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ENZYME-LINKED IMMUNOSORBENT ASSAY, ELISA

III. Quantitation of Specific Antibodies by Enzyme-Labeled Anti-Immunoglobulin in Antigen-Coated Tubes¹

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A sensitive and simple method for the quantitative determination of antibodies is reported. Tubes coated with antigen are incubated with antiserum followed by an enzyme-labeled preparation of anti-immunoglobulin. The enzyme remaining in the tubes after washing provides a measure of the amount of specific antibodies in the serum. Coating of polystyrene tubes with antigen is described, as well as the preparation of specifically purified antibodies against rabbit IgG, and their conjugation to alkaline phosphatase.

When rabbit antisera against human serum albumin or against the dinitrophenyl group (DNP) were incubated in tubes coated with antigen, less than 1 ng/ml of specific antibody could be detected in both systems. Antibodies in unknown sera could be quantitated by comparison with a standard antiserum. The results obtained compared well with those obtained with other methods for antibody determination.

In the DNP system, the specificity of the reaction was assessed by inhibition with hapten. The reaction of immune serum against DNP with DNP-protein, adsorbed to the tubes, was completely inhibited by hapten in solution.

Enzyme-conjugated antibodies have been used by several workers for localization of antigens and antibodies in tissue sections (1), as well as in immunoelectrophoresis and diffusion (2, 3). We have used enzyme-conjugated antigen for the quantitative determination of antigen in solution in a competitive immunoassay. A few ng/ml of rabbit IgG could be determined by means of alkaline phosphatase coupled to purified IgG (4, 5). Recently, van Weemen and Schuurs described a similar technique for determination of human chorionic gonadotropin (6). In this assay peroxidase was used as the marker of the antigen.

We now report the use of alkaline phosphatase in an assay for determination of antibodies. The principle of this assay is similar to the radioallergosorbent test, RAST (7). Antigen on a solid phase is allowed to react with

antiserum. After washing off excess protein, enzyme-conjugated anti-immunoglobulin is added. The uptake of label to the solid phase gives a measure of the amount of specific antibodies in the serum. With rabbit antisera against the hapten dinitrophenol (DNP), or against human serum albumin (HSA), it was possible to detect nanograms per milliliter of specific DNP and HSA antibodies, respectively. For solid phase we have used disposable polystyrene tubes, to which the antigen is physically adsorbed. This procedure was introduced by Catt and Tregear for the radioimmunoassay of human placental lactogen and human growth hormone (8).

MATERIALS AND METHODS

Antigens. HSA was kindly supplied by AB KABI, Stockholm, Sweden. Histone (HIS) was extracted from calf thymus with trichloroacetic acid according to de Nooij and Westenbrink (9). Bovine γ globulin (BGG) was prepared from bovine serum by precipitation with

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33% saturated ammonium sulfate. DNP-HSA, DNP-HIS and DNP-BGG were prepared by coupling sodium dinitrobenzene sulfonate to the three proteins, respectively (10). The degree of substitution, expressed as micromols of DNP per milligram of protein N, was 4.3 for DNP-HSA, 1.3 for DNP-HIS and 2.1 for DNP-BGG. The DNP-content was determined spectrophotometrically, by assuming a molar absorbance for ϵ -DNP-lysyl residues of 17,530 at 360 nm. Determination of protein N was done according to Schiffman *et al.* (11).

Rabbit antisera. Anti-HSA serum was obtained by immunizing rabbits with up to 10 monthly intramuscular injections of 10 to 30 mg HSA in Freund's complete adjuvant (FCA) (Difco Laboratories, Detroit, Mich., U. S. A.). Serum was collected from after the third injection. The amount of specific HSA antibodies in the serum was 5.2 mg/ml, as determined by quantitative precipitation. One-tenth milliliter of anti-HSA serum and 0.2 ml of double dilutions of HSA in 0.05 M Tris-HCl in 0.15 M NaCl (pH 8.0) were incubated at 37°C for 1 hr and at 4°C for 40 hr. The washed precipitates were dissolved in 0.5% sodium dodecyl sulfate and analyzed spectrophotometrically at 280 nm, by assuming the absorbance for 1% HSA to be 5.8 and for 1% IgG, 13.8 respectively.

Anti-DNP serum. Rabbits were immunized by two intramuscular injections of 5 mg DNP-BGG in FCA, 8 weeks apart. The amount of anti-DNP in the one hyperimmune serum used here was 2.5 mg/ml as determined by a modification of the Farr technique (12) (Å. Larsson, unpublished). Twenty-five microliters of normal rabbit serum, 25 μ l of anti-DNP serum and 25 μ l of 6.4×10^{-8} M tritium-labeled ϵ -DNP-amino caproic acid were incubated for 30 min at room temperature in 11- x 80-mm polystyrene tubes. The anti-DNP serum was diluted with 1% HSA in phosphate-buffered saline (PBS) (pH 7.2) and the hapten with PBS. The labeled hapten was prepared according to (13). One hundred microliters of 88% saturated ammonium sulfate were then added and the contents of the tubes were mixed immediately. After 16 hr at 4°C the tubes were centrifuged in the cold at 3000 rpm for 30 min. The precipitates were washed once with 3 ml cold 50% saturated ammonium sulfate. The amount of radioactivity in the dissolved precipitates was determined by liquid scintillation counting.

Sheep antibodies against rabbit IgG. A sheep was immunized with the γ globulin fraction of rabbit serum in FCA. The γ globulin fraction was prepared by precipitation with 33% saturated ammonium sulfate.

For isolation of specific sheep anti-rabbit IgG antibodies, an immune adsorbent was prepared by cross-linking rabbit IgG with glutaraldehyde (A. G. Merck, Darmstadt, W. Germany) according to Avrameas and Ternynck (14). This rabbit IgG was obtained from the γ globulin fraction mentioned above after further purification by means of batch adsorption at pH 6.8 on DEAE-cellulose (Serva, Heidelberg, W. Germany) and gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Fifty milliliters of the sheep anti-rabbit globulin serum were stirred with 100 mg of unsolubilized rabbit IgG for 16 hr at 4°C. The immune adsorbent was washed with 0.9% NaCl until the absorbance at 280 nm of the centrifuged washings had reached background value. The antibodies were then eluted from the sorbent with 3- x 4-ml 0.1 M glycine-HCl (pH 2.8). The combined eluates were filtered through a 0.8- μ Millipore filter and solid Tris was added to neutralize the filtrate. The solution of specific sheep anti-rabbit IgG antibodies was dialyzed against PBS, concentrated to 5 mg/ml by ultrafiltration, and finally stored frozen (-80°C) until used.

Alkaline phosphatase conjugated to sheep anti-rabbit IgG was prepared essentially as described for the conjugation of the same enzyme to rabbit IgG (4, 5). The enzyme preparation used was alkaline phosphatase, Sigma type VII, (Sigma Chemical Company, St. Louis, Mo., U. S. A.) of specific activity 310 to 380 U/mg. It is a suspension of the enzyme in 2.6 M ammonium sulfate. Three-tenths milliliter of this suspension, containing 1.5 mg protein, was centrifuged in the cold for 10 min at 1000 rpm. The supernatant was discarded and the pellet was mixed with 0.1 ml of the solution of specific sheep anti-rabbit IgG antibodies, 5 mg/ml. After dialysis overnight against PBS, 10 μ l of glutaraldehyde were added to a final concentration of 0.2%. After 2 hr at room temperature, the solution was diluted to 1 ml and again dialyzed against PBS. Conjugates thus prepared were stored in the cold (4°C) after dilution to 10 ml with 5% HSA in 0.05 M

Tris-HCl (pH 8.0) containing 0.001 M MgCl_2 and 0.02% NaN_3 .

Antigen-coated tubes. Disposable polystyrene tubes (11- x 55-mm) (Hegerplastics AB, Stallarholmen, Sweden) were coated with antigen in the following way: 1 ml of a solution of the antigen in 0.1 M sodium carbonate buffer (pH 9.6) with 0.02% NaN_3 was added to each tube. The tubes were incubated in a water bath at 37°C for 3 hr. The tubes were stored with the antigen solution in the cold until used.

Immunoassay. Before assay the desired number of antigen-coated tubes were washed with 0.9% NaCl, containing 0.05% Tween 20. The tubes were filled three times with the washing solution, allowed to stand for a couple of minutes each time and emptied by suction.

Rabbit sera were diluted with PBS containing 0.05% Tween 20 and 0.02% NaN_3 . Tween 20 was used to avoid nonspecific adsorption. One milliliter serum dilution was added to each tube. The tubes were incubated in a roller drum for 6 hr at room temperature. After washing, 1 ml of the conjugate diluted 1:500 with PBS-Tween was added. The tubes were then incubated overnight (16 hr). Excess conjugate was washed out and the amount of alkaline phosphatase bound to the tubes was determined by using *p*-nitrophenylphosphate (NPP) (Sigma) as a substrate. To each tube was added 1 ml 0.05 M sodium carbonate buffer (pH 9.8) containing 1 mg/ml NPP and 10^{-3} M MgCl_2 . The enzyme hydrolyzes bound phosphate and liberates yellow *p*-nitrophenolate, which is measured in a spectrophotometer at 400 nm. The increase in absorbance with time is linear up to approximately one. After suitable incubation times at room temperature the enzyme reaction in individual tubes was stopped by the addition of 0.1 ml 1 M NaOH. In most experiments, absorbances obtained from spectrophotometer measurements were then extrapolated to the same time, usually 60 or 100 min.

RESULTS

In order to allow as much specific antibody binding as possible, there should be an excess of antigen present on the tubes during incubation with antiserum. However, this is difficult to achieve since the amount of antigen that can bind to the plastic tubes is limited. More-

over, some of the adsorbed antigen is released from the tubes during the incubations (5) and the amount released is dependent on the total amount adsorbed. Released antigen will inhibit the reaction of the antibodies with antigen on the tubes. To investigate the influence of the amount of antigen added during the coating procedure on the uptake of specific antibodies, we coated the tubes with solutions of antigen of different concentrations. Two different dilutions of each antiserum were applied. The results are shown in Figure 1. The uptake of specific antibodies to the tubes passed a maximum as the concentration of antigen in the coating solution increased. With higher dilutions of the antiserum this maximum seemed to move to lower concentrations of antigen. This was most apparent in the HSA-anti-HSA system (Fig. 1A). When tubes were coated with DNP-HIS or DNP-HSA by adding comparable amounts of DNP (assessed by absorbance at 360 nm), the basic DNP-HIS was better adsorbed to the polystyrene than the acidic DNP-HSA (Fig. 1B). Unless otherwise stated, the tubes for further analyses were coated with 1 $\mu\text{g/ml}$ HSA and 2.6 $\mu\text{g/ml}$ DNP-HIS ($A_{360} = 0.01$), respectively.

The influence of the duration of various incubations on the uptake of conjugate was investigated with tubes coated with HSA (2 $\mu\text{g/ml}$) and two dilutions of anti-HSA, respectively. Coated tubes incubated with anti-HSA in the roller drum were taken after various times, washed with NaCl-Tween and stored at 0°C. The conjugate, diluted 1:500, was added simultaneously to all tubes. Incubation with conjugate lasted 16 hr. The result is shown in Figure 2. When the incubation time with anti-HSA was 16 hr and the incubation time with the conjugate was varied, the result in Figure 3 was obtained. The curves show that 6 hr incubation with antiserum and about 8 hr incubation with conjugate give optimal binding of enzyme to the tubes. For practical purposes, overnight incubation with conjugate was used.

The dose response curves for the immunologic reactions and their specificities are demonstrated by the results shown in Figure 4. The curves obtained with the two antisera, each reacted with its specific antigen, respectively, nearly coincide at high dilutions. Due to higher concentration of specific antibodies, the anti-HSA serum should have given rise to

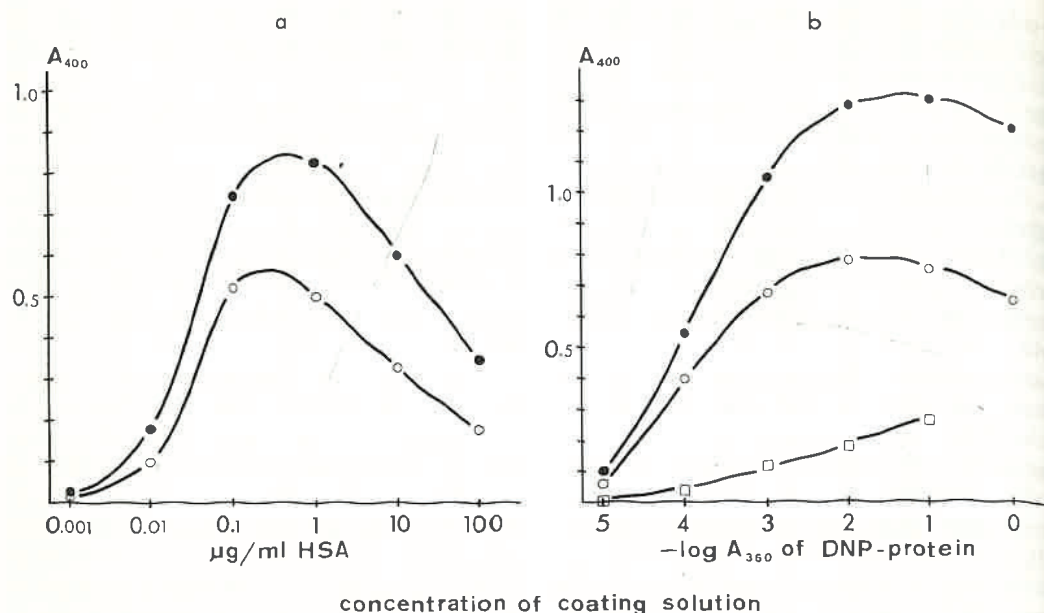


Figure 1. Antigen titration. Tubes were coated with solutions of antigen of different concentrations and incubated with dilutions of antiserum, followed by incubation with enzyme-conjugated anti-IgG. *a*, ●, Absorbances obtained with anti-HSA, diluted 10^{-5} . Enzyme-substrate incubation for 15 min. ○, Absorbances obtained with antiserum diluted $5 \cdot 10^{-7}$. Enzyme-substrate incubation for 120 min. *b*, ●, Tubes coated with DNP-HIS and incubated with anti-DNP serum, diluted $5 \cdot 10^{-5}$. Enzyme-substrate incubation for 10 min. ○, Tubes coated with DNP-HIS and incubated with anti-DNP, diluted 10^{-6} . Enzyme-substrate incubation for 90 min. □, Tubes coated with DNP-HSA and incubated with anti-DNP, diluted $5 \cdot 10^{-5}$. Enzyme-substrate incubation for 10 min.

higher enzyme activities than the anti-DNP serum when tested at corresponding dilutions. Instead, the anti-HSA curve flattened out earlier than the anti-DNP curve. This may reflect a relative shortage of antigenic determinants in HSA-coated tubes.

When dilutions of normal rabbit serum were incubated in untreated tubes, no enzyme activity was obtained. This indicates that even at a rather high serum concentration there is no unspecific adsorption of IgG to the tubes. With tubes coated either with HSA or with HIS, a low background was obtained with normal serum added in high concentrations. A higher background was regularly obtained in tubes coated with DNP-protein. This was seen with all normal sera tested (Fig. 4).

Dilutions of the anti-HSA serum, containing 5.2 mg/ml of specific antibodies, were used to prepare the standard curve shown in Figure 5A. With this curve the amount of HSA antibodies in a rabbit hyperimmune serum against whole human serum was estimated to 5.7 ± 0.3

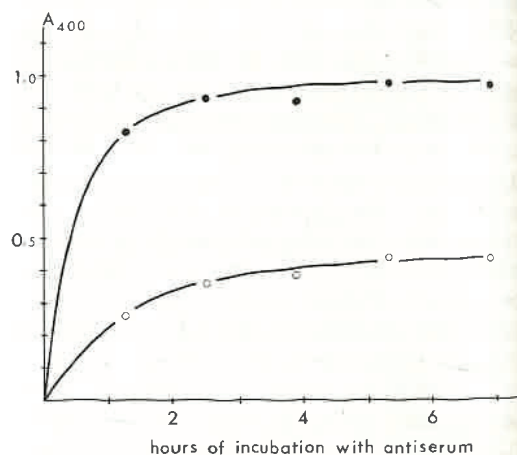


Figure 2. Kinetics of the binding of antibodies to tubes coated with antigen. Specific uptake of conjugate to tubes coated with HSA, incubated with antiserum for various times and followed by 16 hr incubation with conjugate. ●, Anti-HSA, diluted $5 \cdot 10^{-5}$. Enzyme-substrate incubation for 10 min. ○, Anti-HSA, diluted 10^{-6} . Enzyme-substrate incubation for 90 min.

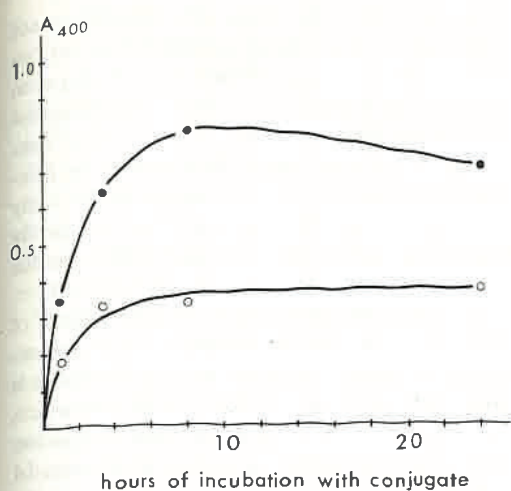


Figure 3. Kinetics of the binding of enzyme-conjugated anti-IgG to antigen-coated tubes after incubation with antiserum. Specific uptake of conjugate to tubes coated with HSA, incubated for 16 hr with antiserum and followed by incubation with conjugate for various times. ●, Anti-HSA, diluted $5 \cdot 10^{-5}$. Enzyme-substrate incubation for 5 min. ○, Anti-HSA, diluted 10^{-6} . Enzyme-substrate incubation for 60 min.

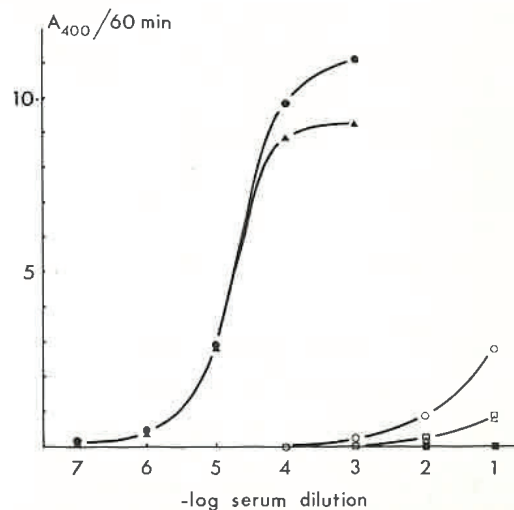


Figure 4. Dose-response curves for normal or immune sera added to tubes coated with different antigens (for details see text). Anti-DNP serum in tubes coated with DNP-HIS, ●. Anti-HSA serum in tubes coated with HSA, ▲. Normal serum in tubes coated with DNP-HIS, ○, in tubes coated with HSA, Δ, in tubes coated with HIS, □, and in untreated tubes, ■.

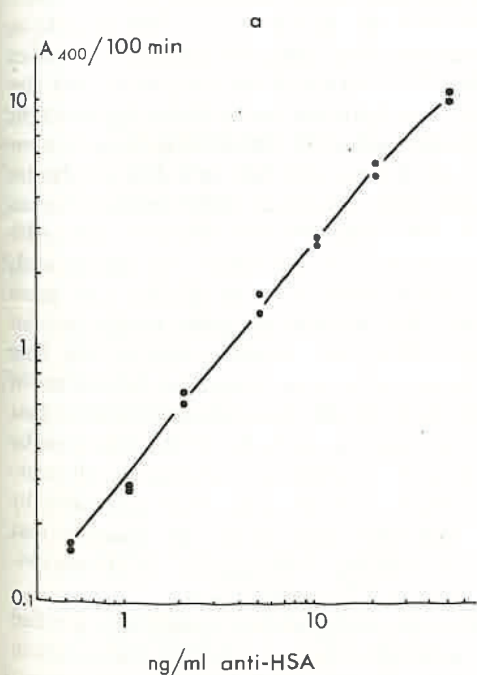
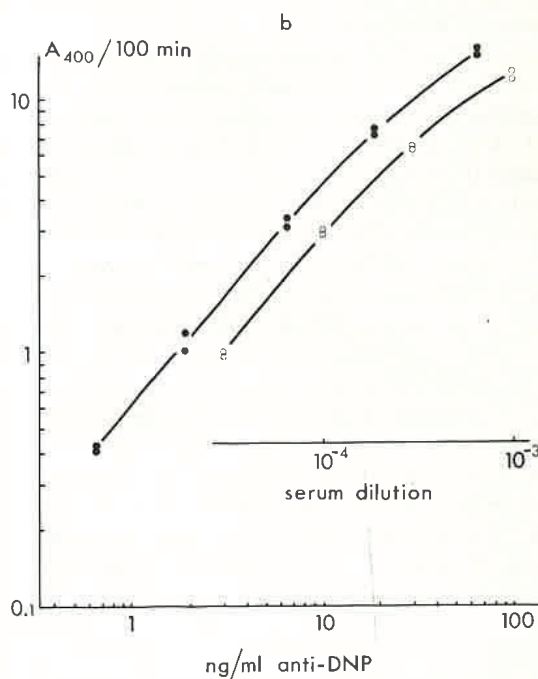


Figure 5. Standard curves for determination of specific antibodies. a. ●, Dilutions of standard anti-HSA serum, 5.2 mg/ml HSA antibodies. b. ●, Dilutions of standard anti-DNP serum, 2.5 mg/ml DNP antibodies. ○, Dilutions of an anti-DNP serum collected 13 days after one injection of 5 mg DNP-BGG.

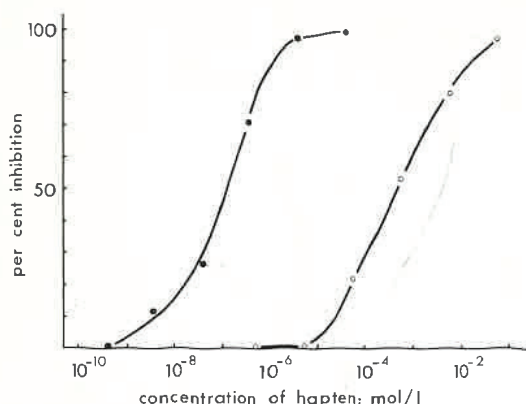


Figure 6. Inhibition of the binding of DNP antibodies to tubes coated with DNP-HIS by ϵ -DNP-lysine, ●, or by DNP, ○. Five-tenths milliliter of anti-serum, diluted 10^{-5} , was incubated together with 0.5 ml of different concentrations of either of the two haptens.

% inhibition

$$= 100 [1 - (\text{antibody binding in the presence of hapten}) / (\text{antibody binding in the absence of hapten})]$$

mg/ml (four determinations). In quantitative precipitation with HSA, the estimated antibody concentration was 5.7 mg/ml.

The anti-DNP serum which contained 2.5 mg/ml of specific antibodies according to the modified Farr technique was used as standard for determination of antibodies in a serum taken from the same rabbit 13 days after the first injection of 5 mg DNP-BGG in FCA (Fig. 5B). According to enzyme-linked immunosorbent assay (ELISA) the antibody content of this serum was $52 \pm 5 \mu\text{g/ml}$ (six determinations). In the modified Farr technique, the antibody concentration of this serum was $36 \pm 3 \mu\text{g/ml}$ (four determinations).

The reaction of anti-DNP serum with DNP-HIS-coated tubes could be completely inhibited by the addition of hapten. This is shown in Figure 6. Five-tenths milliliter of a 10^{-5} dilution of the anti-DNP serum was incubated together with 0.5 ml of a dilution of the haptens DNP and ϵ -DNP-lysine, respectively.

DISCUSSION

The original RAST technique (7), which has become important in screening allergies, makes use of allergens coupled to cellulose or

Sephadex particles. Although ELISA is not restricted to the use of coated tubes (4), we have chosen this type of immune adsorbent to simplify the washing procedure. Proteins, glycoproteins and probably many other substances as well can easily be adsorbed to polystyrene. However, as the antigens are only adsorbed to the solid phase and not covalently bound, the tubes are not particularly stable and this increases the error of the assay (5).

As indicated above, low concentrations of antibodies may not be detected if the concentration of antigen used for coating the tubes is either too high or too low. It is important, therefore, to find optimal antigen concentrations for every system as shown in Figures 1A and 1B.

In the present experiments, there was a good correlation between the concentrations of HSA antibodies in an antiserum against human serum, determined with ELISA and quantitative precipitation, respectively. This was expected since the hyperimmune serum contains mainly antibodies of IgG-class and of high affinity. The correlation between the amount of DNP antibodies in an early immune serum as assessed with either ELISA or the Farr technique was not equally good. The ELISA, as performed herein, measured mainly antibodies of IgG-class, although the conjugate used also reacts with IgM due to antibodies against light chain determinants. The Farr technique, however, should measure IgG and IgM antibodies equally well. Since an early immune serum like the one analyzed here should contain relatively more IgM antibodies, the higher antibody value obtained with ELISA was unexpected. The reasons for these results are unknown. Since the modification of the Farr technique used here measures antibodies of relatively high affinity only, it is possible that the discrepancies were due to the detection by the ELISA of additional antibodies of somewhat lower affinities than those detectable by the Farr technique. It is also possible that ELISA measures "nonspecific" DNP-reactivities like those discussed below.

Although prepared from specifically purified IgG antibodies, some conjugates had a certain reactivity against DNP-HIS but not against HIS or HSA. This DNP-reactivity seemed to increase upon storage of the antibodies used

for conjugation to enzyme. It could not be absorbed with unsolubilized DNP-HSA. Similarly normal rabbit serum also reacted with tubes coated with DNP-HIS. This reaction could not be inhibited by either ϵ -DNP-lysine or DNP in solution (unpublished). This indicates either the presence of natural antibodies of very low affinity or a nonspecific reaction against DNP similar to that shown by some conjugates.

The reaction of the hyperimmune anti-DNP serum was completely inhibited by low concentrations of free hapten. Since the immunogen was DNP coupled mainly to the ϵ -amino group of lysyl residues of BGG (10), the antibodies produced have a higher affinity for ϵ -DNP-lysine than for the DNP-molecule alone. The difference in inhibitory capacities (mol/l hapten necessary for 50% inhibition) reflects the difference in affinities of the antibodies for the two haptens. The ELISA may be used in inhibition studies of other systems as well. Thus it should be useful for the detection of antigenic determinants on molecules in solution or on cell surfaces, or for the investigation of antibody specificities or affinities.

Enzyme-conjugated proteins prepared with glutaraldehyde are very stable. The stability of the conjugates used in this investigation has not been separately studied. However, conjugates which have been in use for up to 4 months have given almost identical standard curves when tested with the same standard sera. No significant decrease in enzyme activity and/or immune reactivity of the conjugates has been noticed during these relatively short time periods. We do not expect the stability of these conjugates to be inferior to that of the conjugate prepared from alkaline phosphatase and normal rabbit IgG, which was used for more than 1 year (5). The amount of conjugate added to the system was chosen ar-

bitrarily to give a satisfactory sensitivity of the techniques. This amount was lower than the saturating dose. By adding more conjugate, its specific binding is increased and antibody can therefore be detected at even lower concentrations. Since the indicator reaction is a catalytic reaction, the product of which accumulates with time, another way to increase the sensitivity is simply to extend the duration of the enzyme reaction.

In conclusion, we believe that the introduction of enzyme-labeled reagents in quantitative immunologic techniques will prove very valuable. The ELISA is as sensitive as the corresponding radioimmunoassays and has the advantages of more stable reagents and fewer requirements of specialized equipment.

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