suppress the background rate associated with zero antigen, and together or separately may permit more highly sensitive assays.

Enzyme-enhancement immunoassay is also applicable to serological measurements. In the method described in Figure 6, there are two potential problems. First, the partially purified antigen needed for preparation of the conjugate is often not available. Secondly, succinylated anti-IgG may cause interfering changes in turbidity because of precipitin formation with nonspecific immunoglobulins. Nevertheless, the method has potential application where specific antigens can be isolated, particularly when specific IgM or IgA is to be determined, rather than the more abundant IgG.

Alternatively, antibodies may be determined by using succinylated antibodies that compete for antigenic sites on complexes of antigen with enzyme-antibody conjugate. These conjugates have been prepared with antigen that is only 2% pure, and probably even cruder material could be used. Moreover, this method eliminates concerns about nonspecific immunoprecipitation. The procedure requires antibodies that have specificities very similar to the analyte antibody. The availability of hybridoma techniques to produce monospecific antibodies is expected to reduce the complexity of this problem. However, there may be a poor assay response when there is only one or very few determinants on the antigen to which the patient's antibodies will bind.

Enzyme-enhancement immunoassays afford great simplicity and automatability, and in assays for antigens they completely obviate the use of antigen as a reagent. Moreover, because they take advantage of the proximity of determinant sites on an antigen, it may be possible to establish the proximity of specific determinants by using two different specific antibodies. These methods are applicable to both antigens and antibodies, and are expected to find significant utility in the clinical laboratory.
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References