Chapter 3 : Immunological Assay

PART I : Antigen

An antigen is a substance that when introduced into an animal with a functioning immune system, can elicit a specific immune response. If the response leads to a state of immunity, the antigen is said to be *immunogenic*. An antigen that produces a state of specific tolerance is called a *tolerogen*.

I. Types

1. proteins	strong immunogenicity
2. polysaccharides	generally weak
3. nucleic acid	weak

*Glycoproteins, lipoproteins, and nucleoproteins give strong immunogenicity.

*For peptides with mol mass less than 2 kDa, a good way to elicit immune response is by the aid of a **carrier** that is coupled with the synthetic peptide.

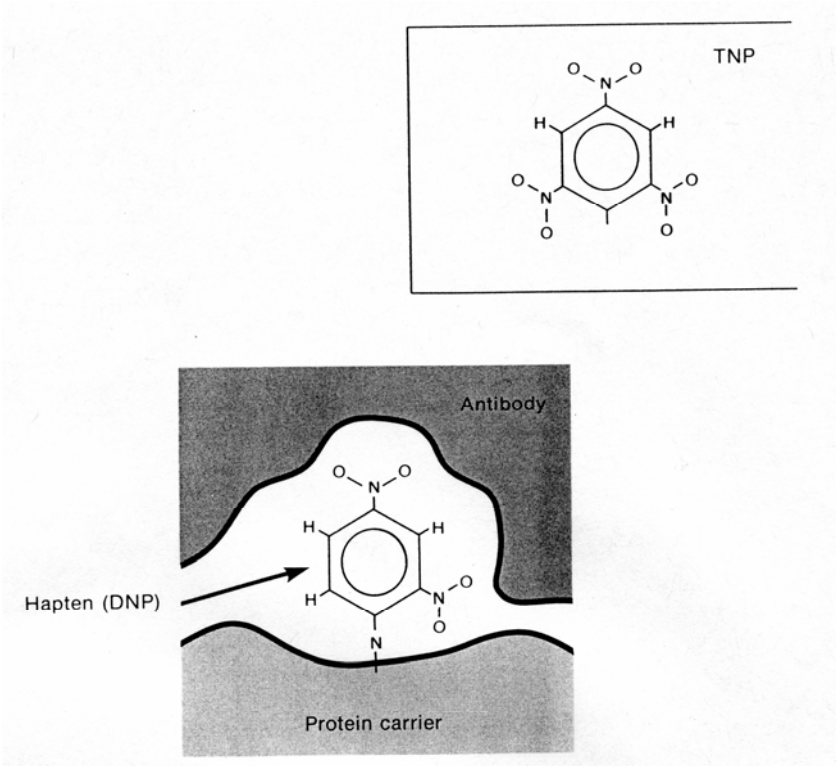

Figure 1.3 *The dinitrophenyl (DNP) group makes up a major part of the antigenic determinant to which the antibody binds. The antibody will also bind to the hapten coupled to a different protein carrier. Inset: the structure of the related trinitophenyl (TNP) group. Most anti-DNP antibodies will also bind to TNP and vice versa.*

II. Purity

*Is it easier to purify a protein than to purify a subset of antibodies ?

*Protein samples should be **homogeneous**. If not, at least it should be **a single species**.

*When a protein sample is limited, you must decide whether to inject the protein into animals or attempt to determine its amino acid sequence. Which is better?

*For amino sequencing, a purified protein more than 20 pmol (50 kDa) that is 1 μg of the protein is necessary to give a reliable result. With the obtained sequence of a unknown protein, one can 1) compare the sequence with known sequences in the protein data bank. 2) may design oligonucleotide primers. 3) may synthesize peptide from which antibody is produced.

III. Dose

	suggested	minimum
rabbits	0.5-1 mg/injection	10 μg/injection
mice	50-100 μg/injection	1 μg/injection

IV. Concentration of the antigen

*The higher the concentration, the better the immunogenicity. a good vaule is 5 (w/v), that is 50 μg/mL.

V. Forms of the antigen

1. aggregate form, a form much more immunogen than soluble molecules.
2. coupling with cells, carrier proteins, or agarose beads
3. self-polymerization by using chemical cross-linkers or partial denaturation of the protein

VI. Sources of the antigen

1. from a cloned DNA sequence
2. from purified proteins of raw materials

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1. From a cloned DNA sequence

1) design synthetic peptides

*choose **hydrophilic amino acid** and **proline** residues, **C-terminal** or **N-terminal region**, or choose amino acid in **more flexible regions**.

*__optimal size of a peptide is 10-15 residues; 6 residues is the least.__ Longer peptides may increase difficulty to synthesize and may be more difficult for coupling reactions.

*make multiple small peptides.

*advantage--- produce site-specific antibodies.
disadvantage---do not recognize native protein.

2) bacteria expression

*antigens as forms of fusion proteins are overexpressed in bacteria by using expression vectors such as B-Gal, trp fusion vectors, or T7 vectors.

*advantage----better chance to recognize native protein
disadvantage---some will be difficult to express in *E coli*.

2. From purified proteins of raw materials

*proteins may be purified from chromatography, HPLC, or from 2D-PAGE. 2D-PAGE is probably the most efficient, time-saving technique to get enough amount of purified proteins ready for injection or sequencing.

*Protein samples can be purified in a preparative fashion by 2D-PAGE. The procedure is briefly outlined below:

1) 1-2 mg of total protein is loaded into each of the IEF gels.
2) After the second dimension of 2D-PAGE, the gels are stained with Coomassie Blue.
3) Protein spots of interest are excised and the gel slices are rinsed by ddH_2O.
4) The gel slices are stored at -70℃.

PART II : Antibody Molecules

I. Antibody structure

*Antibodies are a large family of **glycoproteins** that share key structural and functional features. Functionally, they can be characterized by their ability to bind both to antigens and to specialized cells or proteins of the immune system. Structurally, antibodies are composed of one or more copies of a characteristic unit that can be visualized as forming a Y shape.

*The two arm domains that carry the antigen binding sites are known as **Fab fragments**, and the protein domain that is involved in immune regulation is termed the **Fc fragment**. The region between the Fab and Fc fragments is called the **hinge**. The hinge segment allows lateral and rotational movement of the two antigen binding domains.

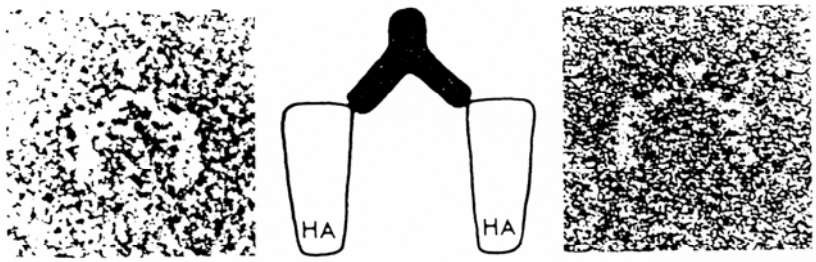


FIGURE 2.1
Two influenza hemagglutinin (HA) molecules bound by an Fab molecule. (Adapted from Wrigley et al. 1983.)

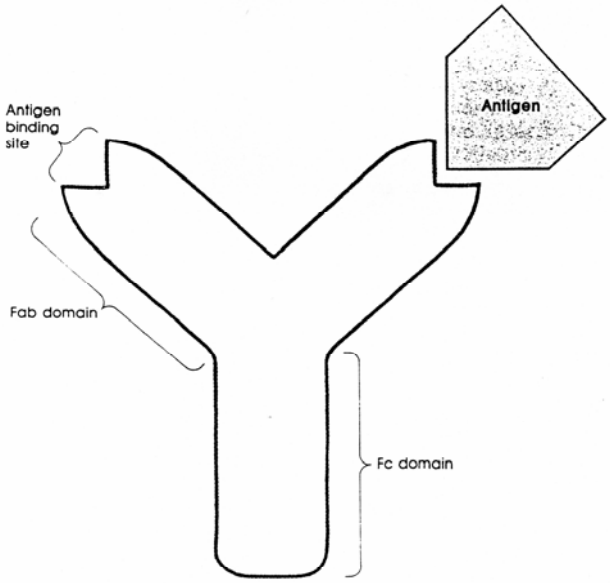


FIGURE 2.2
Antibody domains.

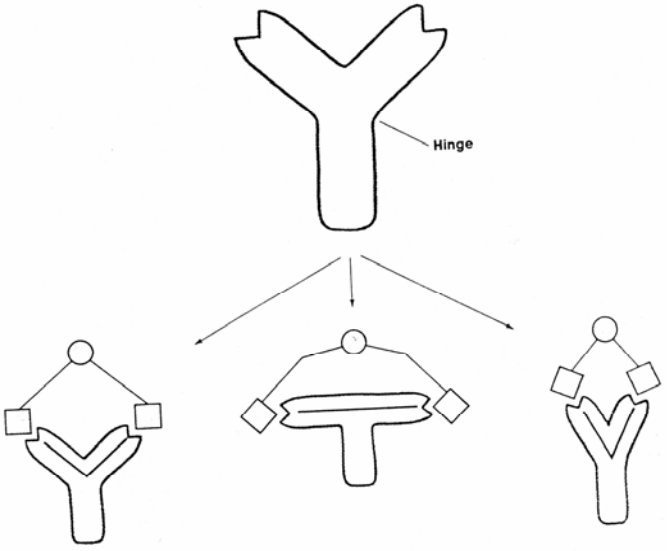


FIGURE 2.3
Hinge and flexibility.

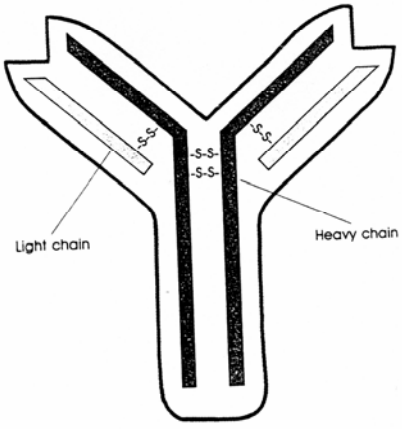


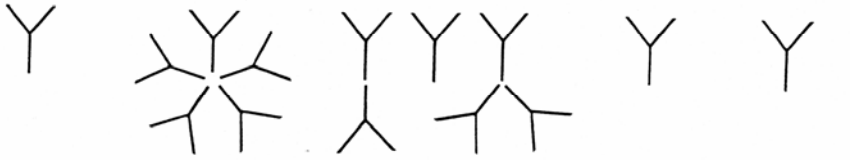
FIGURE 2.4
Heavy and light chains combine to form antibody.

*Each Y contains four polypeptides---two identical copies of a polypeptide known as the heavy chain and two identical copies of a polypeptide called the light chain. The two heavy-chain polypeptides in the Y structure are identical and are about 55 kDa. The two light chains are also identical and are about 25 kDa. The four polypeptide chains are held together by disulfide bridges and noncovalent bonds.

*Antibodies are divided into five classes, lgG, lgM, IgA, lgE, and lgD, on the basis of the number of Y-like units and the type of heavy chain polypeptide they contain.

***The most abundant antibodies in serum are lgG antibodies** that contain only one structural Y unit. lgG molecules have three protein domains (2 Fab and I Fc domains); Fab domains are identical and form the arms of the Y. Each arm contains a site that can bind to an antigen. The third domain (Fc domain) forms the base of the Y, and this region is important in immune regulation.

TABLE 2.1
Classes of Antibodies

Characteristics	IgG	IgM	IgA	IgE	IgD
Heavy Chain	γ	μ	α	ε	δ
Light Chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Molecular Formula	$\gamma_2\kappa_2$ or $\gamma_2\lambda_2$	$(\mu_2\kappa_2)_5$ or $(\mu_2\lambda_2)_5$	$(\alpha_2\kappa_2)_n$ [a] or $(\alpha_2\lambda_2)_n$	$\varepsilon_2\kappa_2$ or $\varepsilon_2\lambda_2$	$\delta_2\kappa_2$ or $\delta_2\lambda_2$
Y Structure					
Valency	2	10	2, 4, or 6	2	2
Concentration in Serum	8–16 mg/ml	0.5–2 mg/ml	1–4 mg/ml	10–400 ng/ml	0–0.4 mg/ml
Function	Secondary response	Primary response	Protects mucous membranes	Protects against parasites (?)	?

[a] n = 1, 2, or 3.

II. Primary amino acid sequences of light and heavy chains

*Light chains are approximately 220 amino acids long and can be divided into two regions, each about 110 amino acids in length. The amino-terminal half of the sequence is heterogeneous that is known as the **variable (V) region**. The carboxy-terminal half is known as the **constant (C) region**.

*There are only two types of light chains: κ and λ chains whereas different types of heavy-chains are assigned by different classes of antibodies, for instances, γ chains for lgG; ε chain for lgE.

*The sequences of the lgG heavy chains have also shown that there are four subclasses of γ chains: IgG_1, IgG_{2a} IgC_{2b}, and IgG_3. The IgG heavy chains are

approximately 440 amino acids long and are divided into one V region and three C regions.

*The varible regions of one heavy chain and one light chain combine to form one antigen binding site. Most of the variability occurs in three short regions (**hypervariable regions**) of each chains. The heterogeneity of the variable regions provides the structural basis for the large repertoire of binding sites used by an animal to mount an effective immune response.

*These hypervariable regions form the majority of contact residues for the binding of the antibody to the antigen. They are referred to as the **complementarity determining regions or CDRs**.

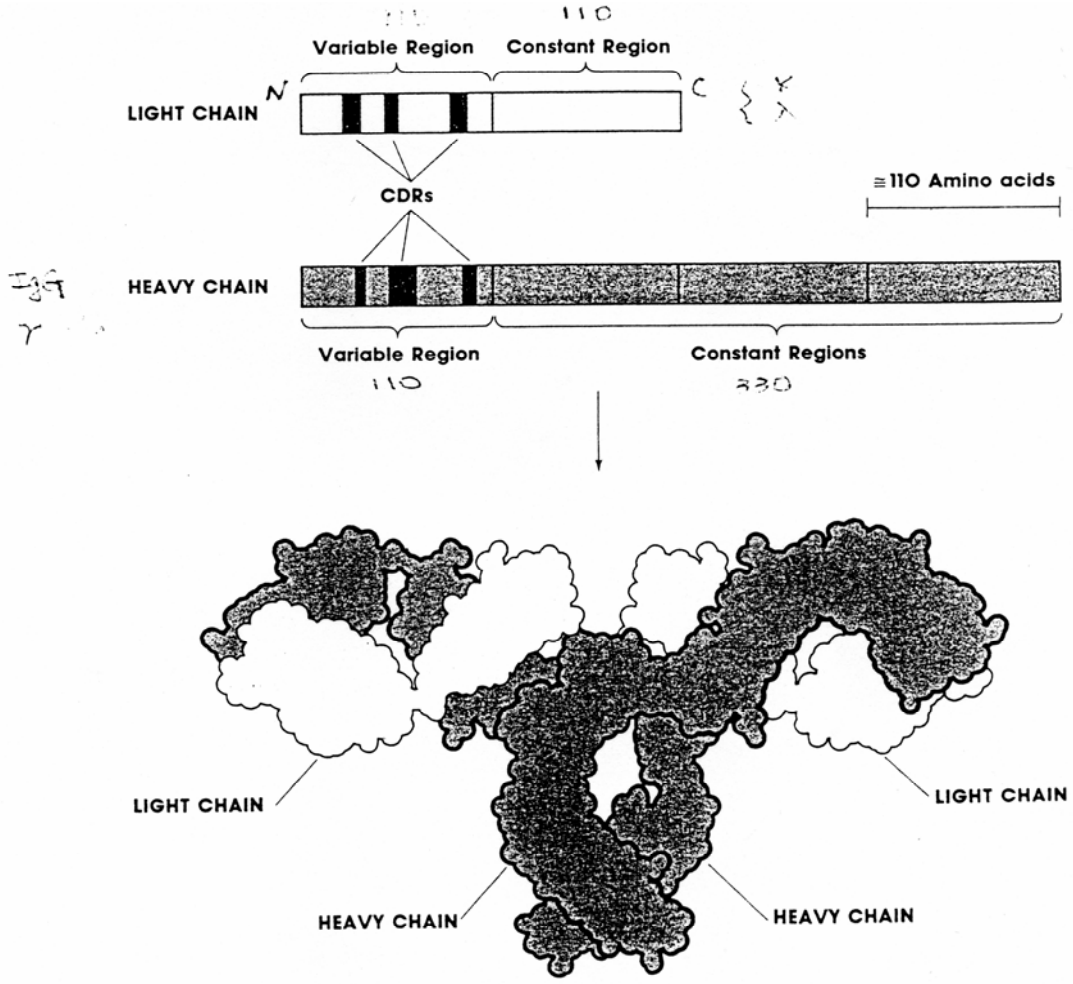


FIGURE 2.5
Light- and heavy-chain structure. (Adapted from Silverton et al. 1977.)

III. DNA rearrangements and additional special mechanisms creat a vast number of antigen binding sites

*There are about **50**-100 variable, 12 D and **4** J chain regions that form the heavy-chain gene cluster on chromosome 12 in the mouse. Because the recombination events are not precise, ther D regions can code for **36** different sequences. Having multiple regions helps to generate a large number of heavy chains, with at least **7200** different possibilities for homogenous mice.

*Recombination between the **variable(V)**, **D**, and **J** regions produces a large number of different heavy- and light-chain polypeptide sequences. The variable regions themselves contain the coding sequences for CDR I and CDR 2 of both the heavy and light chains. The third CDR is produced by the recombination junctions of the sequences that encode amino acids for the third CDR. Therefore, recombination provides both different assortments of the various CDRs and, because the recombination is not always precise, diversity in the third CDR.

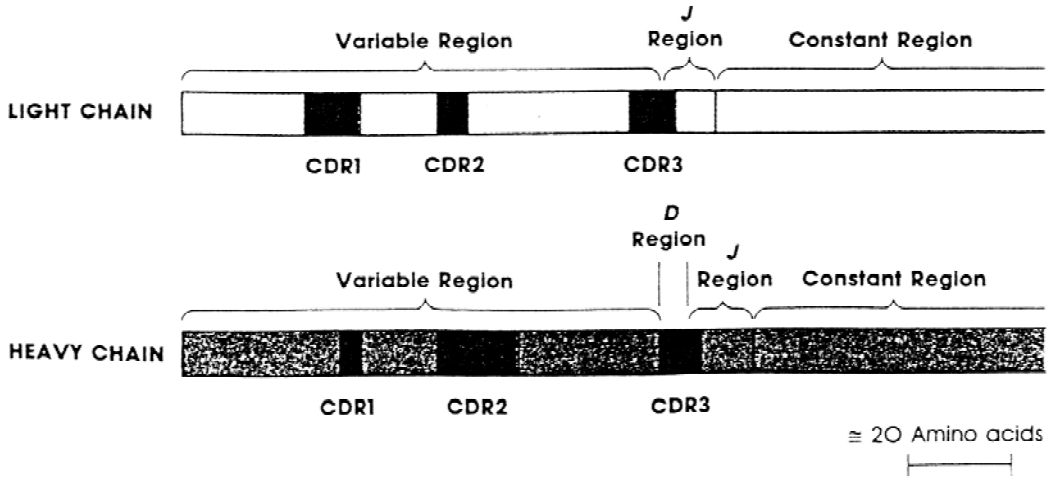


FIGURE 2.9
Heavy- and light-chain rearrangements both reassort and restructure CDRs.

*A vast number of antigen binding sites are created by 1) gene recombination between the variable(V), D, and J regions. 2) recombination of hypervarible regions (or CDRs). For the mouse, there are at least **800** potential κ chains, **five** λ chains, and **7200** heavy chains. If all light chains have a chance to pair with all heavy chains in the production of the antigen binding site, there are potentially 5.8×10^6 ([800+5] x 7200) binding domains.

*Another mechanism also increases antibody diversity. Great than half of the heavy- or light-chain variable regions carry **point mutations** in the antigen binding regions.

Although the mechanisms that generate these mutations are not known, they appear to provide a system that Fine-tunes the binding site, thus creating a better fit for the antigen-antibody interaction. This process is known as affinity maturation.

*In all, it is because of 1) recombination events, 2) junctional diversity, 3) mutation, 4) association of different light and heavy chains that human immunity is able to respond to a large number of antigens.

PART III : Immunizing Animals

Antibody-antigen interactions

*__The antigen binding site of an antibody is formed by the variable regions of the heavy and light chains.__ The hypervariable regions are known as the **complementarity determining regions** (CDRs). There are six CDRs, three on each chain, and they form discrete loops anchored and oriented by the framework residues of the variable domains.

*The region of an antigen that binds to an antibody is called an **epitope**. The size of an epitope is governed by the size of the combining site that was thought to be relatively small. The site was visualized as a cleft or pocket into which the epitope docked. Small changes in the epitope structure can prevent antigen recognition.

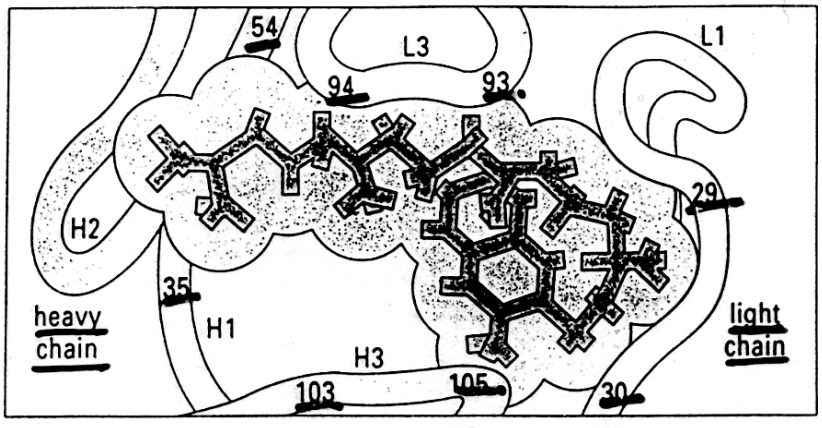
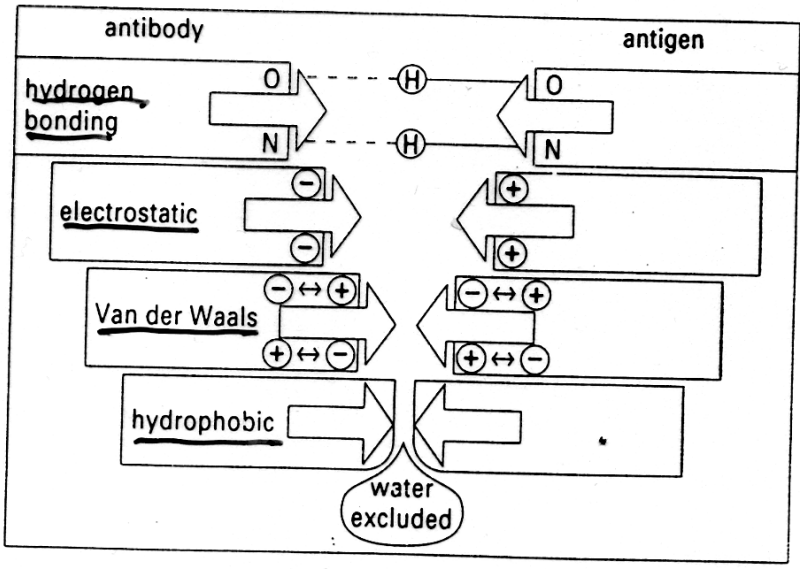


Fig. 6.1 The antigen molecule nestles in a cleft formed by the heavy and light chains, called the antibody combining site. The example shown is based on X-ray crystallography studies of human IgG (the myeloma protein NEW) binding γ-hydroxyl vitamin K. The antigen makes contact with 10–12 amino acids in the hypervariable regions of both heavy and light chains. The numerals refer to amino acids identified as actually making contact with the antigen.

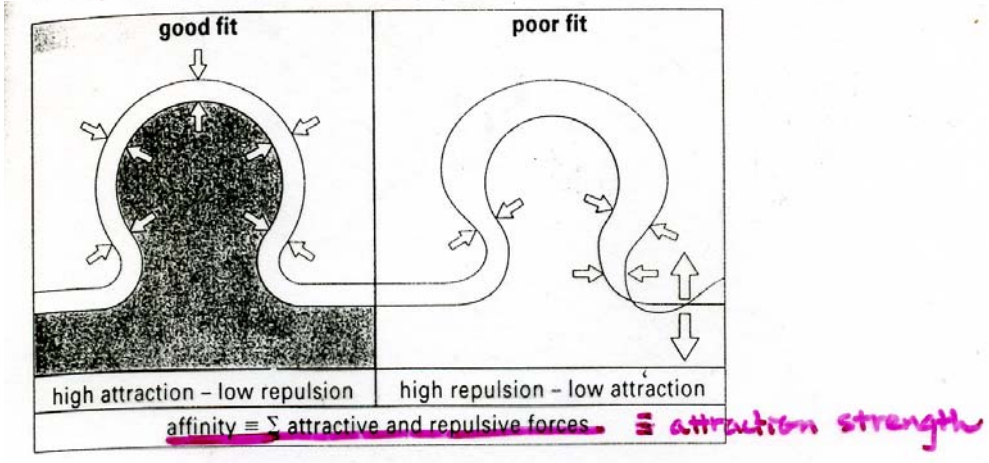
*Because antibodies can recognize relatively small regions of antigens, occasionally they can find similar epitopes on other molecules. This forms the molecular basis for **cross-reaction**.

*The antibody-antigen complexes held together by **multiple, noncovalent bonds**. These noncovalent interactions include hydrogen bonds, van der Waals forces, ionic pairing, and hydrophobic interactions. These bonds can occur between side chains or the polypeptide backbone.



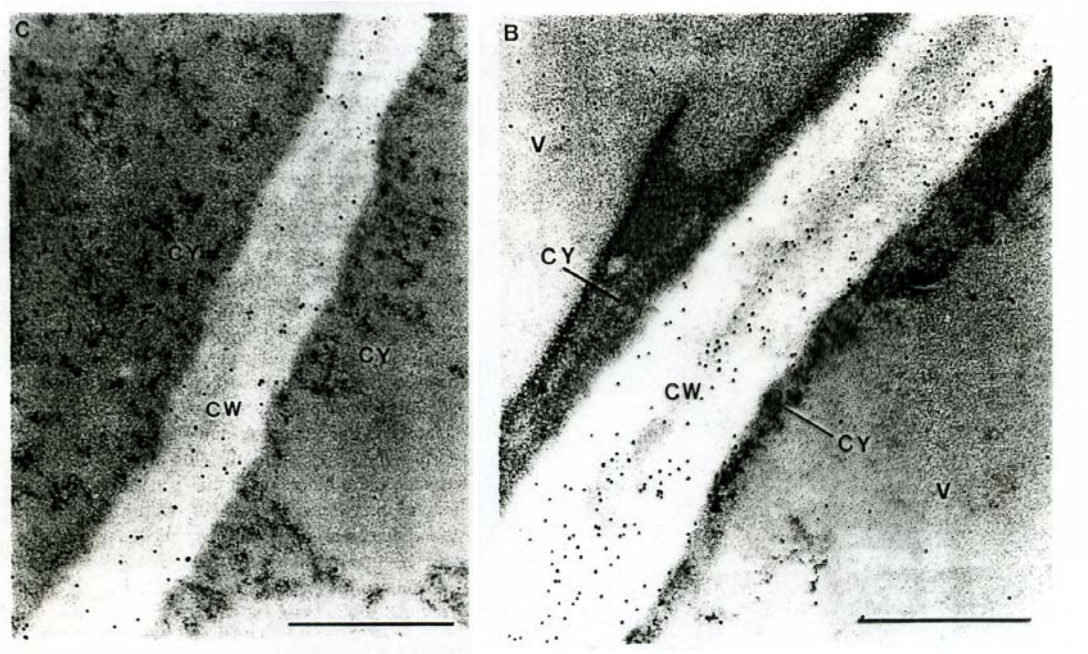
***Affinity is a measure of the strength of the binding of an epitope to an antobody.**
The affinity constant K_A = [Ab-Ag]/[Ab]'[Ag]. In practical terms, affinity describes the amount of antibody-antigen complex that will be found at equilibrium. High-affinity antibodies will bind larger amounts of antigen in a shorter period of time than low-affinity antibodies.

Fig. 6.5 The affinity with which antibody binds antigen results from a balance between the attractive and repulsive forces. A high affinity antibody implies a good fit and conversely, a low affinity antibody implies a poor fit.



*Affinity strength of the Ab-Ag complex is determined by a combination of several attraction forces such as H-bonds, ion-pairing, van der Waals, and hydrophobic interaction. **The affinity constant for antibody-antigen interactions is affected by temperature, pH, solvent, and ionic strength.**

***The affinity of monoclonal antibodies can be determined exactly, but the affinity of polyclonal antibodies cannot.** Because monoclonal antibodies are homogeneous. Polyclonal sera contain complex mixtures of antibodies of different affinities, therefore, the affinity of such sera cannot be exactly determined.



***Titer** is determined by the reciprocal of the highest dilution in which agglutination is obtained.

***Avidity** is a measure of the overall stability of the complex between antibodies and antigens. The overall stability of an antibody-antigen interaction is governed by three major factors, the intrinsic affinity of the antibody for the epitope, the valency of the antibody and antigen, and the geometric arrangement of the interacting components.

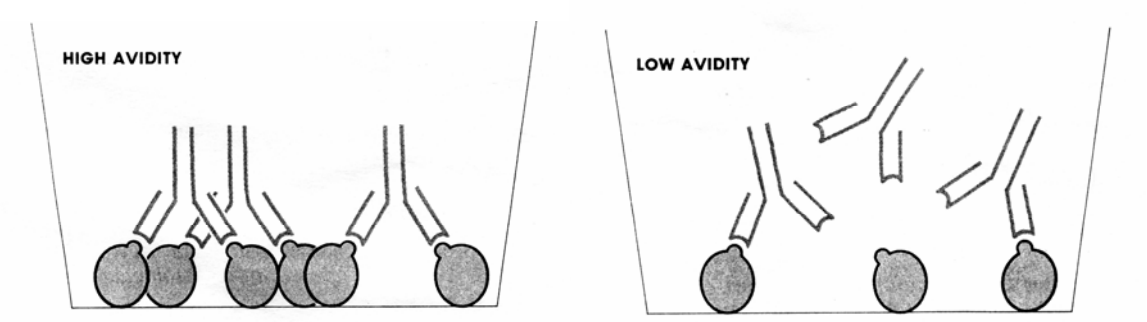


FIGURE 3.5
Bivalent binding to antigens immobilized on a solid phase

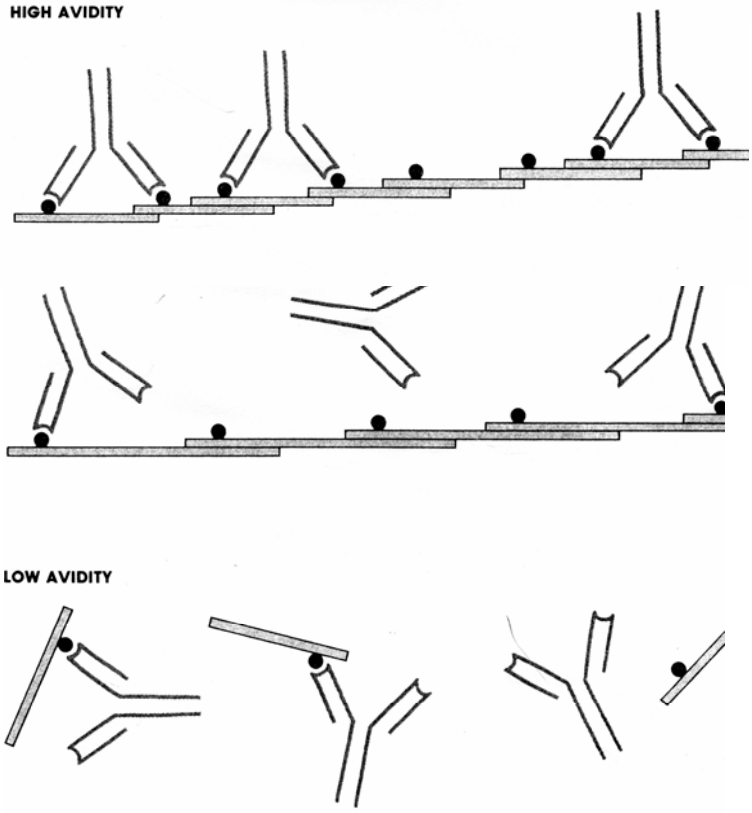
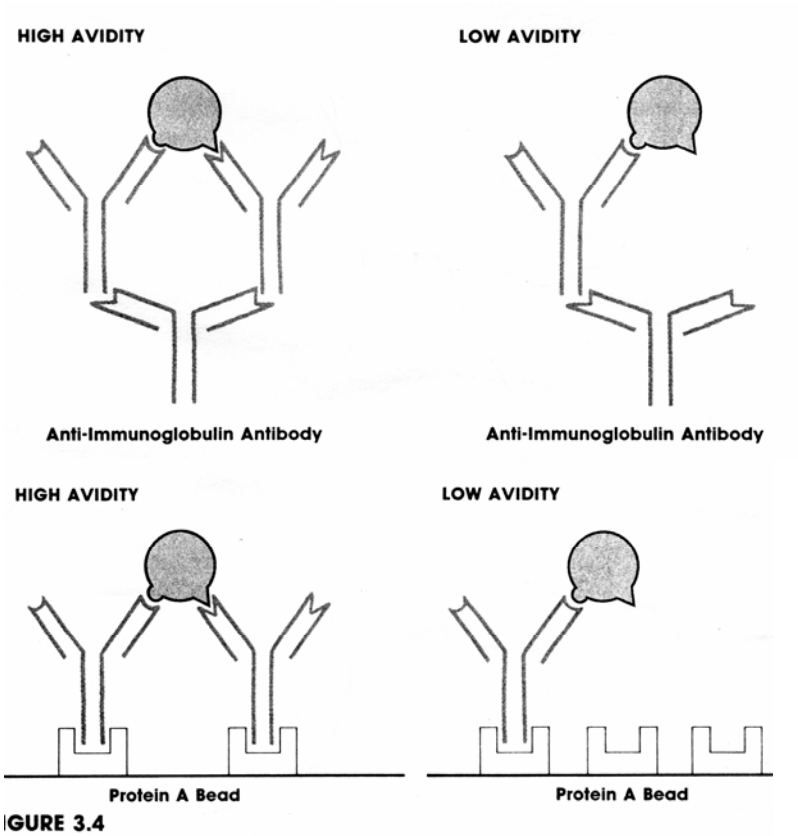


FIGURE 3.2
Bivalent binding of antibodies to polymeric antigens increases the avid



IGURE 3.4
olyclonal antibodies binding to multivalent antigens provide good target
econdary reagents.

Immunization

1. Choice of animals

rabbits	12-week old --- used for polyclonal antibody production
mice	> 6 week old---- used for polyclonal antibody production
rats	6-8-week old --- used for monoclonal antibody production

2. Numbers of used animals

at least two animals should be used.

3. Anethesia

ether	inhalation
sodium pentobarbitone	injection
fentanyl/fluanisone	major surgery
fentanyl/droperiodol	major surgery

4. Routes of injection

rabbits	multiple subcutaneous sites
mice	intraperitoneal injection
chicken	multiple intramuscular sites

5. Boosts

rabbit	4-6-week interval
mice	3-week interval

6. A Practical procedure for immunization

1) Gel slices are thaw at room temperature.
2) Gels are ground in phosphate buffer saline (PBS).
3) Sample in PBS is mixed with an equal volume of Freund's adjuvant. Freund's **complete adjuvant** contains two major components: (1) **mineral oil form** w/o emulsion and (2) **dead bacteria** stimulate inflammatory reaction.
4) Vortex sample vigorously until a thick emulsion develops.
5) Transfer the emulsion into a syringe; avoid air bubbles.
6) Sample is injected into the subcutaneous area (rabbits) or into muscle (hens).
7) Second injection will be administered three weeks after the first injection.
8) For the second injection, sample in PBS is mixed with an equal volume of **incomplete adjuvant** (without containing bacteria).

9) Bleed rabbits or take eggs a week after the second injection. The maximal volume to be collected from a rabbit at one time is 50 ml. Bleed rabbits once a week. One may continue to collect blood for four weeks.
10) The third injection is performed 4 weeks after the second injection.
11) Bleed rabbits or take eggs a week after the third injection.

Injection scheme

day 0	1st injection (with complete adjuvant)
day 21	2nd injection (with incomplete adjuvant)
day 28	1st bleeding
day 49	3rd injection (with incomplete adjuvant)
day 56	bleeding

The four phases of a primary antibody response

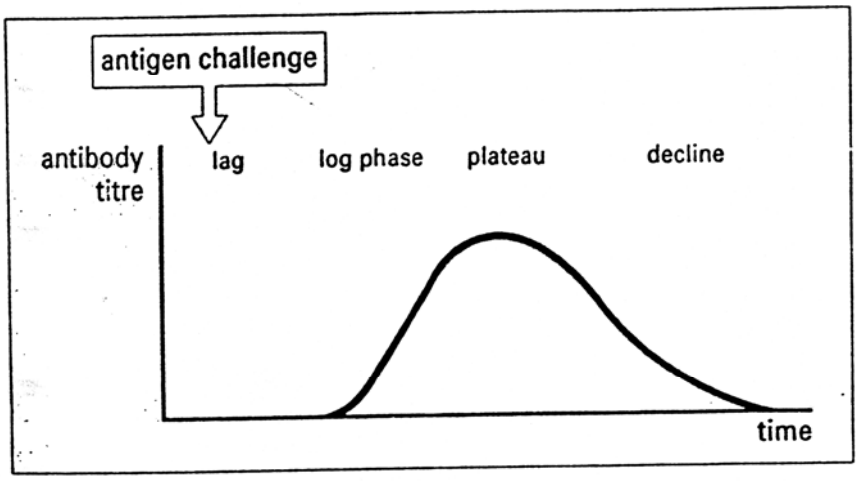


Fig. 7.24 Following antigen challenge the antibody response proceeds in four phases:
1. a lag phase when no antibody is detected
2. a log phase when the antibody titre rises logarithmically
3. a plateau phase during which the antibody titre stabilizes
4. a decline phase during which the antibody is cleared or catabolized.

The actual time course and titres reached will depend on the nature of the antigenic challenge and the nature of the host.

Primary and secondary antibody responses

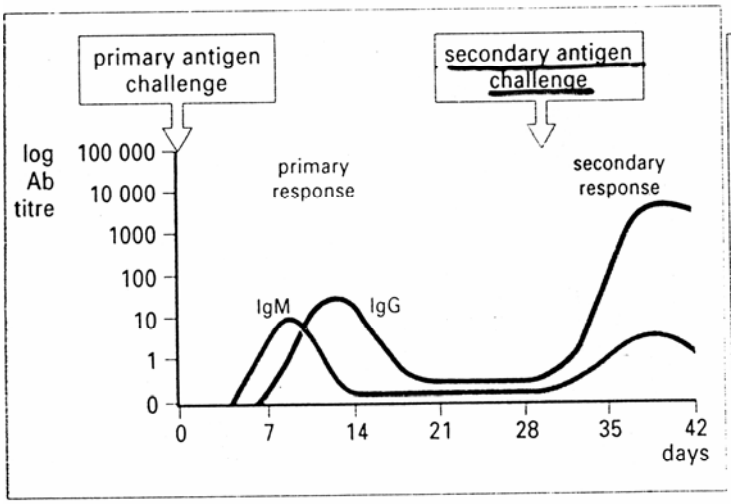


Fig. 7.25 In comparison with the antibody response following primary antigenic challenge, the antibody level following secondary antigenic challenge in a typical immune response:
1. appears more quickly and persists for longer
2. attains a higher titre
3. consists predominantly of IgG.

In the primary response the appearance of IgG is preceded by IgM.

Affinity maturation

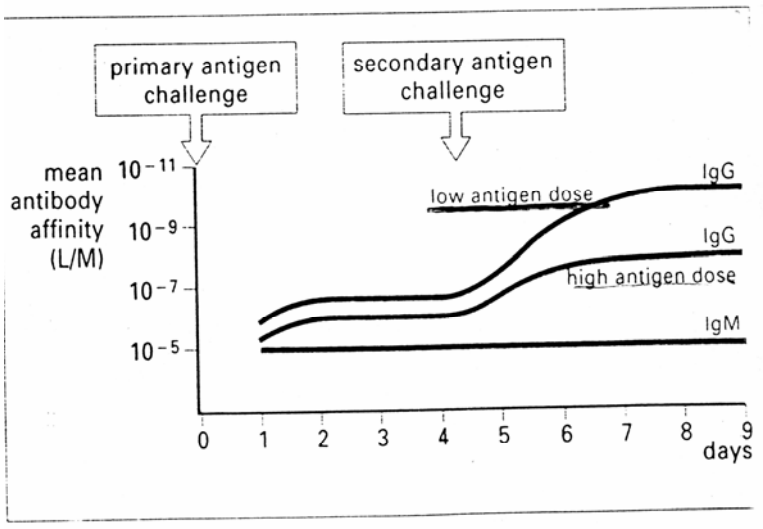


Fig. 7.26 The average affinity of the IgM and IgG antibody responses following primary and secondary challenge with a T-dependent antigen are shown. The affinity of the IgM response is constant throughout. The affinity maturation of the IgG response depends on the dose of the secondary antigen. Low antigen doses (low [Ag]) produce higher affinity immunoglobulin than high antigen doses (high [Ag]).

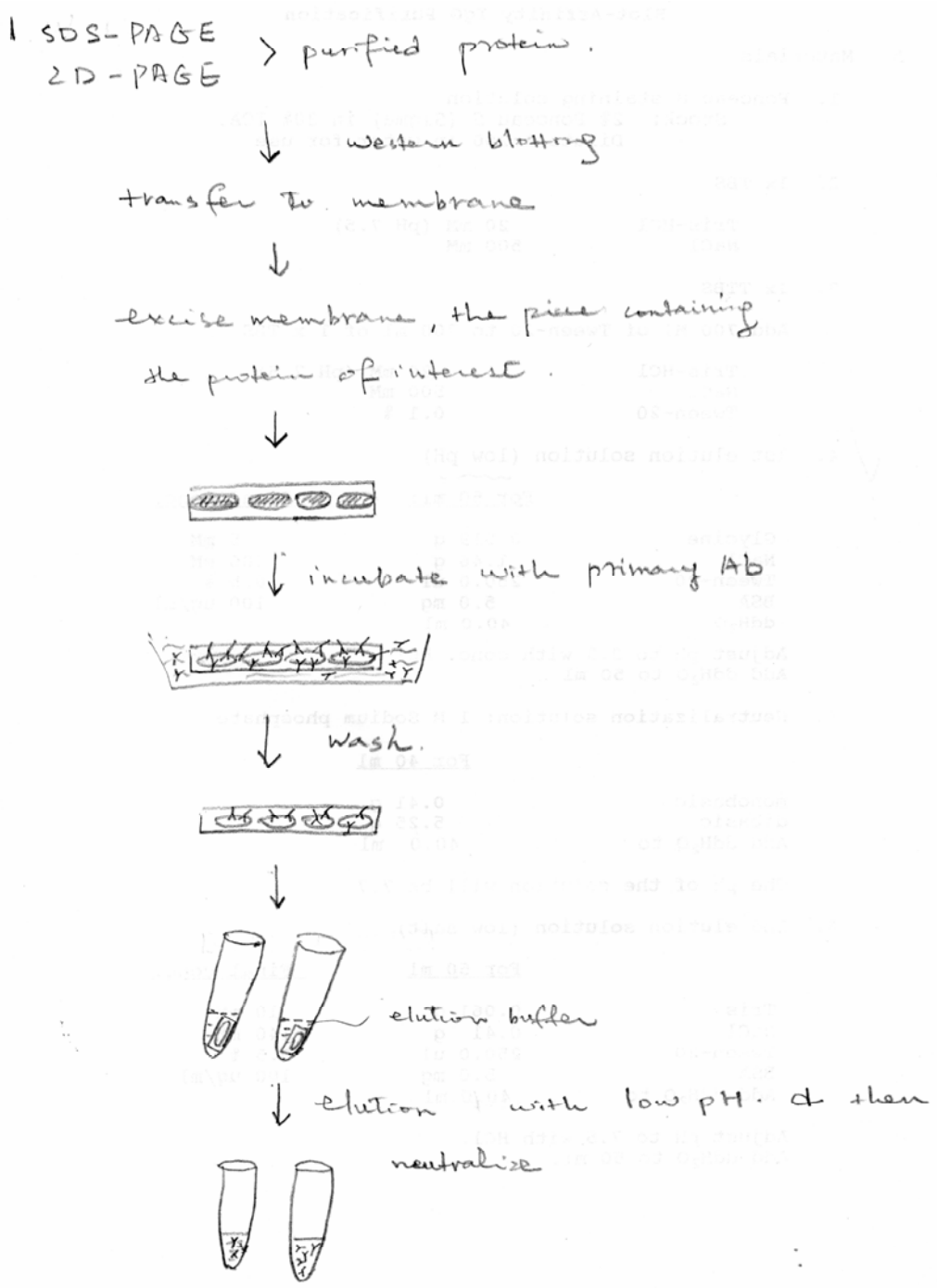
PART IV: Immunological Techniques

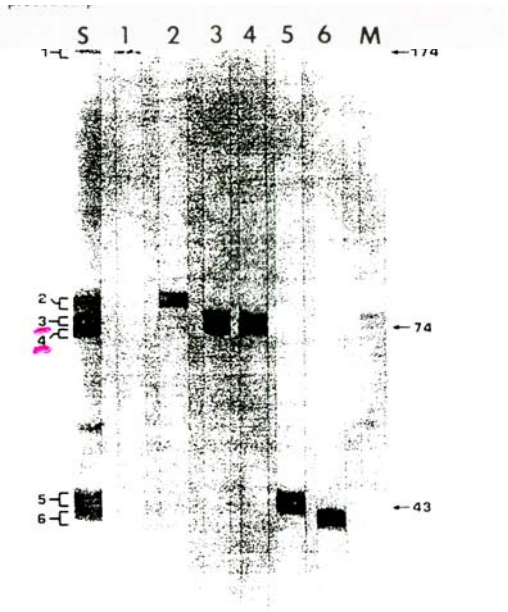
1. Affinity-purified antibodies

*Use affinity column chromatography or blot-affinity purification method.

*elution solution: 1) use extreme pH;

2) use chaotropic buffer such as 4 M, NaSCN, 6 M urea, 5 M guanidine-HCI



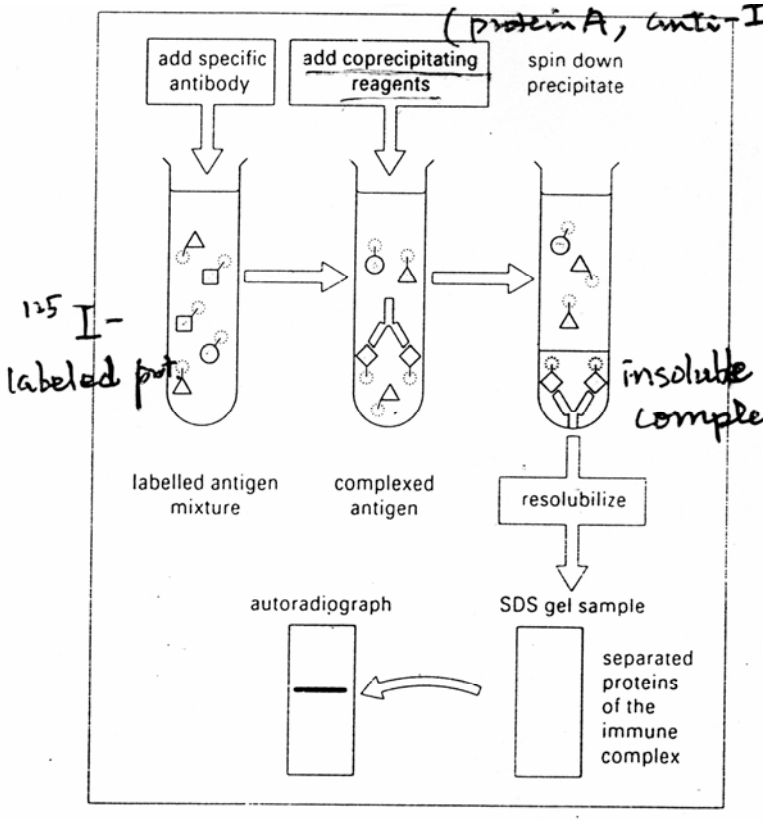


2. Immunoprecipitauon

*lmmunoprecipitation is used to detect and quantitate target antigens in mixtures of proteins. It is extremely sensitive and is capable of detecting as little as 100 pg of radiolabeled protein.

*When coupled with SDS-polyacrylamide gel electrophoresis, the technique is ideal for analysis of the synthesis and processing of foreign antigens expressed in procaryotic and eucaryotic hosts or in in vitro systems.

*The use of **protein A-agarose** beads or immunoprecipitin (protein A on bacteria membrane) in order to precipitate the antigen-antibody complexes for analysis.



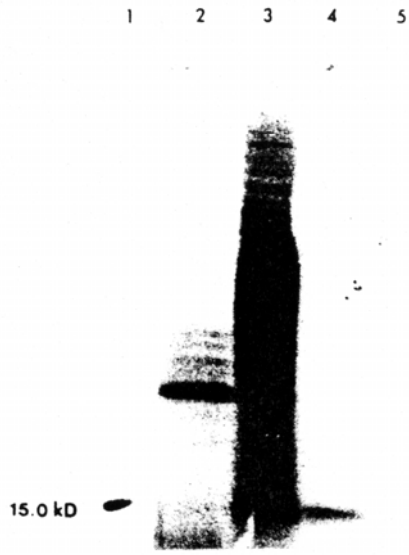
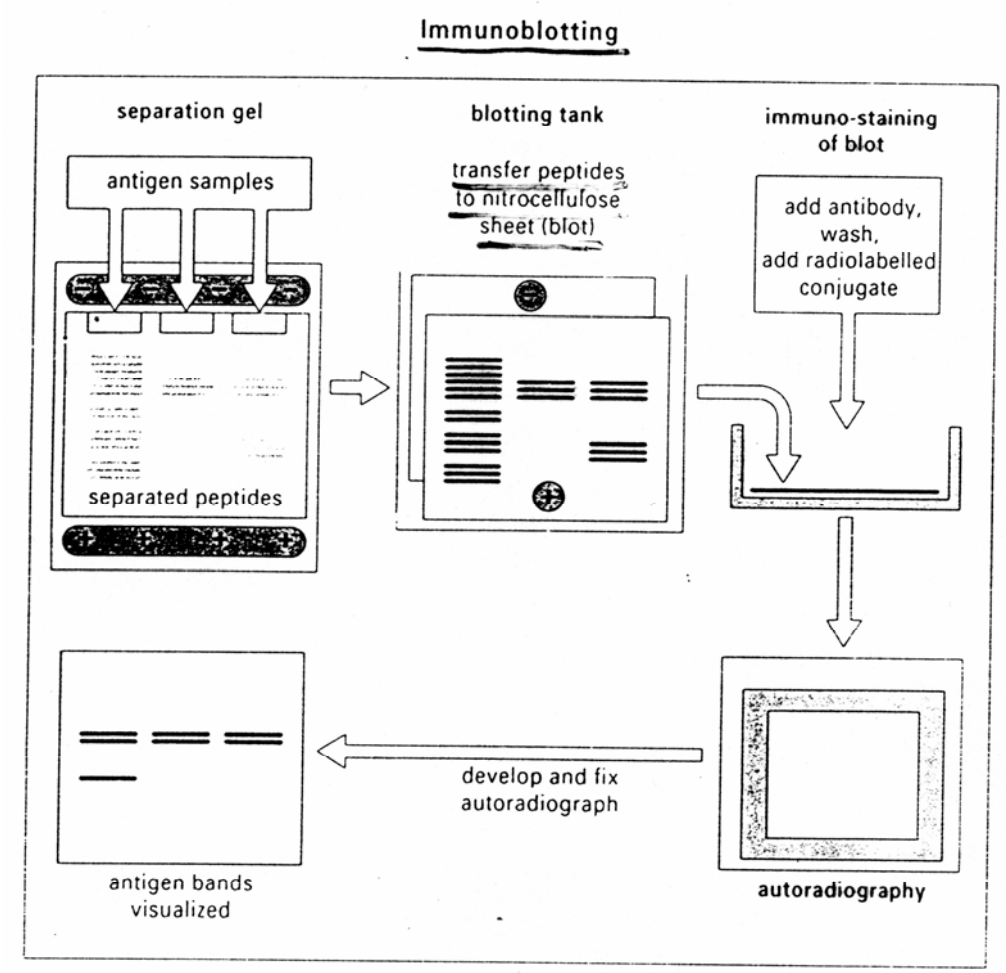


Figure 6. SDS-PAGE analysis of *in vitro* translation of total RNA from 15- to 20-mm lily anthers and immunoprecipitation of the LLA-15 protein. Lane 1 is a Coomassie blue-stained gel of 5 μg of purified LLA-15c–e proteins. Lanes 2 through 5 are *in vitro* translations that were carried out in the rabbit reticulocyte lysate with no lily anther RNA (lane 2) or with 5 μg of total RNA from 15- to 20-mm anthers (lanes 3–5). Total translation products (lane 3) were immunoprecipitated by anti-LLA-15 antiserum (lane 4) and by preimmune serum (lane 5). The translation products were fractionated by SDS-PAGE and fluorographed. The exposure was for 2 d at −80°C.

Figure 7. 2D-PAGE analysis of lily anther LLA-15 proteins synthesized *in vitro*. Total RNA isolated from 15- to 20-mm anthers was translated in the rabbit reticulocyte lysate. The translation products were immunoprecipitated by anti-LLA-15 antiserum, fractionated by 2D-PAGE, and fluorographed. The exposure was for 10 d at −80°C.

3. Immunoblotting (Western blotting)



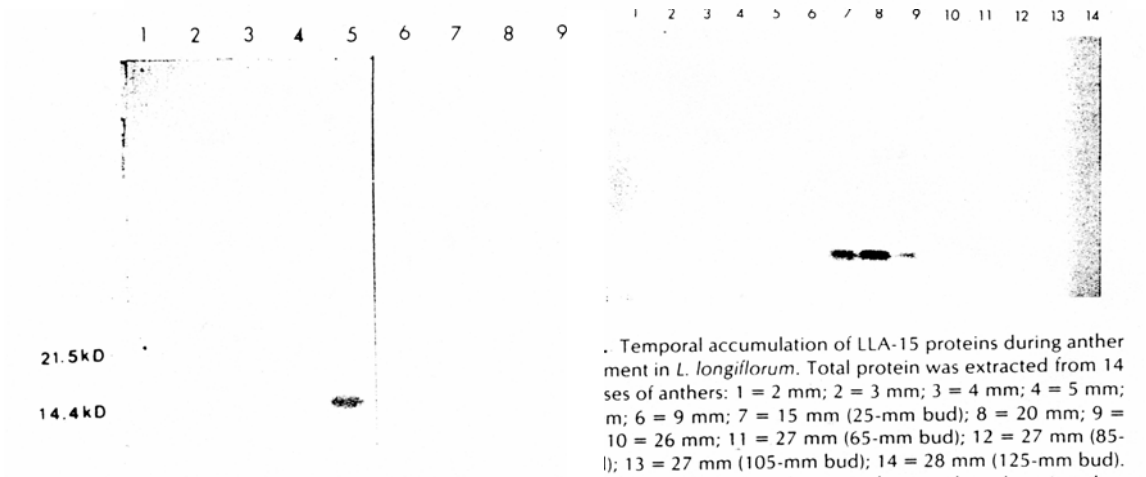
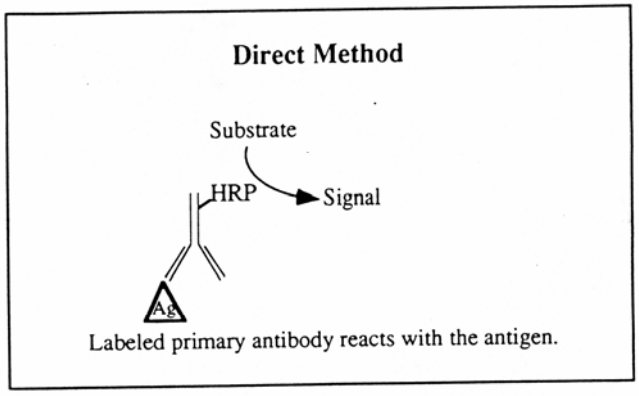


Figure 3. Immunoblot analysis of the distribution of LLA-15 in *L. longiflorum* organs. Total protein was extracted from vegetative and floral organs of 25-mm buds. Approximate of total protein from each organ was fractionated by SD electroblotted onto nitrocellulose, and immunochemically using monospecific anti-LLA-15 antibodies. Foliar leaves roots (lane 2), stems (lane 3), bulb leaves (lane 4), anthers filaments (lane 6), tepals (lane 7), ovaries (lane 8), styles and stigmas (lane 10). Positions of molecular mass mar

. Temporal accumulation of LLA-15 proteins during anther ment in *L. longiflorum*. Total protein was extracted from 14 ses of anthers: 1 = 2 mm; 2 = 3 mm; 3 = 4 mm; 4 = 5 mm; m; 6 = 9 mm; 7 = 15 mm (25-mm bud); 8 = 20 mm; 9 = 10 = 26 mm; 11 = 27 mm (65-mm bud); 12 = 27 mm (85-l); 13 = 27 mm (105-mm bud); 14 = 28 mm (125-mm bud). mately 12 μg of total protein from each anther size class tionated by SDS-PAGE, electroblotted onto nitrocellulose, nunochemically detected using monospecific anti-LLA-15 es. Three anther developmental phases (I–III) are indicated ne size classes (33). Phase I corresponds to the premeiotic Phase II corresponds to a period of microspore develop-nd major cytological changes in the wall layers. Phase III onds to pollen maturation. Positions of molecular mass are indicated.

1) ELISA (enzyme linked immunosorbent assay)--- detected by enzymes
2) RIA (radioimmunoassay) ---detected by isotopes
3) FIA (fluorescence immunoassay) ---detected by fluorescence

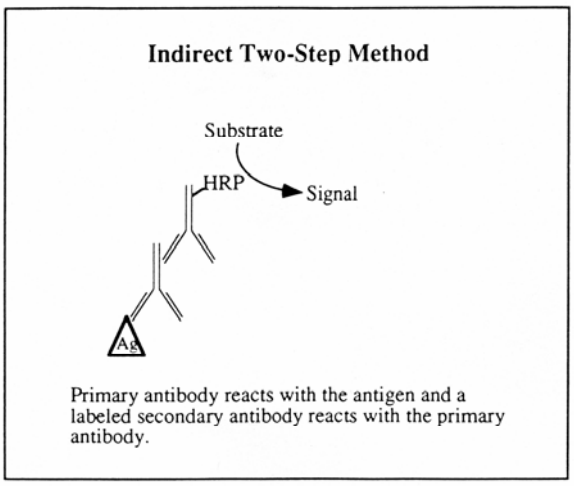
***Direct immunodetection** --- such as fluoresceinated antibodies



advantages: (1) quick method because only one antibody is used
(2) cross-reactivity of secondary antibody with the primary antibody is eliminated
(3) double staining is easily achieved using different labels on primaryantibodies from the same host

disadvantages: (1) immunoreactivity of the primary antibody may be reduced as a result of labeling
(2) labeling of every primary antibody is time consuming
(3) no flexibility in choice of label from experiment to expriment
(4) little signal amplification

***Indirect immunodetection** -- such as fluoresceinated anti-Ig; enzyme-linked Ig; enzyme-linked protein A; or biotinylated Ig and enzyme-labeled avidin system



advantages: (1) versatility because the same labeled secondary antibody can be used with different primary antibodies.
(2) immunoreactivity of the primary antibody is not affected by labeling.
(3) increased sensitivity

disadvantages: (1) icross reactivity may be a problem
(2) precision may be lost in electron microspore application.

Avidin-biotin immunodetection (an example)

There are several ways in which the avidin-biotin interaction can be used as a detection system. The method of detection can be chosen based on its convenience or its detection sensitivity. The three basic designs are the labeled avidin-biotin (LAB), the hndged avidin-biotin (BRAB) system and the avidin-jbiotin complex (ABC) system. These methods can be used in ELISA, blotting, or immunohistochemical staining techniques.

Figure 8: *The reaction scheme for all the interacting components involved with the LAB complex.* (Labeled Avidin Biotin complex)

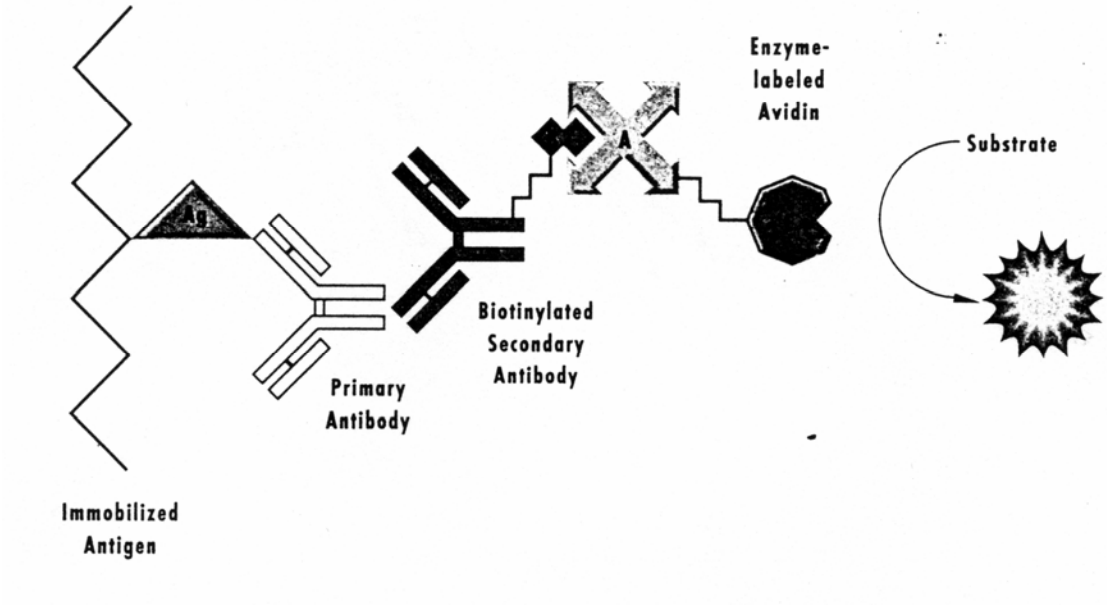


Figure 9: *The BRAB system.* (Bridged Avidin Biotin system)

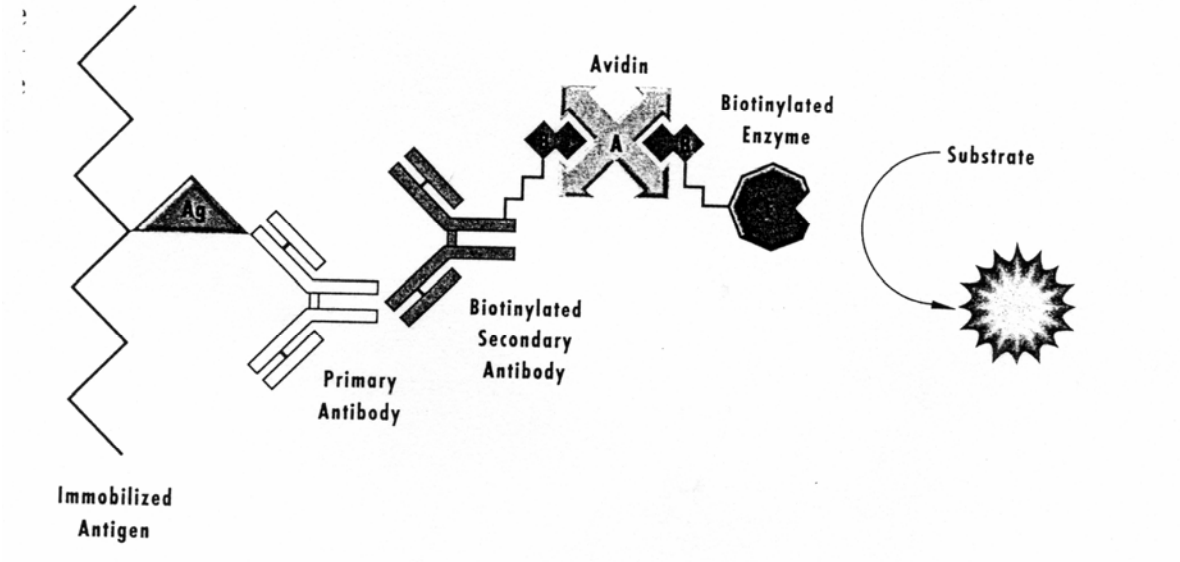
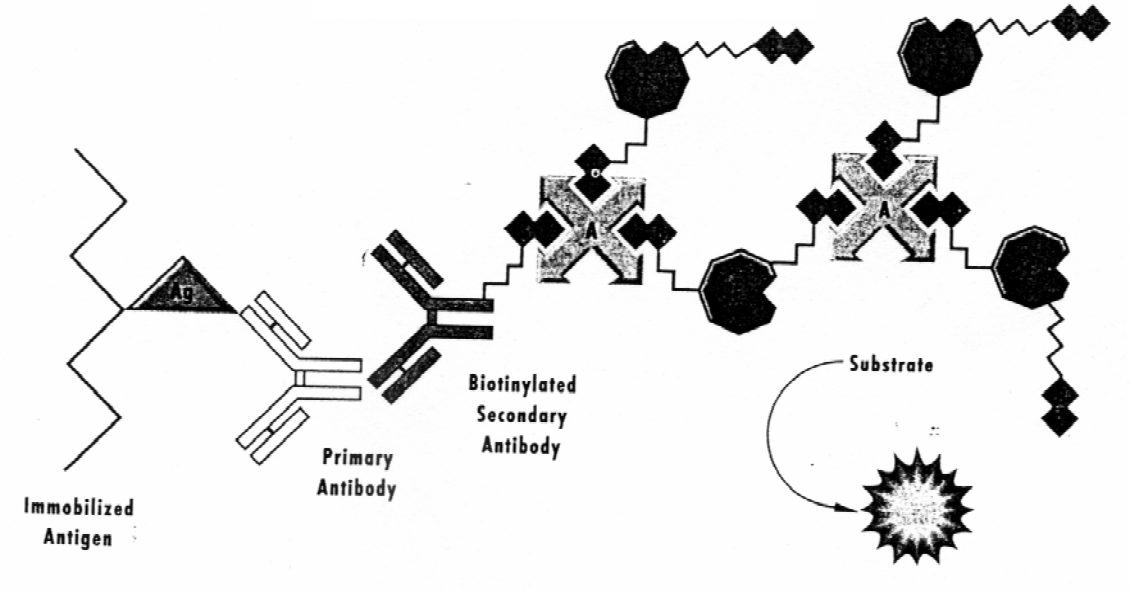
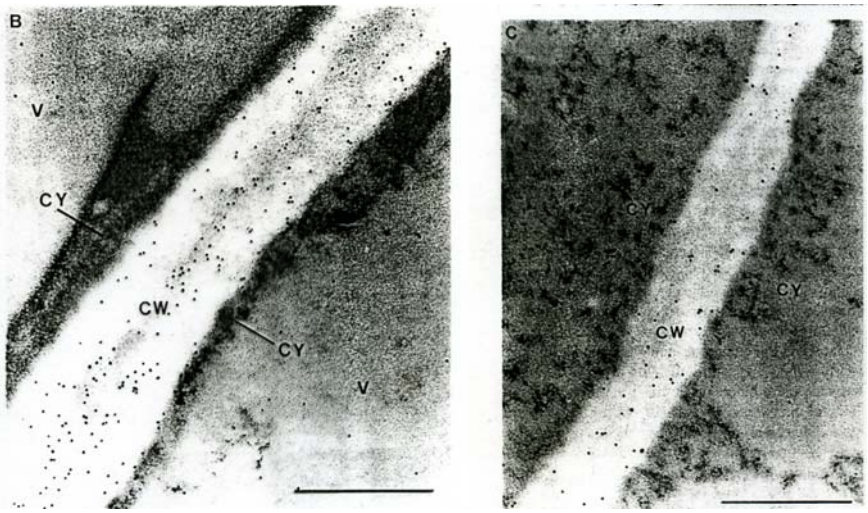


Figure 10: *The ABC system.* (Avidin-Biotin Complex)



4. In situ localization

1) visualized by **fluorescent** reagents; **gold particles**; **isotope**



2) **nonradioactive labeling**

Standard procedure of nonradioactive DNA labeling

(1) random labeling of hexanucleotides

* Incorporation of a nucleotide analog (**digoxigenin-11-dUTP**) into DNA by the random primed labeling technique.
* The labeling reaction incorporates a digoxigenin moiety every 20th to 25th nucleotide in the newly synthesized DNA.

(2) hybridization of filter-bound DNA with the digoxigenin-labeled probe.

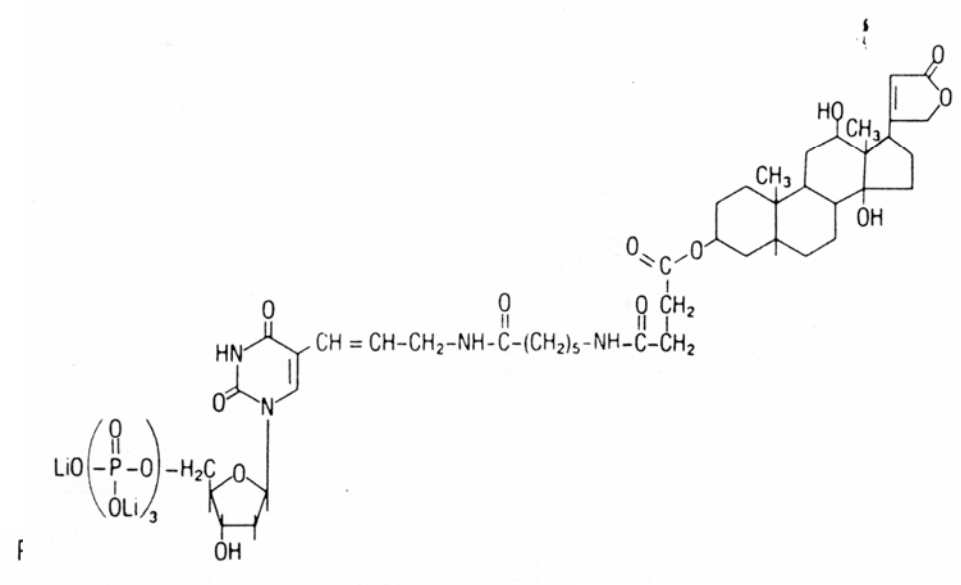
(3) location of the digoxigenin-labeled probe with an antibody-enzyme conjugate (anti-digoxigenin-alkaline phosphatase).

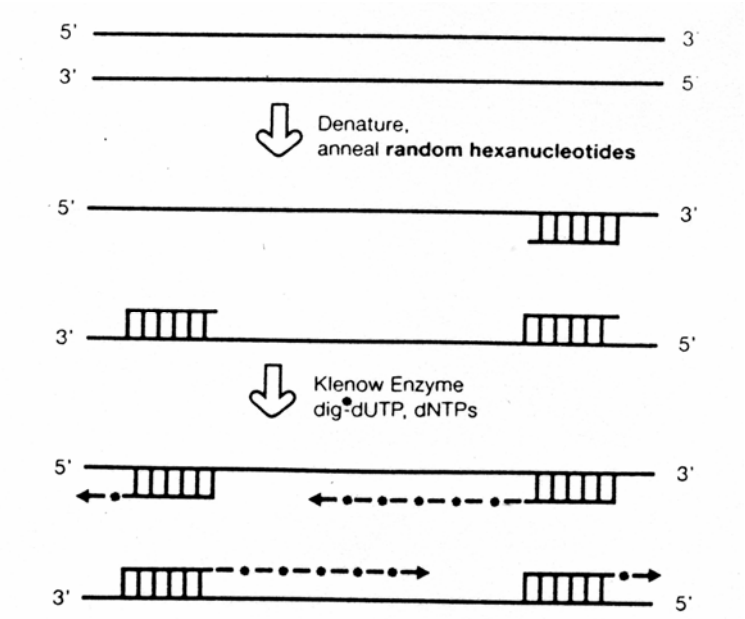
(4) detection of the DNA-digoxigenin-antibody-enzyme congugate complex by an enzyme-linked color reaction.

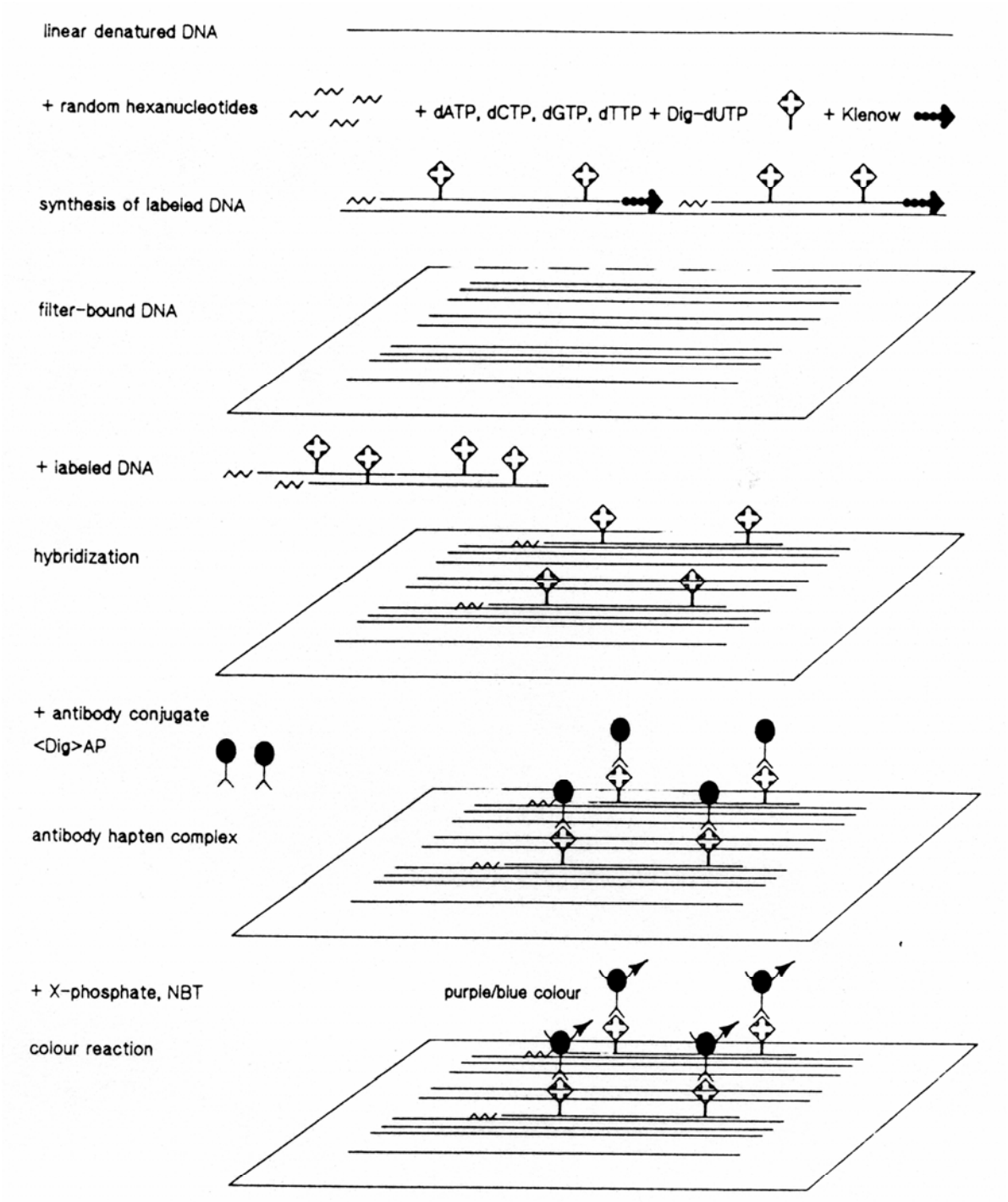
* The color reaction is initiated at alkaline pH by the addition of BCIP and NBT. A blue precipitate starts to form within a few minutes and continues up to 3 days.

*The color reaction is based on the oxidation of **BCIP** to indigo after the release of the phosphoryl group. In a simultaneous coupled reaction, **NBT** is reduced to diformazan. Each process leads to the formation of blue insoluble color precipitates. The precipitate adheres directly to the membrane, producing a blueish color on nitrocellulose, and a brownish color on nylon.

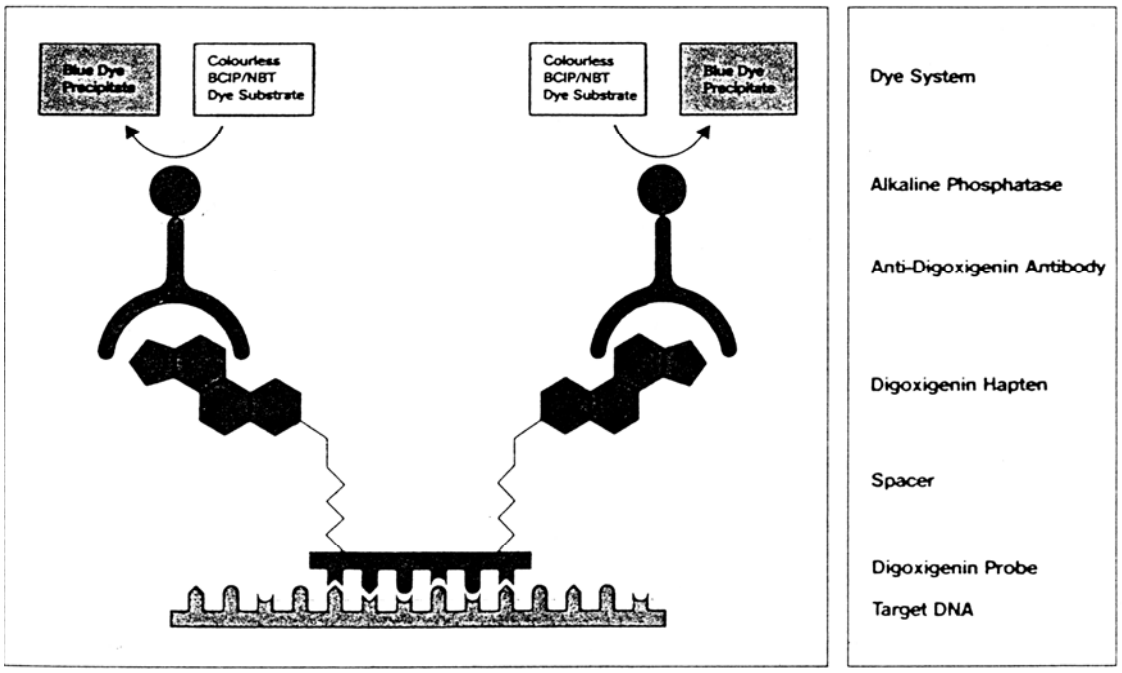
Structure of Digoxigenin-11-dUTP.







A Labeling and Detection Kit.



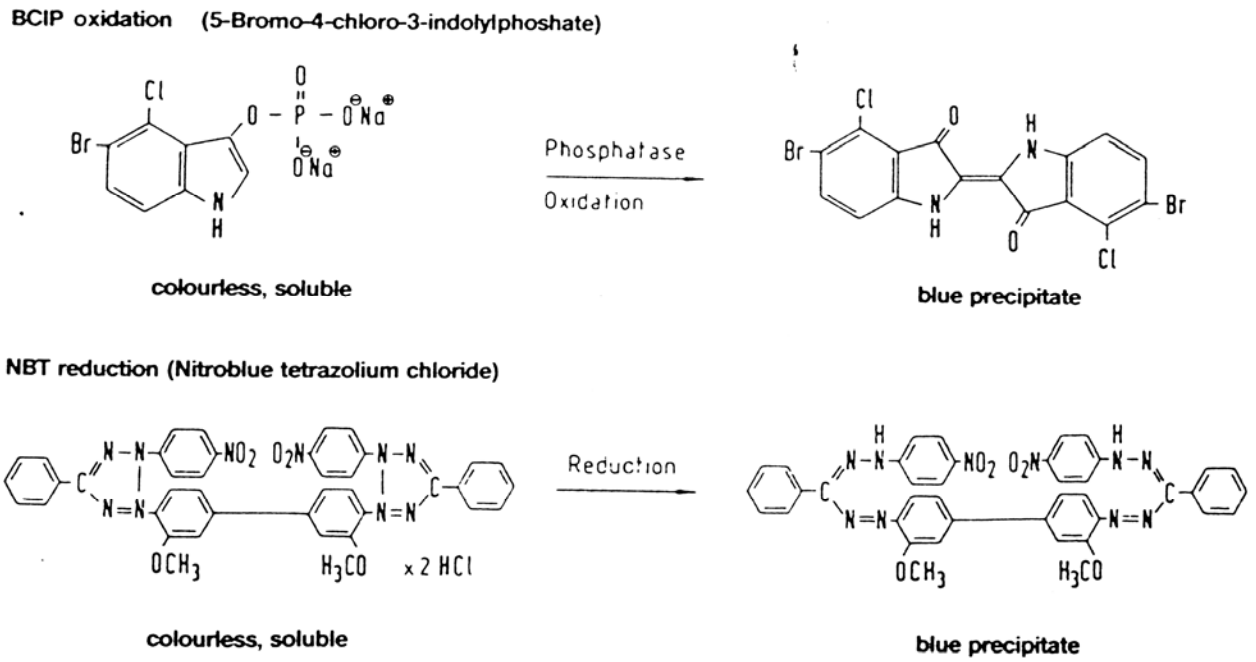


Figure 17. Substrates and reaction products of alkaline phosphatase catalyzed color reaction with BCIP/NBT.

5. Immunoscreening

*Ideally, an antibody used for screening of expression libraries should be 1) polyclonal or a pool of monoclonal antibodies, 2) of high titer 3) specific for conformation-independent epitopes that are displayed on both native and denatured forms of the protein.

*To determine whether the available batches of antisera are suitable as probes, serial dilutions of several independent antisera should be tested for the following properties:

1) Reactivity with the protein of interest on a Western blot.
2) If antisera react with several different proteins, the antibody should be further purified by affinity chromatography on columns of the immobolized antigen.
3) Affinity-purified antibodies are usually preferred because low backgrounds may be generated than whole sera.
4) Production of weak or undetectable signals when reacted with bacteria expressing the sequences of the vector alone.

*The probability of identifying a recombinant clones of interest is only **1/6** taking into account the fact that a given DNA fragment can be inserted into the vector in two possible orientations and that only one of the three potential reading frames is correct for expression.

*Antibody reactive sequences should be tested for the presence of at least one other distinguishing property that the desired sequence may reasonably be expected to have. These distinguishing properties may be unusual mRNA or encoded protein sizes, regulated expression in appropriate cell types, expression of the expected gene product.

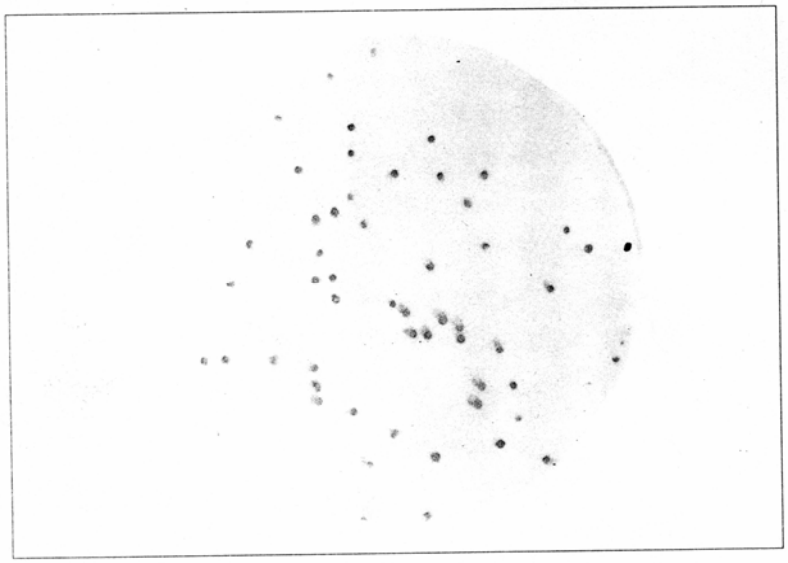


Figure 32.
Detection of λgt 10 phages containing a pBR 322 insert.