# Enzyme immunoassays in diagnostic medicine

## Theory and practice *

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*Serological methods are playing an increasingly important role in the diagnosis and epidemiological assessment of diseases. Simple, inexpensive methods for large-scale application are urgently needed. The enzyme immunoassay methods developed recently and reviewed here hold great promise for application in a wide variety of conditions. Under laboratory conditions they can be as sensitive as radio-immunoassay, but they can also be adapted as simple field screening procedures. These methods are based on the use of antibodies or antigens that are linked to an insoluble carrier surface. This is then used to "capture" the relevant antigen or antibody in the test solution and the complex is detected by means of an enzyme-labelled antibody or antigen. The degradation of the enzyme substrate, measured photometrically, is proportional to the concentration of the unknown "antibody" or "antigen" in the test solution. The application of these techniques to endocrinology, immunopathology, haematology, microbiology, and parasitology is reviewed.*

At present there are two widely accepted assays that employ labelled antibodies and antigens. They are immunofluorescence, in which a fluorescent dye is conjugated to the antibody, and radio-immunoassay, in which isotopes are attached to antibodies or antigens. In practice immunofluorescence is not easy to quantify for antibody assays, since it depends on subjective visual assessments of fluorescence and the results are usually expressed as the serial dilution of serum that gives the least fluorescence. Radio-immunoassay, on the other hand, is highly sensitive and permits precise quantification. However, the isotope labels may decay rapidly, so that the conjugates have a short shelf-life; complex equipment is necessary for their assessment; and, because of the medical hazards, they must be handled only by highly trained personnel.

The introduction of enzyme immunoassays, pioneered by Engvall & Perlmann (11, 12) [a] and Van Weemen & Schurs (31, 32), offered an attractive alternative to the labelled antibody/antigen methods mentioned previously. Enzyme immunoassays employ antibodies or antigens conjugated to enzymes in such a way that the immunological and enzymatic activity of each moiety is maintained. These assays give objective results and are extremely sensitive, although, at present, they are not usually quite as precise as radio-immunoassay. The reagents present no health hazards, they are stable, and have long shelf-lives. Moreover, the estimation of results can either be visual or be made with a rather simple spectrophotometer of the type found in most laboratories. The range of application of enzyme immunoassays is potentially as wide as that of radio-immunoassay and they may also reinforce or replace other serological tests, such as complement-fixation, haemagglutination, and immunofluorescence.

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[a] These assays are generally known as ELISA (enzyme-linked immunosorbent assays), but for the purposes of this paper the generic term enzyme immunoassays will be employed.

### PRINCIPLES

*Detection of antibody using enzyme-labelled anti-globulins* (indirect method—Fig. 1)

The antigen is coupled to a solid-phase support and the sera thought to contain antibody are incubated in this sensitized carrier. Excess serum components are washed away and then the enzyme-labelled

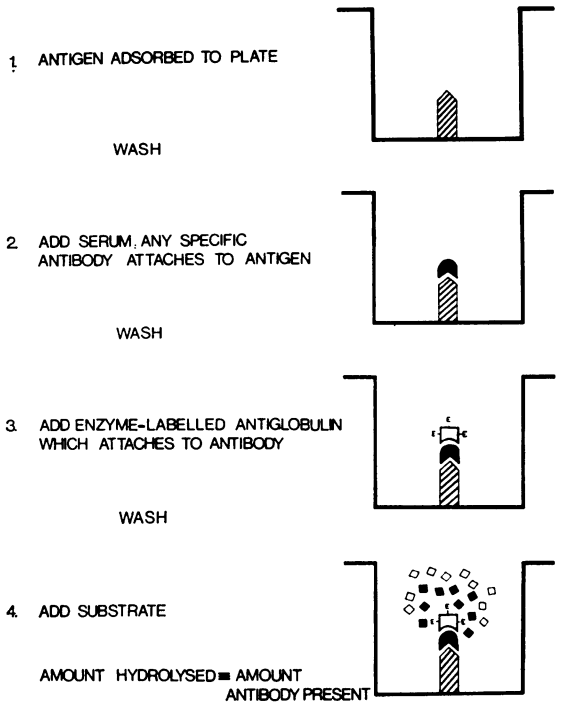


Fig. 1. The principle of the enzyme-labelled antiglobulin method for measuring antibody (indirect method).

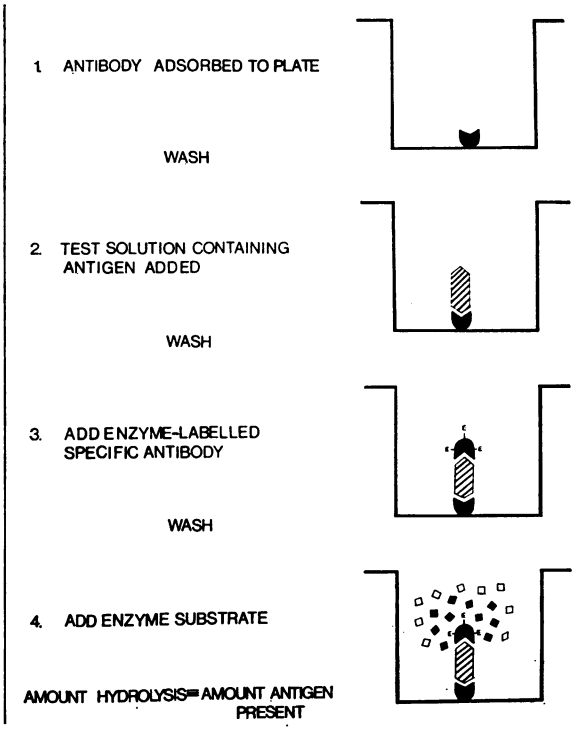


Fig. 2. The principle of the double-antibody method for measuring antigen.

antiglobulin (conjugate) is added. The conjugate will become attached to the antigen–antibody complexes on the carrier surface, and the amount of conjugate attached is measured by the amount of substrate that it degrades.

This is a very useful method, since a single enzyme-labelled antihuman globulin can be used to detect any human antibodies regardless of the disease state that is being investigated. For more sophisticated investigations it is of course possible to use separate enzyme-conjugated antisera to human IgG, IgM, and IgA, and so class-specific antibodies can be detected and measured.

*Detection of antigen by the double-antibody method* (Fig. 2)

In this variation immunoglobulin containing specific antibody is used to sensitize the carrier surface. The solution containing the antigen is then incubated with the sensitized surface and the excess solution is washed away. A conjugate consisting of enzyme-labelled specific antibody is then added and this becomes attached to the antigen already "captured" by the sensitized surface. After incubation, the excess conjugate is washed away and the amount attached is measured by the rate at which it degrades added substrate.

*Detection of antigen by the labelled antigen competition method* (Fig. 3)

Again, immunoglobulin containing specific antibody is attached to the solid carrier surface. This time a mixture of solution thought to contain antigen and enzyme-labelled antigen are incubated in various proportions on the carrier. The amount of enzyme-labelled antigen attached is again measured by the rate of hydrolysis of its substrate. The more antigen there is in the unknown solution, the less labelled antigen will be attached.

In all these methods the end result is a change in colour of the enzyme substrate. This can be measured accurately in a spectrophotometer.

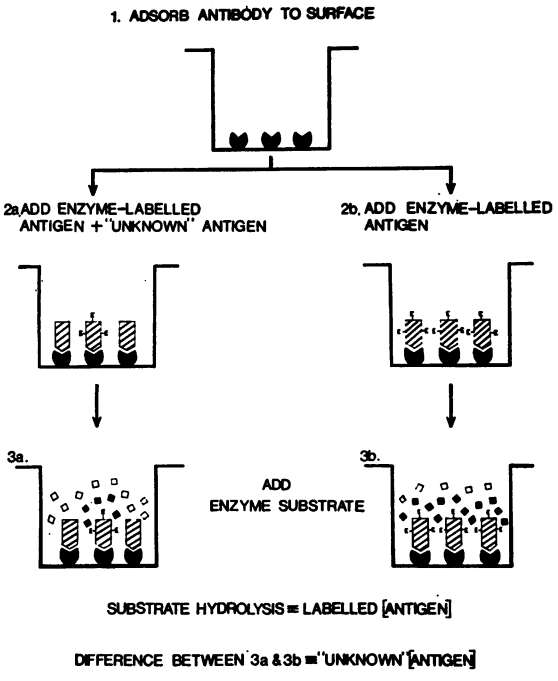


Fig. 3. The principle of the enzyme-labelled antigen competitive assay for measuring antigen.

## GENERAL CONSIDERATIONS

### *The carrier surface*

The carrier may be beads, tubes, or plates. Some workers have used Sepharose beads, since these permit covalent linking of antigen or antibody to the surface (7, 8); however, they are less suitable for large-scale use than are the polystyrene tubes used by Engvall & Perlmann (11, 12) or polystyrene microhaemagglutination plates (35). The latter are particularly suitable for large-scale use because they are cheap and small quantities of reagents can be used. Both polystyrene tubes and plates are satisfactorily coated with antigen or antibody by passive adsorption. Solutions containing 1–10 mg/litre of the specific protein usually give adequate coating.

### *Coating the carrier with antigen and antibody*

For most tests, only crude mixtures of antigens are available. For these, the only practicable way of determining the correct amount for coating is to carry out chequer-board titrations against positive and negative sera. The antigen dilution that gives the best discrimination between the positive and negative sera is used in subsequent tests. Where pure antigens are available, absolute measurements of the optimum coating quantities can be determined and used for later batches of antigen. Similarly, for antibody coating of plates, if specific antibody is used, an absolute, measured quantity can be used each time. However, for convenience it will often be found that the whole immunoglobulin fraction of an antiserum can best be employed. In the latter case, a chequer-board titration must be carried out on each new antiserum.

### *Test parameters*

It is easiest to determine the optimum incubation times and temperatures for each new system by trial and error. For convenience, in our laboratory, plates are usually sensitized overnight at 4°C and serum and conjugate incubation times of 2 h each are used. However, in many systems, adequate sensitization is achieved after 1 h at 37°C and an incubation time of 1 h at 37°C is often satisfactory. Shorter times tend to yield less accurate results.

The washing procedures are critical and it is important to ensure that all wells or tubes are treated in exactly the same way.

### *Conjugates*

Engvall & Perlmann (11, 12) favoured alkaline phosphatase as an enzyme marker. It has high activity, and the chosen substrate is cheap and nontoxic, with a bright yellow reaction colour that can be assessed visually or in an inexpensive spectrophotometer. Conjugates of alkaline phosphatase can be stored at 4°C with sodium azide preservative.

Peroxidase was shown long ago by Avrameus & Uriel (1), Nakane & Pierce (24), and Nakane (23) to be a good choice of enzyme for conjugation. Again, it has high activity, is cheaper than alkaline phosphatase, and yields a visible (brown) reaction product.

The choice of enzyme is largely a matter of personal preference. Conjugates of either that are available at present are not entirely satisfactory, since they contain varying amounts of polymers and unconjugated proteins.

In tests employing labelled antibodies, it is often possible to label the whole immunoglobulin fraction with the enzyme. However, to ensure specificity, pure antibodies, prepared by affinity chromatography, may be preferable in some instances.

It is always best to use the most highly titred antisera that are available, because this allows the conjugates to be used in a more dilute form and so requires less enzyme, which is the most expensive reagent in these tests.

*Standardization*

Adequate standardization is the key to high precision in enzyme immunoassays, and this can be achieved only by employing reference preparations.

In tests for antibody, a reference sample consisting of a pool of sera from a group of individuals with a high antibody titre should be used. This should be made into aliquots and preserved, preferably in the lyophilized state. Dilutions of this sample and dilutions of a reference negative serum must be included in each group of tests.

Antigens are often more difficult to standardize, unless they are available in a purified form. For crude antigens, activity in the test is their most important property and can best be expressed in terms of a reference preparation, even though this may not itself be pure. Biochemical characterization—e.g., protein content—is often misleading, since it frequently does not indicate biological activity.

## RESULTS

The results can be expressed in a variety of ways, as the following examples indicate.

(*a*) Under carefully controlled conditions, the results may be expressed in absorbance values, and this is the easiest method available. In antibody assays the range of absorbance values given by a normal uninfected population is determined. A value indicative of infection is then chosen above the control level. Sera are classified as positive if above and negative if below that value. The values for " positive " and " negative " subjects will frequently overlap slightly. For example, the distribution of absorbance values of a group of Brazilians without Chagas' disease (open columns) is plotted in Fig. 4. It can be seen that virtually all were under 0.4, so this was chosen as the level above which samples would be considered as positive. The solid columns show the distribution of values for a group of Brazilians with Chagas' disease. All the values except one were above the negative level.

(*b*) In terms of reference samples Fig. 5 shows a standard curve for enzyme immunoassay of Factor VIII (blood clotting factor) related antigen determined in a double-antibody test. Values of unknown samples are read off this standard curve.

(*c*) All sera can be titrated by serial dilution. An absorbance value is then chosen and the dilution of serum yielding such an absorbance value is the " titre ".

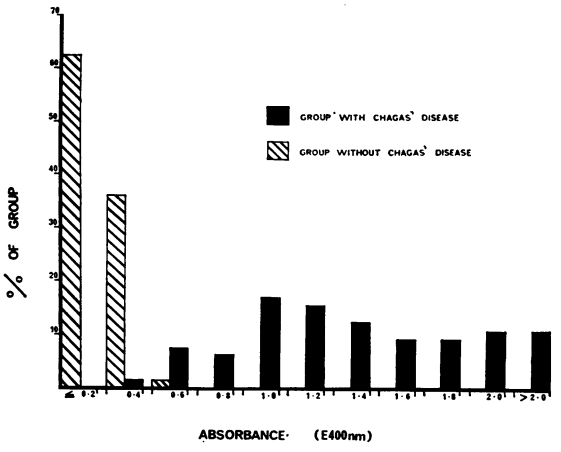


Fig. 4. Results of enzyme immunoassays with *Trypanosoma cruzi* antigen, showing the distribution of values of Brazilians with Chagas' disease and of uninfected Brazilians.

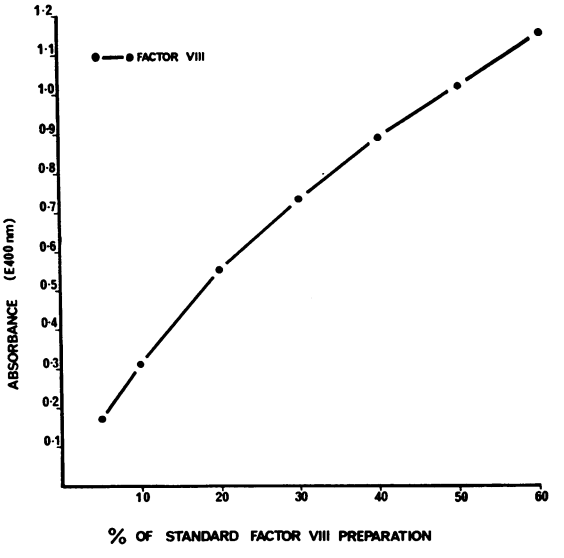


Fig. 5. Standard curve of enzyme-immunoassay values obtained in double-antibody method with dilutions of a reference preparation of Factor-VIII-related antigen. Plates are coated with rabbit antiserum to Factor-VIII-related antigen, the test sample is added, and alkaline-phosphate-labelled rabbit antiserum to Factor-VIII-related antigen is the indicator reagent.

## PRACTICE OF ENZYME IMMUNOASSAYS

The practical details of three methods of microscale immunoassays are shown in Table 1 and are supplemented by the notes given below. We have

Table 1. Microhaemagglutination-plate enzyme immunoassays for antibody and antigen

| Antibody assay using enzyme-labelled antiglobulin | Antigen assay by double-antibody method | Antigen assay by labelled antigen competitive assay |
|---|---|---|
| (1) Sensitize wells overnight with 0.3 ml of antigen in coating buffer at 4°C. | Sensitize wells overnight with 0.3 ml of immunoglobulin containing specific antibody (diluted in coating buffer) at 4°C. | Sensitize wells overnight at 4°C with immunoglobulin containing specific antibody diluted in coating buffer. |
| *wash* | *wash* | *wash* |
| (2) Add 0.3 ml of test serum diluted in PBS-Tween. Incubate for 2 h at room temperature. | Add 0.3 ml of solution thought to contain antigen, diluted in PBS-Tween. Incubate for 2 h at room temperature. | Mix together the solution containing antigen to be measured and enzyme-labelled antigen, both at various dilutions in PBS-Tween. Incubate in tubes or unsensitized plates for at least 30 min at room temperature. |
| *wash* | *wash* | |
| (3) Add 0.3 ml of enzyme-labelled antiglobulin diluted in PBS-Tween. Incubate for 3 h at room temperature. | Add 0.3 ml of enzyme-labelled immunoglobulin containing specific antibody, diluted in PBS-Tween. Incubate for 3 h at room temperature. | Transfer 0.3 ml of the above-mentioned mixture to each well in sensitized plates. Incubate for 3 h at room temperature. |
| *wash* | *wash* | *wash* |
| ↓ | ↓ | ↓ |
| (4) Add 0.3 ml of enzyme substrate solution (4-nitrophenyl phosphate for alkaline phosphatase conjugates; 5-aminosalicylic acid and hydrogen peroxide for peroxidase conjugates). | | |
| (5) Stop reaction by adding 0.05 ml of 3 mol/l NaOH to each well. | | |
| (6) Read absorbance of contents of each well in a spectrophotometer, at 400 nm for alkaline phosphatase conjugates and at 449 nm for peroxidase conjugates. | | |

found these methods to be effective in all the systems outlined in Table 2.

A complete list of all solutions and chemicals employed is given in the Annex.

*Sensitization of plates*

The wells in microhaemagglutination plates (Cooke Microtiter M29AR) [a] are sensitized by adding 0.3 ml of antigen (or antibody) in coating buffer and then incubating the plates at 4°C overnight. The plates are then washed and are ready for immediate use. Sensitized plates can be stored satisfactorily at −70°C.

*Washing*

This consists in emptying the plates, refilling all wells with phosphate-buffered saline with Tween 20 (PBS-Tween) from a wash bottle, and gently agitating the plates for 3 min. This procedure is repeated three times and after the final wash the plates are shaken dry and the next stage is begun at once.

[a] Dynatech Laboratories, Billingshurst, Sussex, England.

*Conjugates*

We employ alkaline phosphatase conjugates or horse-radish peroxidase conjugates.

*Enzyme-substrates*

For alkaline phosphatase conjugates the substrate used is 4-nitrophenyl phosphate, which gives a yellow colour on hydrolysis, with peak absorbance at 400 nm. With peroxidase conjugates 5-aminosalicylic acid + hydrogen peroxide is a suitable substrate. The brown reaction product shows maximum absorbance at 449 nm.

*Substrate reaction time*

(*a*) The reaction can be stopped after a given time—e.g., 30 min or 1 h (shorter times lead to inaccuracy).

(*b*) The reaction can be stopped in all wells when the absorbance of a reference positive sample reaches a predetermined value (e.g., 1.0). This is determined by monitoring the reaction at time intervals on a series of wells containing the positive reference sample.

Table 2. Materials that adsorb satisfactorily to polystyrene microhaemagglutination plates

| | |
|---|---|
| **Viruses** | |
| rubella | extracts from infected tissue culture |
| herpes simplex | |
| cytomegalovirus | |
| measles | |
| influenza | |
| coxsackie B | |
| Japanese B encephalitis | infected mouse brain extract |
| **Bacteria** | |
| *Treponema* | sonicated organisms from infected rabbits |
| *Brucella* | disrupted organisms from agar slopes |
| *Salmonella* | lipopolysaccharide and phenol water extracts |
| cholera vibrios | lipopolysaccharide and synthetic 'O' antigen |
| **Protozoa** | |
| *Entamoeba histolytica* | lysed, sonicated organisms, axenic culture |
| malaria parasites | sonicated infected erythrocytes |
| trypanosomes | lysed sonicated organisms from infected animals or cultures |
| *Toxoplasma gondii* | lysate or cultured organisms |
| **Helminths** | |
| *Trichinella spiralis* | disrupted larvae from infected rats |
| *Schistosoma mansoni* | extracts of sonicated eggs or adult worms |
| filarial parasites | saline extract of adult worms |
| **General** | |
| immunoglobulins | 18% sodium sulfate precipitates |
| specific antibody | affinity chromatography purified |
| DNA | calf thymus extract |

(*c*) The reaction can be stopped when the contents of each well reaches a suitably dense colour. The absorbance is then read and the reaction time is noted. The reaction rate/unit time is then calculated for each well. Obviously this is not suitable for large-scale use.

## APPLICATIONS

### *Endocrinology*

Van Weemen & Schurs (31, 32) and Van Weemen (30) used competitive inhibition immunoassays in their successful studies on the measurement of human chorionic gonadotrophin, luteinizing hormone, and estrogens. They found that the enzyme immunoassay has almost the same sensitivity as radio-immunoassays and, at least in the case of human chorionic gonadotrophin, a high level of precision was achieved. Some problems were encountered that will need to be overcome before the assays are suitable for routine laboratory use. Attempts have also been made by Miedema et al. (22) to measure insulin, but so far these have not been followed up.

### *Immunopathology*

Engvall & Perlmann (11, 12) first described the enzyme-linked immunosorbent assay (ELISA) for the measurement of IgG in rabbits, and Hoffmann (15) used it to quantify human IgE. It seemed to be sensitive in the 1–100 $\mu$g/l range. Pesce et al. (25) have recently introduced the method as an alternative means of measuring DNA antibodies in lupus erythematosus. Their results were promising, but considerable variability was encountered. Belanger et al. (3) and Maiolini & Masseyeff (21) quantified $\alpha$-fetoprotein by means of the competitive methods with labelled antigen or with a labelled antiglobulin.

### *Haematology*

Holm et al. (16) were not satisfied with enzyme immunoassay for measuring antibody to erythrocyte antigens since, surprisingly, they did not find it sensitive enough. However, we have been able to use the double-antibody technique to measure Factor VIII-related antigen in plasma (Fig. 5). The enzyme immunoassay is as efficient as the tedious electrophoretic technique at present in use and is more suitable for large-scale use. Preliminary experiments show that fibrin degradation products can be measured in the same way (Table 3).

Table 3. Enzyme immunoassay of fibrinogen [a]

| | mg of fibrinogen/litre | | | | |
|---|---|---|---|---|---|
| | 1 | 5 | 10 | 50 | 100 |
| enzyme immunoassay value: E 400 nm | 0.44 | 0.74 | 0.88 | 1.06 | 1.17 |

[a] Plates: coated 5 h at 37°C with 10 mg/l immunoglobulin containing antibody to fibrinogen; plasma incubation: 18 h at 4°C; conjugate: antifibrinogen serum labelled with alkaline phosphatase.

Table 4. Microhaemagglutination-plate enzyme immunoassay for syphilis (enzyme-immunoassay value: E 400 nm) [a]

| Subject | Serum dilutions | | | | | |
|---|---|---|---|---|---|---|
| | 1/200 | 1/400 | 1/800 | 1/1600 | 1/3200 | 1/6400 |
| syphilitic patient | 1.04 | 0.66 | 0.48 | 0.46 | 0.33 | 0.2 |
| uninfected individual | 0.38 | 0.35 | 0.23 | 0.21 | 0.18 | 0.18 |

[a] Antigen: sonicate of *T. pallidum* diluted 1 : 400, incubated overnight at 4°C; sera incubated for 2 h at room temperature; alkaline phosphatase–antihuman globulin conjugate incubated for 3 h at room temperature.

*Microbiology*

The really explosive growth in the application of enzyme immunoassays has been in the measurement of antibodies in infectious diseases. Carlsson et al. (5, 6) were the first to apply the method in bacteriology when they showed that human antibodies to *Salmonella* 'O' antigen could be fairly accurately measured. There was greater sensitivity than by the Widal reaction and the results were reproducible. Holmgren & Svennerholm (17) were also able to adapt the enzyme immunoassay to cholera and they made measurements of antibodies in the different immunoglobulin classes after immunization. The method has been found to be useful also in the study of antibodies to *Escherichia coli* (19) and *Brucella* (9). Veldkamp & Visser (33) obtained very promising results with the tube test for *Treponema*.

In collaboration with Dr P. O'Neill we also applied the microplate enzyme immunoassay to syphilis serology. Representative results are illustrated in Table 4. These were obtained with sera from a proved case of syphilis and from a negative control individual. Enzyme-labelled anti-IgM conjugates may well be useful in the field of syphilis serology.

There are many possible applications of enzyme immunoassay in virology. Voller & Bidwell (34) surveyed about 200 people for rubella antibody. They found a good correlation between the enzyme immunoassay results and the traditional but more cumbersome haemagglutination inhibition (HI) technique. Recently we have carried out microplate enzyme immunoassays for IgM antibody to rubella. The results are shown in Table 5. It can be seen that the patient who had no detectable antibody to rubella before immunization produced an IgM response during the first month but 2 months later most of his antibody was IgG. The patient with serum positive for rubella at the time of immunization did not give a detectable IgM response.

Table 5. Results of microhaemagglutination-plate enzyme immunoassays for rubella antibody (enzyme immunoassay value: E 400 nm) [a]

| Subject | Days after vaccination | | |
|---|---|---|---|
| | 7 | 28 | 70 |
| *Patient 1* | | | |
| (serum negative for rubella before vaccination) | | | |
| polyspecific conjugate | 0.29 | 0.26 | 1.22 |
| anti-IgM conjugate | 0.27 | 0.27 | 0.18 |
| *Patient 2* | | | |
| (serum positive for rubella before vaccination) | | | |
| polyspecific conjugate | 0.31 | 0.69 | not done |
| anti-IgM conjugate | 0.06 | 0.05 | |

[a] Plates coated with antigen overnight at 4°C; dilution of serum incubated at room temperature (25°C) for 2 h; conjugate reacted 2 h at room temperature; substrate time: 30 min.

The enzyme immunoassay has been compared with the HI test and the neutralization test normally used for detecting antibody to Japanese B encephalitis virus (Table 6). It distinguished the acute and convalescent phases of the disease as well as the HI and neutralization tests did.

The microplate enzyme immunoassays may be suitable for screening for other viral diseases, and many antigens are already commercially available. In Table 7 the enzyme immunoassay values are given for dilutions of sera from patients with cytomegalovirus, coxsackie B, herpes simplex, measles, and adenovirus. Control sera from normal individuals

Table 6. Results of haemagglutination inhibition, enzyme-linked immunosorbent assays, and neutralization tests on sera from patients with Japanese B encephalitis [a]

| | Acute sera | | | Convalescent sera | | |
|---|---|---|---|---|---|---|
| | HI | ELISA | NT | HI | ELISA | NT |
| 1 | 640 | 0.62 | 2200 | 2560 | 1.35 | 4400 |
| 2 | 640 | 0.65 | 2200 | 2560 | 1.6 | 4400 |
| 3 | 40 | 0.37 | 280 | 320 | 0.76 | 2200 |
| 4 | 80 | 0.67 | 550 | 640 | 1.3 | 2200 |
| 5 | 10 | 0.49 | 69 | 2560 | 1.7 | 4400 |
| 6 | 20 | 0.43 | 140 | 80 | 1.1 | 550 |
| 7 | <10 | 0.11 | 63 | 640 | 0.58 | 3500 |

[a] See footnote to Table 5.

Table 7. Enzyme immunoassay values (E 400 nm) for serum dilutions from patients with various viral infections [a]

| Virus antigens | Sera [b] | Serum dilution | | | |
|---|---|---|---|---|---|
| | | 1:100 | 1:200 | 1:400 | 1:800 |
| adenovirus | + | 1.1 | 0.93 | 0.69 | 0.47 |
| | − | 0.18 | 0.1 | 0.14 | 0.14 |
| coxsackie | + | 0.59 | 0.34 | 0.27 | 0.16 |
| | − | 0.19 | 0.14 | 0.08 | 0.04 |
| cytomegalovirus | + | 1.02 | 0.87 | 0.57 | 0.49 |
| | − | 0.05 | 0.06 | 0.09 | 0.07 |
| herpes simplex | + | 0.73 | 0.71 | 0.47 | 0.38 |
| | − | 0.02 | 0.02 | 0 | 0 |
| measles | + | 1.3 | 1.15 | 0.9 | 0.78 |
| | − | 0.4 | 0.35 | 0.27 | 0.18 |

[a] Plates coated with antigen overnight at 4°C; dilution of serum incubated at room temperature (25°C) for 2 h; conjugate reacted 2 h at room temperature; substrate time varied according to antigen system.

[b] Each positive serum was obtained from a patient with a proved infection of the relevant virus.

were tested in each case and it was possible to differentiate clearly between patient and control in each of these viral infections.

An obvious application of the assay is for the measurement of hepatitis B antigen. For this purpose we used the double-antibody method in experiments set up jointly with Professor A. Zuckerman. Goat anti-HBsAg immunoglobulin was used to coat the plates and the same goat immunoglobulin was labelled with enzyme to detect the fixed antigen. Although most hepatitis B patients could be detected, there were both false negative and false positive results. Further studies are in progress.

*Parasitology*

Ljungstrom et al. (20) and Ruitenberg et al. (27) introduced the ELISA method to parasitology when it was used in the serodiagnosis of *Trichinella* infections. Later Ruitenberg et al. (26, 28, 29) evaluated the test in great detail for its practical value in detecting trichinosis in pigs and they found it better than immunofluorescence. Engvall & Ljungstrom (10) also used ELISA in a longitudinal study of immunoglobulin class-specific antibodies in people infected with *T. spiralis*. It was as sensitive as passive agglutination and more sensitive than immunofluorescence.

Bout et al. (4) reported that ELISA was specific in schistosomiasis, but Huldt et al. (18) were rather less enthusiastic in their initial studies—even though the latter group used an antigen purified by affinity chromatography.

Farag et al. (13) also reported excellent results with ELISA for hydatid disease in man, with a specific antigen; with a less purified antigen more cross-reactions were reported.

Bartlett et al. (2) also used ELISA in onchocerciasis, but they found widespread cross-reactions with other nematode antigens, which made interpretation difficult.

Voller et al. (35) introduced the microplate enzyme immunoassay in a small seroepidemiological study of malaria. The results made it possible to discriminate between areas where malaria was endemic and areas from which the disease had been eradicated. In a small study in Papua New Guinea, McGregor and Voller were able to identify malarious areas by the enzyme immunoassay result. Voller et al. (36) also used the macro-ELISA test in tubes to survey groups of malaria-infected people in Iran and Tanzania. Almost all those with proved malaria were detected.

Better tests are needed for American and African trypanosomiasis, and here the microplate method (38, 39) has proved to be very satisfactory. A good correlation was found between the immunofluorescent antibody technique and the enzyme immunoassay in African trypanosomiasis, and the latter detected most cases of the American form (Fig. 4).

Toxoplasmosis is another disease in which sero-

logy is the only practicable means of diagnosis, and enzyme immunoassays have been applied by Bout et al. (4) and by Voller et al. (37). Although the two groups used slightly different methods, they both agreed that the results obtained with the enzyme immunoassay generally agreed well with those of other serological tests, such as passive haemagglutination, the dye test, and immunofluorescence. Enzyme immunoassay was certainly easier to perform than the other two tests.

## ACKNOWLEDGEMENTS

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*Annex*

## MATERIALS USED IN THE STUDY

**Coating buffer** (pH 9.6

| | |
|---|---|
| $Na_2CO_3$ | 1.59 g |
| $NaHCO_3$ | 2.93 g |
| $NaN_3$ | 0.2 g |
| Distilled water | 1000 ml |

**PBS-Tween** (pH 7.4)

| | |
|---|---|
| NaCl | 8.0 g |
| $KH_2PO_4$ | 0.2 g |
| $Na_2HPO_4.12H_20$ | 2.9 g |
| KCl | 0.2 g |
| Tween 20 | 0.5 ml |
| $NaN_3$ | 0.2 g |
| Distilled water q.s. | ad 1000 ml |

**Conjugates**

Enzyme-labelled antihuman immunoglobulin reagents will be used for most purposes. We usually employ alkaline phosphatase and the conjugation is as follows.

*Enzyme: alkaline phosphatase.* From calf intestinal mucosa. Ammonium sulfate suspension. Type VII, 1000 units/mg protein (P-4502 [a]).

*Antiserum: rabbit antiserum to human IgG-immunoglobulin fraction (product 62-144 [b]).*

(1) Add 2 mg of immunoglobulin in 1.0 ml of PBS to 5 mg of enzyme and mix at room temperature. Dialyse for 18 h against PBS at 4°C with changes of buffer.

(2) Add 25% glutaraldehyde to yield a final concentration of 0.2%. Incubate 1–2 h at room temperature, then dialyse overnight at 4°C against PBS with several changes of buffer.

(3) Transfer the dialysis sac containing conjugate to 0.05 mol/litre Tris buffer pH 8.0 and dialyse overnight at 4°C with buffer changes.

(4) Dilute the dialysis sac contents (conjugate) to 4.0 ml with 0.05 mol/litre Tris buffer (pH 8.0) containing 1.0% bovine serum albumin and 0.02% $NaN_3$. This is the stock conjugate. Store in the dark at 4°C until used.

The working strength of the conjugate is determined (*a*) by testing dilutions of it in enzyme immunoassays on plates coated with 100 $\mu$g/l human immunoglobulin and (*b*) by testing dilutions of it in an indirect enzyme immunoassay with the antigen that will be used subsequently. Usually conjugates can be diluted 1:500 or more for use.

On the day of the tests the required amount of conjugate is diluted in PBS-Tween to the working strength.

**Substrate**

*For alkaline phosphatase conjugates*

4-nitrophenyl phosphate 1 g/litre in 10% diethanolamine buffer is used. This is prepared as follows.

*10% diethanolamine buffer*

| | |
|---|---|
| diethanolamine | 97 ml |
| $H_20$ | 800 ml |
| $NaN_3$ | 0.2 g |
| Add a 1 mol/l solution of HCl to give pH 9.8 | |
| Distilled water q.s. | ad 1000 ml |

[a] Sigma London Chemical Co., Kingston, Surrey, England.

[b] Microbiological Associates, Bethesda, MD, USA.

*4-nitrophenyl phosphate*

Sigma 104 phosphatase substrate tablets [a] are stored at −20°C in the dark until use. Immediately before use, one tablet (5 mg) is removed from the deep freeze and is dissolved in 5 ml of the 10% diethanolamine buffer at room temperature.

*For horse-radish peroxidase conjugates*

Hydrogen peroxide and 5-aminosalicylic acid.

[a] Sigma London Chemical Co., Kingston, Surrey, England.

*Aminosalicylic acid*

8 mg of 5-aminosalicylic acid are dissolved in 10 ml of warm distilled water. This solution is cooled and stored at 4°C. Immediately before use, an aliquot of the solution is warmed to room temperature and a 1 mol/litre solution of NaOH is added to give pH 6.0.

*Substrate solution*

1 ml of 0.05% hydrogen peroxide and 10 ml of aminosalicylic acid solution are mixed. This is the final substrate and is used immediately.

## RÉSUMÉ

TITRAGES IMMUNO-ENZYMATIQUES POUR LE DIAGNOSTIC MÉDICAL: THÉORIE ET PRATIQUE

Pour les titrages immuno-enzymatiques, on utilise des conjugués constitués d'anticorps (ou plus rarement d'antigène) lié à une enzyme, qui est le plus souvent une peroxydase ou une phosphatase alcaline.

Le principe de l'épreuve est le suivant: l'antigène (ou l'anticorps) est fixé à un support insoluble tel qu'une plaque de polystyrène pour microhémagglutination. On ajoute ensuite la solution à éprouver, et si elle contient de l'anticorps (ou de l'antigène) celui-ci se combine avec la surface solide sensibilisée. Le matériel qui n'a pas réagi est éliminé par lavage. On ajoute alors le conjugué enzymatique. Il s'agit souvent d'une antiglobuline marquée par une enzyme, ce qui convient à la détection d'un anticorps dans la solution à éprouver. Il peut s'agir aussi d'un anticorps spécifique marqué par une enzyme, ce qui convient pour détecter les antigènes solubles dans la méthode à double anticorps. On ajoute ensuite le substrat de l'enzyme, en le choisissant de manière telle que l'hydrolyse s'accompagne d'un changement de couleur. La vitesse de dégradation, qui peut être mesurée par la méthode photométrique ou appréciée à l'œil nu, est proportionnelle à la concentration de l'antigène (ou de l'anticorps) recherché dans la solution à éprouver.

La méthode fondée sur l'utilisation de l'antiglobuline marquée par une enzyme se prête bien à la détection et à la mesure des anticorps dans les maladies infectieuses. Les applications du titrage immuno-enzymatique à l'endocrinologie, l'immunopathologie, la microbiologie et la parasitologie sont passées en revue. Une attention particulière est accordée à la détection des anticorps contre divers virus: rubéole, cytomégalovirus, herpès, rougeole, et encéphalite japonaise B. Il est également possible de mesurer des anticorps appartenant à des classes particulières d'immunoglobuline (par exemple, des anticorps contre la rubéole, le cytomégalovirus). On souligne les avantages du titrage immuno-enzymatique par la méthode à double anticorps en hématologie, notamment pour la mesure de l'antigène lié au Facteur VIII et du fibrinogène. La détection de l'antigène de l'hépatite B est également mentionnée.

## REFERENCES

1. AVRAMEUS, S. & URIEL, J. *Comptes rendus hebdomadaires des séances de l'Académie des sciences*, **262**: 2543 (1966).
2. BARTLETT, A. ET AL. *Zeitschrift für Tropenmedizin und Parasitologie*, **26**: 370 (1975).
3. BELANGER, L. ET AL. *Clinica chimica acta*, **48**: 15 (1973).
4. BOUT, D. ET AL. *Lille médical*, **20**: 561 (1975).
5. CARLSSON, H. E. ET AL. *Infection and immunity*, **6**: 703 (1972).
6. CARLSSON, H. E. ET AL. *International archives of allergy and applied immunology*, **48**: 485 (1975).
7. DEELDER, A. M. ET AL. *Annals of the New York Academy of Sciences*, **254**: 119 (1975).
8. DEELDER, A. M. & STREEFKERK, J. G. *Experimental parasitology*, **37**: 405 (1975).
9. ENGVALL, E. & CARLSSON, H. E. *In:* Immunoenzymatic techniques. Paris, INSERM, 1976 (in press).
10. ENGVALL, E. & LJUNGSTROM, I. *Acta pathologica et microbiologica scandinavica*, **83**: 1 (1975).
11. ENGVALL, E. & PERLMANN, P. *Immunochemistry*, **8**: 871 (1971).
12. ENGVALL, E. & PERLMANN, P. *Journal of immunology*, **109**: 129 (1972).

13. FARAG, H. ET AL. *Biomedicine express*, **23**: 276 (1975).
15. HOFFMAN, D. R. *Journal of allergy and clinical immunology*, **51**: 303 (1973).
16. HOLM, G. ET AL. *Scandinavian journal of immunology*, **3**: 173 (1974).
17. HOLMGREN, J. & SVENNERHOLM, A. M. *Infection and immunity*, **7**: 759 (1973).
18. HULDT, G. ET AL. *Annals of tropical medicine and parasitology*, **69**: 483 (1975).
19. JODAL, U. ET AL. *International archives of allergy and applied immunology*, **47**: 537 (1974).
20. LJUNGSTROM, I. ET AL. *Proceedings of the British Society of Parasitology*, **69**: XXIV (1974).
21. MAIOLINI, R. & MASSEYEFF, R. *Journal of immunological methods*, **8**: 223 (1975).
22. MIEDEMA, K. ET AL. *Clinica chimica acta*, **40**: 187 (1972).
23. NAKANE, P. K. *Annals of the New York Academy of Sciences*, **254**: 203 (1975).
24. NAKANE, P. K. & PIERCE, G. B. *Journal of histochemistry and cytochemistry*, **14**: 929 (1966).
25. PESCE, A. J. ET AL. *Clinical chemistry*, **20**: 353 (1974).
26. RUITENBERG, E. J. ET AL. *Bulletin of the World Health Organization*, **51**: 108 (1974).
27. RUITENBERG, E. J. ET AL. *In:* Proceedings of the Third International Congress of Parasitology. Munich, 25-31 August 1974. Vienna, Facta, 1974, Vol. 3, p. 1203.
28. RUITENBERG, E. J. ET AL. *In:* Immunoenzymatic techniques. Paris, INSERM, 1976 (in press).
29. RUITENBERG, E. J. ET AL. *Medikon Nederland*, **4**: 30 (1975).
30. VAN WEEMEN, B. K. Enzyme immunoassays. Groningen, 1974 (thesis).
31. VAN WEEMEN, B. K. & SCHURS, A. H. W. M. *FEBS letters*, **15**: 232 (1971).
32. VAN WEEMEN, B. K. & SCHURS, A. H. W. M. *FEBS letters*, **24**: 77 (1972).
33. VELDKAMP, J. & VISSER, A. M. *British journal of venereal diseases*, **51**: 227 (1975).
34. VOLLER, A. & BIDWELL, D. E. *British journal of experimental pathology*, **56**: 338 (1975).
35. VOLLER, A. ET AL. *Bulletin of the World Health Organization*, **51**: 209 (1974).
36. VOLLER, A. ET AL. *British medical journal*, **1**: 659 (1975).
37. VOLLER, A. ET AL. *Journal of clinical pathology*, **29**: 150 (1976).
38. VOLLER, A. ET AL. *Lancet*, **1**: 426 (1975).
39. VOLLER, A. ET AL. *Zeitschrift für Tropenmedizin und Parasitologie*, **26**: 247 (1975).