METHODS OF BIOCHEMICAL ANALYSIS

Edited by DAVID GLICK

VOLUME 33

PREFACE

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned not only with the results in the developing fields but also with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, applier, the teacher, and the student.

It is particularly important that review services of this nature should include the area of methods and techniques because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore, an emphasis on methodology and instrumentation is a fundamental need in order for material achievement to keep in sight of the advance of useful ideas.

The volumes in this series are designed to try to meet the need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological, and if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, and so on.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate, these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critial evaluation of the various approaches, and a presentation of the pro-

cedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analysis.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, and point of view. It is the Editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the Editor to make this series of volumes as useful as possible and to this end suggestions will be always welcome.

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Protein Blotting: A Manual

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I. SCOPE

Over the past 10 years, blotting procedures have become an essential element in the biochemical analysis of DNA, RNA, proteins, and lipids.

As could be expected, numerous articles have been written on various technical aspects of these methods and many more have appeared in which blotting, of one type or another, has been employed as a means to study biological problems.

The subject of protein blotting has already been reviewed (Gershoni and Palade, 1983; Haid and Suissa, 1983; Gooderham, 1984; Symington, 1984; Towbin and Gordon, 1984; Bers and Garfin, 1985; Gershoni, 1985; Beisiegel, 1986) and the main points of this technique are well established. Therefore, this chapter is not intended to be an updated and extensive review of the literature, but rather to provide a practical description of how to blot and analyze proteins. By no means should the protocols given here be regarded as the best possible approach. They have been selected because they are generally simple and reliable. They can almost certainly be improved upon and should be adapted to the specific needs of the system being tested. The concepts described should enable the reader to determine whether a given biological system is amenable to blot analyses. Each section deals with a particular step of blotting, and examples are given to demonstrate the variety of approaches and applications that have been adopted. Obviously, the examples cited are only a representative selection of the many articles published.

II. HISTORICAL PERSPECTIVE

The combined use of sodium dodecylsulfate (SDS) with polyacrylamide gel electrophoresis (PAGE), and discontinuous buffer systems (Laemmli, 1970; Neville, 1971) provides the investigator with the means to evaluate the purity or the complexity of protein mixtures being studied. The resolution of the constituents of the samples analyzed has been increased considerably by the development of two-dimensional gel electrophoresis (O'Farrell, 1975). Nonetheless, identification and characterization of individual peptides require the ability to further probe the electrophoretogram. Thus, various overlay techniques have evolved (Adair et al., 1978; Burridge, 1978; Glenney and Weber, 1980; Carlin et al., 1981; Snabes et al., 1981; Adair, 1982). Overlay of gels with antibodies or lectins, for example, allows the identification of antigens or glycoproteins, respectively. The manipulation of the gels, however, is often cumbersome and not always sufficiently sensitive. Therefore, it was a marked improvement when blot techniques, originally developed for the analysis of DNA (Southern, 1975), were applied to proteins as well (Erlich et al., 1979; Renart et al., 1979; Towbin et al., 1979; Bittner et

al., 1980; Bowen et al., 1980). Rapidly, numerous protocols evolved in which almost every possible element that could be modified, was. Various gel systems have been used ranging from acrylamide to agarose, from SDS denaturing to isoelectrofocusing. Different immobilizing matrices were developed, and changes in the buffer systems have been made. Probing the blots has been accomplished with a diversity of ligands, antibodies, and lectins, as well as with nucleic acids. Even the conditions for washing and blocking the filters have been examined. However, no ideal procedure has emerged and most probably none will. Therefore, only guidelines can be prescribed, to be custom tailored to one's specific needs.

Curiously, "blottologists" are notorious for their need of jargon and the field has been flooded with lab slang. Many of the terms range from the misleading to the uninformative and a few are simply distasteful. Nonetheless, without going into the origins of the terms that have evolved, there are a few that have become generally accepted. Thus, Southern, Northern, and Western blots refer to the blot analysis of DNA, RNA, and protein, respectively. "Immunoblotting" has become the generic term for the analysis of Western blots with antibodies. Dot blotting is the analysis of macromolecules applied directly to the immobilizing matrix as opposed to transferring them from a gel. Table I presents a collection of blot terms and their meanings.

II. A TYPICAL BLOT EXPERIMENT

Before considering the different parameters that can affect blot analysis, it is useful to outline a typical experiment.

In this example, a crude protein mixture is to be analyzed for the purpose of detecting a particular polypeptide, which is "the antigen" for a given antibody.

The protein mixture is first separated into its constituents, most commonly on a SDS-polyacrylamide gel. After electrophoresis, part of the gel may be stained with Coomassie brilliant blue to serve as a reference, and the remainder is used for blotting. A piece of membrane filter, usually nitrocellulose, is applied to the gel and this assembly is then secured in a cassette, which is placed into a transfer apparatus (ostensibly a Plexiglas tank equipped with two electrode arrays). Electrotransfer is performed for a number of hours, and then the gel and filter are removed.

The blotted gel may be stained to determine the efficiency of protein elution, while the blot is quenched in a buffer, containing protein and/

TABLE I

An Unabridged Glossary of Blot Terms

Block: See **quench**.

Blot: *n.* The product of blotting, that is, the transferred immobilized electrophoretic pattern, also referred to as "replica." *v.* The process of transferring macromolecules from gels to an immobilizing matrix. Thus, DNA blotting, RNA blotting, and protein blotting deal with the transfer and immobilization of DNA, RNA, and protein respectively.

Blotch: An unsuccessful blot (D. Lester, personal experience).

BLOTTO: Bovine lactotransfer technique optimizer, that is, nonfat dry milk used as a quencher (Johnson et al., 1984).

Capillary blotting: Blotting according to Southern (1975), that is, transferring when the driving force for elution is the convection (movement of fluid) through the gel and filter due to the capillarity of absorbent paper placed on top of the immobilizing matrix.

Colony blot: A technique in which colonies of bacteria grown on an immobilizing matrix (usually nitrocellulose) are probed with DNA or RNA probes or specific antibodies to detect desired recombinant transformed bacteria. In the latter case the bacteria are transformed with recombinant-expression vectors and are grown under inductive conditions (Stanley, 1983).

Convection blotting: See **capillary blotting**.

Detergent blot: A technique developed to detect membrane proteins. A gel containing detergent (e.g., Nonidet P-40) is placed between the acrylamide gel to be blotted and the immobilizing matrix. The hydrophobic integral membrane proteins are trapped in the mid-gel while the more hydrophilic proteins pass through this barrier and are caught on the membrane filter (Ito and Akiyama, 1985).

Dot blot: The process of analyzing samples that are directly applied to immobilizing matrices. Application may be achieved with a micropipette, and the droplets (2–3 μl) thus form dots. Vacuum manifolds have been developed and are commercially available to perform dot-blot analyses of larger volumes of samples (Hawkes et al., 1982).

DBM: Diazobenzyloxymethyl paper, an immobilizing matrix in which diazo groups are covalently linked to cellulosic paper. The diazo groups provide the ability to covalently immobilize the blotted macromolecules (Alwine et al., 1979).

DPT: Diazophenylthioether paper (Reiser and Wardale, 1981; Reiser and Stark, 1983); see **DBM**.

Diffusion blotting: The process of transferring macromolecules from gels to immobilizing matrices by way of diffusion. In such instances two identical blots can be obtained (Bowen et al., 1980).

Eastern blotting: Blotting of proteins from isoelectrofocusing gels (Reinhart and Malamud, 1982b).

EITB: Enzyme-linked immunoelectrotransfer blot technique (Tsang et al., 1983).

Electroblotting: The electrophoretic transfer of macromolecules from chromatographic gels to immobilizing matrices.

Golden blot: The process of analyzing a blot with a colloidal gold probe (Brada and Roth, 1984).

HRP: Horseradish peroxidase, the enzyme commonly used in enzyme-linked assays.

Hybridization: A term derived from nucleic acid analyses in which the probe binds to the immobilized DNA or RNA via hydrogen bonds of specific base pairing. It is sometimes misused in referring to the binding of proteins or ligands to blotted proteins (e.g., antibodies with their respective antigens).

TABLE I (*continued*)

Immunoblot: The process of analyzing a blot with an antibody for the purpose of detecting an antigen.
Immunogold: Colloidal gold associated with an antibody or *S. aureus* protein A to be used as a detecting reagent for the localization of an immobilized antigen.
LPS blotting: Transfer and immunodetection of lipopolysaccharides (Bradbury et al., 1984).
Native blot: The process of transferring proteins from isoelectrofocusing gels (Reinhart and Malamud, 1982a).
NC: Nitrocellulose membrane filter.
Northern blot: RNA blot.
Overlay: The process of probing a blot, that is, incubating a blot in a solution containing a probe. Originally derived from assays in which the gel was maintained stationary and horizontal and actually covered with a minimal layer of fluid containing a probe.
PCM: Positively charged nylon membrane filter.
Prehybridization: see **quench**.
Quench: The process of sequestering or blocking all unoccupied potential binding sites of an immobilizing matrix for the purpose of preventing nonspecific background.
Replica: A transferred immobilized electrophoretic pattern, that is, a blot.
Southern blot: DNA blot. The term was originally derived from the publication of E. M. Southern (1975) describing the technique of the analysis of blotted DNA from agarose gels.
Squash blot: The process of analyzing the DNA or RNA content of squashed immobilized flies (Tchen et al., 1985).
Stool blot assay: The DNA analysis of bacterial colonies derived from stool samples (Laiata et al., 1985).
WELLA: Western enzyme-linked lectin analysis, that is, enzyme-conjugated lectin overlay of protein blots (Reading and Hickey, 1985).
Western blot: Protein blot (Burnette, 1981).
Vacuum blot: The process of accelerated transfer of proteins from gels to immobilizing matrices by employing a vacuum (Perferoen et al., 1982).

or nonionic detergents. Quenching is followed by reacting the blot with the probe, in this case the antibody. The incubation (1–2 h) is normally performed in a quenching buffer. Then the blot is washed in buffer and reacted with a labeled second antibody in quench solution. The second antibody may be radioiodinated or enzyme-linked. *Staphylococcus aureus* protein A has also been extensively used in place of the second antibody. After the blot has been washed, thus removing the unbound second antibody, the antibody-antigen complex can be detected. When radiolabeled ligands are used, autoradiography is employed. Should the second antibody be enzyme-linked, the blot is incubated in a solution containing the corresponding substrate to give a colored precipitate at the position of the immobilized antigen.

In such procedures nanogram levels of antigens can be detected. Quite obviously, there are many variables that can influence the quality of the final result. Before dealing with some of these, the issue of whether the system being studied is amenable to blot analysis must be addressed.

IV. TO BLOT OR NOT TO BLOT . . . THAT IS THE QUESTION

Often the final goal of a specific project is "crystal clear" and one can envision the use of protein blotting as the ultimate means for proving a point. However, translating the anticipated "figure" into reality is sometimes problematic. This may be due to incompatibility between the blot procedure used and the system being studied. Preliminary tests should be conducted to determine working conditions before a complete blot experiment such as that described above is attempted. The importance of this step cannot be overemphasized. This is especially true when novel assays are being developed or new reagents are being introduced.

The simplest means for determining initial conditions is to do a series of dot blots (Hawkes et al., 1982). In such assays, droplets of samples are directly applied to an immobilizing matrix. This is then processed through quenching, probing, and detection. Based on the signal-to-background ratio obtained and the specificity of the signal, one can determine the optimal conditions to be used when blotting a gel. A general dot-blot protocol is given in Section XIV. 1. The use of dot blots as a first step preceding gel blotting is a common practice (e.g., Glenney et al., 1983; Vissing and Madsen, 1984; Gershoni et al., 1985a), and considerable information can be gained by carefully designing a well controlled experiment.

1. Quantitative Dot Blotting

A corollary to the simple dot-blot procedure is to apply serial dilutions of a sample to one filter. This approach enables the determination of detection limits for a particular protocol. Thus, even when using an enzyme-linked probe that generally renders qualitative information, one can obtain fairly quantitative results by gauging the range of dilutions used. This simple assay can be surprisingly effective (see, e.g., Leary et al., 1983; Hsu, 1984; Jahn et al., 1984; Gershoni et al., 1985a, 1986; Handman and Jarvis, 1985; Kumar et al., 1985; Nakamura et al., 1985).

2. Dot Blotting as a Screening Assay

Often the objective is to screen a series of samples, aliquots, or fractions against one probe, or various probes against one protein sample (see Glenney et al., 1983; Littauer et al., 1986). For this, a number of commercial devices have been produced. In principle, they consist of a manifold that allows actual vacuum-mediated filtration through the immobilizing matrix. A large piece of immobilizing matrix (~9 × 15 cm) is placed into a Plexiglas manifold, which forms well-separated filtration chambers. Some machines have the appearance of a typical 96-well tissue culture plate. The manifold may be used for the application step, each "well" for a different sample. Afterward the dotted filter is removed from the manifold and processed as described in Section XIV.1. Alternatively one can treat each sample separately, retaining the filter in the assembly and performing the different washes, and so on, by vacuum filtration through the matrix. Manifolds can be extremely useful for the application of relatively large volumes of dilute samples to a discrete "spot."

In summary, dot blotting should be a first approach for testing the suitability of blotting for the particular question on hand. By modifying the conditions and designing a well-controlled assay, dot blotting might even be found to be sufficient in itself. If, on the other hand, gel transfers are required, the gel system and how it can affect blotting should be considered.

V. GELS

There are a number of aspects regarding the type of gel and its composition that can influence a blot experiment. Some of these are discussed in this section.

1. Sample Preparation

Once a specific signal has been obtained in a dot-blot experiment, one may want to ascribe the binding activity to a discrete component of the protein mixture. This requires resolution of the protein sample into its constituents, a matter conveniently accomplished by gel electrophoresis. Often, the process of solubilizing the sample may already be detrimental. The mere dissociation of subunits disrupts quaternary interactions that could be crucial to the maintenance of functional or recognizable configurations (see Islam et al., 1983; Thorpe et al., 1984). It is advisable

to use conditions under which the protein sample is denatured as little as possible. Unfortunately, one cannot always run nondenaturing gels, and most often, efficient resolution of membrane proteins is achieved only in the presence of anionic detergents (e.g., SDS). Moreover, reducing reagents such as 2-mercaptoethanol or dithiothreitol are also commonly required for optimal separations as they prevent aggregation via interchain disulfide bridges. Therefore, a positive dot-blot experiment does not necessarily promise successful overlays of gel transfers.

The components of sample buffers (i.e., SDS, 2-mercaptoethanol, EDTA, urea, etc.) should be tested for their individual and joint effect on the proteins being studied. This can be accomplished by running dot blots. Protein samples are suspended in various concentrations of the different sample buffer constituents. These samples are then applied as dots to a blotting matrix and tested for their ability to bind the probe. In this way optimal conditions for sample preparation can be established. One should be aware, however, of the possible effects perturbants may have on the adsorption of the "dot" to the filter. This obviously would affect the intensity of the signal, not because of loss of essential structure but rather because of reduced amounts of protein present on the filter. This last issue can be monitored by using radiolabeled protein to test the effect of various reagents on the adherence of the protein to the filter.

2. Acrylamide Concentration

The gel itself may affect the final results of an experiment in a number of ways. The concentration of acrylamide and cross-linker will dictate the dimensions of the pores through which the proteins must migrate. The denser the gel, the more difficult it will be for proteins to be eluted (DuBois and Rossen, 1983; Gershoni and Palade, 1983). Practically speaking, this becomes appreciable only for high molecular weight (> 90 kDa) proteins. Running "gradient gels" can help in this respect because more efficient elution for high molecular weight proteins is achieved in the areas of low acrylamide concentration, while reasonable resolution of low molecular weight proteins is ensured by the ever-increasing gel density. Other approaches have involved the use of reversible cross-linkers in the gel (Renart et al., 1979; Tas et al., 1979; Bolen et al., 1982), composite agarose-polyacrylamide gels (Elkon et al., 1984), protease nicking of the high molecular weight protein during blotting (Gibson, 1981), or introduction of low concentrations of SDS in the transfer buffer (Erickson et al., 1982; Nielsen et al., 1982).

3. Transfer of Fixed Gels

The question of how to preserve a gel before blotting sometimes arises. In fact, the easiest solution to this problem is to blot immediately after gel electrophoresis. Yet, if for some reason one needs to delay blotting for a time, this too is possible.

For short periods of time (up to 2 h), leave the gel in its glass plates and store them at 4°C. Should gels need to be stored for substantially longer periods, it is advisable to fix them until blotting can be performed. This is achieved by placing the gel in a "destain" solution (e.g., 25% isopropanol : 10% acetic acid in water), which will preserve the electrophoretic pattern indefinitely. Just before blotting, the gel is incubated in transfer buffer until it completely reaches equilibrium with the alkaline pH. Washing (3–4 × 10 min) and shaking in a reasonable volume (100–200 ml) of transfer buffer is usually sufficient. Then blotting proceeds as normal.

Should the transfer efficiency be markedly reduced, further equilibration in transfer buffer may help. On the other hand, fixation tends to precipitate the protein bands and also removes SDS. Some proteins may not be sufficiently negatively charged upon equilibration, a problem easily solved by introducing 0.1% SDS to the equilibration solution.

From the discussion above it is clear that Coomassie brilliant blue stained and destained gels are amenable to blotting (Jackson and Thompson, 1984; Jackson et al., 1985). In such cases the stained proteins are efficiently transferred and retained on the immobilizing matrix, and a Coomassie brilliant blue pattern on the nitrocellulose sheet is observed. One ought to be aware that the dye is eluted off the protein bands much quicker than the proteins themselves. Thus the dye pattern should not be taken as an indication of transfer efficiency. In addition, the presence of the dye may interfere with subsequent analyses (Lin and Kasamatsu, 1983). If this is the case, Coomassie brilliant blue stained patterns can be decolorized with dimethyl sulfoxide prior to blotting. Interestingly, Perides et al. (1986) have reported that fixed, stained, and dried gels can still be blotted; however a 50% drop in efficiency compared to fixed gels is observed.

The transfer of silver-stained gels is a bit more complicated. Silver staining is not always run to completion; thus a particular band may be only partially stained. That fraction which is not complexed with silver will electroelute, while the "stained" portion will remain fixed in the gel (N. Reiss, Weizmann Institute, personal communication).

4. Isoelectrofocusing and Two-Dimensional Gels

Two special cases of gels that have been used for blot analyses are one- and two-dimensional gels in which isoelectrofocusing has been performed. The subject of blotting two-dimensional gels has been extensively reviewed (Symington, 1984; see also Anderson et al., 1982). Generally speaking, when the second dimension is performed in SDS, the final two-dimensional gel can be regarded as any other SDS gel. Gradient electric fields should be used in electrotransfer of two-dimensional gels only when one of the dimensions is based on differences of molecular weight; otherwise a uniform electric field should be employed (see Section VII.2.A.a).

For isoelectrofocusing gels, and agarose gels in general, it has been found that the preferred modes of transfer are by diffusion and by capillarity (Reinhart and Malamud, 1982a,b; Grace et al., 1985; Handman and Jarvis, 1985; Hoffman and Jump, 1985). Efficient transfer can be achieved by simply applying a filter to the agarose or acrylamide gel and stacking Whatman filter paper and a weight onto the gel/immobilizing matrix assembly. Attempts to remove the agarose gels from their plastic supports to allow electrotransfer seems to be more work than is necessary and of no advantage. This is especially true in light of the fact that efficient transfer by diffusion is possible within an hour or two. Nishizawa et al. (1985) have used specially reinforced polyacrylamide gels, which allow subsequent electroblotting without the need to remove the mechanical support.

VI. THE IMMOBILIZING MATRIX

The process of blotting entails the transfer of macromolecules to an immobilizing matrix. Matrices can be divided into two general types, microporous membrane filters and cellulose-based papers (see Presswood, 1981). The most commonly used material is nitrocellulose membrane filters (NC), and probably this should be taken as a first choice in setting up a blotting experiment.

There are a number of factors that can determine the suitability of one matrix over another and they should be considered.

1. Chemical Composition

A preferred matrix should adsorb the protein samples efficiently and bind the subsequent probe as little as possible. The first requirement

relies on the chemical or physical interactions between the protein and the filter medium, the second is achieved by quenching (see Section IX).

Most proteins at pH values above 7 are negatively charged. It is therefore surprising that NC (also negatively charged) is such an efficient blotting matrix. Clearly hydrophobic interactions play a major role in the adsorbance of protein to NC. This is demonstrated by the fact that nonionic detergents (e.g., Triton X-100) are quite effective in removing bound protein from NC (Gershoni and Palade, 1982; Kakita et al., 1982; Lin and Kasamatsu, 1983; Flanagan and Yost, 1984). This fact should be taken into consideration when incubation and washing solutions are designed, since the presence of excessive amounts of detergent can remove up to 80% of the bound protein. Fixing blotted molecules to NC can, of course, help overcome the loss of this protein during processing. Alcohol/acetic acid, as well as glutaraldehyde, have been used (Gershoni and Palade, 1982; Jahn et al., 1984; Faye and Chrispeels, 1985). Chemical cross-linking (Kakita et al., 1982) and ultraviolet irradiation (Cannon et al., 1985) have also been employed.

Another consideration that should be taken into account in choosing a matrix is the amenability to selective staining of the transferred protein pattern. NC seems to be the easiest filter to deal with in this respect, whereas the nylon-based membranes (e.g., Zetabind, Zetaprobe, Nytran, and Genescreen) are problematic with regard to stainability (see Section VIII).

The main drawbacks of NC are its moderate binding capacity for protein (~80–100 $\mu g/cm^2$) and the reversibility of this association. It should be noted that not all "NC" filters are pure nitrocellulose, and some manufacturers incorporate cellulose acetate in their NC products (e.g., the Millipore HAWP series). This modification decreases the protein-binding capacity even further. Therefore, other media have been used, which have better binding characteristics.

Alwine et al. (1979) introduced diazo-modified cellulosic papers (DBM and DPT papers; see Table I). These filters offer a covalent linkage of the transferred protein to the paper (Renart et al., 1979; Reiser and Wardale, 1981; Renart and Sandoval, 1984). This would appear to be a significant improvement, especially since the reactive side group can be chemically quenched after transfer. However these papers are a bit more awkward to use compared to NC. First, only a precursor of such papers, the amino derivative, can be stored. The filters are then activated by nitric acid treatment just before blotting. Because the diazo groups are labile and highly reactive with amine-containing molecules (e.g., glycine), transfer must be performed quickly

(within a matter of hours) and usually in borate or phosphate buffers (Bittner et al., 1980; Olmsted, 1981; Reiser and Wardale 1981; Symington et al., 1981; Renart and Sandoval, 1984). This last requirement demands that most "Laemmli" gels (Laemmli, 1970) be preequilibrated in borate or phosphate buffers to remove the otherwise reactive Tris and glycine. Regrettably, the binding capacity of the diazo papers is not significantly different from that of NC. When covalent binding is important, the diazo papers are useful. This has been shown by Olmsted (1981), who used blotting as a means for purifying monospecific antibodies (see Section XIII.1.A). Cyanogen bromide papers (Clarke et al., 1979), which also provide covalent immobilization, have been used for blotting as well (Newman et al., 1981).

Positively charge-modified nylon-based membrane filters (PCM) have been introduced as an efficient blotting matrix because they have an exceptionally high binding capacity (>500 $\mu g/cm^2$) and practically irreversible binding; moreover, the filters require no preactivation (Gershoni and Palade, 1982). However the attributes of PCM turn out to be its main drawback. Efficient quenching of these filters is sometimes difficult to achieve. It has been found that relatively high concentrations (5–10%) of quenching materials, and elevated temperatures (40–50°C) may be required to reduce nonspecific background. Moreover, most dyes that stain protein (e.g., Coomassie brilliant blue, Amido black, or Ponceau S), stain these filters equally well, so destaining becomes impractical. Nonetheless, PCM has been found to be extremely useful where reliable quantitative blotting is necessary, and especially where the probe is itself positively charged (e.g., Gershoni et al., 1983). For example, the ^{86}Ru overlays of dot blots shown in Figure 1 are practical only on PCM. In addition, the analysis of small peptides appears most efficient with PCM. There have also been instances of specific proteins not being adsorbed by NC, and in such cases use of PCM is absolutely essential (Miribel et al., 1986). As experience in working with PCM is increasing, the technical problems associated with it are being solved. Milk solutions (15–20% in buffer) seem to be rather effective for quenching PCM, and a number of protein stains compatible with nylon have been developed (e.g., Kittler et al., 1984; Kumar et al., 1985; Moeremans et al., 1986). It is noteworthy that PCM has been found to be an excellent matrix for nucleic acid blotting.

Other matrices that have been used are cellulose acetate (Schaltman and Pongs, 1980), and most interesting are the modified glass fiber filters (Vandekerchkhove et al., 1985; Aebersold et al., 1986), which have been developed to allow microsequencing of blotted bands.