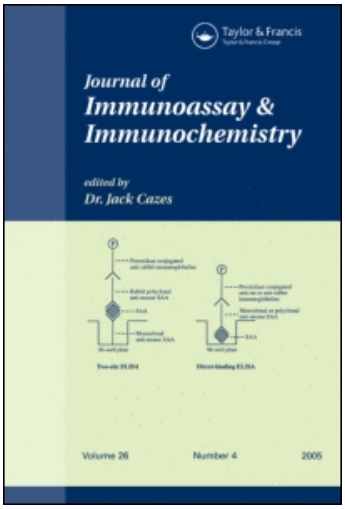

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Characterization of the Soluble Immune Complex (EIC) of the Amplified Enzyme-Linked Immunosorbent Assay (a-ELISA) and an Evaluation of this Assay for Quantitation by Reaction Stoichiometry

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CHARACTERIATION OF THE SOLUBLE IMMUNE COMPLEX (EIC) OF THE AMPLIFIED ENZYME-LINKED IMMUNOSORBENT ASSAY (a-ELISA) AND AN EVALUATION OF THIS ASSAY FOR QUANTITATION BY REACTION STOICHIOMETRY[1]

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ABSTRACT

The molecular composition of the soluble enzyme immune complex (EIC) of alkaline phosphatase (AP) and anti-AP which comprises the detection system of the amplified ELISA (a-ELISA) was investigated. The EIC appeared relatively homogenous in sucrose density gradients and sedimented as a protein of 600-650 K daltons. Based on size and the results of double-lable experiments, the EIC was shown to be composed of two moles of anti-AP and three moles of AP. During reaction with substrate at pH 9.6, greater than 50% of the AP is released as free enzyme and the released enzyme has the same activity as enzyme found in the EIC. The maximum yield of EIC is produced by solubilization of the antibody-AP equivalence precipitate with a 9-fold excess of the amount of AP required for precipitation at equivalence. EICs show no significant loss of activity when stored for one year at 4°C, -20°C or -70°C. The EIC is most stable during long term storage (five years) in 50% glycerol at -20°.

Over the linear region of titration curve for dimeric and monomeric M315, the ratio of AP or EIC to M315 fails to show a constant stoichiometry. Using ^{131}I-EIC and ^{125}I-M315, it was determined that the lack of a constant stoichiometry in the linear region was due to differences in the amount of enzyme bound. Hence, stoichiometric quantitation of the primary antibody is not possible using the current a-ELISA.

(KEY WORDS: ELISA, STOICHIOMETRY, QUANTITATION, IMMUNE COMPLEX, M315, STABILITY)

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INTRODUCTION

Enzyme-linked immunosorbent assays (ELISAs) (1) are widely used in immunobiology as serological assays but have also been used for the quantitation of antibodies in absolute, gravimetric units, i.e. mg/ml. A variety of techniques including the immunoglobulin standard method (2-5), reference standard method (6-7) and immunoglobulin inhibition method (8) have been employed; the advantages and disadvantages of these techniques are reviewed elsewhere (9, 10). All three methods are an indirect measure of the *in situ* bound antibody and the disadvantages of them originate from this fact. If the stoichiometry of the ELISA reaction sequence was constant, i.e. that a quantitative relationship existed between the terminal enzyme activity and the amount of bound primary antibody, a reliable method of quantitation could be developed. Such constancy would be expected only if: (a) the size and isotype of the primary antibody was constant, (b) the size and composition of the enzyme complex was uniform, (c) the enzyme activity of the conjugate was stable over long periods of time, and (d) the terminal reactants were used in excess. If all of these requirements are satisfied by antibodies of a given isotype and size, the same number of enzyme molecules should be bound per primary antibody. Therefore enzyme activity per mole of primary antibody could be used as a standard for each assay and antibody concentration calculated as a function of the moles of enzyme bound.

An essential ingredient for such an approach to quantitation is the requirement for a conjugate of reproducible homogeneity and stability. A number of different methods have been used for preparing antibody-enzyme detection reagents. Conventional antibody enzyme conjugates are covalent and glutaraldehyde is the most common agent used for conjugation either using a one (11) or two step (12) procedure. It is known that glutaraldehyde conjugation of antibodies and enzyme results in decreased activity of both reactants (13) and we have shown them to be heterogeneous in size and composi-

tion (14). Those methods utilizing hetero-bifunctional reagents to cross-link enzymes and antibodies (13) or those dependent on coupling via the carbohydrate groups of horse-radish peroxidase (15) are more controllable and result in more homogeneous conjugates although an evaulation of the enzyme or antibody denaturation which occurs using these techniques is poorly documented. In addition to covalent antibody-enzyme conjugates, our laboratory develped an amplified ELISA (a-ELISA) employing a soluble, noncovalent immune complex (EIC) as the detection system (2, 5, 7, 10, 16, 17). This EIC is formed between alkaline phosphatase (AP) and rabbit antibody to AP, and unlike glutaraldehyde conjugates, sediments as a single peak in sucrose density gradients (17).

The a-ELISA has shown itself to be a sensitive (<30 pg of antibody; 5) and adaptable assay in the measurement of anti-hapten, anti-protein, anti-bacterial and anti-allergic antibodies in at least six animal species (10). The relative homogeneity of the EIC, its apparent stability, and the amplified sensitivity of the a-ELISA make it a good candidate for testing whether quantitation of specific antibodies by reaction stoichiometry is feasible. This report presents data on the size and characteristics of the EIC and its stoichiometric relationship in the detection of both monomeric and polymeric IgA antibodies to dinitrophenol (DNP) using a microELISA system. Data are also presented on the stability of the EIC during storage under various conditions.

MATERIALS AND METHODS

Source of antisera and antigens

Rabbit and guinea pig anti-AP and goat anti-rabbit IgG and rabbit anti-guinea pig IgG, i.e. "bridging antibodies" were prepared as previously described (10, 16). Rabbit anti-M315 was a gift from Dr. Richard Lynch, Dept. of Pathology and Microbiology, University of Iowa Medical School. Gelatin (Gel; BBL, Cockeyville, MD) was conjugated with 3 times recrystallized dinitro-

benzene sulfonate (DNBS; Eastman Kodak, Rochester, NY) according to the method of Eisen (18). Unbound DNP residues were removed by dialysis.

Preparation and storage of the enzyme immune complex (EIC).

Routine preparation of the EIC has been previously described (10, 16, 17). Briefly, 50 μg of AP was mixed with the volume of rabbit or guinea pig anti-AP necessary to produce an equivalence immunoprecipitate. The reactants were incubated for one hour at RT, overnight at 4°C and the resulting precipitate washed twice in cold PBS. The immune precipitate was then resolubilized in a nine-fold excess of free enzyme. Such preparations do contain free enzyme but no free anti-AP. In experiments to determine the optimal production of the EIC, the excess of free enzyme added to resolubilize the immune precipitate was varied from one to eighteen fold. In some experiments ^{131}I-AP was used to make ^{131}I EIC. This EIC was utilized in experiments to study the ratio of terminal enzyme to the primary antibody being measured in the ELISA. Double-labeled EIC was prepared using rabbit ^{125}I-anti-AP and ^{131}I-AP. This EIC is designated as $^{125/131}$I-EIC.

Aliquots of EIC's produced using guinea pig anti-AP and AP (gpEIC) were stored under various conditions for one year in PBS or in various solutions and at different temperatures for five years (see Table 2). gpEIC activity was determined by titration against rabbit anti-guinea pig IgG as the solid phase capture antibody (see below) or by using the a-ELISA to detect rabbit IgG (an antigen) adsorbed on plastic. Samples were also analysed by SDG and the gpEIC activity detected using solid phase capture antibody.

Sucrose density gradient (SDG) analysis of EICs.

Analysis of EICs were performed in 10-40% sucrose gradients. Samples were centrifuged for 19 hr at 4°C at 208,000 x g in a Beckman L5 ultracentrifuge using 4.4 ml cellulose nitrate tubes made for the SW60Ti rotor. Tube contents were fractionated using a paraffin oil fractionator which when coupled to an

air-tight Hamilton syringe, allowed collection of forty 100 µl, or twenty, 200 µl, fractions. The SDG fractions of the various EIC's were analyzed using goat anti-rabbit IgG (capture antibody) which had been adsorbed to Immulon 2[a/] microtiter plates. After allowing sufficient time for the fractions (diluted in PBS-T) to bind the capture antibody, the plates were washed and the AP activity associated with rabbit IgG (i.e. EICs) measured in each fraction after addition of the substrate p-nitrophenyl phosphate (PNPP; 1 mg/ml). The total enzyme activity in the same fractions, i.e. free AP and AP complexed with anti-AP, was measured by allowing an aliquot of each SDG fraction to react with PNPP. All reactions were measured using a Dynatek 580 Plate Reader. Similar tests were performed using the $^{125/131}$I-EIC to determine the ratio of AP to anti-AP in the EIC fracionated by SDG although in these situations, Immulon 2 Removawells[a/] were used and both the amount of enzymatic activity and I-125 or I-131 radiactivity, measured in each well.

Iodination

Purified M315, and dM315 and the globulin fraction of rabbit anti-AP were iodinated using the chloramine-T method. Two mg of protein was iodinated with 1 mCi of Na^{125}I at 4°C using 20 µl Chloramine-T (10 mg/ml). After 30 min, 20 µl of sodium metabisulfite (6.7 mg/ml) was added. Five minutes later, 100 µl of 0.1 M KI was added and the free iodine was removed by desalting on a Sephadex G-25 column or by dialysis. AP was labelled with ^{131}I using the same procedures.

The specific activities of the mM315, dM315 and anti-AP were 250.1 cpm/ng, 211.0 cpm/ng and 235 cpm/ng, respectively. The specific activity of the ^{131}I-AP was 318 cpm/ng at the initial determination of specific activity. Precipitablity by trichloracetic acid was determined for mM315 (96.2%), dM315 (97.6%), anti-AP (94.6%) and AP (96.3%). Binding of labelled proteins

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to DNP-gelatin, DNP-*E. coli* and to anti-M315 (capture antibody method, see above) was determined. The behavior of equivalent quantities of each preparation before and after radiolabelling in the a-ELISA was evaluated. The labelled EIC was titrated to demonstrate that it produced reactions equivalent to those seen with unlabelled EIC.

Determination of the alkaline phosphatase activity in the EIC.

Solid-phase capture antibody (goat anti-rabbit Ig) was used to immobilize the EIC (see above). Release of enzyme from the solid phase capture antibody or EIC was determined following incubation in substrate buffer without substrate. The buffer was removed and the AP activity remaining on the solid phase was determined. The enzyme released into buffer was further characterized by measuring its ability to be bound to solid-phase anti-rabbit IgG or normal goat globulin in a solid phase capture antibody assay. The specific enzymatic activity of free and bound enzyme was determined in samples of ^{131}I-EIC by comparing enzyme activity with enzyme weight (calculated from specific radiological activity) as determined by radioactivity in a Packard Autogamma 800. Comparisons were also made between labelled enzyme before and after its incorporation into the EIC.

Methodology of the a-ELISA

The a-ELISA was performed as described (2, 16) and reviewed (10) elsewhere. In the model system used in this research, DNP-gelatin (500 ng/ml) in 0.1 M carbonate buffer, pH 9.6, was adsorbed to Immulon 2 microtiter plate wells or Removawells. Dilutions of the primary antibody (mM315 or dM315) were then added in PBS-T, incubated overnight at 4°C, the plates washed and a 1:500 dilution of rabbit anti-mouse IgA was added. After incubation for 2 hr at RT, a dilution of rabbit IgG ("bridging" antibody) was added in PBS-T and incubated 2 hr at RT. After washing, a dilution (usually 1:1000) of the EIC was added in PBS-T, the plates incubated for 2 hr at RT and after washing, PNPP added to

develop the p-nitrophenyl color reaction. mM315 and dM315 were prepared as previously described (14).

To determine the stoichiometry of the a-ELISA, ^{125}I-M315 was used as the primary antibody and ^{131}I-EIC was used as the detecting reagent. Primary binding and EIC binding were determined by counting the Immulon 2 Removawells after completion of the a-ELISA.

Results

Characteristics and optimal preparation of EIC.

The results obtained when the equivalence EIC precipitate was solubilized with increasing amounts of AP are presented in Figure 1. Increasing the amount of added AP up to an excess of 9-fold, over that needed for equivalence, increases the amount of EIC; addition of a greater excess produces a reduction in the amount of EIC. Fractionation by SDG and identification of the EIC using a capture antibody, shows that regardless of the amount of excess enzyme, the size of the resulting EIC remains constant. The activity of these various EICs in an ELISA assay paralleled their SDG behavior (Fig. 2).

The size of the EIC estimated from SDG is 600-650 k daltons (Fig. 1). The composition of the EIC in each fraction obtained by SDG was calculated from the ratio of ^{125}I to ^{131}I captured by solid-phase goat anti-rabbit IgG (Fig. 3). Using data on specific activity, a ratio of AP to antibody in the EIC of 1.5:1 was determined.

Dissociation of EIC during incubation in substrate buffer

Figure 4a demonstrates the loss of AP from the solid phase and its presence in solution with time of incubation in substrate buffer. Figure 4b demonstrates that in the same buffer system, the enzyme reaction is linear after a three minute lag phase. Table I demonstrates that the enzyme

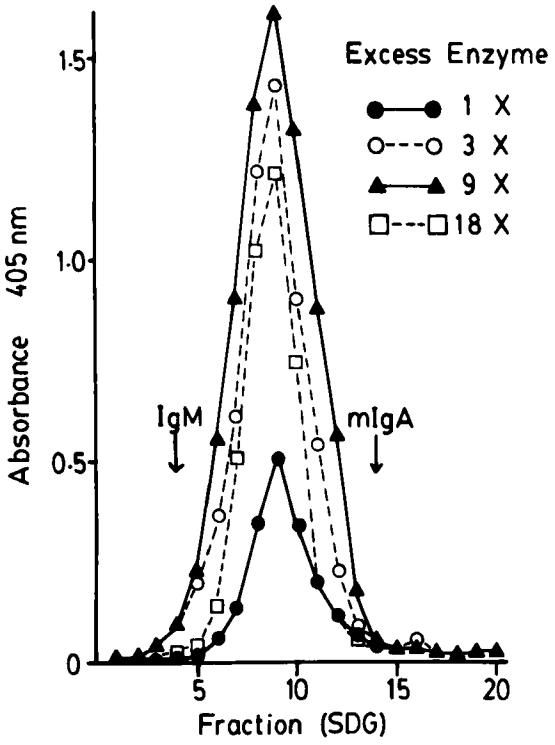

Figure 1. Immunologic and molecular characteristics of EICs solubilized using different amounts of excess AP. Excess is relative to the amount needed to form the equivalence precipitate. The sedimentation positions of the EICs in SDG were determined using solid-phase capture antibody specific for rabbit IgG. The sedimentation behavior of molecular weight markers is indicated (IgM, 1000 Kd; IgA, 160 Kd). Legend on figure.

released into the buffer is no longer bound to anti-AP but is free enzyme.

Stability of the EIC during long-term storage.

When EICs were stored for one year in PBS at different temperatures we failed to see significant differences in activity (Table 2). Table 2 also presents data on the activity of gpEIC stored for five years under seven different conditions. These data show that EIC stored at -20° and stabilized with either 20% sucrose or 50% glycerol, retained similar activity. EICs

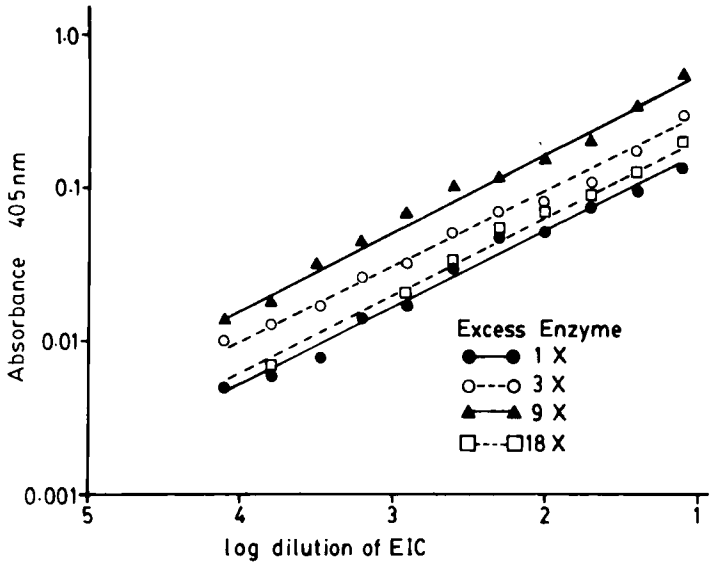

Figure 2. Immunologic activity of EICs prepared by solubilization of the equivalence AP-anti-AP precipitate using various amounts of excess AP. Preparations are the same as those studied in Figure 1. A single concentration of M315 was tested using the a-ELISA while the various EICs were titrated. Legend on figure.

stored in either Tween or PBS at -20° lost much more activity. When stored at 4°, samples stored in 50% glycerol were as active as those stored at -20° in glycerol or sucrose. Samples stored under other conditions at 4°, including sucrose, show a much greater loss of activity.

The EICs stored for long periods under different conditions were also evaluated for their molecular characteristics. These tests indicated that the long-term loss of activity seen in samples stored in glycerol or sucrose (-20°) was accompanied by a tendency to dissociate to more heterogeneous complexes, especially those of a smaller molecular size (Fig. 5). EICs stored at 4° showed even greater loss of molecular integrity. Consistent with data in Table 2, EICs which lost their activity upon storage produced no detectable activity after fractionation by SDG.

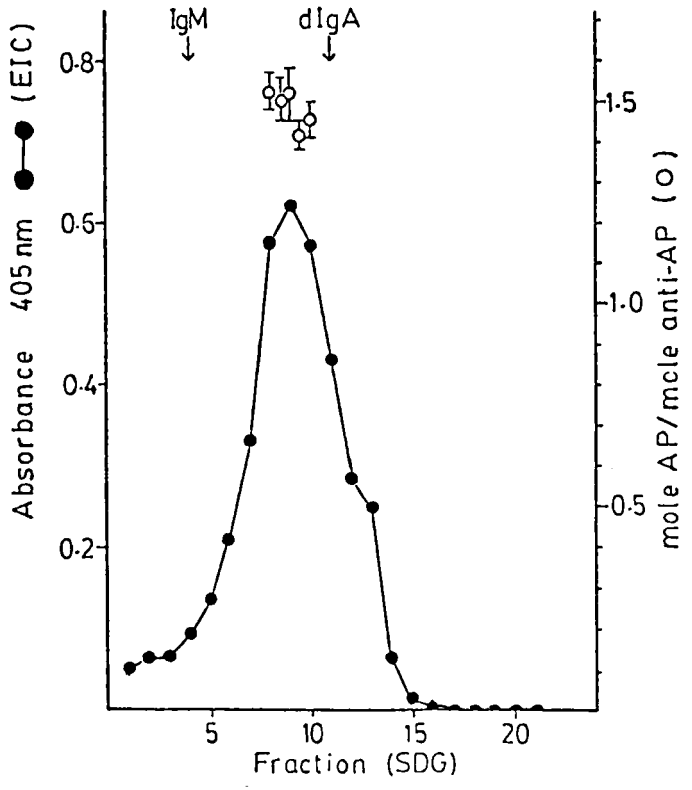

Figure 3. The characterization of double-radiolabelled ($^{125/131}$I) EIC fractionated on SDG. The fractionated EIC was assayed using an anti-IgG capture antibody on the solid phase. Molar ratios of AP: anti-AP in the various SDG fractions which contained the EIC were calculated from the specific activities of the labelled components which bound to the capture antibody. The sedimentation behavior of reference proteins is shown comparatively (IgM, 1000 Kd; dIgA = 320 Kd; mIgA = 160 Kd). Error bars depict standard deviation.

Ratio of enzyme primary antibody.

A second requirement for quantitation of antibodies by reaction stoichiometry, is the maintenance of a constant ratio of enzyme to primary antibody over the linear region of the ELISA titration curve. As the molecular size of primary antibody might be expected to influence this ratio, we choose to study this using mM315 and dM315 which have the same intrinsic affinity of

1×10^7 $1M^{-1}$. Both ^{125}I-M315 preparations behaved similarly to their unlabelled counterparts when tested in the a-ELISA (14).

In other studies (14) we have shown that the ratio of primary antibody bound to primary antibody added gave a slope of 1.0 over a 3-log range, indicating a constant percentage of primary binding over the linear region of the a-ELISA. We also showed that this represented 100% of the active antibody in the preparations used. On the contrary, titration curves produced by ELISA, including the a-ELISA, are sigmoidal and when the same preparations of antibody were assayed, the linear region of the plot was but 1½ logs in length and had a slope of less than 1.0 (14). In investigations reported here we focused exclusively on the linear region of the ELISA titration using ^{131}I-EIC and ^{125}I-M315 (Fig. 6). These data show that the ratio of ^{131}I-EIC to ^{125}I-M315 decreased throughout the linear region of the ELISA titration for both mM315 and dM315.

DISCUSSION

The use of stoichiometry for quantitation of the a-ELISA is an inviting possibility. The adaptability of the assay (10, 17), high sensitivity (5) economy of enzyme (unpublished data) combined with the nature of the EIC, justify this enthusiasm. For such a system to work, the preparation of a detection system or adaptation of an existing one, which is of constant and reproducible composition is required. The EIC of the a-ELISA appeared to be a likely candidate based on preliminary estimates of its homogeneity (17). In addition, the non-covalent nature of the EIC could mean that the enzymic activity of the AP in such a conjugate would be less altered than enzymic activity in a covalently-linked antibody-enzyme conjugate. Finally, because the pH optimum for AP (the enzyme used in the EIC) is about 10, dissociation of the immune complex might occur during the substrate reaction resulting in the liberation of free unaltered enzyme.

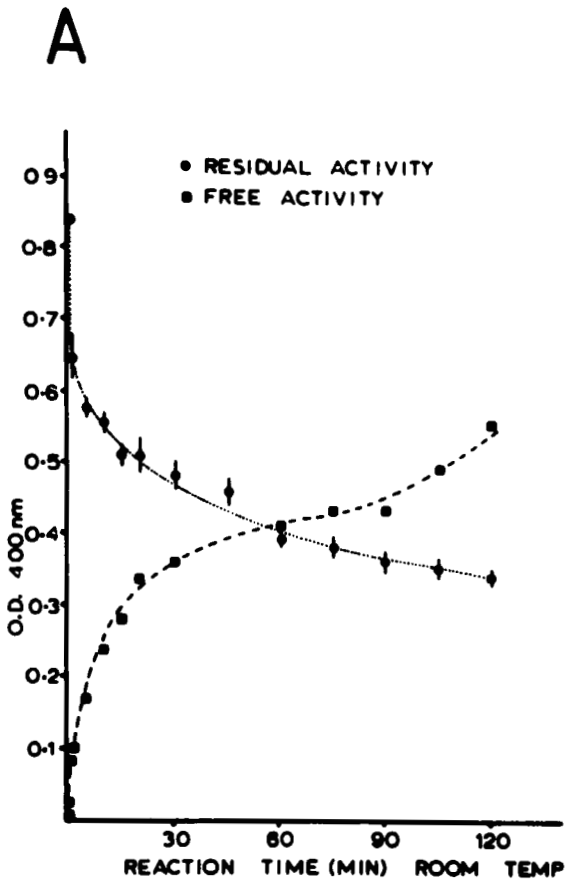

Figure 4. The dissociation of AP from the solid-phase during the substrate reaction step in the a-ELISA. 4a. The relationship of residual (bound to solid-phase) and free (released into substrate buffer) AP activity at various times during the 2 hr incubation step. 4b. Kinetics of the AP activity of residual (EIC) and released (free) AP during the first 90 min of substrate reaction. The slopes of the kinetics plots are not statistically different.

Data presented in this report fits the prediction that the EIC does indeed dissociate during the course of the substrate reaction; 50% dissociation occurs within the first 60 minutes (Fig. 4a). This released enzyme was not complexed with the anti-AP (Table 1), and there was no increase in specific enzymatic activity upon release (Fig. 4b). The stoichiometric quantitation of

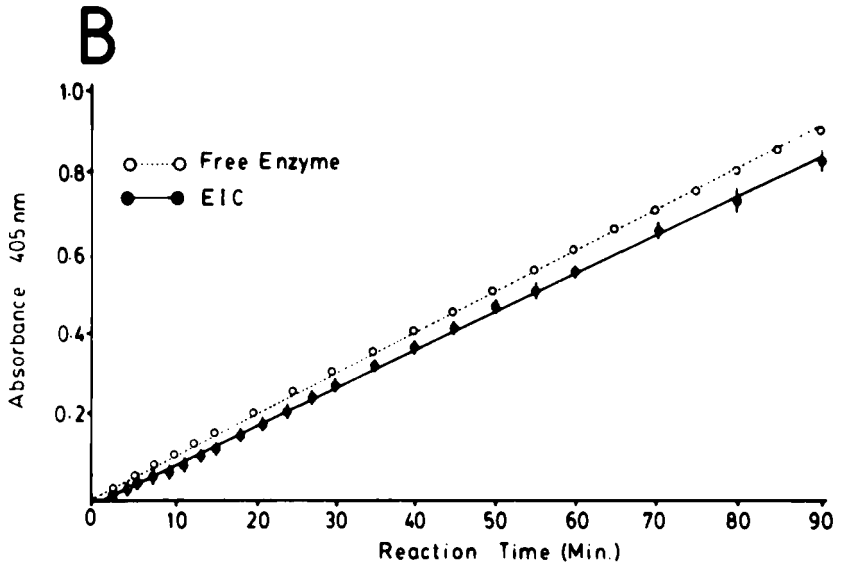

FIG. 4 (continued)

ELISAs would also require that the composition of the EIC be known and reproducible and that it remain stable with time. The 1.5:1 ratio of enzyme:anti-enzyme in the EIC, along with its location in the SDG between dIgA and IgM, suggests an EIC with a molecular weight of 600-650 K daltons and a probable composition of three enzyme and two antibody molecules (Fig. 2). A similar complex has been described in the peroxidase-anti-peroxidase system used in immunohistochemistry despite a different method of preparation (19). The size of the EIC appears to be comparatively homogeneous as opposed to glutaraldehyde conjugates (14, 20, 21) and its size is not altered by changes in the amount of enzyme used for resolubilization of the complex during its production (Fig. 1). Optimal production of the EIC is achieved with the addition of a nine-fold excess of AP over the amount needed for equivalence precipitation.

The practical use of the a-ELISA in stoichiometric quantitation requires that the EIC of the a-ELISA be stable for a convenient period of time. Data

Table 1: Characterization of released enzyme

Solid Phase Reagent	Absorbance ± S.E.[a/]
Anti-AP	0.721 ± 0.032 (A)
Bridge	0.055 ± 0.004 (B)
Normal goat globulin	0.000 ± 0.001 (B)

a/ Values followed by the same letters (A or B) are not significantly different at the p=0.05 level as measured by analysis of variance using Tukey's test.
S.E. is the standard error of the mean.

Table 2. Comparative activity of EICs stored one and five years under different conditions

Treatment of EIC	Dilution giving $OD^{405} \approx 0.5$	Relative activity[c/]
4°C, 1 yr.	4300	1.0
-20°C, 1 yr.	4300	1.0
-70°C, 1 yr.	4300	1.0
PBS, 4°C, 5 yr.	1330	0.31
0.1% Tween-20, 4°C, 5 yr	ND[d/]	ND
20% sucrose, 4°C, 4 yr.	1900	0.44
0.1% Tween-20, -20°C, 5 yr	1050	0.24
20% sucrose, -20°C, 5 yr	2500	0.58
50% glycerol, -20°C, 5 yr	2300	0.54
New EIC in 50% glycerol	4300	1.00

c/Relative activity = $\frac{\text{dilution of stored EIC giving } OD^{405}=0.5}{\text{dilution of new EIC giving } OD^{405}=0.5}$

d/An OD^{405} of 0.5 was not reached during equal time.

presented confirm our preliminary findings on the stability of the EIC (17) and further show that long-term storage (5 years) at 4 °C results in nearly total loss of activity for samples stored in PBS, 0.05% Tween or 20% sucrose while those in 50% glycerol retained 50% of the activity of fresh EIC. At -20°, storage in 20% sucrose was as effective in preserving activity as storage in 50% glycerol. While total activity was not different between EICs stored in glycerol at -20° and 4°, EICs stored at 4° showed a loss of molecular

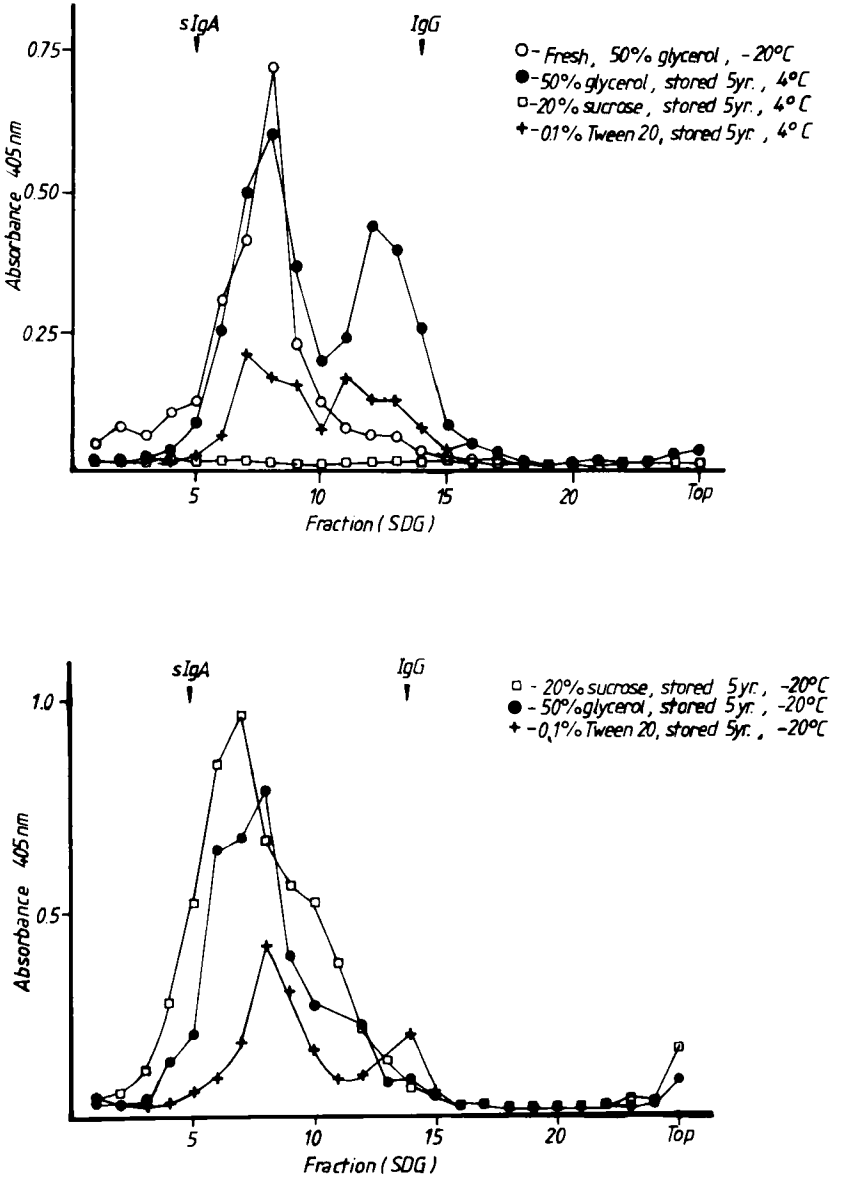

Figure 5. The sedimentation behavior in SDG of gpEICs stored for five years under different conditions. gpEICs were detected using rabbit anti-guinea pig globulin as the solid-phase capture antibody. Legend on figure.

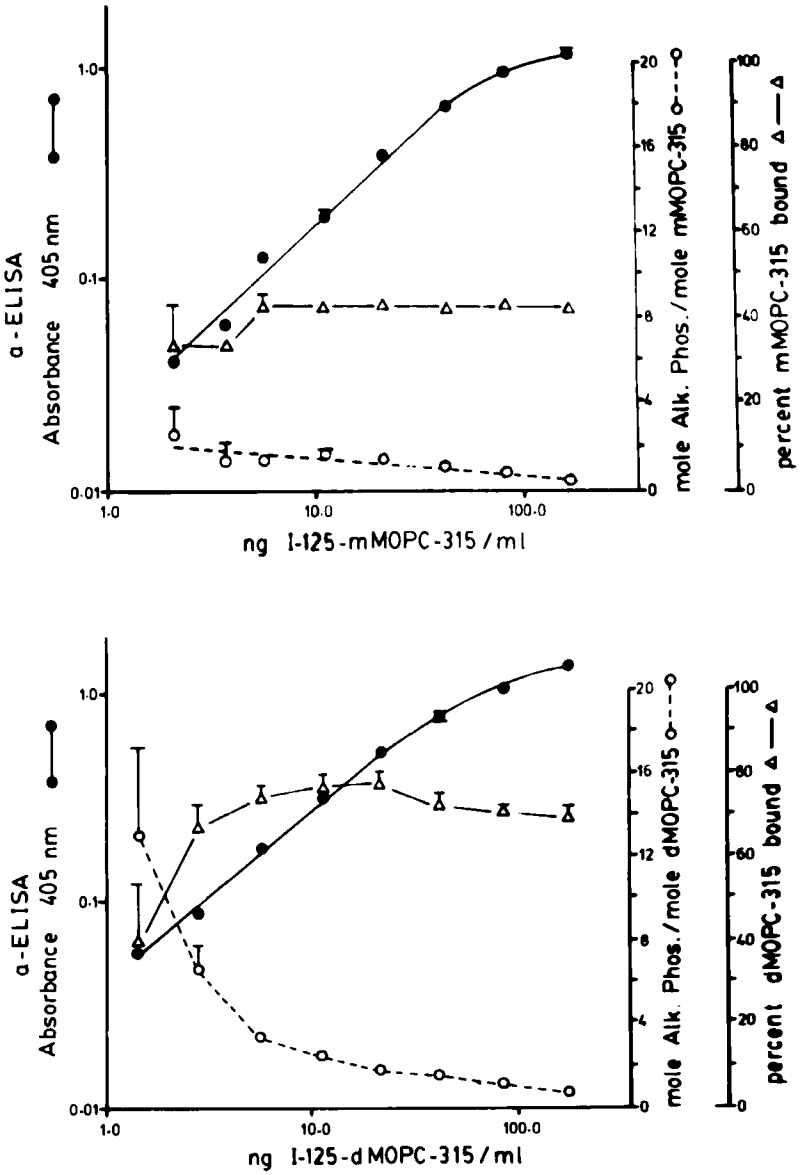

Figure 6. The relationship between the percentage of primary antibody bound in the linear region of the a-ELISA (Δ—Δ) and the amount of AP in the EIC bound (o------o). The solid symbols (●——●) show the linear region of the titration plot for dM315 anti-DNP (top) and mM315 anti-DNP (bottom) as measured using the a-ELISA.

homogeneity, especially the appearance of lower molecular weight complexes (Fig. 5). As a result of these studies we now store our EICs at -20° in 50% glycerol.

The relative homogeneity of the EIC and its known composition means that theoretically, a minimum of three moles of AP could be indirectly bound to one mole of primary antibody and if this ratio, or one that is empirically established, would remain constant over the linear region of the ELISA titration plot, a stoichiometric method of quantitation could be developed. When we measured the ratio of ^{131}I-EIC to iodinated M315 (primary antibody) over the linear region of the ELISA titration plot, two phenomena were noteworthy: (a) the percentage of bound mM315 or dM315 over this region was constant and (b) the ratio of enzyme to M315 in this region decreased with increasing amount of bound M315. This progressively changing enzyme:primary antibody ratio in the region where the binding of primary antibody was constant (linear region) could result from (a) gradual steric inhibition of EIC binding with increasing input of antibody, i.e. in the direction of the "plateau region" of titration, (b) progressive, non-specific binding of EIC at high dilutions of primary antibody, i.e. in the direction of the "tail" of the sigmoidal curve or (c) a combination of the two phenomena. There is evidence that the plateau regions of ELISA titration curves are the consequences of steric hindrance (14) and that non-specific binding can explain the lower "tail" of the sigmoidal curve (22). In any case, the lack of a constant ratio of enzyme to primary antibody in the a-ELISA currently makes the use of a stoichiometric method of quantitation invalid.

Although our data fail to validate the use of the a-ELISA for stoichiometric quantitation of primary antibody, our findings do extend our knowledge of the optimal preparation and storage of EICs, provide data on the homogeneity and composition of the EIC, and for the first time, reveal the molar relationship between the amount of enzyme and the amount of primary antibody which is bound to solid-phase antigen throughout the ELISA titration plot.

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Appendix

Abbreviations used in this paper: a-ELISA, amplified-enzyme-linked immunosorbent assay; AP, alkaline phosphatase; dM315, dimeric M315; DNP, dinitrophenyl; DNP-gel, DNP-gelatin; EIC-alkaline phosphatase-rabbit anti alkaline phosphatase soluble immune complex; ELISA, enzyme-linked immunosorbent assay; Gel, gelatin; gpEIC, EIC made using guinea pig rather than rabbit anti-AP; IgA, immunoglobulin A; M315, a mouse myeloma IgA secreted

by the MOPC 315 plasmacytoma which is specific for the hapten DNP; mM315, monomeric M315; PBS, 0.01 phosphate buffered saline, pH 7.1; PBS-T, PBS containing 0.05% Tween-20. PNPP, p-nitrophenyl phosphate, substrate for alkaline phosphatase; SDG, sucrose density gradient ultracentrifugation.