Ag-Ab interactions are reversible and non-covalent.

The forces that contribute to the strength of interaction include:

- Hydrogen bonds
- Ionic bonds
- Hydrophobic interactions
- Van der Waals interactions (outer electron clouds of two atoms)

All are relatively weak in an aqueous environment, so close structural complementarity is necessary. All of these interactions are effective over a distance of approx. 1 x 10^-7 mm (1 angstrom).

Strength of interaction of a single Ag-binding site and an epitope is referred to as affinity.

\[ \text{forward rate constant} \quad k_1 \]
\[ \text{Ag + Ab} \quad \rightleftharpoons \quad \text{Ag-Ab} \quad \text{reverse rate constant} \quad k_{-1} \]

\[ \frac{k_1}{k_{-1}} = K = \text{association constant} \]

\[ K = \frac{k_1}{k_{-1}} = \frac{[\text{Ag-Ag}]}{[\text{Ag}][\text{Ab}]} \]

- \( k_1 \) is in L/mol/sec (second order)
- \( k_{-1} \) is in 1/sec (first order)

Small haptens can have \( k_1 \) as high as 4 x 10^8 (fastest possible is 10^9 L/mol/sec (i.e., diffusion limited)).

\( k_1 \) can vary a lot. The ratio \( k_1 / k_{-1} \) is what determines affinity.

Low affinity, \( k_1 / k_{-1} = K = 10^4 - 10^5 \) L/mol

High affinity, \( k_1 / k_{-1} = K = \text{as high as} \ 10^{11} \) L/mol

You can measure the affinity of Ab and a small diffusible molecule by a procedure called equilibrium dialysis (Figure 6-2).

Two equal compartments are separated by a semi-permeable membrane.

Ab at known concentration is in solution on one side only. Radiolabeled hapten (small diffusible molecule) is on the other side. Allow to come to equilibrium.

At that time, the concentration of unbound ligand will be equal on both sides of the membrane, but there will be more ligand on the antibody side due to the antibody-bound ligand.
The higher the affinity of the antibody, the more antibody-bound ligand there will be.

\[ K = \frac{[\text{Ag-Ag}]}{[\text{Ag}][\text{Ab}]} = \frac{r}{(n-r)(c)} \]

Where 
- \( r \) = ratio of the concentration of bound ligand to the total Ab concentration
- \( c \) = concentration of free ligand
- \( n \) = number of binding sites per antibody

You can rearrange this equation to give the Scatchard equation:

Obtain values of \( r/c \) by repeating equilibrium dialysis at different initial concentrations of ligand.

\[ \frac{r}{c} = Kn - Kr \]

Then plot \( r/c \) as a function of \( r \):

If \( K \) is a constant (i.e., the antibody is a single pure monoclonal antibody and all the antigen binding sites are identical) this plot is a straight line with:

- Slope = -K
- Intercept = n

As the concentration of unbound ligand increases, \( r/c \) approaches zero and \( r = n \).

For heterogeneous, polyclonal antibodies, \( r/c \) vs. \( r \) gives a continuous curve.

You can determine the average affinity constant, \( K_0 \), by determining \( K \) when the antigen-binding sites are half-filled.

\[ K_0 = \frac{1}{(2 - 1)c} = \frac{1}{c} \]

\( r = 1 \) for bivalent antibody

What happens if the antigen is not small enough to diffuse through a semi-permeable membrane? How can you measure affinity of an antibody or T cell receptor for a protein antigen?

We can use an apparatus called a BiaCore which utilizes a property of certain materials called surface plasmon resonance. Let’s say we want to measure the affinity of bovine serum albumin (BSA) and Fab fragments directed...
against BSA. First we couple BSA to a chip made from a material that exhibits surface plasmon resonance. We then flow a solution of known concentration of Fab anti-BSA over the chip and measure this property. As the FAB binds non-covalently to the BSA on the chip, the surface plasmon resonance increases to a maximum. From the shape of the curve, the rate constant of binding (the on rate) can be calculated.

Then, we replace the fluid flowing over the chip with buffer that lacks Fab anti-BSA. Gradually the Fab bound to the BSA on the chip will dissociate. From the shape of the curve and time it takes to return to baseline, you can determine the off rate constant. The ratio of on/off rate constants equals the association constant \( K_A \).

![Surface plasmon resonance](image)

### Avidity

- **Avidity** – when multivalent antibody binds to an antigen with multiple repeating epitopes, interaction by one antigen-binding site increases the chance of binding by the other site. This gives a true measure of the antibody’s tendency to dissociate.

High avidity can compensate for low affinity. For example, IgM antigen-binding sites are generally lower affinity than IgG, but multivalency gives it high avidity.

### Precipitates of antibodies with antigens

Before all of the fancy assays were developed for observing antigen-antibody interactions, **precipitations** formed when antibodies and antigens were mixed was a major tool. A number of these assays are described in the text.

When polyclonal antibodies and an antigen with multiple epitopes are mixed in solution, they interact with each other to form complexes. The degree to which insoluble antigen-antibody complexes form, resulting in a flocculent precipitate that settles to the bottom of the tube, depends on the extent of lattice formation (see Figure 6-4). If there is too little or too much antigen relative to the amount of antibody present, the extensive lattice needed to make insoluble complexes cannot form and little or no precipitate results. However, as the ratio of antibody to antigen mixed together approach an ideal equivalence point, the precipitate becomes so extensive that nearly all of the antigen and antibody drop out of solution.

This principle of equivalence makes possible the **Ouchterlony double diffusion method** shown in Figure 6-5, and the **immuno-electrophoresis** technique shown in Figure 6-6.

You should understand both of these methods. In them, antigen diffusing from a well in a agarose gel diffuses towards antibody in another well (Ouchterlony method) or in a trough (immuno-electrophoresis). As the diffusion zones of antigen and antibody overlap, a visible precipitate forms a band or an arc in the gel in the zone of equivalence. In the case of the Ouchterlony method, the shape of the bands or arcs that form can provide information on the similarity or differences of the antigens in adjacent wells.

The difference in sensitivity of these older methods and newer ones like radioimmunoassay or ELISA assay is very great.

Precipitation assay in solution (Fig. 6-4) can detect 3-20 micrograms of antibody nitrogen/ml (antibody nitrogen is a way of quantitating protein);

Sensitive assays like RIA and ELISA detect as little as \( 10^{-4} \) micrograms of antibody nitrogen/ml.
You should also understand the agglutination and agglutination inhibition tests shown in Figures 6-7 and 6-8 of the Kuby text.

**Solid phase radioimmunoassay (RIA) and ELISA assays**

These assays make use of the fact that proteins (and therefore, antibodies and protein antigens) have a tendency to stick to certain kinds of plastic. You therefore can add a specific antibody to wells of a plate made of such plastic, and about the same amount of antibody will stick to each well. You can then add an irrelevant protein (BSA or even powdered milk) to the wells to block any other sites on the plastic that might bind the antigen when you add it. Then you can add the antigen and quantitate it, either by an intrinsic label such as radioactive iodine that can be attached to the antigen (this is done in a RIA, or by another method (see ELISA below).

**Figure 6-9** illustrates a competitive RIA to detect and quantitate hepatitis B surface antigen (HbsAg) in human serum. It is a diagnostic test for hepatitis. It measures the ability of the HbsAg in serum to compete for a standard amount of radiolabeled HbsAg used in the test. In order to use the test for quantitation, a standard curve must be constructed every time the assay is done. This means that known amounts of HbsAG must be added to a number of wells, and the degree of inhibition of binding of radiolabeled HbsAG must be plotted as a function of the known amounts of unlabeled HbsAg added to each well. The assay is only accurate in the linear portion of the standard inhibition curve. Values for the amount of HbsAg in various unknown serum samples tested can be interpolated from the standard curve.

In ELISA assays (for enzyme-linked immunosorbent assays), either antibody or antigen can be stuck to the plastic well, depending upon the geometry of the assay to be performed. Three different geometries that you should know are shown in **Figure 6-10**. The competitive ELISA assay shown in part (c) of the figure accomplishes the same thing as the RIA to quantitate HbsAg that is described above. As for the competitive RIA, a standard inhibition curve must be included every time this ELISA assay is done.

The distinguishing feature of ELISA assays is that they employ an antibody to which an enzyme such as horseradish peroxidase or β-galactosidase has been covalently conjugated. At the end of the assay, a chromogenic substrate that yields visible color when acted upon by the enzyme provides a measure of the amount of the antibody still adhering to the well.

**Western blot assay**

This is a variant of the ELISA assay in that it employs antibody conjugated to an enzyme. Let’s say you want to see if a particular protein is present in a protein mixture. You electrophorese the mixture on a gel so that the proteins separate on the (general) basis of molecular weight (e.g., SDS gel). You then transfer the proteins to a membrane sheet such that their positions relative to each other are preserved. You then bathe the membrane in solution containing the antibody-enzyme conjugate. Wherever the protein lies on the sheet, the antibody will bind specifically. You then wash off the excess antibody and bathe the sheet in a solution containing a substrate that forms a colored insoluble product at the site where the antibody is bound to the protein antigen. Thus, if the protein was present in the mixture, a colored band appears on the sheet at a position corresponding to the protein’s molecular weight.

**Immunofluorescence assays**

In the RIA we saw that you can radiolabel an antigen (e.g., HbsAg) or, in other instances, an antibody. In the ELISA and Western blot assays, an enzyme is covalently conjugated to the antibody, and the amount of antibody is quantitated by a chromogenic substrate.

In the case of **immunofluorescence**, a fluorophore is conjugated to the antibody, and when illuminated with light from a laser, the bound antibody emits visible light that can be observed through a fluorescence microscope or quantitated using a photomultiplier.

**Figure 6-14** of the Kuby text shows how direct conjugation of the fluorophore to the antibody of interest can show the distribution of the corresponding antigen on the cell surface or, if you permeabilize the cell, inside the cell. It
also shows how you can first bind the anti-antigen antibody to the cell, wash away the unbound antibody, and then visualize the bound antibody using a fluorochrome-labeled anti-isotype antibody. These are very powerful techniques for use with cell-associated antigens.

**Flow cytometry and the fluorescence activated cell sorter (FACS)**

A cartoon showing how this instrument works is shown in Figure 6-15. Cells in suspension and already reacted with fluorochrome-conjugated antibodies are passed through the light of one or more lasers that illuminate the stream at a point. Multiple antibodies, each labeled with a fluorochrome that emits a different color visible light may be used to label cells in a complex population.

As a cell passes by the laser and is illuminated, a record of a number of parameters related to that cell is stored in a computer.

- A value for **low angle light scatter** that is proportional to cell size.
- A value for **light scatter at 90° to the direction of laser illumination**. This value is a function of cell shape and granularity, and helps to distinguish cell types.
- A value for each color fluorescent antibody that has been used to stain the cells. **Up to four different colors can be measured**. The photomultiplier detectors that collect and quantitate each color of light are arranged such that they collect fluorescent light emitted **at 90° to the direction of laser illumination**.

After the experiment, all of this information can be displayed graphically to analyze the cell content of the ell population being studied.

During the experiment, the instrument can **very rapidly** compute the properties of the cells passing by the laser. If a cell conforms to a set of parameters of size and fluorescence preset by the operator, the instrument can sort those cells into a collection tube for future use by the investigator. This “sorting” of cells can be achieved by isolating the cells of interest in tiny droplets of fluid generated after passing the laser by an ultrasonic nozzle vibrator.

Cells obtained in this way can be kept alive and sterile, and used for many different **in vitro and in vivo assays**.

**Use of magnetic bead-labeled antibodies to isolate cells**

Anti-isotype antibodies can be covalently conjugated to magnetic beads. If a population of cells is reacted with an antibody of the correct isotype (for example, mouse IgG1), magnetic beads containing the correct anti-isotype antibody can be added and cell-bound antibody will adhere to the beads. A magnet applied along the side of the tube can then be used to cause these cells to accumulate along the tube wall. Non-labeled cells can then be removed and the labeled cells recovered. Alternatively, the method can be used to **deplete** cells reactive with a particular antibody. In this case, the cells that **do not** adhere to the beads are the ones of interest.