DEVELOPMENT AND MECHANISMS OF SILVER STAINS FOR ELECTROPHORESIS

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Silver stains permit the detection of nanogram amounts of proteins and nucleic acids in gels or membranes. These silver stains have been adapted from histological and photographic photochemical protocols. The basic mechanism of the visualization of protein and nucleic acids by silver staining involves the reduction of ionic to metallic silver. Staining properties of individual amino acids, homopolymers, and small peptides, have been used to demonstrate the importance of the basic amino acids, lysine and histidine, and the sulfur containing amino acids in the silver staining of proteins while the purines have proven to be important in the staining of nucleic acids. Many silver stains demonstrate reproducible curvilinear relationships between silver densities and protein and nucleic acid concentrations. Their sensitivity and reproducibility permits their use in quantitative analysis. By utilizing sets of operationally constitutive proteins for the normalization of intra-gel stain intensities, quantitative comparisons of protein concentrations have been made in electrophoretograms from complex biological fluids or cellular extracts. These ultrasensitive silver stains have also permitted the discovery of disease associated spinal fluid proteins in a number of central nervous system diseases.

Silver staining was introduced as a general protein detection method for proteins separated by polyacrylamide gel electrophoresis in 1979 (32, 52). It has provided an increase in the sensitivity of protein detection by more than 100 fold over that attained by the most commonly used organic protein stain, Coomassie Blue (32, 52).

The observation that silver nitrate, the main ingredient of silver stains, can stain or blacken when in contact with organic substances, including the human skin, is credited to Count Albert von Bollstadt in the twelfth century (11). Although experimentation was performed with silver nitrate between the twelfth and the nineteenth centuries, it wasn’t until the middle of the nineteenth century that this property of silver nitrate to stain organic substances was exploited by modern science. The first modern application of silver nitrate as a stain was Krause’s use of it to stain fresh tissues for histological examination in 1844(30). By 1873, Golgi followed by Cajal, were utilizing silver stains to revolutionize the understanding of the anatomy of the central nervous system (5, 19, 22). The turn of this century was also a period of intense development of photographic methods, based on silver compounds which had been discovered to be light sensitive. Cajal adapted many of the then newly developed photochemical methods to produce histological stains (5).

Histological silver stains were first used to detect specific proteins separated electrophoretically. Frederick in 1963 first used a histological silver stain, a
modified Gomori method, to study phosphorylases separated on polyacrylamide gels (15), and Kerenyi and Gallyas adapted another histological silver stain in 1972 to study cerebrospinal fluid proteins electrophoresed in agarose (28, 29). This latter stain did not achieve widespread acceptance, perhaps because it did not work well in polyacrylamide. Hubbell et al. in 1979 utilized a histological silver stain, developed to visualize nucleoli, to detect nucleolar proteins separated on polyacrylamide (26).

The adaptation of the de Olmos histological silver stain as a general protein stain by Merril and Switzer 1979, and the recognition of the large quantitative gain in sensitivity that silver staining offers, over the commonly used organic stain (32, 52), has stimulated the recent widespread use of silver staining for the detection proteins separated by polyacrylamide gel electrophoresis. This use of silver has been enhanced by the development of numerous simplified silver stain protocols (9).

GENERAL SILVER STAIN PROTOCOLS

These silver stain protocols can be divided into three categories: first, the diamine or ammoniacal silver stains, second, the non-diamine stains including stains based on photographic chemistry, and third, stains based on the photodevelopment or photoresolution of silver ions to form a metallic silver image.

Diamine silver stains were first employed in histological studies of nerve fibers (3). The formation of diamine silver complexes, with ammonium hydroxide, offers a means of stabilizing silver ions in an alkaline environment. The actual silver ion concentration is usually very low in these stains, as most of the silver is bound in the diamine complexes (42). The first histological stain to be used for the general detection of proteins separated by polyacrylamide gel electrophoresis was a diamine stain (32, 52). Diamine stains tend to become selectively more sensitive for glycoproteins if their concentration of silver ions is decreased. However, this specificity can be minimized, if the stain is to be used as a general protein stain, by maintaining a sufficient sodium to ammonium ion ratio in the diamine solution (1).

In some applications, the specificity of the diamine stains has been purposefully enhanced, as in the adaptation of a diamine histological silver stain to visualize neurofilament polypeptides in electrophoretograms of spinal cord homogenates (18).

In the diamine stains, the ammoniacal silver solution must be acidified, usually with citric acid, for image production to occur. The addition of citric acid lowers the concentration of free ammonium ions, thereby liberating silver ions to a level where their reduction, usually by formaldehyde, to metallic silver is possible. The citric acid also participates in the reduction of silver ions.

Many of the non-diamine chemical development silver stains were developed by adapting either silver based photographic protocols (34–37, 41) or histochemical stains (28, 29) for use as protein and nucleic acid stains. These stains rely on the reaction of silver nitrate with protein or nucleic acid sites in acidic conditions, followed by the selective reduction of ionic silver to metallic silver by formaldehyde in alkaline conditions. Sodium carbonate and/or hydroxide or other bases are used to maintain an alkaline pH during development. Formic acid, produced by the oxidation of formaldehyde, is buffered by the sodium carbonate or some other basic compound in these stains.

Photo-development stains utilize energy from photons of light to reduce ionic to metallic silver. Scheele in 1777 recognized that the blackening of ionic forms of
silver by light was due to the formation of metallic silver (12). This ability of light to reduce ionic to metallic silver was adapted by William Fox Talbot, in 1839, as the basis of a photographic processes that dominated photography from its introduction until 1862, when photo-development was replaced by "chemical development" processes (43). The use of photo-reduction provides a rapid and simple, yet sensitive silver stain for detecting proteins separated by gel electrophoresis (38, 39).

Most chemical development stains require a minimum of two solutions, in addition to solutions used to fix the proteins or nucleic acids in the gels. This requirement for multiple solutions in the chemical development stains is a result of their use of alkaline solutions in the chemical-development methods. The presence of silver ions and an organic reducing agent in an alkaline solution often results in the uncontrolled reduction of silver. However, since light can reduce silver in an acidic solution, a photo-development stain may utilize a fixation solution followed by a single staining solution.

Photo-development and chemical-development methods have been combined to develop a silver stain which can detect proteins and nucleic acids in the nanogram range, in under fifteen minutes, with minimal background staining on thin membranes (40). This stain utilizes: copper acetate, a metal salt that is both a good fixative (50) and a silver stain enhancer, silver halide, to provide a light sensitive detection medium, and to prevent the loss of silver ions from membranes or thin layer plates; photo-reduction, to initiate the formation of silver nucleation centers; and chemical-development, in a solution containing the reducing reagents hydroquinone and formaldehyde, to provide a high degree of sensitivity by depositing additional silver on the silver nucleation centers. The stain’s rapidity of action, and its ability to stain samples spotted on membranes, such as cellulose nitrate, has afforded new opportunities to investigate the mechanisms of silver staining.

SILVER STAIN MECHANISMS

The basic mechanism underlying protein detection by the silver stains involves the reduction of ionic to metallic silver. Detection of proteins in the gel or on membrane requires a difference in the oxidation-reduction potential between the sites occupied by proteins and adjacent sites of the gel or membrane. If a protein site has a higher reducing potential than the surrounding gel or matrix, then the protein will be positively stained. Conversely, if the protein site has a lower reducing potential than the surrounding gel or matrix, the protein will appear to be negatively stained. These relative oxidation-reduction potentials can be altered by the chemistry of the staining procedure. Proteins separated on polyacrylamide gels have been shown to stain negatively if the gel is soaked in the dark in silver nitrate followed by image development in an alkaline reducing solution (such as Kodak D76 photographic developer) (37). However, by treating the gel with potassium dichromate prior to the silver nitrate incubation followed by development of the image in an alkaline reducing solution (utilizing formaldehyde as the reducing agent), a positive image is produced (37). Positive images may also by obtained by substituting potassium ferricyanide (34), potassium permanganate (2), or dithiothreitol (41) for potassium dichromate in this stain. It has been suggested that dichromate, permanganate and ferricyanide enhance the formation of a positive image by converting the protein’s hydroxyl and sulfhydryl groups to aldehydes and
thiosulfates, thereby altering the oxidation-reduction potential of the protein. The formation or presence of aldehydes has often been suggested as being essential for silver staining in certain histological stains. However, it has been shown that neither aldehyde-creating or aldehyde-blocking reagents appreciably affect silver staining in many of these histological stains (54). In some cases there is evidence that silver staining depends on physical interface phenomena. In one study, the silver staining of tissue fibrils, as observed in the electron microscope, appeared to depend on whether the fibrils were in an ordered or random array (54).

Dithiothreitol, a reducing agent, also creates a positive image, perhaps by maintaining the proteins in a reduced state. However, other reducing agents, such as beta-mercaptoethanol, do not enhance positive image formation. Alternatively, all of the positive image enhancing compounds may simply act as nucleation centers for silver reduction after forming complexes with the proteins (9).

In the photo-development silver stains, light photons are utilized to liberate electrons. The mechanism is probably best described by the Gurney-Mott photo-chemical theory: wherein a liberated photoelectron combines with a silver ion to form metallic silver. It is generally accepted that a single silver atom will rapidly undergo oxidation, reverting to a silver ion, unless additional silver atoms are formed nearby. Once a critical number of silver atoms are formed in a local region, they become stably reduced and can then serve as an autocatalytic center for the reduction of additional silver ions. The rate of silver ion reduction after the photocatalytic formation of stable metallic silver depends on the electron availability or the local oxidation reduction potential (23).

In the photo-development stains, the gel is impregnated with a fixative containing chloride ions, as silver chloride is more sensitive to light than silver nitrate. Some insight into the underlying mechanisms has been gained from the observation that when a gel that has been treated with a fixative, containing a small amount of sodium chloride, is transferred into a silver nitrate solution, a fine bluish-white precipitate of silver chloride forms throughout the gel, except for those regions containing proteins, which appear clear. However, with illumination by a light source of sufficient intensity, the clear regions containing the proteins will darken (40). The formation of the characteristic black or brown images in the regions of the gel containing protein, by the photo-reduction of silver ions on exposure to light, indicates that the silver ions were not excluded from the previously clear protein containing regions (37, 40). Further evidence that the silver ions are not excluded from gel regions containing electrophoresed proteins has been obtained by placing a polyacrylamide gel into a radioactive silver nitrate solution (110mAg). At very low concentrations of silver nitrate, (10^-9 M) sufficient silver is bound to the proteins to visualize their positions by autoradiography. At higher concentrations of silver, similar to that used in the silver stains, 0.1 M, the distribution of silver was fairly uniform. In no case was there evidence for the exclusion of silver ions (37). Evidence for the presence of chloride ions in the clear zones is more indirect. However if chloride ions were excluded from the stain protocol, there was a significant loss of the stain’s sensitivity.

Lack of a visible silver chloride precipitate in gel regions containing relatively high concentrations of proteins may be due to altered solute structures affecting interactions between the silver and chloride ions. Biological molecules often re-
organize solvent molecules in the neighborhood of reactive groups. X-ray diffraction studies of proteins have demonstrated immobilized water oxygen atoms, such that they are visible in specific positions in electron density maps (56). Effects of proteins on surrounding solvents may be augmented by the physical organization of the proteins themselves, as in the previously discussed electron microscopic observations which suggest that the degree of silver staining depends on whether fibrils are arranged in random arrays or in organized bundles (54).

PROTEIN GROUPS REACTIVE IN SILVER STAINING

In studies of amino acid homopolymers, individual amino acids, nucleotides, nucleosides, and purine and pyrimidine bases, Merril and Pratt observed that only cysteine, cystine, and the purine bases, adenine and guanine, stained with the combination photo-chemical-development silver stain (40). Staining was also observed with the homopolymerls poly-methionine, and the hydrophilic basic amino acid polymers: poly-lysine, poly-arginine, poly-histidine, and poly-ornithine (40). This staining of the basic amino acids and methionine in their homopolymeric form, but not as individual amino acids, may indicate the need for cooperative effects of several intramolecular functional groups to form complexes with the silver.

Silver staining of the basic homopolymers of histidine, arginine, and ornithine, has also been observed by Heukeshoven and Dernick, although they did not report staining of the basic amino acid homopolymer poly-lysine (44). The role of the basic amino acids in silver staining has been further strengthened by the observation by Nielsen and Brown that the basic amino acids: lysine, arginine, and histidine, in both free and homopolymeric forms produce colored complexes with silver (25).

Heukeshoven and Dernick also reported silver staining of the homopolymers of glycine, serine, proline and aspartic acid (44) while Nielsen and Brown reported the formation of colored silver complexes with: aspartate, and tyrosine (25). Staining of these homopolymers was not observed in the study of Merril and Pratt (40), and metal binding studies have failed to demonstrate metal interactions with the side-chain hydroxyl groups of serine, threonine or tyrosine (16). These discrepancies concerning the non-basic amino acids may be due to differences in the staining procedures employed; the Heukeshoven and Dernick study stained homopolymers on polyacrylamide gel while Nielsen and Brown studied formation of silver-amino acid complexes in solution. Both of these studies used formaldehyde in an alkaline sodium carbonate solution for image development. Merril and Pratt utilized photo-development in acidic conditions followed by chemical-development with a combination of light, hydroquinone and formaldehyde (40).

The importance of the basic and the sulfur containing amino acids has been corroborated by observations with purified peptides and proteins of known amino acid sequence (40). Leucine enkephalin, which has neither sulfur containing nor basic amino acids did not stain with silver, while neurotensin which also has no sulfur containing amino acids but does have three basic amino acid residues (one lysine and two arginines) did stain. Gastrin produced a weak staining reaction. It lacks basic amino acids but it has one sulfur containing amino acid, methionine. Oxytocin stained fairly vigorously. It also has no basic amino acids but it does have two sulfur containing cysteines. The staining reaction of angiotensin II was rather anomalous. It produced a negative stain rather than a positive silver stain despite
its two basic amino acids, arginine and histidine. Other polypeptides tested; insulin somatostatin, alpha-melanocyte stimulating hormone, thyrocalcitonin, aprotinin, vasoactive intestinal peptide and ACTH, contained both basic and sulfur containing amino acids and they all produce positive silver staining reactions (40).

The importance of the basic amino acids has been further substantiated by evaluations of the relationship between the mole percentage of specific amino acids in a protein and the ability of that denatured protein to stain with silver. The best correlations were observed when a comparison was made between the slope of the linear portion of a denatured protein's staining curve and the protein's mole percentages of the basic amino acids, histidine and lysine (40). Similar correlations were observed by Dion and Pomenti (7). Dion and Pomenti have suggested that this correlation may be due to interactions between lysines and glutaraldehyde, which was used in their stain protocol. Bound glutaraldehyde could supply aldehyde groups to facilitate the reduction of silver ions. While this mechanism may play a role in the stain protocol employed by Dion and Pomenti (7), it is are unlikely to be a factor in the Merril and Pratt protocol, since that protocol did not employ glutaraldehyde (40). Dion and Pomenti also had suggested that alkaline conditions may be important for the formation of silver complexes with lysine and histidine. However, the Merrill and Pratt protocol utilized acidic conditions (39).

Studies concerning the mechanisms of Coomassie dye staining of proteins have indicated a similar importance for the basic amino acids. Polypeptides rich in lysine and arginine are aggregated by Coomassie G dye molecules, suggesting that the dye interacts with the basic groups in the polypeptides (46). Also, studies of proteins with known sequences have demonstrated a significant correlation between the intensity of Coomassie blue staining and the number of lysine, histidine and arginine residues in the protein (53).

Studies utilizing native, undenatured, proteins have failed to display significant correlations between the specific amino acid mole percentages and their ability to stain with silver (40). This lack of significant correlation is probably due to the inaccessibility of many of the potentially active amino acid side chains in undenatured native protein structures.

The significant correlation of silver staining intensity to the mole percentage of the basic amino acids lysine and histidine in a protein is most likely due to the electron donating abilities of the amino groups and imidazole groups at the termini of the lysine and histidine side chains. The N-terminal amino groups and the amino groups involved in peptide bonding are in themselves insufficient for visualization with silver stain. If they were capable of independently reducing silver ions, all peptides, proteins, and amino acids would stain positively. Amino and imidazole groups at the termini of amino acid side chains are capable of interacting cooperatively intramolecularly to bind silver ions, while peptide bonding and N-terminal amino groups are not as capable of such interactions.

The guanidine group in arginine's side chain proved to be less active than either the amino or the imidazole groups in the side chains of lysine and histidine respectively. Arginine's correlation coefficients were not found to be significant in studies of comparing staining densities to mole percent of arginine. This lack of activity of the guanidine group may have been, in part, responsible for the negative staining reaction of the peptide angiotensin II which contains the two basic amino
acids arginine and histidine (one residue of each). However, neurotensin, which contains two arginine residues and one lysine residue, stained fairly well. This difference in the staining between angiotensin II and neurotensin may provide further evidence concerning the importance of cooperative metal binding effects. In angiotensin II the arginine residue is separated from the histidine by three residues, while in neurotensin the two arginines are adjacent to each other and only one residue separates them from lysine.

Of the nonpolar and uncharged polar amino acids, only the sulfur containing amino acids, methionine, cysteine and cystine, showed any silver staining reactivity with the Merril-Pratt Protocol (40). Cysteine and cystine were the only amino acids to stain as an individual amino acids and they may account for the silver staining properties of the peptide oxytocin. Oxytocin contains no basic amino acids and its only sulfur containing amino acids are two cysteine residues. The ability of cysteinyI side-chains to form complexes with silver ions is well known.

It has been suggested that the ability of reducing agents [including: thiosulfates, sulfides, borohydrides, cyanoborohydrides, mercaptoethanol, thioglycolic acid, cysteine, tributylphosphine reducing metal salts (such as FeCl₂, SnCl₂ and TiCl₄) and dithiothreitol] to intensity silver stains may be related to the generation of thiol groups in cysteine residues (41). However, proteins that contain no cysteine or proteins with an alkylated cysteine(s) were also affected by these reducing agents in some stain protocols (44).

Methionine's ability to participate in the silver staining process has been demonstrated by silver staining of methionine homopolymers. Methionine may also be responsible for the staining of the peptide gastrin. Gastrin contains no basic amino acids and only one methionine residue. In general the thioether sulfur atoms in the methionine residues are weaker electron donors than the sulfhydryl sulfur atoms in the cysteine residues (16).

Insignificant staining correlations have been observed between staining densities and mole percentages of the sulfur containing amino acids methionine and cysteine (40). This observation may indicate a relatively minor silver staining role for the sulfur containing amino acids in proteins with large numbers of basic amino acids. However, this poor staining correlation is somewhat of a paradox since polymethionine stains with a higher silver density than the basic amino acid homopolymers (40). This paradox could be explained by a requirement for cooperative intramolecular effects between sulfur atoms and silver ions which is disrupted in heteropolymers.

NUCLEIC ACID GROUPS REACTIVE IN SILVER STAINING

In studies of silver-staining reactions with nucleic acids and their precursors, nucleic acid polymers stained fairly well; however of the nucleic acid precursors, only the purine bases, adenine and guanine, demonstrated a positive staining reaction (40). Neither the nucleosides nor the nucleotides of adenine or guanine stained. Adenine and guanine have free amino groups in the C-6 and C-2 positions, respectively. Given the active metal-binding role of amino groups in the proteins, one might have expected these amino groups to be important in the silver-staining reaction of these bases. However, if those amino groups were major factors in the staining reaction, then the nucleoside and nucleotide derivatives of these bases should
have stained. The amino group in a purine base may be hindered in forming complexes with metal ions because the potential free electron pair of the amino group is needed to stabilize the electron deficient purine ring (13). Given this theoretical argument against a simple involvement of the amino group and the observation that the nucleotides and nucleosides do not stain, an explanation of the staining reaction of the free purine bases may involve the nitrogen atom in the N-9 position in the purine ring. The guanine and adenine N-9 position have previously been implicated as the favored binding site for copper ions (13). However, when a purine is incorporated in a nucleoside, nucleotide, or nucleic acid the N-9 nitrogen atom is bound to a ribose or deoxyribose sugar and incapable of binding a metal ion. This inaccessibility of the N-9 nitrogen would account for the lack of staining by the purine nucleosides or nucleotides.

It may be that the N-9 nitrogen atom is important for the silver staining of free purines while in nucleic acid polymers cooperative effects of other groups or sites are necessary. It has been suggested that adenine's affinity for silver involves a cooperative effect in which a two silver ion bridge is formed between the amino group of one adenine residue and the N-1 atom of another adenine residue (13). In support of this suggestion methylation of adenine's amino group decreases its affinity for silver (13).

Additional sites on the bases which have been shown to complex with metal ions include the N-3 site of the pyrimidines, and the N-3 site of the purines. Of the three potential metal-binding sites in nucleic acids, the base, the ribose and the phosphate, the ribose is the weakest electron donor while the phosphate is the strongest (13). However, a study of stability constants of silver complexes formed with adenosine, AMP, ADP, ATP and DNA, has denigrated the role of the phosphate group in silver binding. A number of investigators have suggested that the binding of metal ions by nucleic acids may require the formation of large chelate rings by the simultaneously binding of oxygen in the phosphate groups and a nitrogen in the purine or pyrimidine base structures with the metal ions (13).

COLOR EFFECTS WITH SILVER STAINS

Silver stains generally produce monochromatic brown or black colors with most proteins. However, Goldman et al. in 1980 noted that certain lipoproteins tend to stain blue while some glycoproteins appeared yellow, brown or red (20). This color effect is most likely an analogue of Herschel's observation in 1840, he projected the spectrum of visible light obtained by passing sunlight through a prism onto a silver chloride-impregnated paper, the colors of the spectrum appeared on the paper, particularly a “full and fiery red” at the focal point of the red light (24). Since these observations by Herschel it has been found that the color produced depends on three variables: the size of the silver particles, the refractive index of the photographic emulsion or electrophoretic gel, and the distribution of the silver particles. In general, studies with photographic emulsions have shown that smaller grains (less than 0.2 microns in diameter) transmit reddish or yellow-red light, while grains above 0.3 microns give bluish colors, and larger grains produce black images (33). Modifications of the silver staining procedures, such as lowering the concentration of reducing agent in the image development solution, prolonging the development time, adding alkali, or elevating the temperature during staining will often enhance
color formation. Some silver stain protocols have been developed to produce colors that may aid in identification of certain proteins (25, 47, 48). Production of color with silver stain depends on many variables. Nielsen and Brown have shown that charged amino acid side groups play a major role in color formation (25). However, variations in protein concentration and conditions of image development may also produce color shifts, confusing identification. Furthermore, color-producing silver stains tend to become saturated at low protein levels and often produce negatively stained bands or spots. These factors tend to make quantitative analysis more difficult.

**SPECIFICITY OF SILVER STAINS**

Silver stains have demonstrated their capability as general protein and nucleic acid (4, 21, 51) detecting stains. However, some silver stains have displayed considerable specificity. Hubbell et al. stained nucleolar proteins with a histological stain (26), while Gambetti et al. adapted a silver stain specific for neurofilament polypeptides (18). Some silver stain protocols also detect lipopolysaccharides (55), and polysaccharides (8). In a study of erythrocyte membrane proteins, sialoglycoproteins, a silver stain protocol was used to stain lipids yellow while the other membrane proteins were counterstained with Coomassie Blue (55). All silver stains do not detect proteins such as calmodulin or troponin C. However, pretreatment with gluteraldehyde often permits positive silver staining of these proteins (49). Histones may also fail to stain with silver. Fixation with formaldehyde coupled with simultaneous prestaining with Coomassie Blue partly alleviates this problem. However, even with this fixation procedure sensitive for histones was decreased 10-fold compared with detection of neutral proteins in the silver stain protocol employed (27). Another example of differential sensitivity has been demonstrated in a study which utilized four different silver stain protocols to stain salivary proteins. Different protein bands were visualized with each of the stains (8).

**SENSITIVITY OF SILVER STAINS**

Silver stains offer the most sensitive non-radioactive method for the post electrophoretic detection of proteins or DNA separated by gel electrophoresis. They are 100-fold more sensitive than the Coomassie stains for most proteins (32, 36, 37, 52). Chemical-development silver stains are in general, more sensitive than photo-development silver stains. This loss in sensitivity may be compensated for by the ability of photo-development stain to produce an image within 10 to 15 minutes after gel electrophoresis (39). Unfortunately, photodevelopment often produces negatively stained protein bands. The presence of negative and positive regions, in photo-developed or color-enhanced silver stained gels makes quantitative analysis difficult (39).

**QUANTITATION WITH SILVER STAINS**

A reproducible relationship between silver stain density and protein and nucleic acid concentrations has been found with most silver stain protocols. The linear portion of this relationship extends over a 40-fold range in concentration, beginning at 0.02 nanograms per mm² for most proteins (36–38, 40, 52). Protein concentrations greater than 2 ng/mm² generally cause saturation of silver images, resulting in
non-linearity above that concentration. Saturation can usually be recognized by bands or spot with centers which are less intensely stained than the regions near the edges. This effect is similar to the "ring-dyeing" noted with some of the organic stains. An often quoted report by Poehling and Neuhoff (45) states that "Silver does not stoichiometrically stain proteins, unlike Coomassie Blue". However, their silver-stain data actually is linear over a 30-fold range in protein concentration, while their Coomassie Blue data is only linearity over a 20-fold range (9, 36).

Employment of curve-fitting techniques can be used to extend the quantitative range of the silver stains, as described by Coakley and James (6) for analysis of the similar curvilinear relationship found in the Folin-Lowry method of protein estimation (31). With careful measurement of total stain densities, estimates of relative protein concentrations have been made over a 220 fold concentration range with six purified proteins (40).

Plots of silver stain densities versus protein concentrations produce different staining curves for each proteins studied (36, 37, 40, 52). Protein specific staining curves have also been observed with the organic stains, including Coomassie Blue (14, 53) and with most protein assays such as the commonly used Lowry protein assay (31). These curves are governed by the basic mechanisms underlying the detection and assay methods. The fact that each protein produces a unique density versus concentration curve in these studies, illustrates a dependence of the detection reaction on specific reactive groups contained in each protein. Furthermore, the occurrence of protein-specific curves argues against a stain mechanism that depends on some fundamental subunit common to all proteins, for example the peptide bond, or a unique element in each protein, such as the terminal amino acid. A stain that depended on a subunit, such as the peptide bond, would result in similar staining curves for all proteins, when the density of staining for each of the protein bands or spots was plotted against the mass of protein contained in each of the bands or spots. Similarly, a stain that was based on a reaction with a unique element in each protein, for example the terminal amino group, would produce similar plots for each protein when the stains densities were plotted against the number of molecules contained in each band or spot.

The occurrence of these protein specific staining curves with silver staining suggests that in general quantitative inter-gel comparative studies should limit comparisons of protein to homologous bands or spots on each gel. For example, the actin spot on one gel should be compared with an actin spot on another gel, the actin spot should not be compared with a transferrin spot. These limitations to homologous comparisons are also applicable to most of the organic stains, including the Coomassie Blue stains. If it is necessary to use these stains quantitatively to estimate general protein concentrations, the importance of the basic amino acids, particularly lysine and histidine in the staining reaction indicates the need for a careful choice of a "standard protein". A protein containing an abnormally large number of stain reactive groups would produce a curve which would tend to underestimate the concentration of proteins containing normal numbers of reactive groups. A similar correlation between the intensity of Commassie Blue staining and the number of basic amino acids in proteins caused Tal et al. to suggest the use of egg white lysozyme rather than the more commonly used bovine serum albumin as a protein standard (53). This suggestion is based on their observation that the basic
amino acid content of proteins ranges between 10–17 mole percent, with a modal content of 13 mole percent (53). Egg white lysozyme has a basic amino acid mole percent of 13.2 while bovine serum albumin has a basic amino acid content of 16.5 mole percent. For similar reasons, egg white lysozyme may also prove to be an optimal standard for quantitative silver stain applications.

REFERENCES