Journal of Chromatography, 569 (1991) 175–196
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5961

Review

Staining and quantification of proteins separated by polyacrylamide gel electrophoresis

I. SYROVÝ*

Institute of Physiology, Czechoslovak Academy of Sciences, Videnska 1083, 142 20 Prague 4 (Czechoslovakia)

and

Z. HODNÝ

Research Institute of Gerontology, Malacky (Czechoslovakia)

(First received February 7th, 1991; revised manuscript received April 31st, 1991)

ABSTRACT

The present review concentrates on techniques for the staining and quantification of proteins separated by polyacrylamide gel electrophoresis. Staining with organic dyes has been used for approximately thirty years; the silver staining technique was introduced in 1979. The problems of silver staining are presented separately because the mechanism of this staining is in principle different from staining with organic dyes. Less attention has been devoted to quantification of two-dimensional gels, because this autoradiography is preferred because of its high sensitivity and fewer problems with accurate quantification in contrast to silver staining.

CONTENTS

0378-4347/91/$03.50

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue
CBB-G	Coomassie Brilliant Blue G-250
CBB-R	Coomassie Brilliant Blue R-250
1-D, 2-D	One-dimensional, two-dimensional
DTT	Dithiothreitol
M_r	Relative molecular mass
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid

1. INTRODUCTION

The exact quantification of the components present in complex biological material is one of the most difficult tasks in protein chemistry. Polyacrylamide gel electrophoresis (PAGE) and staining of proteins is relatively easy to perform, but quantification of separated proteins is often necessary. It is quite easy to measure stained protein bands with a densitometer, however, it is much more difficult to evaluate the measured absorbance and to obtain reliable quantitative data reflecting the amount of the protein actually present. There are specific problems concerning quantification following one- and two-dimensional gel electrophoresis. To quantitate two-dimensional electrophoresis is much more difficult, and this is one of the reasons why great inequality exists between the numbers of papers dealing with one- and two-dimensional gel electrophoretic quantification. Next we will discuss aspects which are important for quantification of proteins separated by PAGE and subsequently stained. The mechanism of silver staining is in principle different from staining with organic dyes and therefore these two approaches are dealt with separately. Protein databases and computer-assisted analysis of electropherograms were reviewed in 1989 [1,2]. Gel electrophoresis of nucleic acids was reviewed by Gersten and Zapolski [3]. Detection of enzymes, lipoproteins and glycoproteins is not included in this review.

2. STAINING OF PROTEINS BY ORGANIC DYES AND THEIR QUANTIFICATION

2.1. Choice of stain

Commonly used stains in protein electrophoresis include Amido Black 10B, nigrosine, Procion Blue RS, Alcian Blue, Fast Green FCF, Coomassie Brilliant Blue G-250 (CBB-G) and Coomassie Brilliant Blue R-250 (CBB-R). In addition, a panoply of methods based on "silver" staining of proteins has been described. Staining with CBB-R and CBB-G is more sensitive when compared with other stains; the sensitivity of silver staining is still higher. Staining with dibromotrisulphofluorescein [4] permits detection of high protein concentrations only.

Staining of proteins is based on the binding of the dye with specific groups of the protein; the detailed mechanism is frequently not clear.

Probably all protein-staining procedures have some disadvantages. Amido Black stains proteins metachromatically, frequently resulting in gels which contain bands of many different shades of blue, black and brown. CBB is very sensitive in detecting minute amounts of protein, but it deviates noticeably from Beer's law at high protein concentration.

A simple method for protein staining with Fast Green was described by Gorovsky *et al.* [5]. Staining does not deviate appreciably from linearity in amounts up to 150–200 μg on 6-mm gels; 1–3 μg of protein can be easily detected.

There are numerous modifications of staining with CBB-G or CBB-R. The former is only slightly soluble in 12% trichloroacetic acid (TCA); the latter is virtually insoluble in water. The extinction coefficient for CBB-G is higher than that of CBB-R, and because of this CBB-G is more sensitive [6]. Staining of gels with CBB-G is described in refs. 6–10. The method used by Diezel *et al.* [8] is based on staining from a suspension, which doet not enter the gel. Staining with CBB-R is described in refs. 10–18.

A very important prerequisite for quantitative measurement is the complete staining of a protein band throughout the entire gel. This is, however, not obtained with all modifications of staining. It is recommended that completeness of staining is controlled by cross-sectioning a parallel gel. Incomplete cross-sectional staining represents a serious problem if a high concentration of protein is present on the gel. For this purpose the procedure of Neuhoff *et al.* [18] is recommended; by reducing the methanol concentration in the staining solution to 25% it is possible to increase considerably protein staining with CBB-R throughout the gel.

It is generally accepted that the lower limit of relative molecular mass (M_r) for fixation and staining of proteins after PAGE is approximately 10 000, but methods are available allowing staining of polypeptides down to an M_r of 3000, and, after pretreatment of the gel with glutaraldehyde, polypeptides with an M_r of only 1000 can also be stained with picomole sensitivity [19,20].

Destaining of gels can be performed on routine basis, but it is time-consuming.

It is also well known that a part of the dye, bound to the protein in the staining step, is lost during destaining in order to obtain a clear background, and that on prolonged storage the protein stain leaches out. Several methods are available for obtaining a clear background in staining proteins on the gel and thus eliminating the destaining step completely [8,14,18,21]. Elimination of the destaining step was achieved by using 12.5% TCA as a solvent of CBB, in which the dye is relatively insoluble and from which it is adsorbed onto the protein bands only. Also, diluted perchloric acid is used as solvent of CBB. Neuhoff *et al.* [6] tested more than 600 variations with many different acids and additional compounds to find an optimal staining procedure with low background. This was performed with phosphoric acid and CBB-G in the presence of ammonium sulphate in order to optimize the colloidal state of the dye.

A one-step staining procedure with low background which can be followed by silver staining if increased sensitivity is desired was described by Zehr *et al.* [22]. The procedure is based on staining in the presence of low concentrations of CBB-R in 10% ethanol–5% acetic acid in water.

2.2. Sensitivity of protein staining

The ideal staining procedure has to be sensitive enough to allow estimation of proteins in the microgram range. In addition, complete staining is mandatory for quantitative evaluation. Gels of 2 mm thickness are not suitable for quantitative evaluations; an upper limit of thickness of 1 mm is recommended. Practically all modifications of staining with CBB dyes are sensitive enough to detect 0.2–1 μg of any protein in a sharp band, and staining is quantitative to 15 μg for some proteins.

The highly sensitive protein staining with CBB dyes described by Neuhoff *et al.* [6] allows the sensitivity of protein staining to be increased to a detection limit of 0.7 ng bovine serum albumin per mm^2, which is close to the sensitivity of silver staining. An improved procedure for staining of proteins following separation in polyacrylamide gels which utilizes the colloidal properties of CBB-R and CBB-G enables the detection of even 0.1 ng of protein [18]. Optimal staining of proteins on a clear background was obtained with phosphoric acid and CBB-G in the presence of ammonium sulphate; under these conditions the dye is completely transferred into its colloidal form and the colloidal state of the dye is optimized.

2.3. Variation of the response of different proteins with CBB

It is well known that CBB-R interacts in different ways with different proteins; the same holds true to some extent for other dyes [18,23,24]. Because different dyes react with different amino residues in proteins, it is clear that values obtained with one dye can be compared with those obtained with another with limitations. The reason for these differences has not yet been fully explained. However, signif-

icant differences in the response of various proteins have been reported [25–27]. Considerable differences in the staining intensity of different proteins were also found when Fast Green was used [28]. Tal *et al.* [29] investigated the binding of CBB-R to the following proteins: cytochrome C, lysozyme, RNase, trypsin, pepsin, pepsinogen, gramicidin S and poly(L-lysine). Their results indicated that a different number of dye molecules is bound to each of these proteins and that the amount of the dye bound depends on the protein's basic amino acid content (about 1.5–3 dye molecules per charge). Lysozyme and cytochrome C had the highest response curves with CBB-G, as well as with CBB-R, whereas pepsin has the lowest with both ligands; the curves of the other proteins were in between (Fig. 1).

2.4. Prestaining methods for immediate visualization of proteins in PAGE

Several procedures have been described for protein staining before PAGE. Griffith's method [30] uses Remazol (a reactive dye) for staining proteins upon denaturation before sodium dodecyl sulphate (SDS) PAGE. The protein is heated in the presence of Remazol Brilliant Blue R and analysed by electrophoresis. Consequently, the migration of the coloured protein bands may be followed visually during their migration through the gel. Datyner and Finnimore [31] used a cationic dye of unspecified composition for prestaining proteins prior to electrophoresis. According to these authors, the procedure is reproducible, and sensitivity of 0.2 μg with selected proteins and peptides was obtained by the digestion of serum albumin with cyanogen bromide.

Varghese and Dywan [32] used CBB-R for simultaneous staining of proteins during PAGE in acidic gels by countermigration of the dye. CBB-R was added to

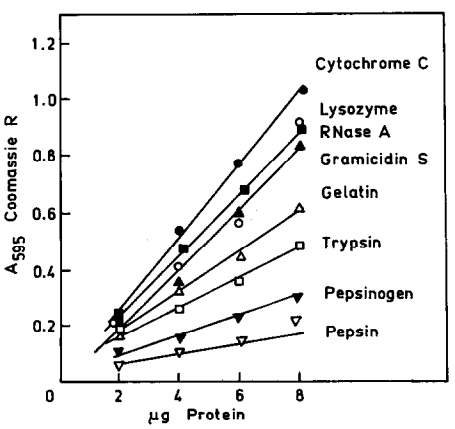

Fig. 1. Dimethyl sulphoxide extraction of CBB-R bound by various proteins. Proteins were subjected to electrophoresis on polyacrylamide gels in 6-mm tubes (from ref. 29 with permission).

the cathodic chamber and electrophoresis was carried out until the dye migrated to the top of the gel. Calf thymus, whole histone and cytochrome C were stained by this procedure, and the results obtained were similar to those obtained by staining after electrophoresis. It is obvious that other reactive dyes also could be used for protein staining before electrophoresis. However, optimum conditions have yet to be found.

2.5. Quantification of proteins separated by PAGE

Separation of proteins and their staining and densitometric measurements are all considered as more or less routine techniques, but it is quite difficult to obtain values which really correspond to the actual amount of proteins present in individual bands. Each step in the procedure involves several factors which contribute to difficulties in obtaining reliable quantitative data.

The measurement of absorbance is correct only if there is a linear correlation between stain density and absorbance. Empirically obtained calibration curves may be also used for the correlation between stain density and absorbance, but this is quite laborious. Protein staining following electrophoresis depends on the particle density (local concentration of protein molecules) in such a way that densely packed proteins are not stained as well as loosely packed proteins; this is probably due to the fact that at a high protein concentration not all possible binding groups of the protein can be reached by the stain. Fishbein [33] came to the conclusion "that progressive compactation of the protein bands of shorter migration distances is the responsible factor, preventing the stoichiometric uptake of dye molecules once a critical protein concentration has been exceeded".

Many problems arise from reproducibility of staining and destaining. Different proteins exhibit different colour values after reacting with a particular stain. For quantitative measurement washing procedures should be avoided and staining procedures without background staining should be used. A clear background is an obvious prerequisite for increasing the signal-to-noise ratio in densitometry.

Neuhoff *et al.* [10] evaluated essential problems in quantification of proteins following staining with CBB dyes, especially (a) dependence of colloidal staining on protein particle density and polyacrylamide concentration, (b) dye diffusion in protein staining, (c) completeness of staining and (d) quantitative densitometry.

It was demonstrated that the local particle density of a protein in a given gel is of critical importance because it determines its stainability; depending on local protein concentration, the dye binding to the same amount of a given protein differs significantly. The stainability of proteins using colloidal staining procedures with CBB dyes depends also on the pore size of a given gel used for electrophoresis and because of this calibration curves for determinations have to be prepared [6]. Quantitation with the use of densitometry is only possible by determining the protein amount of each single measuring point, on the basis of calibration curves.

Loading of the gel is also important. Pepe and Drucker [34] showed that when loading of the gel varies the measured myosin/actin mass ratio also varies (whole myofibrils were used for PAGE) (Fig. 2).

According to Neuhoff *et al.* [10], gradient gels are not suited for quantitative protein densitometry via staining, because in a gel of low acrylamide concentration the diffusion of free dye molecules is faster than in a gel of high concentration.

2.5.1. One- and two-dimensional densitometry

For quantitative measurements fast, high-resolution (performance) densitometers capable of digitizing two-dimensional (2-D) PAGE gels for subsequent computer-aided image analysis and the establishment of protein databases are available. With modern instruments it is possible to resolve more than 4000 grey levels and to measure a signal-to-noise ratio of more than 2500:1.

It is the usual practice in densitometry to scan only once a one-dimensional (1-D) gel. There are many reasons why this is not sufficient. A protein peak is never absolutely homogeneous. The obtained data depend also on the size of the measuring area (Fig. 3). Therefore 2-D data acquisition is recommended also for 1-D PAGE gels. For 2-D measurement the optimal measuring area has to be a circle with a diameter of about 0.2 mm.

Up to several thousand proteins can be resolved by 2-D PAGE, but quantitative analysis represents a more formidable task. There are two alternative methods of performing quantitative analysis. Either quantitative densitometers are

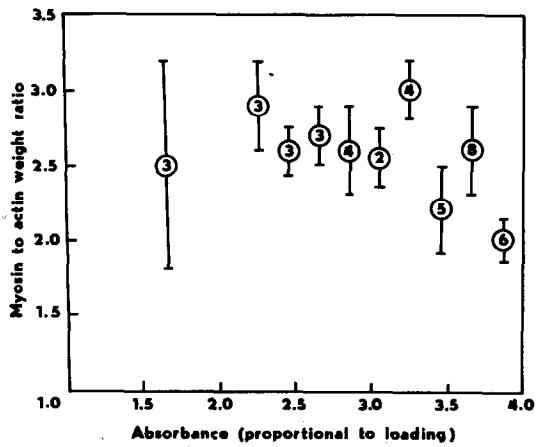

Fig. 2. Myosin-to-actin mass ratio as a function of loading. The myosin-to-actin mass ratio is plotted as a function of absorbance, where absorbance is the setting required on the Gilford spectrophometer to bring the height of the myosin peak to a given position. Therefore absorbance is proportional to loading. Absorbances in the range of 2.2–3.3 optical density units were used in this work. The number of measurements for each point is given in the circle and the fiducial marks indicate the range of values obtained (form ref. 34 with permission).

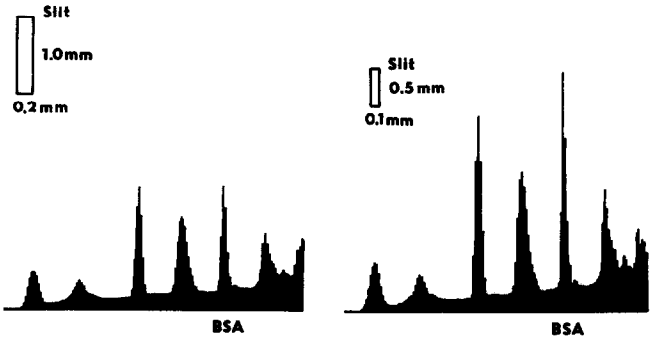

Fig. 3. Densitograms obtained from the same lane of a 1-D PAGE with low-molecular-mass marker proteins. The step size is 0.1 mm in both scans. Observe the influence of the size of the measuring area on the resulting densitogram (from ref. 10 with permission).

used or gels are scanned using a laser scanner or another type of optical reader and the files are transferred to a microcomputer. Evaluation of 2-D gels involves the following steps: individual gels are scanned, polypeptide spots are found and their locations, shapes and intensities are measured. Refs. 35–44 can serve as typical examples of automatic computer-aided evaluation of 1-D and 2-D electrophoretic gels.

Quantitative evaluation of silver-stained 2-D gels is, according to some authors, much more difficult than that gels stained with CBB. Nevertheless, useful data can be obtained, as shown by Yang *et al.* [45]. Proteins of fibroblasts from control subjects and patients with inherited diseases were separated by 2-D PAGE. After silver staining, the electrophoretic gel was subjected to semi-automatic digitizer–personal computer analysis; scanning with an image sensor video camera connected to a digitizer was followed by quantitation and statistical analysis with a personal computer. A total of 247 spots was examined. Quantitative variations were found in 9 spots; in three spots slight variations in molecular mass were observed.

Perhaps it is not necessary to emphasize that reliable identification of individual spots in 2-D maps is inevitable before quantitation can be considered. A number of computer programs exist to compare protein patterns on 2-D PAGE gels, such as TYCHO [46], AUTOMATCH [42], GIPSY [43], HERMES [47], GELLAB [48] and others [38,49–53].

2.5.2. Detection of proteins separated by electrophoresis with the use of UV

Detection of protein bands can also be achieved by UV absorbance measurement [54]. Hjerten [54] used UV detection of proteins separated by high-performance electrophoresis in a gel rod of polyacrylamide of small diameter (0.05–0.3 mm). In other studies [55,56] with polyacrylamide gels, proteins migrated out of the gel during electrophoresis and the protein zones were transferred continuously to the cuvette of a UV monitor and detected at 230 nm. Quantita-

tive measurement during PAGE as well as detection of protein bands can be done also by a modernized schlieren optics system. This has the advantage that no distortion of the electrophoretic pattern occurs when compared with the staining process. Protein bands containing as little as 0.1–0.3 μg can be detected [57], which is comparable to that of staining by CBB.

2.5.3. Quantification of stained proteins based on dye elution

Major sources of error in quantitative densitometry are protein variations in dye binding, inaccuracy in integrating small areas and the limited range over which detection of the dye is proportional to protein present. 1-D gels, which contain only limited number of protein bands, can alternatively be quantitated by analyses of the eluted dye.

Fenner *et al.* [58] have shown that the CBB-R dye can be extracted from stained bands after cutting and macerating of the gel pieces with 25% pyridine in water. However, this technique also has certain limitations, due, for example, to the variability in the number of the dye-binding sites on proteins. By this method 1–100 μg of protein can be analysed. According to the authors, precise results for two proteins differing ten-fold or more in molecular masses on a single gel can be obtained. Medugorac [59] used Fast Green for staining of myosin and its subunits on SDS-electrophoresis gels. The dye was eluted with 0.1% SDS and absorbance measured at 623 nm. A quantity of 2–100 μg of protein can be determined.

2.6. Separation artifacts

The existence and danger of separation artifacts is frequently neglected. It is not very easy, especially in 2-D PAGE, to obtain identical results and identical protein pattern in two subsequent experiments, performed under "identical" conditions, and the same is true for inter-institutional comparison.

Artifacts may be due to a step-like polymerization at the origin of the gel, and thus protein, *e.g.* in the form of an artificial triplet, can be observed. Another possibile origin of separation artifacts is insufficient separation of proteins, and this may result from a curvature on top of the stacking gel.

In 2-D PAGE it is absolutely necessary to check by control staining whether the isoelectric focusing gel is completely eluted, since otherwise, especially when different samples are compared, any interpretation of the 2-D pattern is meaningless [60].

In 2-D PAGE greasy streaks are frequently visible, and it is questionable whether this streaking is due to the presence of separated proteins or whether some proteins are selectively retained. Streaks in 2-D PAGE gels may be caused by the formation of stable micelles, consisting, for example, of protein, ampholytes and SDS, or may be related to the stacking procedure. Avoiding streaking and evaluation of such gels is difficult.

In 2-D protein PAGE numerous artifactual spots exist, not because substances

TABLE I

APPLICATIONS OF SILVER-STAINING METHOD (SELECTED)

Compound(s)	Technique	Staining method	Comments	Ref.
Proteins, RNA, DNA	PAGE	Acidic	Sodium thiosulphate as enhancing agent	89
Proteins	2-D ELFO	Acidic	Large gels, enhancement by sodium dithionite	83
Proteins		Alkaline		90
Proteins, nucleic acid	ELFO → blotting TLC	Acidic	Membranes, thin-layer plates	71
Proteins	SDS-PAGE	Alkaline	Large-pore polyacrylamide gels	91
Proteins	IEF, agarose gels			92
Proteins	SDS-ELFO		Double staining by silver and CBB G 250	93
Proteins	IEF, agarose gel		Modified method of Kerènyi and Gallyas [94]	95
Proteins	Immunoelectrophoresis	Alkaline	For detection of antigen–antibody immuno complexes on agarose gels	96
Histones	PAGE	Alkaline	For lysine-rich histones	97
Glycoproteins	SDS-PAGE	Acidic	Enhancement by carbohydrate-binding dyes	98
Glycoproteins, polysaccharides	SDS-PAGE	Alkaline	Periodic acid–silver stain	99
Sialoglycoproteins	SDS-PAGE	Acidic	Double staining (silver + CBB), coloured staining for O-linked glycoproteins	79
Lipopolysaccharides	SDS-PAGE	Alkaline	Periodic acid pretreatment	100
DNA, lipopolysaccharides	PAGE		Thick gels	101
Nucleic acids	–	Acidic	Stained on nitrocellulose	102
DNA	PAGE	Acidic	Quantification study	103
DNA	PAGE	Alkaline	Detection of picogram amounts of DNA	104
DNA native, denatured	Agarose gels	Alkaline	Modified method of Kerènyi and Gallyas [94]	105
RNA	ELFO, agarose gels	Alkaline	Denatured RNA on agarose gel	106
RNA + proteins	PAGE	Alkaline and acidic	Double-staining techniques for RNA and proteins in the same gels	107
tRNA	2-D ELFO	Alkaline	Comparison of three acidic methods	108

other than proteins are revealed by staining, but as a result of irregularities in the proteins themselves. Inhomogeneities in the gel matrix and in the distribution of the protein stain and residual protein left behind by many of the migrating protein spots produce in the pattern numerous small areas of slightly increased amounts of protein (artifactual spots) not recognizable with the eye but detectable with a sensitive densitometer.

3. DETECTION OF PROTEINS BY SILVER STAINING

3.1. General

The applicability of the most commonly used organic dyes for protein staining in gels has several limitations which have been specified in the preceding section of this review. In particular, the relatively low sensitivity, which precludes detection of trace amounts of proteins in the sample, needs to be emphasized. Other detection methods, such as autoradiography, are more sensitive, but their application is frequently limited to model systems only.

These difficulties were partially eliminated when in 1979 Switzer *et al.* [61] described a staining method the sensitivity of which was comparable to that attained by autoradiography. Their staining procedure, derived from the histological protocol, is based on visualization of the protein bands in the gel by selective reduction of silver ions bound on proteins. In the following ten years the method was (in parallel with the increasing amount of information concerning the chemistry of the staining mechanism) frequently innovated, simplified, made more inexpensive and adapted for various kinds of biological material analysed (Table 1). Hundreds of original papers are available today. The purpose of this review is to highlight some of the progress made during the last decade towards understanding of silver staining and its quantification. More detailed information concerning the mechanism of silver staining can be found in the recent review of Rabilloud [62].

Most of the methods described up to now fall into two categories:

(a) The acidic methods (first described by Merril *et al.* [63] are characterized by using silver nitrate solution in water (*i.e.* in a weak acid environment).

(b) The alkaline methods (first described by Oakley *et al.* [64] are based on gel impregnation with the diammine complex of silver nitrate in a highly alkaline environment.

Reduction of silver ions to metallic silver is currently carried out with dilute formaldehyde solution either at alkaline pH (usually the case in most of the acidic methods) or at the acidic pH (usual in the alkaline methods); however, other developers have also been proposed [65]. The general (partly simplified) scheme of the whole procedure is shown in Fig. 4. Various modifications differ substantially mainly in the "enhancing step" which is necessary for obtaining the high sensitivity in all of the acidic methods; with the alkaline methods it can, however, be omitted.

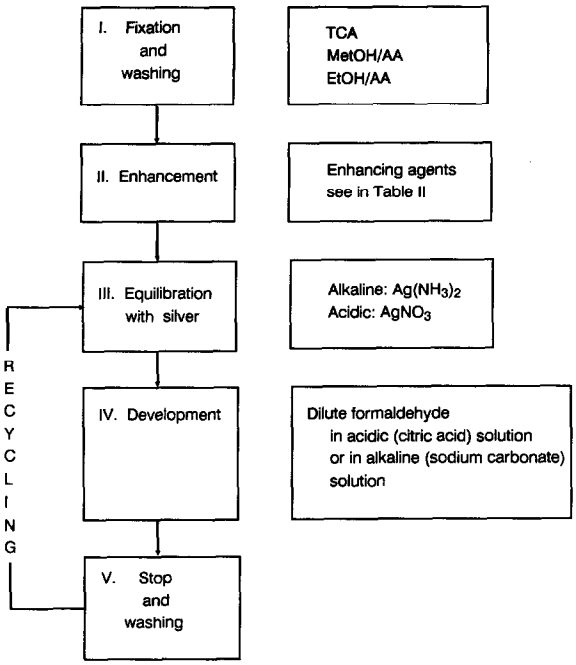

Fig. 4. General scheme of the silver-staining procedure (steps/most frequently used compounds).

3.2. Mechanism

The mechanism of silver staining is not yet completely understood. Elucidation of this process has been attempted by many investigators, however, some results are not always fully compatible (probably depending on the procedure used). For further information in this respect the reader is referred to refs. 62 and 66–72.

It is generally accepted that charged amino acids (either basic —lysine [66,67,71] and histidine [66,67,69,71]—or acidic— glutamate and aspartate [67,69]— as well as sulphur-containing amino acids (cysteine and methionine [71]) participate in silver protein staining, but their contribution to protein stainability differs according to the method used (for review see ref. 73); other amino acids, however, are stained in their homopolymeric form as well (arginine [67,69,71]; ornithine [69,71]; glycine, serine, proline [69]; tyrosine [67]). Final stainability is probably influenced by cooperative effects between individual amino acids in the protein involved [71]. Thus it is obvious that the main observed differences in the staining intensity between individual proteins depend on the primary and probably tertiary structure of the particular stained protein.

As fas as the chemical basis of this process is concerned, the amino acid side-chains bind silver ions during the impregnation step; silver–protein complexes

formed are successively reduced by a reducing agent to metallic silver. The silver–protein deposits are seen in gels as bands or spots of various colours, mostly brownish. The mechanism of formation of silver deposits in protein zones is quite complex; it can be affected by numerous factors at any stage of the staining procedure.

It has been postulated that the reduction kinetics of silver ions is the key factor in silver staining [62]; it depends mainly on free silver ion concentration in the gel and on the chemical nature of the reducing agent. The reaction is pH-dependent and is accelerated as pH rises. In general, silver complexes are less easily reduced than free silver ions. Furthermore the redox potential of silver ions bound in the form of salts or complexes to the different amino acid side-chains varies. The binding affinity as well as the reduction rate during development are different for individual silver-binding groups.

Silver ions are present in the gel after impregnation in three forms; free, complexed to some groups of polyacrylamide gel and complexed to proteins. All three forms are reducible during development but their "reducibility" is different. Loosely bound silver complexes (and free silver ions) in the gel matrix are more easily reducible than those attached to the protein, which may lead to negative staining of protein zones when the washing step between silver impregnation of the gel and its development is omitted. Insertion of the washing step reverses this negative staining to positive imaging of protein zones. According to Rabilloud [62] the reversal is due to easier leakage of silver ions from the gel matrix than from protein zones during washing; consequently the silver ion concentration in the gel matrix is lower in comparison with the protein zone.

The reduction rate of silver ions (and the final silver image) is influenced by diffusion processes between gel and staining solutions during development (according to the diffusion hypothesis of Rabilloud [62], *i.e.* by diffusion of silver ions out from the gel, by diffusion of the reducing agent into the gel, movement of the pH gradient, etc. It is understood that these processes are considerably influenced by gel thickness.

Although the chemical basis of both categories of methods is analogous, there are some substantial differences. In alkaline methods silver appears in the form of the diammine complex, which reacts probably with the ε-amino group of lysine and the –SH group of cysteine. High pH moreover partially hydrolyses the polyacrylamide matrix and liberates new groups, which are responsible for background staining [74]. The washing step in the alkaline procedure is not so critical, because leakage of silver complexes from protein zones is lower because of the relatively low solubility of the silver–diammine complex in water. On the other hand, in acidic methods fast leakage of silver ions from protein zones occurs during the washing step and therefore washing has to be short and its duration always the same in different experiments in order to achieve comparable results.

In acidic methods, however, binding of silver ions to $-NH_2$ and –SH groups at weakly acidic pH is weaker compared with alkaline staining; on the other hand

formation of silver salts of COO^- groups and the immino groups of the histidine imidazole ring represents a complementary process involved in staining in acidic media.

3.3. Sensitivity

In contrast to the most commonly used dye, CBB, the detection limit of which in its highly sensitive modification (CBB-G [10]) for bovine serum albumin is about 0.7 ng/mm^2, the sensitivity of the commonly used silver-staining methods is at least ten-fold higher (0.05 ng/mm^2 for this protein [75]). However, the sensitivity of CBB dyes is more uniform for various proteins; variations in sensitivity between individual proteins are expressed more in silver staining and fluctuate over two orders of magnitude. Moreover, the sensitivity of individual proteins is strongly dependent on the staining procedure used. As an extreme example of this phenomenon let us mention that a particular protein (calmodulin or troponin C) may be stained negatively by using one method but positively with another [76]. This different staining behaviour of various proteins is conditioned by intrinsic factors, such as structural differences, binding capacity for various ligands such as metals [77,78], lipids [79], or by post-translational modifications [79,80], and probably by other factors as well. In other words it reflects the very protein structure, as already mentioned. This fact is very important for quantitative evaluation.

The sensitivity of most polyacrylamide silver staining methods is limited by the formation of background staining, which also disturbs reproducibility and quantification.

Optimal staining pattern depends on the ratio of the reduction rate of silver ions present in protein zones to the reduction rate of silver ions in the protein-free gel matrix [62]. For this reason considerable attention has been directed to finding which factors are responsible for background staining and to specifying such conditions in which background staining is minimized [81]. It is known, that some compounds (*e.g.* riboflavin, glycine, SDS, etc.) increase background staining and prolonged washing after the fixation step is necessary to clarify the background. Heukeshoven and Dernick [69] recommended the use of an ethanol–acetic acid bath as fixative instead of the frequently used methanol–acetic acid; methanol-containing fixative must be subsequently washed from the gel with an ethanol-containing solution.

The hypothesis that amide groups (and probably carboxy, hydroxy and keto groups liberated in gel matrix by hydrolysis of a methylenebisacrylamide cross-linking agent at high alkaline pH) may be in part responsible for background staining led to the development and utilization of new types of cross-linking agents such as 1,4-bisacrylpiperazine [74]. At the same time it was found that the increasing ratio of methylenebisacrylamide to acrylamide increases background staining [74]. It is also necessary to find the proper balance between the concen-

tration of silver in the impregnating solution, development time, concentration of the reducing agent and pH of the developer, particularly with respect to the gel thickness [81]. One of the efficient ways of suppressing background staining and increasing sensitivity is the introduction of an "enhancing step" into the procedure (most frequently before impregnation by silver). There exist three ways of enhancing which are based on (1) increasing silver binding, (2) preventing background development and (3) increasing latent image formation. These three categories of enhancing agents, according to their assumed chemical mechanism of function, are surveyed in Table 2 [62].

The less effective approach to remove background once developed is based on its destaining with photographic "Farmer reducer" (this concentrated solution contains approximately 30% potassium ferricyanide, 60% sodium thiosulphate and 10% sodium carbonate). However, this procedure affects not only the background but also silver deposition in the protein bands. Farmer reducer is frequently used for background destaining in "recycling" methods [69]. In this procedure stained gel is partially destained (by Farmer reducer) and again overstained by new impregnation with silver (the procedure is repeated from step III in Fig. 4). Sodium thiosulphate here acts also as an enhancing agent for subsequent staining. This finding led to simplification of this procedure [82] and new enhancers (such as sodium dithionite) were introduced [83].

TABLE 2

AGENTS FREQUENTLY USED IN ENHANCING STEPS [62]

Enhancement by	Used compounds
Increased silver binding (amplification)	Anionic compounds (SDS, CBB) Aromatic sulphonates (sulphosalicylic acid)
Preventing background image development (contrast)	Oxidizing agents (potassium permanganate, potassium dichromate)
Favouring latent image formation (sensitization)	Sulphiding agents Thiourea Thiosulphate Tetrathionates Dithiothreitol Reducing agents Aldehydes (glutaraldehyde, formaldehyde) Inorganic reducers (borohydrides, dithionite)

3.4. Quantification

Although silver staining is an excellent method of detecting proteins because of its high sensitivity, there are problems with quantitative evaluation if required. Problems of this kind also exist with other colloidal dyes and concern differences in the spatial arrangement of proteins in the gel, differences in molecular mass, availability and number of the amino acid side-chains [10], etc. The band width of the electrophoretically separated proteins of the same concentration is different with different proteins and increases to a different extent with increasing protein loading, depending on the nature of the protein separated. Some of the proteins tend to form more diffuse bands with increasing concentration, while others aggregate into more concentrated zones [84]. For this reason it is necessary to relate the staining intensity (concentration of protein or optical density) to the area of the protein band for more correct quantitative evaluation.

Other factors emerging from the very nature of silver staining make quantitative evaluation difficult. As mentioned above the staining pattern of a given protein is determined by its amino acid composition, conformational arrangement in the gel, bound ligands, etc., and by the complexity of the whole procedure. Thus differences in the stainability of individual proteins are more pronounced in silver staining than, for example, in CBB staining, and for good reproducibility attention must be paid to maintaining the same conditions during the whole process. Coomassie Blue is nearly linear in binding to different proteins, and so densitometry of Coomassie Blue-stained gels can be used to reveal the stoichiometry, *e.g.* of multi-subunit proteins.

The extent of linearity in silver staining is characteristic of a given protein and ranges between 0.02 and 6 ng/mm^2 [75]. Differences between various proteins are 1–3 orders of magnitude (*i.e.* the extent of linearity is approximately in the 10-fold to 500-fold range of concentration of various proteins [71]). The slope of the staining intensity (density per nanogram of protein) is also characteristic for each protein [75] (Fig. 5). Non-linearity occurs when the concentration of the protein exceeds about 2 ng/mm^2. This is probably due to the saturation of the protein band by silver, which can be avoided by shorthening the developing time, and a quantitative response may be achieved at much higher loads; at the same time, however, the sensitivity is lowered [85].

Cross-sectional studies of silver-stained gels proved that it is very difficult to achieve staining of the protein band throughout the whole thickness of the gel (especially using gels $>$ 1 mm thick) without corresponding background staining [81]. The use of thin gels is more suitable if quantification is required.

Merril and Pratt [71] described a photochemical method with a wider range of linearity (for most non-denatured proteins from 5 to 790 ng). In this method proteins were stained after their transfer onto nitrocellulose membrane. Provided that quantitative transfer of all proteins from the gel to the membrane occurred, the above-mentioned procedure could be used to advantage for quantitative eval-

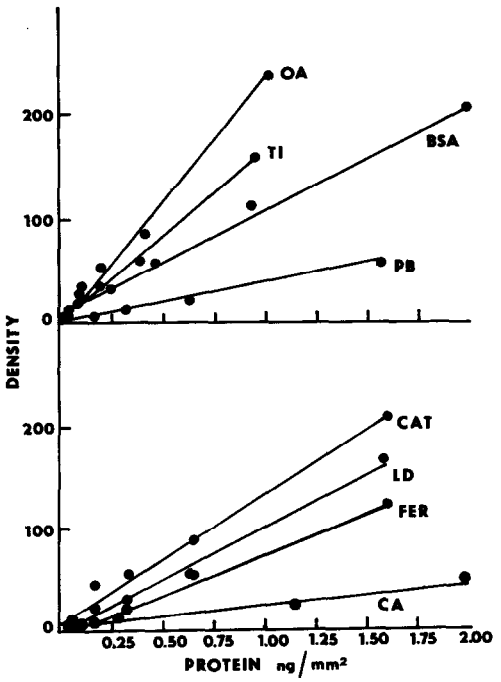

Fig. 5. Different slope of silver image density (optical density × mm^2) *versus* concentration (ng/mm^2) of eight purified proteins: OA = ovalbumin; TI = trypsin inhibitor; BSA = bovine serum albumin; PB = phosphorylase B; CAT = catalase; LD = lactate dehydrogenase; FER = ferritin; CA = carbonic anhydrase (from ref. 75 with permission).

uation in both denaturing and non-denaturing conditions, and thus difficulties due to diffusion processes in the gels and/or non-uniform background staining in gradient gels may be eliminated.

Wedrychowski *et al.* [77] showed that one cause of the negative staining of certain proteins may be contamination of the sample by nucleotides or by metal salts which compete for the silver-binding sites. This leads to dark background and negative staining of proteins. This effect can be eliminated by intensifying the washing procedure after fixation or by pretreating the gel with sodium thiosulphate just prior to the silver staining step (as described by Otsuka *et al.* [78], for carboxymethylated metallothioneins, proteins typical in their ability to bind heavy metal ions).

Although silver-stained proteins usually appear as dark-brown bands or spots on gels in most described methods, procedures have been developed in which coloured staining of proteins occurs [67,70,86]. The differences in colour may cause difficulties in subsequent quantitation, as previously described [87] and therefore monochromatic procedures are preferred.

The production of coloured bands depends on the amount of protein loaded, the temperature and pH of the developer, the concentration of the reducing agent

in the developer and on the developing time [70,72]. Increased protein concentration, decreased concentration of the reducing agent and increased developing time favour the occurrence of coloured bands. The presence of specific amino acids [67,70,71] or various covalently and/or non-covalently bound ligands [79,80,86] is responsible for variations in colours between various proteins as well, but no systematic survey of the role of these factors is available so far. Merril *et al.* [72] elegantly showed, by electron-microscopic study, that the resulting protein colour depends on the size of the silver grains formed in the gel.

Sammons *et al.* [86] proposed that some post-translational modifications of proteins may also influence silver staining (metachromatism of protein spots). This proposal was exploited in the "double staining" technique of Deh and Dzandu and co-workers [79,80] for specific (coloured) detection of lipoproteins and sialoglycoproteins by using a modification of the method of Merril *et al.* [75]. We found differences in the behaviour of glycated proteins. The acidic method (according to Morrissey [88]) with the omission of dithiothreitol (DTT) from the enhancing step was used. It was found that many proteins which are stained negatively or with low sensitivity by this procedure are stained positively if the proteins are preincubated before electrophoretic run with various reducing sugars

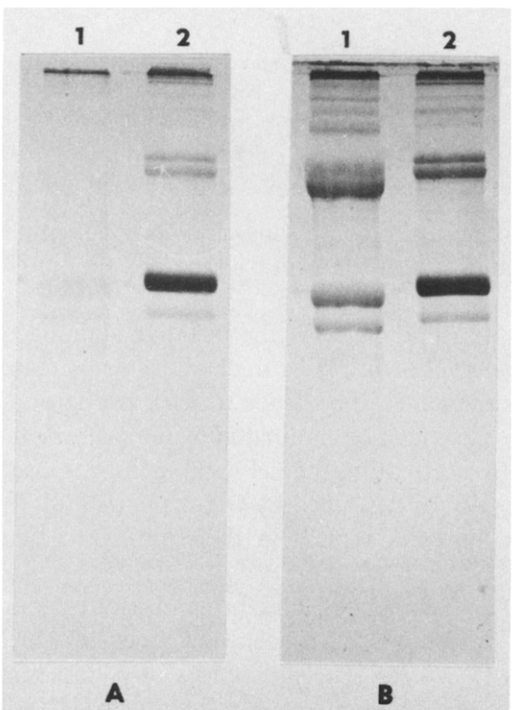

Fig. 6. Silver staining of glycated proteins. (A) Acid-soluble collagen (rat tail tendon) non-glycated (lane 1) and glycated by glyceraldehyde (lane 2). (B) The same gel overstained by CBB-R. (Staining procedure according Morrissey [88] with omission of DTT from the enhancing step.)

such as glucose, fructose, ribose and glyceraldehyde (our unpublished results, Fig. 6). When the original method, with DTT, was used, the end-point staining intensity of glycated and non-glycated proteins was quite similar (Fig. 7), but differences in the appearance between glycated and non-glycated protein bands during development were observed. Our explanation of this phenomenon is that glycation influences either the reduction rate of silver complexes probably (1) by cooperative effects between the bound sugar and the amino acid side-chains or (2) by conformational changes of proteins due to the glycation process, and/or the formation of new reactive groups derived from attached sugar contributes to the total silver-binding sites on the protein.

Silver staining is an efficient and highly sensitive method of qualitative or semiquantitative detection of proteins, at least in the field of the 2-D techniques. The stain dependence on specific reactive groups requires careful selection of standard proteins for good quantitative evaluation. The different content of specific reactive groups present in every protein is responsible for specific protein-staining curves (ref. 71 and Fig. 5). Thus for the purpose of quantitation and for inter-gel comparisons a suitable internal standard has to be used. Tal *et al.* [29] proposed the use of egg white lysozyme in Coomassie staining methods, because its content of basic amino acids (binding CBB) is more representative for most

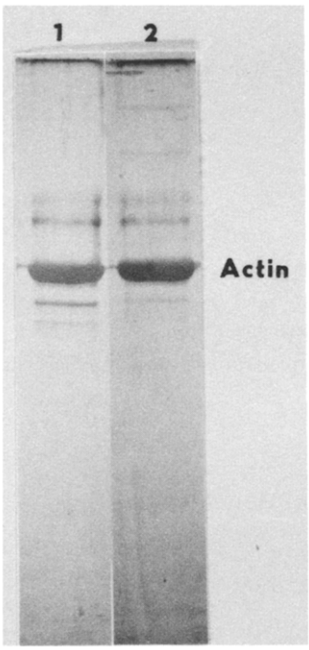

Fig. 7. Silver staining of non-glycated and glycated skeletal muscle actin by glyceraldehyde. No substantial differences in staining intensity between unmodified and modified actin bands are seen. Lanes were dissected from photography of the same gel. (Staining procedure according Morrissey [88] without omission of DTT.)

proteins when compared with frequently used bovine serum albumin (BSA). From the same reason Merril and Pratt [71] recommended lysozyme as a suitable standard for silver-staining procedures. However, the question remains whether this protein can be used in both, *i.e.* alkaline and acidic silver staining methods. For more precise quantitation of a given protein the use of the same purified protein co-electrophoresed in the gel should be used.

4. CONCLUSION

Both classical dye staining and silver staining have certain limitations, and none of these techniques is universal. The main advantage of staining with organic dyes is its simplicity (one-step procedures are described), and problems with reproducibility are relatively small. However, the low sensitivity of these stains is disadvantageous, especially in 2-D gels. The sensitivity of silver staining is approximately 100-fold higher, but this type of staining requires at least three steps and many problems with reproducibility and quantification may occur. It can be expected that the more precise knowledge of chemical events involved in silver staining will lead to further increases in sensitivity and to broader applicability for quantitative purposes.

5. ACKNOWLEDGEMENTS

The authors thank Mrs. Jaroslava Bemova, Mrs. Jitka Tomanova and Mrs. Zdena Polakova for technical assistance. The authors wish to express their gratitude to Dr. Zdenek Deyl for helpful discussions.

REFERENCES

1 J. E. Celis, P. Madsen, B. Gesser, S. Kwee, H. V. Nielsen, H. H. Rasmussen, B. Honore, H. Leffers, G. P. Ratz, B. Basse, J. B. Lauridsen and A. Celis, in A. Chrambach, M. J. Dunn and B. J. Radola (Editors), *Advances in Electrophoresis*, Vol. 3, VCH, Weinheim, 1989, p. 1.
2 M. J. Miller, in A. Chrambach, M. J. Dunn and B. J. Radola (Editors), *Advances in Electrophoresis*, Vol. 3, VCH, Weinheim, 1989, p. 182.
3 D. M. Gersten and E. J. Zapolski, in A. Chrambach, M. J. Dunn and B. J. Radola (Editors), *Advances in Electrophoresis*, Vol. 4, VCH, Weinheim, 1989, p. 50.
4 L. Orstein, *Ann. N. Y. Acad. Sci.*, 121 (1964) 321.
5 M. A. Gorovsky, K. Carlson and J. L. Rosenbaum, *Anal. Biochem.*, 35 (1970) 359.
6 V. Neuhoff, R. Stamm and H. Eibl, *Electrophoresis*, 9 (1985) 427.
7 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
8 W. Diezel, G. Kopperschläger and E. Hofmann, *Anal. Biochem.*, 48 (1972) 617.
9 N. Malik and A. Berrie, *Anal. Biochem.*, 49 (1972) 173.
10 V. Neuhoff, R. Stamm, I. Pardowitz, N. Arold, W. Ehrhardt and D. Taube, *Electrophoresis*, 11 (1990) 101.
11 F. de St. Groth, R. G. Webster and A. Datyner, *Biochem. Biophys. Acta*, 71 (1963) 377.
12 T. S. Meyer and B. C. Lambert, *Biochim. Biophys. Acta*, 107 (1965) 144.
13 J. Maizel, *Science*, 151 (1966) 988.

14 A. Chrambach, R. A. Reisfeld, M. Wyckoff and J. Zaccari, *Anal. Biochem.*, 20 (1967) 150.
15 G. Fairbanks, T. L. Steck and D. F. H. Wallach, *Biochemistry*, 10 (1971) 2606.
16 O. Vesterberg, *Biochim. Biophys. Acta*, 243 (1971) 345.
17 D. Rodbard and A. Chrambach, *Anal. Biochem.*, 40 (1971) 95.
18 V. Neuhoff, N. Arnold, D. Taube and W. Erhards, *Electrophoresis*, 9 (1988) 255.
19 H. O. Kratzin, J. Wiltfang, M. Karas, V. Neuhoff and N. Hilschmann, *Anal. Biochem.*, 189 (1989) 1.
20 J. Wiltfang, N. Arnold and V. Neuhoff, in B. J. Radola (Editor), *Electrophoresis Forum 89*, Technische Universität München, Munich, 1989, p. 276.
21 A. H. Reisner, P. Nemes and C. Bucholtz, *Anal. Biochem.*, 64 (1975) 509.
22 B. D. Zehr, T. J. Savin and R. E. Hall, *Anal. Biochem.*, 182 (1989) 157.
23 A. Chrambach, T. M. Jouin, P. J. Svendsen and D. Rodbard, in N. Catsimpoolas (Editor), *Methods of Protein Separation*, Vol. 2, Plenum Press, New York, 1976, p. 27.
24 C. R. Merril, in A. Chrambach, M. J. Dunn and B. J. Radola (Editors), *Advances in Electrophoresis*, Vol. 1, VCH, Weinheim, 1987, p. 111.
25 J. Pierce and C. H. Suetler, *Anal. Biochem.*, 81 (1977) 478.
26 H. Van Kley and S. M. Hale, *Anal. Biochem.*, 81 (1977) 485.
27 S. M. Read and D. H. Northcote, *Anal. Biochem.*, 116 (1981) 53.
28 J. D. Potter, *Arch. Biochem. Biophys.*, 162 (1974) 436.
29 M. Tal, A. Silberstein and E. Nusser, *J. Biol. Chem.*, 260 (1985) 9976.
30 I. P. Griffith, *Anal. Biochem.*, 46 (1972) 402.
31 A. Datyner and E. D. Finnimore, *Anal. Biochem.*, 55 (1973) 479.
32 G. Varghese and A. M. Dywan, *Anal. Biochem.*, 132 (1983) 481.
33 W. N. Fishbein, *Anal. Biochem.*, 46 (1972) 388.
34 F. A. Pepe and B. Drucker, *J. Mol. Biol.*, 130 (1979) 379.
35 P. Mancini, A. Benassi, G. Valli and L. Donato, *Med. Biol. Eng. Comput.*, 16 (1978) 542.
36 N. L. Anderson, J. Taylor, A. E. Scandore, B. P. Coulten and N. G. Anderson, *Clin. Chem.*, 27 (1981) 1807.
37 K. P. Vo, J. Miller, E. P. Geiduschek, C. Nielsen, A. Olson and N. H. Xuong, *Anal. Biochem.*, 112 (1981) 258.
38 M. M. Skulnick, *Clin. Chem.*, 28 (1982) 979.
39 P. Tarroux, *Electrophoresis*, 4 (1983) 63.
40 T. S. Ford-Holevinski, B. W. Agranoff and N. S. Radin, *Anal. Biochem.*, 132 (1983) 132.
41 R. Amess, R. Ramasany and S. D. Spragg, in V. Neuhoff (Editor), *Electrophoresis '84*, Verlag Chemie, Göttingen, 1984, p. 297.
42 M. J. Miller, A. D. Olson and S. S. Thorgeirsson, *Electrophoresis*, 5 (1984) 297.
43 J. J. Tyson and R. H. Haralick, *Electrophoresis*, 7 (1986) 107.
44 D. P. Häder and G. Kauer, *Electrophoresis*, 11 (1990) 407.
45 R. Ch. Yang, A. Tsui and Y. Suzuki, *Electrophoresis*, 10 (1989) 785.
46 J. Taylor, N. L. Anderson, A. E. Scandora, K. E. Millard and N. L. Anderson, *Clin. Chem.*, 28 (1982) 861.
47 P. Vincenos, N. Paris, J. Pusol, C. Gaboriano, T. Rabilloud, J. L. Pennetler, P. Matherat and P. Tarroux, *Electrophoresis*, 7 (1986) 347.
48 P. F. Lemkin, L. E. Lipkin and E. P. Lester, *Clin. Chem.*, 28 (1982) 840.
49 P. F. Lemkin and L. E. Lipkin, *Comput. Biomed. Res.*, 14 (1981) 355.
50 M. J. Miller, P. K. Vo, C. Nielsen, E. P. Geiduschek and N. H. Xuong, *Clin. Chem.*, 28 (1982) 867.
51 W. R. Hruschka, *Clin. Chem.*, 30 (1984) 2037.
52 D. J. Potter, *Comput. Biomed. Res.*, 18 (1985) 347.
53 J. Prehm, P. Jugblut and J. Klose, *Electrophoresis*, 8 (1987) 562.
54 S. Hjerten, *J. Chromatogr.*, 270 (1983) 1.
55 S. Hjerten, *Chromatogr. Rev.*, 9 (1967) 122.
56 M. Zhu and S. Hjerten, in V. Neuhoff (Editor), *Electrophoresis '84*, Verlag Chemie, Göttingen, 1984, p. 110.

57 M. Takazaki and H. Kubota, *Electrophoresis*, 11 (1990) 361.
58 C. Fenner, R. R. Traut, D. T. Mason and J. Wikman-Coffelt, *Anal. Biochem.*, 63 (1975) 595.
59 I. Medugorac, *Basic Res. Cardiol.*, 74 (1979) 406.
60 M. Pöhling and V. Neuhoff, *Electrophoresis*, 1 (1981) 90.
61 R. C. Switzer, C. R. Merril and S. Shifrin, *Anal. Biochem.*, 98 (1979) 231.
62 T. Rabilloud, *Electrophoresis*, 11 (1990) 785.
63 C. R. Merril, D. Goldman, S. A. Sedman and M. H. Ebert, *Science*, 211 (1981) 1437.
64 B. R. Oakley, D. R. Kirsch and N. R. Morris, *Anal. Biochem.*, 105 (1980) 361.
65 C. R. Merril, *U.S. Pat.*, 4 (1983) 405, 720.
66 A. S. Dion and A. A. Pomenti, *Anal. Biochem.*, 129 (1983) 490.
67 B. L. Nielsen and L. R. Brown, *Anal. Biochem.*, 141 (1984) 311.
68 E. Hemplemann, M. Schulze and O. Götze, in V. Neuhoff (Editor), *Electrophoresis '84*, Verlag Chemie, Weinheim, 1984, p. 328.
69 J. Heukeshoven and R. Dernick, *Electrophoresis*, 6 (1985) 103.
70 P. J. Chuba and S. Palchaudhuri, *Anal. Biochem.*, 156 (1986) 136.
71 C. R. Merril and M. E. Pratt, *Anal. Biochem.*, 156 (1986) 90.
72 C. R. Merril, M. L. Dunau and D. Goldman, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 453.
73 C. R. Merril, *Acta Histochem. Cytochem.*, 19 (1986) 655.
74 D. F. Hochstrasser, A. Patchornik and C. R. Merril, *Anal. Biochem.*, 173 (1989) 412.
75 C. R. Merril, D. Goldman and M. L. VanKeuren, *Electrophoresis*, 3 (1982) 17.
76 M. Schleicher and D. M. Watterson, *Anal. Biochem.*, 131 (1983) 312.
77 A. Wedrychowski, R. Olinski and L. S. Hnilica, *Anal. Biochem.*, 159 (1986) 323.
78 F. Otsuka, S. Koisumi, M. Kimura and M. Ohsawa, *Anal. Biochem.*, 168 (1988) 184.
79 J. K. Dzandu, M. E. Deh, D. L. Barratt and G. E. Wise, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 1733.
80 M. E. Deh, J. K. Dzandu and G. E. Wise, *Anal. Biochem.*, 150 (1985) 166.
81 H. M. Poehling and V. Neuhoff, *Electrophoresis*, 2 (1981) 141.
82 C. Damerval, M. LeGuilloux, J. Blaisonneau and D. deVienne, *Electrophoresis*, 8 (1987) 158.
83 T. Rabilloud, G. Carpentier and P. Tarroux, *Electrophoresis*, 9 (1988) 288.
84 W. Quitschke and N. Schechter, *Anal. Biochem.*, 124 (1982) 231.
85 G. G. Giulian, R. L. Moss and M. Greaser, *Anal. Biochem.*, 129 (1983) 277.
86 D. W. Sammons, L. D. Adams and E. E. Nishizawa, *Electrophoresis*, 2 (1981) 135.
87 E. Gianazza, A. Frigerio, A. Tagliabue and P. G. Righetti, *Electrophoresis*, 5 (1984) 209.
88 J. M. Morrissey, *Anal. Biochem.*, 117 (1981) 307.
89 H. Blum, H. Beier and H. J. Gross, *Electrophoresis*, 8 (1987) 93.
90 D. F. Hochstrasser and C. R. Merril, *Appl. Theor. Electrophoresis*, 1 (1989) 35.
91 B. A. Perret, R. Felix, M. Furlan and E. A. Beck, *Anal. Biochem.*, 131 (1983) 46.
92 R. McLachlan and D. Burns, in V. Neuhoff (Editor), *Electrophoresis '84*, Verlag Chemie, Weinheim, 1984, p. 324.
93 H. J. Biel, P. Gronski and F. R. Seiler, *Electrophoresis*, 7 (1986) 232.
94 L. Kerenyi, F. Gallyas, *Clin. Chim. Acta*, 38 (1972) 465.
95 J. A. Black, *Electrophoresis*, 6 (1985) 27.
96 M. Porro, S. Viti, G. Antoni and M. Saletti, *Anal. Biochem.*, 127 (1982) 316.
97 K. Tsutsui, T. Kurosaki, H. Nagai and T. Oda, *Anal. Biochem.*, 146 (1985) 111.
98 G. D. Jay, D. J. Culp and M. R. Jahnke, *Anal. Biochem.*, 185 (1990) 324.
99 G. Dubray and G. Bezard, *Anal. Biochem.*, 119 (1982) 325.
100 C.-M. Tsai and C. E. Frasch, *Anal. Biochem.*, 119 (1982) 115.
101 T. Marshall, *Electrophoresis*, 4 (1983) 269.
102 X. Su, *Anal. Biochem.*, 163 (1987) 535.
103 D. Goldman and C. R. Merril, *Electrophoresis*, 3 (1982) 24.
104 B. Lomholt and S. Frederiksen, *Anal. Biochem.*, 164 (1987) 146.
105 M. Gottlieb and M. Chavko, *Anal. Biochem.*, 165 (1987) 33.
106 R. N. Skopp and L. C. Lane, *Anal. Biochem.*, 169 (1988) 132.
107 A. Paleologue, J. P. Reboud and A. M. Reboud, *Anal. Biochem.*, 169 (1988) 234.
108 G. L. Igloi, *Anal. Biochem.*, 134 (1983) 184.