vestigator to attempt reasonable quantitation, it is essential to keep the exposure time within the linear range of the film (roughly the middle 10-fold from threshold to saturation).

Thus, in the example above (50-fold signal at 2 days), one would need to decrease the exposure intensity (from 50-fold to about 5-fold), which could be done by reducing the exposure time by a factor of 10 (to 4.8 hours). Of course, the labeled tissue may be re-exposed to different x-ray films until a correct exposure is obtained. For emulsion-dipped slides, several series of test slides are developed after different time periods to establish optimal exposure time for the body of the experiment. The photomicrograph in Fig. 3 illustrates the results of such an autoradiographic study, in this case involving the localization of a class of opiate receptors.

Having established a set of binding conditions for a particular ligand and also having produced autoradiograms within the dynamic range of the emulsion, it is now possible to quantitate the binding. Such quantitation is usually undertaken to compare specific binding across anatomical regions, to evaluate receptor subtypes within a tissue region, or to study changes in receptor levels within regions after various treatments. The methods commonly used in quantitation of autoradiographic data involve digitization of the x-ray (or emulsion dipped) signal, and passage of data to a common personal computer. Along with the digitized value of the autoradiographic signal, separate values for the background area, and area containing only nonspecific binding are usually taken. Through subtraction of background and nonspecific binding, an estimate value for specific binding is obtained. By comparing the signal density (per square micron) with densities produced by a series of known radioactive standards, and knowing the specific activity of the ligand, it is possible to calculate the number of moles of labeled ligand per gram of tissue.

**Advantages**

The advantages of receptor autoradiography have become increasingly clear over the last decade, making it a standard technique in a large part of biology. The validity of the method has been supported by data using alternative methods for localization of receptors (or their mRNAs) such as immunocytochemistry and in situ hybridization (see below and Chapter 19, *this volume*). Advantages include:

1. Pharmacological relevance in an anatomical context.
2. Medium to high level of anatomical resolution.
3. Ability to quantitate receptor binding, and thereby estimate number and affinity of receptor binding sites, allowing the study of regulation of receptor systems in a large number of tissues, systems, and conditions.

**Limitations**

1. Because receptor proteins are largely transported along axonal or dendritic processes, much ambiguity can arise in the distinction between neuronal perikarya and other cellular processes.
2. The slice autoradiographic method is limited with respect to complex biochemical techniques. For example, the best binding conditions for the natural ligand may be different than those for a pharmacological analogue, and might be difficult to process on a tissue slice. Moreover, the second messenger system coupling actions of the receptor may differentially affect the affinity of the natural ligand or analogue for the site.
3. Finally, even with our best pharmacological efforts, it is clear that we do not have ligands capable of selectively binding to many of the receptors that have been cloned, and thus it is probable that in some instances more than one receptor type is bound. While this issue is slowly being resolved as new clones and ligands come to the fore, it is important not to lose sight of the fact that one is detecting "binding activity" only, rather than a specific gene product or protein itself.

**IMMUNOCYTOCHEMISTRY**

Immunocytochemistry is a method for studying proteins in an anatomical context through the use of specific antibodies directed to detect a particular antigen in tissue (see ref. 34). Briefly, the tissue to be studied is fixed so that the antigen (protein) is kept in place (and not degraded). An antibody directed against that target protein binds it in a thinly sectioned piece of tissue. After washing to remove excess antibody, a second series of antibodies or other marker protein is used to visualize the original (or primary) antibody, thereby indirectly marking the location of the protein in question. As applied to neuroanatomy, this protein might be a neurotransmitter molecule, neurotransmitter-synthesizing enzyme, receptor, or other protein. In addition to neuronal localization, the technique is also widely used to provide valuable information on glia and other non-neuronal cells.

Prior to addressing the method itself, it is valuable to review some basic aspects of the structure of immunoglobulins (IgG in particular) and the antibody–antigen interaction. Antibodies are divided into five classes, with immunoglobulin G (IgG) type being the most commonly used in immunocytochemical methods. IgGs are 160-kD proteins comprised of two heavy chains and two light chains connected by disulfide bonds (Fig. 4A). The structure of the IgG molecule is divided into three domains: hypervariable, variable, and constant. The hypervariable region is the primary source of antigen-binding specificity. Most of the flexibility of IgG binding is genomically deter-
mained in this region. The variable region contributes additional sources of protein variation in the unique binding qualities of any particular IgG clone. Finally, the constant region is a nonvariable "structural" component of IgG. This region is common to all IgGs in a particular species. It is known that the hypervariable and variable part of the molecule are the receptor portions of the IgG molecule. Fragments of IgG which contain these regions continue to exhibit active binding. In contrast, the constant region of the IgG molecule can itself serve as an antigen. Thus, in an immunocytochemical reaction the very same IgG may bind antigen (at its hypervariable segment) while its constant region acts an antigen for a second antibody (Figs. 4A and 5). Hence, a series of antibodies can be layered, and this amplification can be used to enhance signal strength (35).

An additional set of concepts, that regarding antigen and epitope, warrant discussion. By definition, an antigen is a protein (usually) which can be bound by a specific IgG. An epitope is the few amino acids in the antigen protein to which a specific IgG binds. Figure 4B illustrates the protein sequence with several epitopes (they are often "bends" in the protein. One epitope sequence is highlighted (ABCDE) to indicate the small size of the average epitope (about 4–6 amino acids). Any one IgG molecule (from a single B lymphocyte which itself has been grown in a clonal fashion) binds to only one epitope. In the case of a monoclonal antibody, a single species of specific IgG molecules is used that is specific for a single epitope. A polyclonal antisera contains multiple IgG species which may bind to any of a number of available epitopes. Interestingly, a monoclonal antibody may be very precise, but because the average epitope is small compared to the total size of the protein (e.g., 4–6 out of 400), one must be concerned about the potential for "cross-reactivity" with an identical sequence in another protein. A polyclonal antisera reacts with any of several epitopes on the target protein, but the diversity of IgG types may cause cross-reactivity with similar epitopes on other molecules. Overcoming the problem of cross-reactivity often requires
fairly thoughtful strategies: multiple antisera, biochemical extraction and characterization of antigen, affinity purification of antibody, and so on.

While the production of antisera and preparation of pure proteins are beyond the scope of this chapter, a few points are worth mentioning. The use of an impure protein preparation (containing multiple proteins) to stimulate antibody production will most likely generate an antiserum with IgGs directed against the protein of interest as well as against protein contaminants. Such a serum may bind some, many, or most of the proteins in an antigen mix. Furthermore, if a large excess of this same antigen mixture is incubated with the antiserum (before it is used on the tissue section), as is commonly performed as a control, the specific signal will probably be blocked and the remaining staining inferred to be specific! Thus, use of a dirty antigen for antibody production can be a source of a mixed antiserum and improper controls.

Assuming that one has a clean antigen and a good antiserum, some considerable effort is then put into establishing optimal conditions for the procedure. Probably the most troublesome is the need to establish fixation chemistry conditions. There is a complex list of fixative reagents, buffers, mixtures, and times which can be tested. Generally, most investigators begin with a neutral buffered 4% paraformaldehyde solution, and then vary mixtures of additional reagents (glutaraldehyde, acrolein, picric acid, alcohols, etc.). It is possible to use carbohydrate or lipid fixatives, or even nucleic acid fixatives. The goal is to preserve the antigen in its original cellular context and prevent degradation, while still rendering it "visible" to the antiserum. It is worth noting that cross-linking proteins with fixatives may hide epitopes normally available in the native state; thus, excessive fixation is also a consideration. Once a fixation condition is established, a "working titer" of the antibody is determined. Generally this refers to the dilution of the original antiserum that produces optimal signal while minimizing nonspecific background. Most good antisera can be used in the dilution range of 1:500 to 1:50,000 or more! Other variables include antibody buffer conditions, tissue thickness, and length of incubation. Finally, a detection system (peroxidase histochemistry, fluorescence, etc.) is chosen based on individual needs and level of analysis. This step may also require some fine-tuning.

Method

In the preceding section we have focused on a variety of concepts and issues related to immunocytochemical methods. Here we attempt to present some more detailed considerations concerning application of the technique. The example chosen involves peroxidase histochemistry for visualization, although many of the principles apply to other detection methods as well.

Prior to sectioning, the tissue of interest is usually treated with a protein fixative (e.g., paraformaldehyde). As noted above, the fixation and associated parameters are usually determined empirically. The next step is to dilute the primary antiserum and apply it to the section for 16–48 hours. For the purposes of demonstration, let us begin with a rabbit anti-encephalin IgG applied to a slice of rat caudate. After incubation with this primary antiserum, the tissue sections are thoroughly washed and a second antiserum is applied for a period of few hours, up to 24 hours. This secondary IgG was produced in goat and raised against rabbit IgG (remember, the primary IgG protein acts as antibody when binding encephalin, but it acts as antigen when bound by the goat anti-rabbit IgG). We thus have a section with encephalin bound by a rabbit IgG, which itself is bound by goat IgG directed against rabbit IgG. Following another wash, the next step is the addition of another rabbit IgG for 1–2 hours. This antibody is directed against the enzyme horseradish peroxidase (HRP), but is bound at its constant domain by the remaining binding site of the secondary IgG (goat anti-rabbit). [Note: All IgGs have two binding sites and can thus capture two molecules of antigen.] Following another wash, the tissue is incubated for 1–2 hours in a solution containing the enzyme HRP. [Note: the latter two steps can be replaced with the peroxidase–antiperoxidase (PAP) complex—see ref. 35]. Finally, the tissue is reacted with the HRP substrate hydrogen peroxide in the presence of a chromagen (e.g., diaminobenzidine), resulting in a colored precipitate at the site of this whole antigen–antibody complex. After washing and dehydrating, this precipitate is visible as a brown stain within the antigen-containing structures. The general method can be used at a very high level of resolution (including ultrastructural analysis) and across a large number of proteins (and nonproteins as well). This immunocytochemical sequence, as well as a related strategy (18) involving biotinylated IgGs and the avidin–biotin peroxidase complex (ABC), is outlined in Fig. 5, and an example of its application is shown in Fig. 6.

Technical controls are a very central part of this method. At a basic level it is important to know that the staining seen after the reaction sequence is specific to a particular protein. Criteria that need to be satisfied include the following: (a) absence of staining when the protocol is run with deletion of the primary antibody, (b) absence of staining when antiserum is applied that was preincubated in the presence of a large excess of antigen, and (c) lack of staining with application of the enzyme or substrate alone. (Interestingly, some tissues have endogenous peroxidase activity that can be confused with specific staining following immunocytochemical protocols involving this enzyme; this problem can usually be avoided by pre-treatment with hydrogen peroxide.) In addition, some non-antigen-related signals are considered to be false-positives. It is important to realize that a "nonimmune"
rabbit serum (often used to block nonspecific IgG sites in tissues) is actually loaded with IgGs produced by the rabbit over time, and can therefore be the source of nonspecific labeling. This potential pitfall can be overcome through the use of blocking agents not derived from normal serum (e.g., addition to the incubation mix of carrageenan, a gum that is effective at blocking nonspecific IgG binding sites in tissues). Perhaps the most elaborate control for specificity is the extraction of antigen from tissue, followed by biochemical characterization and quantitation. Other approaches to specificity and precision may be seen in the use of multiple antisera against the same antigen, or even antisera directed against different epitopes on the same antigen.

Advantages

1. Immunocytochemistry is a broad-based and powerful method for analyzing specific biochemical structures and sequences at the cellular level.

2. Multiple cellular compartments may be amenable to study, including the cell body, axons, and dendrites.

3. Minute levels of protein can be visualized in cellular compartments and membranes.

Limitations

1. Perhaps the greatest concern in immunocytochemistry is the question of specificity. It can be quite difficult to prove antigen specificity, particularly in view of the potential for cross-reactivity.

2. Immunocytochemistry is generally not quantitative. There are a number of reasons for this, including multiple epitopes, different affinities for different sizes and sequences of various protein versions, variable fixation, and variable tissue penetration by antisera.

There are several other considerations that can make immunocytochemistry a complex, expensive, and often
acids of all of the opioid peptides are tyrosine-glycine-glycine-phenylalanine. This sequence is found once in the β-endorphin/adrenocorticotropic hormone (ACTH) precursor, three times in the pro-dynorphin precursor, and seven times in the pro-enkephalin precursor! A monoclonal antibody directed against this epitope could potentially find 11 targets in any of three propeptides (see ref. 23). The use of polyclonal antisera can also be somewhat problematic, because they are produced in limited amounts. Moreover, polyclonal antisera can react with different epitopes over different bleeds from the same source.

IN SITU HYBRIDIZATION

In situ hybridization is a method which allows for the detection of mRNA molecules, usually within their cells of origin (10,11,19,30,32,37,38,43). In a sense the method is similar to receptor autoradiography and immunocytochemistry, in that all three techniques rely on some form of binding to form stable complexes. Receptor autoradiography involves binding of receptor by radiolabeled ligand, and immunocytochemistry involves binding of antigen by antibodies. In situ hybridization, binding occurs between mRNA in the cytosol and an externally produced radiolabeled RNA or DNA probe capable of forming a hybrid with it. Detection of the radiolabeled probe is then performed by methods similar to those used in receptor autoradiography (x-ray film or photographic emulsion).

The most important concept for understanding this technique is that of “hybridization.” Two strands of RNA, two strands of DNA, or one RNA and one DNA can bind (or hybridize) through specific hydrogen bonding between chains. In Fig. 7, two strands of RNA are depicted (see inset box). One (AUGCCUCAU) represents a short strand of mRNA; the strand below it (UACGGA-GUA) is complementary to it (called cRNA). Inspection of the figure shows that A always binds U and that G always binds C. In fact, A shares two hydrogen bonds with U (or T in a DNA strand), and G shares three hydrogen bonds with C. While any single hydrogen bond is weak, a series of them can form a quite stable complex. In the example shown in Fig. 7 there are 5 A-U pairs (10 hydrogen bonds) and 4 G-C pairs (12 hydrogen bonds), resulting in a fairly stable hybrid of 22 hydrogen bonds. A stable combination of RNA–RNA strands is easily obtainable by as few as 25–30 bases (50–75 hydrogen bonds). This simple piece of chemistry forms the basis for a large number of molecular biological methods (in situ hybridization, Northern analysis, Southern analysis, cDNA library screening, etc.), not to mention its central role in stabilization of the double-stranded DNA of the genome itself!

The in situ hybridization method is described below. It is not very different from many aspects of receptor