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The immobilization of immunoreactants on solid phases

13.1. Relative merits of solid phases

The simple manipulations required to separate free antibody or antigen from immune complexes immobilized non-covalently on plastic solid phase is probably the most important reason for the rapid increase in popularity of EIA. Desired traits of the solid phase are: (i) high capacity for binding immunoreactants (high surface/volume ratio); (ii) possibility of immobilization of many different immunoreactants; (iii) minimal dissociation; (iv) negligible denaturation of immobilized molecule; and (v) orientation of immobilized antibody with binding sites towards the solution and the Fc to the solid phase (e.g., Section 13.2.2).

Plastic is by far the most popular solid phase, since it makes the procedures extremely simple. However, plastics may also have some important limitatons: (i) they are immunoreactant-consumptive, i.e. often require 10 times more reactants than particulate solid phases or membranes; (ii) the avidity of immobilized antibodies for large antigens decreases by 1–2 orders of magnitude (Zwolinski, G.; Josephson, L.; cited by Parsons, 1981), probably due to the wide spacing of epitopes or paratopes; (iii) the rate of antibody–antigen interactions is slower than in solution or with particulate solid phases (hours instead of minutes), due to the necessity of the free immunoreactant to diffuse to the solid phase (association kinetics is largely dictated by diffusion rate; Section 8.4); and, (iv) few suitable antibod-
ies will be bound to plastic due to its low adsorption capacity per unit area, particularly if the fraction of high-avidity IgG is low. Nevertheless, plastic has proven extremely useful for EIA.

Nitrocellulose membranes are not yet widely used but should replace plastic in many investigations in which: (i) only the presence of an immunoreactant is to be established and not its quantity (though the latter is also possible); (ii) only very small samples (e.g., less than 1 μl) are available; and, (iii) ionic detergent-solubilized antigens are to be tested. Nitrocellulose binds close to 100% of most antigens or antibodies.

Particulate solid phases (agarose, cellulose, polyacrylamide, dextran) are very efficient since they may be dispersed throughout the reaction mixture and have a much higher ratio of surface area/volume. Moreover, the immunoreactant is covalently bound.

13.2. The use of plastics as solid phases

13.2.1. Nature of protein–plastic interaction

Non-covalent adsorption works generally well and is most frequently used. However, up to 68% of non-covalently adsorbed antigen may be desorbed during the test (Engvall et al., 1971; Hermann et al., 1979; Lehtonen and Viljanen, 1980b). This desorption is strongly influenced by the serum used (Dobbins Place and Schroeder, 1982). If no proper care is taken, a whole spectrum of binding, ranging from very tight to rather loose will be obtained. In each step, some of the loosely bound antigen detaches and competes for the immunoreactant added, leading to decreased detectability and increased variation. Two simple measures can be taken to minimize this problem: (i) to use the immunoreactant for immobilization at the appropriate concentration; and, (ii) to wash extensively after immobilization and after every subsequent step (Christensen et al., 1978; Lehtonen and Viljanen, 1980b). As many as 6 washes may be necessary instead of the usual 2 or 3 advocated in standard procedures for the complete
removal of loosely bound immunoreactants. Though covalent linkage improves binding (Hendry and Hermann, 1980; Suter, 1982), this is not always accompanied by a similar increase in detectability.

In a recent study (Viscidi et al., 1984) desorbing activity (up to 60%) was linked to certain fractions in the sample (fecal) with a molecular weight of about 25000 having proteolytic activity since this effect could be reversed with protease inhibitors or a large excess of inert protein.

The non-covalent adsorption process is poorly understood, despite its widespread application. It has been investigated most extensively with respect to the introduction of synthetic materials into the cardiovascular system (Morissey, 1977). Protein adsorption to plastic surfaces is generally attributed to non-specific hydrophobic interactions and is independent of the net charge of the protein, although binding is different and characteristic for each protein (Cantarero et al., 1980). The unique adsorptive behavior of each protein to polystyrene is independent of its pI, charge and molecular weight. Partial denaturation of IgG increases its adsorption, presumably due to an increase in hydrophobicity which, compared to native IgG, increases its detectability in EIA about 5 times (Ishikawa et al., 1980; Conradie et al., 1983).

Up to a certain limit, a constant fraction of the proteins is adsorbed to plastic surfaces. For example, 80% of the IgM but only about 25% of BSA is adsorbed, with a limit of about 1.5 ng/mm² for both proteins, independent of the input. According to Butler (1981), the protein molecules at this limit become equidistantly distributed on the surface and the failure to exceed this coverage (about 1/3 of the surface) is due to steric hindrance.

With an excess of protein, more adsorption occurs than dictated by the binding capacity, due to the formation of multiple layers, stacked on the protein monolayer by protein–protein interactions. Such secondary interactions are not very stable and interfere in the EIA. Early studies of Oreskes and Singer (1961) on the interaction of human IgG with polystyrene indicated that adsorption occurred in two steps, characterized by different binding constants, cor-
responding to protein–plastic and protein–protein interaction. Maximum adsorption was observed at the pI of the proteins probably since the electrostatic repulsion between proteins is the lowest at this pH. Undesired multiple layers are also most difficult to remove at pH values near the pI (MacRitchie, 1972). However, the negative surface charge of the plastic does not interfere with the plastic–protein interaction, pointing to a non-electrostatic adsorption.

An important implication of these studies is that antigen present in a complex mixture binds in a non-competitive manner, up to a limit of about 1.5 ng total protein/mm², above which the binding is no longer representative of the concentration of the various antigens in the sample.

Among 11 different polystyrene microplates (from 4 different companies), Kenny and Dunsmoor (1983) distinguished essentially two types: one which adsorbs albumin poorly and the other which adsorbs it well; IgG is adsorbed well on both types. Plates which bind albumin well are best suited for mixtures of antigens; however, background staining also tends to be higher. A 100-fold excess of a non-specific protein during adsorption prevents the detection of the antigen (Kenny and Dunsmoor, 1983). Some brands of plates bind at least three times more antigen from a dilute solution than others (Signorella and Hymer, 1984). Protein desorption may result not only from disaggregation of multiple layers and proteolysis, but also from true desorption from the solid phase and some protein, such as fibrinogen, may even replace the adsorbed protein on some solid phase (Morrissey, 1977).

Not only do plastic plates exhibit a significant variability among the various lots, but also among the wells of the same plate (Shekarchi et al., 1984). Irrespective of the origin of the plates, the coefficient of variation of absorbance can range from 5% for the wells of one plate to 30% of the other for the same lot (Kricka et al., 1980). Notorious is the 'edge effect' (Chessum and Denmark, 1978; Kricka et al., 1980): wells at the perimeters adsorb more proteins than those in the interior. Many investigators forego the use of almost 40% of the available wells for this reason. This edge effect
has been attributed to differences in surface characteristics of the plastic (Burt et al., 1979) or to thermal characteristics (moulding temperature, cooling) different from those in the interior (Denmark and Chessum, 1978). A critical analysis of this edge effect (Oliver et al., 1981) indicated that thermal gradients during incubation may be responsible: polystyrene is a poor conductor and a thermal gradient may exist between the outer and central wells during an incubation period of 30 min, with initial and final temperatures of 20°C and 37°C, using a routine laboratory incubator. The use of a forced-air incubator may eliminate this edge effect, giving a much lower coefficient of variation and a higher reproducibility. Warming both the plate and the solution to the incubation temperature prior to the addition of the solution seems a simple alternative.

To test for reproducibility, essential for reliable EIA, a solution with a less than saturating concentration of conjugate can be adsorbed. The optical density and standard deviations of all wells of the same plate (perimeter and interior wells) or of different plates are compared. Shekarchi et al. (1984) found well-to-well variation to be of greater statistical significance than the edge effect. Plates are selected with respect to the antigen. Pretreatment of plates with cleaning agents has little effect unless an oily film is present, which can be removed by washing for 5 min with 25% acetone. Soaking of polystyrene in 6 N HCl for 2 h permits the reuse of plates (Shekarchi et al., 1984).

13.2.2. Non-covalent adsorption of antigens to plastic

The attachment of proteins to plastic by non-covalent bonds is often little affected by the buffer. Many different protocols can be indiscriminately used without any significant modification in the procedures. Some recent methods were designed to expose more of the hydrophobic regions of the proteins and seemed to have a beneficial effect on the end-results. The three most important variables for the adsorption of proteins on a solid phase are temperature, time and concentration.
The most widely used coating buffer is 50 mM carbonate, pH 9.6. Other buffers are 10 mM Tris–HCl, pH 8.5, containing 100 mM NaCl or PBS (10 mM sodium phosphate buffer, pH 7.2, containing 100 mM NaCl).

For a new assay it is recommended to investigate if one of the buffers is indeed the most appropriate. The frequent use of a given buffer is not necessarily a proof of its superiority. For example, Barlough et al. (1983) observed that the use of carbonate buffer for the coating with coronavirus antigens produced diffuse and non-specific color reactions both in viral and control experiments. This was primarily due to the high pH and not to the carbonate or the ionic strength. The use of PBS, 0.9% NaCl or deionized water as solvent gave superior results in this system. It is not clear from these studies whether or not the carbonate buffer promoted the stacking of multiple protein layers.

Non-ionic detergents (Triton X-100, Tween 20) should be avoided during coating since they compete strongly with the protein for the solid phase and prevent the formation of hydrophobic interactions.

The conditions most frequently chosen for incubation are overnight at 4°C, in a humid chamber. However, incubation may be shortened by increasing the temperature or the antigen concentration (Fig. 13.1 and 13.2). Adsorption of densonucleosis virus to polystyrene plates (Fig. 13.1), was shown to be optimal at about 10 μg/ml (2 μg/well) and at 37°C at least 4 times shorter sensitization times (less than 20 min) were required than at 4°C. In contrast, for purified IgG, the optimum coating concentration was 1 μg/ml but at least 60 min at 37°C were required (Fig. 13.2). The optimal concentration of antigens or antibodies for coating is commonly between 1 and 10 μg/ml (Engvall, 1980). Coating with complete antiserum (Fig. 13.2) is more complicated since a pronounced optimum is achieved at a dilution of 10⁻⁴. A 10-fold change in this dilution lowers the detection limit by a factor of about 2, whereas at a 100-fold change (i.e., a dilution of 10⁻² or 10⁻⁶) the detectability is 20–30 times less. However, even at its optimum, the detectability with complete antiserum is about 4 times less than with purified IgG (Fig. 13.2).
Fig. 13.1. Coating of polystyrene plates with densonucleosis virus (DNV) at different concentrations (0.2 ml/well) and incubation at 4°C or 37°C. The dilution of antiserum giving an optical density of 0.15 (cut-off value) after 30 min was plotted against the incubation period. For DNV, incubation at 37°C has the advantages that short incubation periods are needed (e.g., 20 min) and that relatively more virus is coated if present in low concentrations (from Tijssen et al., 1982; courtesy Archives of Virology).

In some cases, the detectability of EIA can be increased considerably by partial denaturation of the coating material: solid-phase coating was improved when the antibody was exposed to pH 2.5 (Ishikawa et al., 1980) or to other denaturants (urea, high temperature) prior to coating (Conradie et al., 1983) and an increase in the hydrophobicity of the proteins was proposed to be involved in this phenomenon. Since Fab is much more resistant to denaturation than Fc (Section 8.2) it is conceivable that these treatments expose more hydrophobic regions in the Fc region, which may then be preferentially adsorbed. The Fab segments may thus be more oriented towards the solvent, thus increasing the efficiency of antigen binding. In addition, Conradie et al. (1983) observed that more antibody becomes bound.
Fig. 13.2. Coating of polystyrene with purified IgG (5 mg/ml) or with complete antiserum in serial dilutions. With the latter a very pronounced optimum of dilution is found, whereas with purified IgG a slight 'hook effect' is observed when tested with the corresponding virus in a sandwich assay. However, the use of purified IgG produces higher detectability than that of complete antiserum for coating. Incubation time required for optimum IgG coating at 37°C was longer than for DNV (Fig. 13.1) (from Tijssen et al., 1982; courtesy of Archives of Virology).

Partial denaturation of IgG is carried out with samples containing 10 μg/ml in 50 mM glycine-HCl buffer, pH 2.5, containing 100 mM NaCl, incubated for 10 min at room temperature and neutralized with 500 mM Tris. The sample is then dialyzed against the coating buffer. Alternatively, IgG may be denatured in neutral buffer by the addition of an equal volume of 6 M urea and incubation overnight at room temperature, followed by extensive dialysis. Thermal denaturation is carried out for 10 min at 70°C for sheep antibodies or at 82°C for rabbit antibodies (Conradie et al., 1983). This treatment may be different for IgG preparations from other species.

The treatment of antigens with chaotropic agents (NaSCN, guanidine-HCl) prior to dilution with carbonate buffer and coating may
enhance the detectability of EIA (Inouye et al., 1984). Conroy and Esen (1984) observed that proteins solubilized with 6–8 M urea or 60%-solutions of methanol, ethanol or acetic acid are efficiently coated on plastic. However, 6 M guanidine·HCl in the coating buffer was less suitable and 60% isopropanol or 0.1% SDS prevented binding to plastic.

Adsorption of lipid antigens (mycobacterial glycolipid, cardiolipin) to polystyrene (Reggiardo et al., 1980) is performed at a concentration of 2 μg/ml in a buffer containing sodium deoxycholate (1 mg/ml) for 3 h at 37°C. The addition of 10 mM MgCl$_2$ in the coating buffer (and in subsequent incubations at 20 mM) is beneficial for the coating of lipopolysaccharides (Ito et al., 1980).

Proteins separated by SDS-PAGE (Chapter 16) can directly be coated to plastic by diffusion from the gel following electrophoresis. The gel is incubated for 15 min in water at 37°C. The gel is cut into 1 mm slices which are incubated (in separate wells) with a 20–25-fold excess of carbonate buffer for 24 h at room temperature.

13.2.3. Covalent attachment of antibodies or antigens to plastic

While covalent coupling is obligatory for the coating of solid phases other than plastics, it may also be advantageous for plastics. Some of the methods for covalent linkage of proteins are quite undemanding and convenient for the large-scale preparation of immunoreactant-coated solid phases. Such methods are also necessary for the attachment of antigens which are poorly adsorbed on plastic, such as native DNA, small oligopeptides and haptens.

A simple method for the attachment of most reactants possessing free amino groups, i.e., oligopeptides and haptens (Weigand et al., 1981; Suter, 1982) is the pretreatment of polystyrene with GA (Barrett, 1977). Polystyrene is activated at a relatively low pH where GA has no strong tendency to react or to form polymers (Table 13.1). After the addition of the immunoreactant, the pH is raised to 8 or 9.5 with 100 mM carbonate (Dobbins Place and Schroeder, 1982), to increase the reactivity of GA. Either GA or ethylchloro-
Pretreatment of polystyrene with glutaraldehyde for covalent linkage (adapted from Weigand et al., 1981, and Suter, 1982)

1. Pretreat polystyrene with 0.2% (v/v) GA in 100 mM sodium phosphate buffer, pH 5.0, for 4 h at room temperature.
2. Wash twice with the same buffer.
3. Add the reactant to be coupled (2–10 μg/ml) in 100 mM sodium phosphate buffer, pH 8.0, and incubate for 3 h at 37°C.
4. Wash twice with 0.9% NaCl.
5. Add 100 mM lysine in sodium phosphate buffer, pH 8.0, for 1 h at 37°C.
6. Wash with several changes of PBS, containing 0.05% Tween 20.

In another version of this method (Dobbins Place and Schroeder, 1982) the GA concentration is raised to 2% and, after coating the wells with antigen or antibody, the plates are post-fixed with 0.007% ethylchloroformate in PBS (pH 7.0) for 2 h at room temperature. Formate (ECF) can be used for the treatment of the plates, but the results are better when the two reagents are combined (Table 13.1). The mode of action of ECF is not well understood, but it may make the adsorbed reactant less polar and promote hydrophobic interaction. Microtiter plates thus treated can be stored for at least 4 weeks. Sera which produced strong desorption of non-covalently bound hepatitis antigens did not release the same antigens adsorbed on such treated plates.

Pretreatment of antibodies with GA prior to coating can also decrease their desorption and give a higher detectability in EIA (Parsons, 1981), in particular for plates not irradiated with 60Co by the manufacturers (Beards et al., 1984). GA at 0.2% is mixed with 5 volumes of serum (diluted 2000-fold with 10 mM phosphate buffer, pH 6.0). The mixture is incubated for 20 min at 37°C, diluted with 1/5 volume of 400 mM sodium phosphate buffer, pH 8.5, and used for coating the plates for 1 h at 37°C.

Modified polystyrene beads are offered commercially (Pierce...
Chemical Co.). Derivatized polystyrene beads may contain hydrazide groups, alkylamine groups or Sanger's reagent (about 3 μmole reactive groups per bead) (Fig. 13.3). A typical procedure to link covalently IgG to hydrazide beads is given in Table 13.2. Beads can also be used by the method of Inman (1974) or Sanger (1945).

Several other methods to link covalently antibodies to plastic were compared by Neurath and Strick (1981), such as coupling to partially hydrolyzed nylon with carbodiimides or GA (Hendry and Hermann, 1980), to diazotized polyaminopolystyrene (PAPS; Phillips et al., 1980), or to PAPS with GA (Reimer et al., 1978, Table 13.3); particularly the latter was suitable.

The method of Hendry and Hermann (1980; Table 13.4) is quite simple and attractive for large-scale preparation of antigen- or antibody-coated nylon beads, particularly for antigens which are generally poorly adsorbed. About 30 times more antibody is attached per surface unit of nylon than adsorbed non-covalently on polystyrene.

The increase in detectability of EIA is generally considerably less than the increase in the amount of the immunoreactant bound to the solid phase, possibly due to masking of epitopes.

Fig. 13.3. Modified polystyrene beads for covalent binding of proteins. Proteins can be linked to hydrazide beads (a) with glutaraldehyde or to beads derivatized with alkylamine using succinic anhydride and carbodiimide or Sanger's reagent (Pierce Chemical Co.; Technical Information).
### Table 13.2
Covalent linkage of IgG to hydrazide-derivatized polystyrene beads

1. Add 5 ml of 12.5% (v/v) GA in 50 mM sodium phosphate, pH 7.0, to 25 hydrazide-derivatized polystyrene beads (6.4 mm diameter; from Pierce Chemical Co.) and shake very gently for 2 h at room temperature.

2. Wash on Buchner funnel (without filter paper) with 100 ml water and with 20 ml 100 mM sodium phosphate buffer, pH 6.0.

3. Add GA-activated beads to 2.5 mg IgG dissolved in 5 ml of sodium phosphate buffer, pH 6.0.

4. Add 1 mg NaCNBH$_3$ (step advised by Pierce; may not be necessary).

5. Shake gently overnight at room temperature.

6. Wash beads with 100 ml 100 mM sodium phosphate buffer, pH 6.0, and then with 50 ml 100 mM NaHCO$_3$.

7. Block residual aldehydes with 5 ml 100 mM lysine for 1 h at room temperature.

8. Wash with 100 ml 100 mM Na$_2$CO$_3$ and with 100 ml water and dry beads with paper towel.

9. Store the IgG-beads dry at 4°C.

### 13.2.4. Attachment of antigens or antibodies to plastic using bridging molecules

Antigens which bind poorly to plastic may be attached to the solid phase by a bridging molecule, e.g., a protein with a high affinity for the plastic to which poor binders may be conjugated by any of the methods discussed in Chapter 11. Positively charged compounds, such as protamine sulfate have been used to bind native DNA to polystyrene (see below).

Skurrie and Gilbert (1983) used BSA as the bridging molecule for the binding of rubella antigens. BSA (fraction V) in distilled water is added to the wells and the plates are dried in a water bath at 56°C under a stream of air. An equal volume of ethanol-
**Table 13.3**
Covalent linking of antibodies to polystyrene plates (Neurath and Strick, 1981)

1. Fill in a well-ventilated hood the wells of the microplate with methanesulfonic acid and incubate overnight at room temperature.

2. Empty the wells, wash extensively with distilled water and dry.

3. Place the plates in a heating block (40°C) and fill the wells with a mixture of glacial acetic acid and fuming HNO₃ (1:1).

4. Incubate for 4 h and wash with water until the pH of the wash is 6 or higher.

5. Place plates in the heating block (50°C) and fill the wells with 0.5% Na₂S₂O₄ in 500 mM NaOH and incubate for 1 h.

6. Wash the plates extensively with water, fill the wells with 1% GA in 50 mM phosphate buffer, pH 8.5, and incubate for 2 h at room temperature and then for about 16 h at 4°C.

7. Wash plates, fill each well with a solution of IgG (100 g/ml) in 50 mM phosphate buffer, pH 8.5, and incubate at 4°C overnight.

8. Wash and treat the wells with 1 M glycine–100 mM borate buffer, pH 8.5, for 4 h at room temperature.

9. Wash with Tris-buffered saline (10 mM Tris–HCl, pH 7.2, containing 0.9% NaCl). The plates may be stored with the wells filled with the same buffer, containing 0.02% NaN₃.

acetone (4:1) is then added to each well and left for 30 min at room temperature, followed by three washings with distilled water and air-drying. For the conjugation of the antigen to the BSA coating, the wells are filled with a solution of 1-ethyl-3(3-dimethyl-aminopropyl)carbodiimide-HCl in distilled water (10 mg/ml), incubated for 30 min at room temperature, before the addition of the rubella antigens (25 times diluted from the preparation of Flow Laboratories; otherwise use 5–20 µg/ml) and incubated in a water bath at 45°C for 4 h. The plates are then washed 6 times with water, rinsed with 1% BSA solution and with 7.5% (v/v) glycerol and stored at −70°C or quick-frozen in ethanol/dry-ice prior to transfer to the
Table 13.4
Covalent linking of antibodies to nylon

1. Hydrolyze nylon 6/6 (poly-hexamethylene-adipimide) balls with a diameter of either 3.2 or 6.4 mm (Precision Plastic Ball Co., Chicago, U.S.A., or other suppliers) with 3.5 M HCl for 24 h at room temperature.

2. Wash balls with distilled water, treat for 1 min with concentrated acetic anhydride, wash with distilled water and then with 100 mM carbonate buffer, pH 9.5 for 1 min.

3. Wash with PBS and react either with 4% (v/v) 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate (CMC) 10 min or with 8% (v/v) GA in distilled water (2 h; in the latter case treatment with acetic anhydride in step 2 is omitted).

4. Wash with distilled water and incubate with the solution of antibodies (100 μg/ml per 36 beads) or antiserum (dilution 1:3000) or the antigen preparation (equivalent molar amount) in PBS, pH 7.2, at room temperature for 4 h for the GA-activated nylon or 2 h at room temperature followed by 18 h at 4°C for the carbodiimide-activated nylon.

5. Wash with PBS and then with 2% (w/v) BSA in PBS for 1 h.

*The aldehyde concentration on the beads was found to be much more uniform when the beads were shaken when in acid and aldehyde baths (Perera et al., 1983). Background staining can be decreased significantly by treatments with 500 mM lysine and 5% BSA after coupling of the immunoreactant to the beads.

freezer. Most cross-linkers discussed in Chapter 11 may be used instead of the carbodiimide. In view of the variation in the affinity of polystyrene for albumin (Section 13.2.1), even within the same lot, other proteins may be more suitable.

In contrast to single-stranded DNA, native DNA adsorbs poorly to polystyrene (Engvall, 1976). This problem can be overcome by treating the polystyrene with a 1% solution of aqueous protamine sulfate for 90 min (Klotz, 1982), followed by three washes with distilled water and drying. Other polycationic substances, such as MBSA (Section 5.2.3.3, Ferrua et al., 1983) or poly-L-lysine (type VII B; Sigma), may also be used (Leipold et al., 1983). Poly-L-lysine is suspended in water at a concentration of 1 mg/ml (which may
be lowered at least 10 times without harm) and incubated with the plastic for 2 h at room temperature. The wells are then washed 4 times with water and dried.

DNA may be adsorbed onto such pretreated plastic at a concentration of 10 μg/ml in 50 mM Tris–HCl buffer, pH 7.5, containing 10 mM EDTA and 10 mM EGTA for 60 min. Single-stranded DNA may interfere in the assay for antibodies to native DNA (Klotz, 1982) and may be removed, after washing with PBS for three times, by digestion with S₁ nuclease (100 units/ml; Sigma type III, in 50 mM sodium acetate buffer, pH 4.5, supplemented with 50 mM NaCl, 1 mM ZnSO₄ and 5% glycerol) for 1 h at 37°C. After digestion, the plates are washed with PBS-Tween (Section 14.2)

A variety of bridging molecules can be used to attach cells to plastic surfaces, e.g. antibodies, lectins (phytohemagglutinin), GA, or poly-l-lysine. The method of Heusser et al. (1981) is satisfactory (Table 13.5). The stability of attachment is quite similar with the different bridging molecules, but the cell monolayer should be fixed with GA if the plates are to be stored.

13.2.5. Forms of plastic solid phases

Separation of free immunoreactant from reactants immobilized on plastic is achieved simply by decanting, aspirating or retrieving the solution, in contrast to the often cumbersome techniques used in other systems (precipitation, chromatography, centrifugation, nonspecific adsorption on charcoal, talc or dextran). This simplicity has a direct, positive effect on the reproducibility and sensitivity of the assay.

The forms of plastic supports for EIA may be varied: beads, discs, tubes, plastic-coated toothpicks, matchsticks, and even cocktail stirrers (Shekarchi et al., 1982). Undoubtedly, many other utensils could be used for this purpose. However, the most common supports are polystyrene or polyvinylchloride (PVC) microtitre plates with 12 × 8 wells of 0.3 ml. Although PVC plates may adsorb IgG more
Table 13.5

| Binding of cells to polyvinylchloride microtitre plates |
|-------------------------------------------------------|
| 1. Pretreat plates with GA (0.25%), or poly-L-lysine (10 µg/ml), or phytohemagglutinin (PHA)-P (20 µg/ml; Difco Laboratories) or anti-cell antibodies (20 µg/ml), using as coating solution Dulbecco's PBS, for 1 h at 37°C. |
| 2. Wash the cells extensively with Dulbecco's PBS and suspend a concentration of 1–5 × 10⁶ cells/ml (depending on cell size). |
| 3. Add cell suspension to wells (shake often to prevent concentration differences) and centrifuge plates at 100 × g for 3 min. |
| 4. Post-fix plates with 0.25% GA (if plates are to be stored) by gentle immersion in a 1 litre beaker containing 0.25% GA in Dulbecco's PBS at 4°C for 5 min (prevent air trapping). |
| 5. Wash by repeated immersions in PBS and gentle decanting. |
| 6. Saturate unreacted sites with 1% BSA in Dulbecco's PBS. Incubate for 1 h and wash again. Use egg white (prepared by stirring chicken egg white in PBS for 1 h), followed by centrifugation at 10,000 × g for 15 min) in PBS (A2RO of about 14) for blocking unreacted sites of PHA-coated plates. |

efficiently, they also tend to give higher background levels and release little of the adsorbed immunoreactant (Zollinger et al., 1976). Frankel and Gerhard (1979) used PVC plates for the quantitation of hybridoma proteins.

Among the polystyrene plates, a variation exists in the quantity of additives (lubricants) used in their preparation which may account for some of the differences encountered. Special plates ('immulon') have very little of these lubricants. Nevertheless, it is necessary to establish for each antigen or antibody the most suitable lot. Signorella and Hymer (1984) found that immulon 2 binds three times more prolactin than immulon 1. Most plastics display a negative electrical charge (ζ potential) at their surfaces which is quite often modified during manufacturing by a treatment for a few seconds with an intense electrical field at the solid–solution interface. This potential gives rise to a diffuse double-layer of ions. The ζ potential is also
affected by the adsorbed protein. All these physical parameters may have different but unpredictable effects on EIA (Section 9.2.2) and affect the accuracy of EIA.

The wells of the microtitre plates may have flat, U-shaped or V-shaped bottoms. The choice between these shapes depends on the method used for quantitation of the EIA: with a read-through photometer flat-bottomed wells give less variation, for visual inspection U-shaped wells are better. The microtitre plates have not been designed for the EIA but adapted for this purpose and equipment has been devised for these plates; possibilities for more optimal designs are obvious, e.g. by increasing the surface/volume ratio. Labrousse et al. (1982) used microwells (0.8 mm diameter, 0.8 mm depth) drilled in plexiglass plates with the top of the wells (0.2 mm depth) enlarged to a diameter of 1.5 mm. Such wells have a capacity of about 0.30 µl, in contrast with the 200-µl capacity of the regular plates; however, rapid desiccation poses serious problems. Solutions are added with suitably prepared capillary pipettes. The reduced volume of reagents lowers the limit of the number of molecules which can be detected (i.e. about 10⁶ antibody molecules). The concentration limits for positive detection remain, however, largely unchanged since the surface/volume ratio is hardly affected. It is conceivable to increase this by a different well design. The reagents could then be more effectively used and the reaction product (enzyme signal) would become less diluted. This aspect remains to be explored.

Plastic tubes are also employed. Here, the choice of the plastic is also critical. Polypropylene is preferred over polystyrene, since it is easier to handle and is less breakable. It is mostly used in commercial kits. There is, however, little or no difference in the coating characteristics of the two plastics. The size of the tubes is 10–12 x 70–75 mm, and their form may be conical, round- or flat-bottom. Tubes generally require larger volumes (1 ml) than microtitre plates, but their surface/volume ratio can be increased by rotating almost horizontally in circular test-tube racks. Upon slow rotation, a relatively large surface is covered. Disposable polystyrene
cuvets are used in some cases since they can be directly used with a common spectrophotometer. Volumes of such containers are quite large. Another often used form is beads. Placed in a round-bottom container only slightly larger than the beads themselves, they have a favorable surface/volume ratio. Beads have been popular in RIA and are used by some commercial suppliers.

Shekarchi et al. (1982) used stainless steel microsticks coated with carrier material. Alternatively, plastic microsticks are available from Dynatech Laboratories. Irrespective of their nature (steel, teflon, polystyrene), they may be coated with polycarbonate or nitrocellulose. The latter is applied as a thin film of colloidon diluted with 4 volumes of ether (laboratory grade). The microsticks are dipped in this solution, dried and arranged in a transfer plate designed for use in a microtitre plate. They can be cleaned with 6 N HCl after use.

13.3. Nitrocellulose membranes and paper

Nitrocellulose membranes have found wide application in nucleic acid research, but are also becoming very popular in protein blotting. The nature and requirements of these membranes will be discussed in Chapter 16.

The attractiveness of these membranes resides in the fact that very small amounts of immunoreactants are required, providing thus a method of choice for many experiments, e.g. monitoring column effluents, screening hybridoma clones, analysis of detergent-dissociated complexes. Minute drops (less than 1 µl, containing about 100 pg, or less if antigen is pure) are applied and dried on the membrane, limiting the binding of the antigen in a small area (diameter less than 1 mm). Other binding sites can then be saturated with an unrelated inert protein or other blocking agents. The subsequent immunoenzymatic revelation of the antigen produces an insoluble colored product which precipitates at the site of the enzyme and is viewed against a white background. The discriminatory power
of this technique was claimed to be greater than that obtained in microtitre plates and various substances, such as proteins, nucleic acids, cell membranes, subcellular organelles, fungi, protozoa, bacteria and viruses, were all tightly bound to the membrane (Hawkes et al., 1982). Another advantage of these membranes is that antigens may be bound in the presence of detergents, although a lower efficiency of binding is observed when the concentration of non-ionic detergents (Triton X-100, Tween 80) exceeds 0.01% (Palfree and Elliott, 1982). Jahn et al. (1984) observed that for small proteins this was due to desorbing during washing and incubation steps. A fixation step (15 min in a solution containing 10% acetic acid and 25% isopropanol followed by rinsing in water) after dotting prevented this desorption, and a 7-fold excess of Triton was found to be beneficial if SDS is present in the sample (up to 7% Triton/1% SDS). The suitability of this solid phase is attested by the rapidly growing number of publications describing their use.

Dotting with small volumes may be done directly on the nitrocellulose sheet on which a rectangular grid (3 x 3 mm) has been drawn with a pencil (Hawkes et al., 1982; Rordorf et al., 1983). For large volumes (10-400 µl), discs (≈4 mm) punched from membrane sheets (Palfree and Elliott, 1982) are used. Alternatively, the nitrocellulose sheet can be inserted in a 'Hybridot' or similar apparatus (BRL; Bio-Rad) (Domin et al., 1984) or homemade devices (Smith et al., 1984). The disc method, designed for detergent-solubilized antigens, needs more antigen for detection than the dotting method.

Discs (with an indexing notch), on which several antigens or allergens are dotted separately, allow the simultaneous detection of antibodies against them from a small serum sample (50 µl; Walsh et al., 1984). This permits rapid screening, e.g. for allergens, by applying on the first disc several dots with different antigen mixtures, and detecting the antigens from a positive spot separately on a second disc. The use of 0.1–0.4% zwitterionic detergent in the blotting buffer was found (Mandrell and Zollinger, 1984) to restore partially the antibody-binding capacity of SDS-denatured antigens.

The enzyme of choice for these methods is POase, though APase
may also be used. After reaction, the spots are examined visually for the presence or absence of a reaction. It is, however, also possible to quantitate such spots by reflectance densitometry with a thin-layer plate scanner. Variations can be expected to be high, though this may be reduced by the apparatus which ensures uniform dots. It is also possible, as for the disc method (Section 13.3.2), to use a substrate which yields a soluble product (such as OPD) and to perform the revelation reaction in the wells of a plate.

Paper may also be used as a support. This inexpensive solid phase has the advantage that large amounts of coated solid phase may be prepared.

13.3.1. Dot-binding of antigens to nitrocellulose

Antigens may be bound in small dots to nitrocellulose (Table 13.6), using micropipetting devices. With dilute solutions of antigen, the sample is applied in small successive doses at the same site, allowing the filter to dry between applications. Dry membranes are much more prone to breaks than wet membranes. The baking step in the dotting procedure is necessary to stabilize binding with nucleic acids (Southern, 1975; Thomas, 1980). Instead of blocking with an 'inert' protein, such as BSA or gelatin, Tween 20 at 0.05% can be used (Batteiger et al., 1982) in the solutions of the various immunoreactants, preventing non-specific adsorption. This is particularly advantageous after transferring proteins from polyacrylamide gels (Chapter 16), since protein bands may be stained without the interference of blocking proteins.

Post-fixation with GA (Section 13.3.2) or acetic acid/isopropanol (Section 13.3) is generally not necessary but may be performed when required or if Triton X-100 is added to the immunoreactants.

13.3.2. Binding of detergent-solubilized antigens on nitrocellulose membrane discs

Palfree and Elliott (1982) used nitrocellulose discs to bind detergent-
### Table 13.6

Binding of antigens in small dots on nitrocellulose

| Method          | Details                                                                 |
|-----------------|-------------------------------------------------------------------------|
| **A. Protein dotting** (Hawkes et al., 1982) | 1. Prepare the nitrocellulose sheet by drawing a rectangular grid (3 x 3 mm) with a pencil, wash with distilled water for 5 min and dry at room temperature.  
2. Place small drops (0.1–0.5 μl) of the sample containing 0.1–1.0 mg protein per ml. For less complex mixtures, the concentration may be reduced accordingly.  
3. Dry the filter thoroughly; the membrane may then be stored for several weeks.  
4. Place the membrane in the cold for 5–10 min and wash for 5 min with 50 mM Tris-HCl buffer, pH 7.4, containing 200 mM NaCl (TBS).  
5. Cut squares from wet membrane with a scalpel and ruler and place squares, face upward, into wells of a 96-well tray. (Alternatively, strips can be used and placed in appropriate containers).  
6. Block remaining active sites on the membrane with a 3% solution of BSA in TBS and shake gently for about 15 min. Tween 20 (at 0.05%) is also effective in preventing nonspecific adsorption. |
| **B. Nucleic acid dotting** (Rordorf et al., 1983) | 1. As in step 1 of method A.  
2. Apply spots as in step 2 of method A, using the nucleic acid (single- or double-stranded) solution at a concentration of 80 μg/ml in 20 mM Tris-HCl buffer, pH 7.8, containing 150 mM NaCl, according to Thomas, 1980.  
3. Air-dry membrane and bake for 2 h at 80 °C.  
4. Proceed as in steps 4 through 6 of method A, for the wetting, cutting and blocking of the membrane. |

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Solubilized membrane glycoproteins. Triton X-100 or Tween 80 should not be used over 0.01%, whereas deoxycholate, taurocholate or octylglucoside can.

Small discs (≈4 mm) punched from the nitrocellulose membrane are floated on the surface of distilled water. The moist discs are then placed on absorbent filter paper and evenly soaked by the application of 10 or 20 μl samples. Gentle pressure is sometimes
necessary to assure good contact of the discs with the filter paper. The discs are air-dried and incubated in microtitre trays (96-well, V-bottom) with 0.1 ml 3% BSA in TBS (Table 13.6, step 4) at 37°C for 1 h to block free binding sites.

For optimal fixation with GA, the discs are rinsed briefly with BSA and incubated with 0.1 ml 0.25% GA in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM NaCl (PBS), for 15 min at room temperature. The discs are then rinsed three times with PBS, the residual active GA groups are blocked with 0.1 ml 100 mM glycine (pH 7.5) and 0.1 ml 3% BSA in TBS for 60–100 min at 37°C and washed with the phosphate buffer. This fixation prevents desorption of proteins when Triton X-100 is used.

13.3.3. Covalent coupling to paper

The method of Ceska and Lundkvist (1972) as applied by Pauwels et al. (1977), Lehtonen and Viljanen (1980a,b) and Giallongo et al. (1982) is described here. The cellulose paper from Munktell Swedish Filter Paper or Whatman No. 52 yields about 400 and 330 discs of 6 mm diameter per gram, respectively.

Activation of paper discs with CNBr is not unlike that of Sepharose, as described in Section 7.1.9.2.2., and the same safety precautions should be taken. Activation of paper discs and subsequent coupling of antibodies or antigens is given in Table 13.7.

Lehtonen and Viljanen (1980b) compared the CNBr-activated paper method with non-covalent adsorption on polystyrene and nylon. A considerable desorption occurred both from polystyrene (30%) and nylon (60%) in all steps of the assay; however, very high concentrations of antigens were used for the adsorption (100 μg/ml) which could be responsible, at least in part, for the leakage (Section 13.2.1). With the CNBr-activated paper, 5–10 times more antigen was bound from the same antigen solutions and desorption during the EIA was only 13% of the mean surface concentration of the antigen, with a standard deviation 3–10 times smaller than for the other solid phases. The response ratio (positive/negative sera) with CNBr-
A. Activation
1. Punch paper discs of 6 mm diameter (≈ 2.5–3 mg/disc).
2. Swell 20 g of discs in 200 ml distilled water for 3 min.
3. Add 600 ml of CNBr solution (20 g in 600 ml of distilled water. For precautions, see Section 7.1.8.2.2).
4. Raise immediately the pH to 10.5 and maintain this pH, by adding up to about 100 ml of 1 mM NaOH, for 30 min.
5. Wash discs with the following solutions:
   a. 500 ml 0.005 mM NaHCO₃ (12 times)
   b. 500 ml distilled water (twice).
   c. 500 ml (twice) in graded series of acetone (25, 50, 75%) and four times in acetone.
6. Air-dry at room temperature and store at 4°C.

B. Covalent coupling
7. Dilute 4 ml of purified antibody or the IgG fraction of the antiserum (or the same amount of the antigen preparation) in 40 ml 100 mM NaHCO₃ (4°C).
8. Add 1 g of activated discs to the solution and stir gently for 3 h at 4°C.
9. Wash at room temperature with:
   a. 100 ml 0.5 mM NaHCO₃ (twice for 10 min).
   b. 100 ml 50 mM ethanolamine in 100 mM NaHCO₃ (3 h with gentle stirring).
   c. 100 ml 0.5 mM NaHCO₃ (twice for 10 min).
   d. 100 ml 100 mM sodium acetate buffer, pH 4.0 (once for 30 min).
   e. 100 ml incubation buffer (twice: the incubation buffer used for the subsequent stages contains 50 mM phosphate buffer, pH 7.5, 150 mM NaCl, 0.1% Tween 20 and 0.1% gelatin).
10. Store discs, if not used immediately, in a small volume of the incubation buffer at −20°C.

*The punching of the paper discs may also be done after the covalent coupling of the protein (Lehtonen and Viljanen, 1980b). The use of PBS in the coupling was claimed to be as effective as the bicarbonate buffer.
activated paper was twice as high as with polystyrene, with half the standard deviation of the latter.

Paper can also be activated with 1-(3-nitrobenzyloxymethyl)pyridinium chloride (NBPC), according to the method of Alwine et al. (1979). The nitrobenzyloxymethyl (NBM) paper thus prepared is stable for at least one year and can be reduced to aminobenzylloxymethyl (ABM) paper. Treatment of ABM paper with nitrous acid produces diazobenzylloxymethyl (DBM) paper to which antigens (nucleic acids, proteins) can be covalently linked (Fig. 13.4). In this reaction negatively charged molecules first interact electrostatically with the DBM paper, followed by their essentially irreversible covalent interaction via the azo linkage. This paper is primarily used for the transfer of proteins or nucleic acids from gels (Chapter 16). The preparation of this paper is given in Table 13.8.

13.3.4. Non-covalent dot-immunobinding on paper

Esen et al. (1983) described a method in which ordinary chromatography paper is used for binding of water-insoluble proteins. This material is easier to handle than nitrocellulose, is very cheap, and requires no particular pretreatment. It has, however, a lower detectability. Detection of spotted antigen is as in Table 16.12, but the

![Fig. 13.4. Activation of paper with 1-(3-nitrobenzyloxymethyl)-pyridinium chloride (NBPC) via nitrobenzyloxymethyl (NBM), aminobenzylloxymethyl (ABM) to diazobenzylloxymethyl (DBM)-paper to which antigens may be covalently bound.](image)
A. Preparation of nitrobenzyloxymethyl (NBM) paper
1. Cut Whatman No. 540 paper to fit bottom of a container which is placed in a water bath of 60°C.
2. For each cm² of paper use a solution of 2.3 mg of 1-(3-nitrobenzyloxymethyl)-pyridinium chloride (Sigma) and 0.7 mg of sodium trihydrate in 28.5 µl of distilled water. Pour the solution evenly over the paper and push out any air bubbles (use rubber gloves).
3. Rub solution evenly over the paper until dry. Dry further at 60°C in an oven for about 10 min and then bake the paper for 30-40 min in an oven at 135°C.
4. Wash paper several times with water (total 20 min), and 3 times with acetone (20 min total) and air-dry.
5. Paper can be stored for at least 1 year in refrigerator.

B. Preparation of aminobenzyloxymethyl (ABM) paper from NBM paper
6. Incubate the NBM paper in a fume hood with 150 ml of 20% (w/v) solution of sodium Na₂S₂O₄ in water, for 30 min at 60°C, with occasional stirring.
7. Wash the ABM-paper several times with large volumes of water for a few min until no odor of hydrogen sulfide remains.

C. Preparation of diazobenzyloxymethyl (DBM) paper from ABM paper
8. Wash the ABM paper with at least 100 ml of 1.2 mM HCl (for a 14 × 14 cm paper). Transfer the wet paper directly to ice-cold 1.2 N HCl, using 0.3 ml/cm². Add, with mixing, a solution of NaNO₂ (10 mg/ml) in water prepared immediately before use, in the proportion of 2.7 ml for 100 ml of HCl.
9. Keep paper at least 30 min in this solution at 0–4°C with occasional swirling (after 30 min, a drop of the solution should give a positive (black) reaction for nitrous acid with starch–iodide paper).
10. Keep the DBM paper in acid until the gel is ready for the transferring of proteins or nucleic acids. Before use, wash quickly the DBM paper with ice-cold water and twice with ice-cold transfer buffer (total time 2–3 min).

antiserum is less diluted (100 to 500-fold) and shorter incubation periods (half of those in Table 16.12) are used. Blocking as for nitrocellulose is not required if antisera are diluted less than 1000 times.
The paper discs (diameter 1 cm), cut from Whatman No. 1 chromatography paper (wear gloves!), are placed in a polystyrene microplate so that the center of the discs and of the wells coincide. One to 2 μl of the protein solution (solubilized in 60% ethanol) is spotted (marked with pencil). After drying, the discs can be stored or used directly for EIA; detectability is 1–2 ng/spot.

13.4. Glass as the solid phase for enzyme immunoassays

Antigens or antibodies may be fixed to glass surfaces by heating, fixation with formaldehyde or coupling with GA to aminoalkylsilyl glass rods. Glass is not frequently used in EIA but may carry some advantages in particular situations.

Proteins may be coupled to glass (Robinson et al., 1971; Hamaguchi et al., 1976), details of which are given in Table 13.9. With this method the detectability in EIA, using BGase as marker, is about 30 amole \((30 \times 10^{-18} \text{ mole})\) of antigen per assay tube (1 ml), which is about 10 times better than with Sepharose 4B. The reproducibility of the technique is also greater.

Particulate or non-particulate antigens may be assayed quantitatively on slides by the method of Conway de Macario et al. (1983) which resembles the nitrocellulose dotting technique (Section 13.3.1). A 5-μl drop of the immunoreactant is applied on a circular glass surface (3 mm diameter) delineated by a thin layer of a hydrophobic material and fixed according to Table 13.9B. The immune reactions are then performed by successive incubations with drops of the reagents, with intermittent washings. After the application of the substrate, the enzymatic reaction is evaluated visually (e.g. with a microscope) or measured with a vertical beam spectrophotometer (e.g. Dynatech MR590 minireader). Many samples may be screened in a short time and background staining is very low.
TABLE 13.9
Fixation of antigens or antibodies on glass

A. Coupling of antigens or antibodies to aminoalkylsilyl glass
1. Bake pyrex glass rods (diameter 3 mm, 5 mm long at 500 °C for 5 h).

2. Immerse the rods for 24 h in a 2% solution of 3-aminopropyltriethoxysilane (Sigma) in acetone at 45 °C.

3. Wash the rods with acetone and dry.

4. Immerse the rods in 1% (v/v) aqueous GA for 1 h.

5. Wash the rods with 250 mM sodium phosphate buffer, pH 7.5.

6. Immerse the rods in a solution of the antigen or antibody in the same buffer (2 mg/ml) for 30 min.

7. Wash the rods with the buffer used for EIA.

B. Fixation of antigens as small dots on glass slides
1. Clean the glass slides (printed with circles of 3 mm diameter; e.g., from Flow Lab.) with 95% (v/v) ethanol.

2. Apply the desired amount of antigen (in 5 μl) in each circle and fix as follows (with minimum amounts of antigens between parentheses):
   a. Viruses: 1 μg/circle; dry by evaporation.
   b. Archaea: 1000/circle (prefixed in suspension with 1% FA in PBS); Eubacteria: 250/circle; apply drops to slide and heat back of the slide quickly with a Bunsen burner three times.
   c. Fungi: 5–10000/circle.
   d. Protozoa: 5000/circle; prefix as in b, and fix by drying.
   e. Mammalian cells: 5000/circle; air-dry and add a drop of chilled acetone.
   f. Nonparticulate antigens: 5 μg/circle; dry by evaporation and incubate for 30 min with Dulbecco's PBS.

13.5. Particulate solid phases for enzyme immunoassays

Particulate solid phases have originally been used for the separation of radiolabeled antibody–antigen complex from free labeled antibody in immunometric assays (Wide and Porath, 1966; Woodhead et al., 1974).
13.5.1. Agarose, cellulose, and Sephacryl solid phases

Linking of an immunoreactant to Sepharose, activated with CNBr (Section 7.1.9.2.2), has the advantage that the immunoreactant can be held in suspension by mild agitation but settles quickly in the sucrose separation procedure (Hunter, 1980) without centrifugation. Moreover, Sepharose 4B has large pores and, thence, has a high binding capacity: 10 μl of a Sepharose 4B immunosorbent suspension has about 5–40 times more antigen bound than a paper disc of 6 mm diameter and about 100 times more than a well of a microtitre plate (Giallongo et al., 1982). Non-specific adsorption levels are also low. However, CNBr has two important disadvantages: (i) it is toxic; and, (ii) as CNBr-activated paper (Section 13.3.3), leakage of bound proteins may occur (Corfield et al., 1979).

A useful alternative is the activation of Sepharose with NaIO₄ (Section 7.1.9.2.1; Guthrie, 1961). Agarose (and thus Sepharose) has no abundant vicinal diols and is, therefore, less reactive and binds less immunoreactant than Sephadex, cellulose or Sephacryl (Wright and Hunter, 1982). The structure of Sephadex, however, is quite easily disrupted by oxidation with NaIO₄. For these reasons Ferrua et al. (1979, 1980) and Wright and Hunter (1982) favor the use of Sephacryl or cellulose as particulate solid phase. Activation of cellulose with NaIO₄ has been discussed in Section 7.1.9.2.1. For the activation of Sephacryl (S300), Wright and Hunter (1982) used a lower pH (100 mM sodium acetate buffer, pH 5.0) and 5 mM NaIO₄ instead of the 60 mM used by Ferrua et al. (1979). For cellulose, IgG is used at a concentration of about 8 mg/ml, for Sephacryl at about 2 mg/ml and the amounts for various antigens are similar. The theoretical aspects of this coupling procedure were discussed in Section 11.2.2.

Sephacryl S-1000 may be suitable as solid phase, since this gel is more porous and may better accommodate larger complexes.

Large batches of activated beads may be stored at −20°C without appreciable loss of activity over prolonged periods. For the immobilization of haptens or small antigens on a solid phase spacer molecules,
are recommended (Section 7.1.9.2.3) to prevent the interference by the matrix.

Particulate cellulose may also be activated with NBPC by a method similar to that for the activation of paper (Section 13.3.3; Fig. 13.4). Gurvich et al. (1961) and Hales and Woodhead (1980) described a somewhat different method: 5 g cellulose are suspended in a Petri dish in a mixture of 0.5 g sodium acetate in 2 ml water and 1.4 g NBPC (BDH or Sigma) in 18 ml of ethanol. The slurry is dried in an oven at 70°C and subsequently for 40 min at 125°C. The pale brown nitro-derivative is washed on a sintered glass funnel 3 times with 200 ml benzene and sucked dry. The nitro groups are reduced as in steps 5-7 of Table 13.8. The resulting cellulose is dried and pulverized in a mortar.

For diazotization, 1.5 g CuCl₂ are dissolved in 5 ml water and 75 ml of freshly prepared 1 M NaOH are added with stirring. The blue precipitate is washed with water on two layers of filter paper until the pH of the wash is below 9.0. The precipitate is then dissolved in 40 ml NH₄OH (specific gravity 0.880) to form a saturated solution. Excess of Cu(OH)₂ is removed by centrifugation after at least 15 min of stirring. About 0.5 g of ABM-cellulose is added to this solution with continuous stirring. Any ABM-cellulose which does not dissolve after 15 min is removed by centrifugation, the supernatant is decanted into 1.5 l water and 10% (v/v) H₂SO₄ is added until the dark-blue solution becomes almost colorless (pH below 4). ABM-cellulose forms a white precipitate which is collected after 1 h and washed (by centrifugation) with water to remove all traces of copper. If difficulties arise in sedimenting the precipitate, a few drops of 1 M HCl should be added. The ABM-cellulose is then suspended in 50 ml of 2 M HCl, cooled in an ice bucket and 2 ml 1% NaNO₂ are added drop-wise. The presence of excess of nitrous acid is tested with starch-iodide paper (step 10 of Table 13.8). At the end of the reaction, i.e. after 20 min, about 5 g urea are added to the diazocellulose suspension until the starch-iodide test becomes almost negative. The diazocellulose is then washed by centrifugation (at 4°C!) 3 times with water and 2 times with 200 mM borate
buffer, pH 8.2, and resuspended in this buffer to 10 mg/ml. A small aliquot of this diazocellulose should be tested with β-naphthol which should produce a bright orange color; in the absence of such a reaction, the preparation is not good and should be discarded.

The coupling of the protein to the pale yellow-green diazocellulose occurs almost instantaneously at high pH. The protein should be used at a high concentration (>10 mg/ml) in ice-cold 100 mM borate buffer, pH 8.2, even if it is available only in small amount. The reaction mixture is incubated at 4°C. The color of the cellulose turns to a deeper yellow and only little washing is necessary to eliminate free proteins. Washing is also advised after a prolonged storage (at 4°C).

Antigens bound in the interior of the cellulose are often inaccessible to IgG conjugates.

13.5.2. Protein A-containing fixed bacteria as solid phases

SpA-containing Staphylococci (Section 3.3), fixed with trichloroacetic acid (TCA) or formalin (Section 3.3.1), may also serve as immunosorbent for many mammalian antibodies (Table 7.1). Both fixation procedures are satisfactory but yield products with different properties. Fixation of Staphylococci with hot TCA (Lindmark, 1982) removes the negatively charged cell-wall polymer teichoic acid, producing an IgG-sorbent which can bind 1.4 mg human IgG per ml of a 10% (v/v) suspension of bacteria and is stable for about 5 months. Formalin-fixed bacteria (Kessler, 1976) bind 35% more IgG and are stable for at least 1 year. However, IgG can be eluted quantitatively from TCA-fixed bacteria but not from formalin-fixed bacteria, probably due to the interaction between IgG and teichoic acid, unless 80 mM MgCl₂ is included in the acid buffer.

The production of Staphylococci and their fixation with formalin has been described in Section 3.3.1. For the fixation of the bacteria with TCA, the bacterial suspension is heated in a boiling water bath for 2 min and an equal volume of hot 10% TCA is then added while swirling. The mixture is heated for 6 min at 90°C
and rapidly cooled in an ice bath. The suspension is diluted with 1.2 volumes of cold potassium phosphate buffer, pH 7.4 and the fixed bacteria are collected by centrifugation (6 min 4000 \times g). The bacteria are then incubated in PBS, containing 0.5\% (v/v) Tween 20, for 15 min at room temperature and washed by centrifugation with the same buffer (Lindmark, 1982).

Teichoic acid may also be removed from formalin-fixed bacteria by alkaline hydrolysis (Roger and Garret, 1963).

13.5.3. Other solid phases

The preparation of immunosorbents by direct cross-linking of antigens is generally too costly to be practical (Section 7.1.9.2.5).

Other particulate solid phases include polyacrylamide gels (Dolken and Klein, 1977), bentonite clay (Cheng and Talmage, 1969) and possibly other supports. Generally, these rarely used solid phases are not very practical and find their application only in particular cases.

13.6. Separation principles for the various solid phases

Large solid phases (plastic, glass, membranes, paper) retain the attached specific immunoreactant while unbound and/or unreacted molecules are removed by aspiration or decanting. This simple separation procedure is probably responsible to a large degree for the wide application of these solid phases, despite their lower binding capacity.

Particulate solid phases with a high surface/volume ratio (consequently higher efficiency) give faster reactions since the solid phase is dispersed throughout the reaction mixture, but may suffer from the necessity of more involved separation techniques such as centrifugation, filtration or special procedures (Hunter, 1980). This also makes automation more difficult.

A possibility to circumvent these problems is the use of iron-
containing particles and magnetic transfer devices (Hersh and Yaerv-
baum, 1975; Avrameas, 1977; Smith and Gehle, 1977; Anderson,
1978; Druet et al., 1982). The beads may contain magnetite (Magno-
gel 44, Industrie Biologie Française, Villeneuve la Garenne, France)
or small iron cores (Smith and Gehle, 1977). These may be coated
with a film of polycarbonate by immersing the beads in a 5% solution
of methylene chloride, scattering them on slick paper and drying.
Adsorption or conjugation of antigens or antibodies can then be
performed as in Section 13.2. One bead per well is used in a microtitre
plate.