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THE MECHANISM OF SILVER STAINING OF PROTEIN IN POLYACRYLAMIDE GEL

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The mechanisms of the penetration of silver ions into gels and of the precipitation of metallic silver on protein were studied and the critical parameters for silver staining of proteins in polyacrylamide gels established. A simple procedure was elaborated for development of gels using soluble methylamine–silver complex. The kinetic equation for the rate of precipitation of metallic silver in the gel was deduced and analyzed for conditions with and without limitation by the diffusion of the reactive components in the gel.

Key words: *development, protein, staining, silver, polyacrylamide gel.*

Silver staining of proteins in polyacrylamide gels is comparable in sensitivity to methods using radioactive labels. An advantage of silver staining is that it allows the analysis of natural protein mixtures. In addition, several of the procedures which have been proposed yield different shades of color for different proteins, which is useful for the identification of proteins.

However, the advantages of silver staining are markedly depreciated by its lack of reproducibility. This difficulty is often mentioned by authors who routinely use silver staining methods in their investigations. The complications are apparently associated with the fact that the mechanism of silver staining has not be thoroughly investigated, and, therefore, the parameters which determine the reproducibility of selective metallic silver precipitation on the proteins in the gel are not well defined. Most of the currently-used silver staining procedures are based on one of the two following schemes.

The first scheme [9, 11, 14, 15] includes stages of impregnation of the gel with an alkali-soluble ammonia–silver complex, washing with water, and development in a dilute solution of formaldehyde and citric acid.

The second scheme [7, 8, 12, 13] involves saturation of the gel with $AgNO_3$ solution, treatment with formaldehyde in a solution of strong alkali (0.75 N NaOH), and development by the residual formaldehyde during subsequent changes of the alkaline solution.

The versions of these two schemes used by various authors differ only in the pre-treatment of the gels before saturation with the silver compound, the number of washings, the concentrations of solutions used, and the time of treatment.

The goal of the present work was to elucidate the mechanism of silver staining of protein by analyzing both schemes, making use of the literature and our own observations, and to devise a simple staining procedure free of the disadvantages of the procedures proposed formerly.

MATERIALS AND METHODS

Reagents. Acrylamide (Serva, Germany), 10675); bis-acrylamide (Serva, 29195); Tris (Serva, 37180); glycine (Bio-Rad, USA), 161-0718); SDS (Bio-Rad, 161-0302); TEMED (Reanal, Hungary); ammonium persulfate (Serva, 13375); rectified ethanol; sodium hydroxide (chemically pure); 25% aqueous ammonia (chemically pure); aqueous methylamine (pure), purified by

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distillation (final concentration 9 M); silver nitrate (analytical grade); 37% aqueous formaldehyde (Merck, Germany), 4003); citric acid (chemically pure); rabbit blood serum (75 mg/ml protein). Distilled deionized water was used in all experiments.

Preparation of Polyacrylamide Gel Samples for Silver Staining. Gel-forming solution containing 11.7% acrylamide, 0.30% bisacrylamide, 0.1% SDS, 0.025 M Tris, 0.192 M glycine, 0.2% TEMED, and 0.005% ammonium persulfate was polymerized between two horizontal glass plates with 1.5 mm clearance. In order to achieve the required protein concentration in the gel, a sufficient amount of serum was introduced into the gel-forming solution prior to the addition of ammonium persulfate. Samples (7 x 13 mm) were cut from the gel slabs and used in the following experiments.

Procedure for Staining of Samples. Staining was carried out according to the first scheme. Samples were washed overnight or longer with 50% ethanol, then treated with an ammonia—$AgNO_3$ solution containing NaOH or with a methylamine—$AgNO_3$ solution in the presence or absence of NaOH, washed with water, and developed with a low-concentration solution of citric acid and formaldehyde. After development the samples were fixed in a solution containing 50% ethanol, 1% glycerol, and 1% acetic acid. In order to ensure identical conditions of treatment and effective agitation of the solutions, the development was carried out as follows. Three gel samples (two of which contained different concentrations of protein and the third one without protein served as a control) were placed into 8 mm (internal diameter) by 120 mm tubes with a hole in the bottom. The samples were separated in the tubes by rings cut from silicone hose, which hugged the tube wall and prevented the samples from falling down. The tubes with the samples were placed into the holes of a plate which was connected with a cam on a motor shaft, so that the plate carrying the tubes was subjected to vertical reciprocating movement. The tubes with the samples were then placed into larger tubes (13-14 mm i.d. x 100 mm length) on a rack, the larger tubes containing the solutions for treatment of the samples. The volume of the solution in these tubes was 5.5 ml, i.e. 12 times the total volume of the samples. Treatment of the samples was carried out with continuous mixing achieved by the vertical movement of the tubes with the samples.

Measurement of the Ag^+ concentration (in millivolts) and pH was performed with Ag^+-ion-selective and glass electrodes, respectively, against a calomel reference electrode connected with the measuring cell by a salt bridge containing 0.2 M $NaNO_3$ solution. A calibration curve was plotted to convert from millivolts to molar concentration logarithms.

Staining Procedure for with Methylamine. The procedure was intended for staining of polyacrylamide gel slabs (150 x 150 x 1.5 mm) with acrylamide concentration 11.7% and bisacrylamide concentration 0.30%. All solutions had constant temperature (20-21°C) and volume (400 ml). The gel slab was kept in 50% ethanol overnight or longer and then incubated for 1 h in freshly prepared 3 mM $AgNO_3$ 24 mM methylamine solution. The gel slab was then washed with water for 20 min and developed for 20-30 min with 12 mM formaldehyde 0.34 mM citric acid solution. After development the gel was fixed as described in the previous section. The gel could be stored in the fixing solution for several days. The gel was continuously shaken during the stages of saturation with silver, washing, development, and during the first 10 min of the fixation. All stages of treatment were performed under relatively dim light.

Initiation of Silver Reduction by Colloidal Silver Particles in Tubes. Twenty microliters of 1 M $AgNO_3$ and 0.1 ml of 0.2 M NaOH were added to 0.5 ml of 0.1% albumin solution. A control solution without albumin was prepared simultaneously. The solutions were incubated at 35°C for 20 min. Both tubes were then supplemented with 5 μl of 9 M methylamine solution. As a result, the mixture in the control tube became colorless, whereas that in the tube with the protein acquired an orange-red color of colloidal silver. When adding 8 μl of this solution to 0.4 ml of solution containing 43 mM formaldehyde, 0.5 mM citric acid, and 20 mM $AgNO_3$, one can observe a rather rapid color development caused by growth of microparticles of colloidal silver. When the same procedure is applied to the control solution, the appearance and the development of color takes much more time.

RESULTS AND DISCUSSION

It became obvious even on primary consideration that the precipitation of silver on proteins in polyacrylamide gels is a rather complex chemical reaction with the following characteristics.

1. The reaction is initiated predominantly by proteins.
2. The reaction is heterogeneous since it occurs on the surface of metallic silver particles.
3. The reaction rate depends on the diffusion rate of the limiting component in the gel.

These characteristics are typical of nonequilibrium, nonlinear reactions. In addition, the involvement of a diffusion process makes the reaction sensitive to changes in parameters such as temperature, thickness and density of the gel, concentra-

tion of the cross-linker in the gel (acrylamide/bisacrylamide ratio), and even the ratio of volumes of the gel and the solutions used for its treatment.

Mechanism of Staining According to the First Scheme. The most important aspect of the silver staining process appears to be the extent to which the protein is darkened during the final stage of development of the gel. Therefore, it seemed reasonable to begin our study of the silver staining mechanism with this final step. For this purpose, the influences of the concentrations of the components of the developing solution used in the first scheme on the intensity of darkening of gels with and without protein were investigated.

The conditions of the experiment are listed in Fig. 1. Figure 1a shows that the intensity of staining decreases with increasing citric acid concentration and increases with increasing formaldehyde concentration. The changes observed are associated not only with changes of the optical density, but also with differences in the thickness of the stained layer within the gels (Fig. 1b).

This may be explained as follows. As the developing solution penetrates into the gel, two opposing reactions occur, i.e. dissolution of the priming colloidal silver particles (which were formed at the previous stages) in the acidic medium, and growth of the particles due to reduction of silver ions by formaldehyde. At the initial stage of penetration, when the acidity of the penetrating solution is high, the dissolution of silver particles predominates in the surface layers of the gel. However, the pH of the medium in the gel and in the external washing solution become equal as the alkaline solution diffuses from the gel and the acidic developing solution diffuses into the gel (see Fig. 4 below). As a result, the growth of silver microparticles predominates in the internal layers of the gel, where the pH is not less than the equilibrium pH. This accounts for the decrease of the thickness and the optical density of the stained layer in the gel, including the disappearance of the stained layer as the citric acid concentration is increased and the formaldehyde concentration is decreased in the initial developing solution and, vice versa, the increase of the optical density and thickness of the stained layer up to the often observed expansion of the silver precipitation reaction to the gel surface and external washing solution when the citric acid concentration is decreased and formaldehyde concentration is increased. In fact, the precipitation of silver on the surface of the gel was noted when citric acid was excluded from the developing solution [4].

Another probable cause for the precipitation of silver on the surface of the gel is local alkalization of the washing solution near the surface of the gel because of insufficient mixing of the acidic developing solution and the alkali diffusing from the gel.

Since increasing the formaldehyde concentration decreases the inhibition of the development by excess citric acid, it is important to keep the ratio of the citric acid and formaldehyde concentrations constant in the developing solution to make the staining process stable.

From the explanations given above, it follows also that the development depends on the ratio of the absolute amounts of acid in the developing solution and the residue of alkali in the gel, i.e. not only on the concentrations but also on the volumes of the developing solution and the gel. This means that the development can be controlled also at the initial stages by changing the total alkali content in the solutions of silver complexes with ammonia or organic amines. This conclusion was confirmed by the fact that doubling the concentration of the ammonia–silver solution in an experiment analogous to that shown in Fig. 1 (with 0.24 mM citric acid 6.2 mM formaldehyde developing solution) led to silver precipitation on the gel surface, and halving the concentration of the solution completely inhibited the precipitation of silver. This observation is in agreement with data presented in [10], where the authors mentioned that a change of the NH_3 and NaOH concentration along the border between the regions of dissolution and precipitation of Ag_2O results in no staining of the proteins in the gels at a low NaOH concentration, satisfactory staining at intermediate NaOH concentrations, and is accompanied by silver precipitation on the gel surface at high NaOH concentrations.

Figure 2a shows that increasing the concentration of NaOH at the initial stage and of citric acid at the final stage have opposite effects on silver precipitation in the gel. As in the case of citric acid and formaldehyde, increasing the concentration of citric acid decreases the thickness of the silver-stained protein layer in the gel and increasing the NaOH concentration causes the opposite effect (Fig. 2b), up to the expansion of the silver precipitation reaction to the border between the gel and solution phases. One can therefore conclude that, in addition to maintaining the ratio of citric acid and formaldehyde concentrations in the developing solution, it is very important to keep the ratio of the total alkali content in the amine–silver solution and the absolute acid content in the developing solution constant to achieve reproducible gel development.

However, the effect of increasing the ammonia concentration in the ammonia–silver solution on the development is ambiguous. We found that an excess of ammonia can inhibit the precipitation of metallic silver in the gel, and increasing the ammonia concentration three times (compared to its content in the $Ag(NH_3)_2^+$ complex) completely inhibits the development.

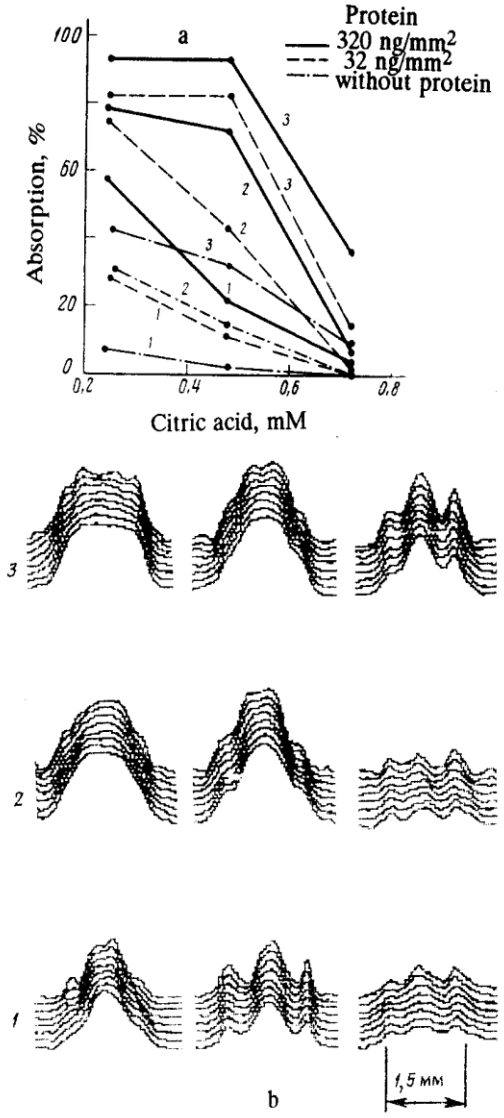

Fig. 1. Influence of the concentrations of citric acid and formaldehyde on the intensity of silver staining of proteins in gels. The samples were incubated for 1 h in solution containing 23.5 mM $AgNO_3$, 50 mM NH_3, and 9.3 mM NaOH, washed for 10 min with water, and developed for 20 min in a dilute solution of formaldehyde and citric acid. The formaldehyde concentrations in the developing solution were 6.2 mM (1), 12.4 mM (2), and 18.6 mM (3). a) Optical absorption by gel. b) The results of scanning cross-sections of developed samples containing 320 ng/mm^2 protein. The peak of the colloidal silver optical density is near the center of the cross section, and edge effects are seen on each side. The numbers of the vertical rows correspond to the numbers denoting the formaldehyde concentrations. The three horizontal columns correspond to the three citric acid concentrations shown in (a).

Such inhibition of development is not observed when an excess of methylamine is used. The different effects of the high concentrations of ammonia and methylamine on development can be explained from analysis of the differences in the titration of $AgNO_3$ solution by ammonia and methylamine (see Fig. 3) and of the expression for the dissociation constant of the silver complexes

$$K_d = [Ag^+][A]^2/[Ag(A)_2^+], \quad (1)$$

where K_d is the dissociation constant and A is ammonia or methylamine.

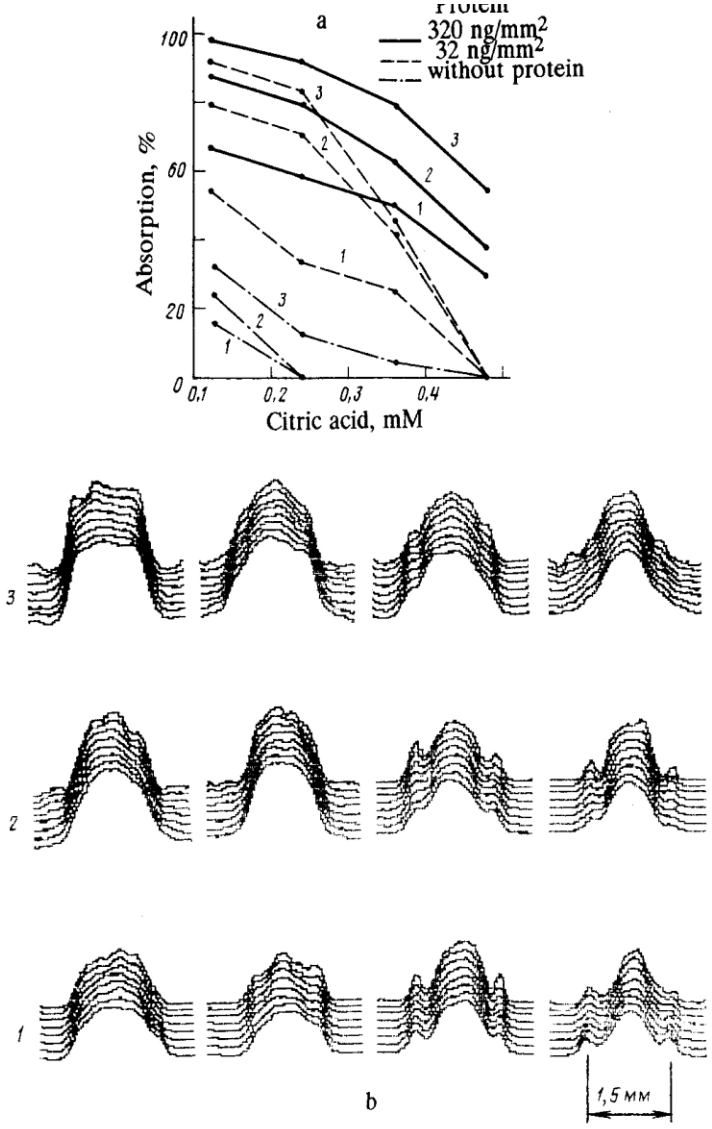

Fig. 2. Influence of the concentrations of citric acid and NaOH on the intensity of silver staining of proteins in gels. The samples were incubated for 1 h in solution containing 3.0 mM $AgNO_3$, 12 mM CH_3NH_2, and various concentrations of NaOH, washed for 20 min with water, and developed for 30 min in a solution of 12.4 mM formaldehyde and various concentrations of citric acid. The NaOH concentrations in the solution of methylamine–silver complex were 0 mM (1), 1.2 mM (2), and 2.4 mM (3). a) Optical absorption by gel. b) The results of scanning cross-sections of stained samples containing 320 ng/mm^2 protein. The peak of the colloidal silver optical density is near the center, and edge effects are seen on each side. The numbers of the vertical rows correspond to the numbers denoting the NaOH concentration. The four horizontal columns correspond to the four concentrations of citric acid shown in (a).

To interpret the titration curves using Eq. (1), let us denote the initial silver ion concentration in the solution as $[Ag^+]_0$ and the ratio of the initial ammonia or methylamine concentration and $[Ag^+]_0$ as n. Then at a small K_d value and $n > 2$ one can assume that $[Ag(A)_2^+] \approx [Ag^+]_0$ and Eq. (1) can be written as

$$K_d = \frac{[Ag^+]((n-2)[Ag^+]_0)^2}{[Ag^+]_0}.$$

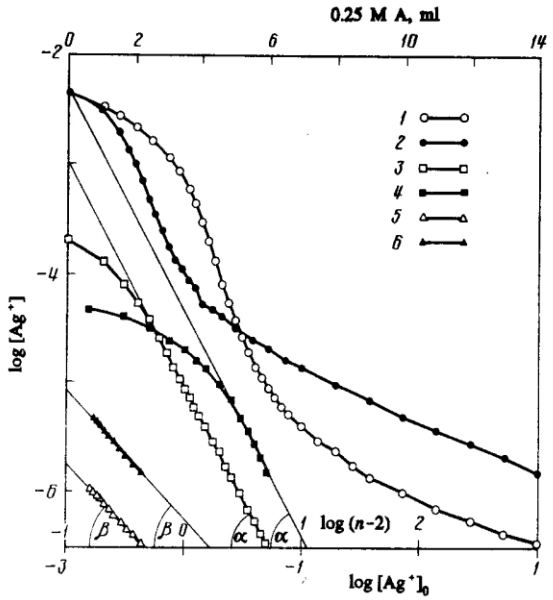

Fig. 3. Titration of 100 ml of 5 mM $AgNO_3$ by 0.25 M solutions of A (NH_3 (1) and CH_3NH_2 (2)). Log $[Ag^+]$ as a function of log $(n - 2)$ for the right branches of the NH_3 (3) (log $K_d = -7.35$; tan $\alpha = 2$) and CH_3NH_2 (4) (log $K_d = -6.6$; tan $\alpha = 2$) titrations curves drawn in accordance with Eq. (3). Log $[Ag^+]$ as a function of log $[Ag^+]_0$ obtained by dilution of the $AgNO_3$ solutions after titration by the NH_3 (5) (log $K_d = -7.4$; tan $\beta = 1$) and CH_3NH_2 (6) (log $K_d = -6.7$; tan $\beta = 1$) solutions at the same n values. The dissociation constants for the silver—ammonia complex and the silver—methylamine complex are found by two independent methods on the basis of the data from the corresponding graphs by the formula log K_d = log $[Ag^+]$ + 2·log $(n - 2)$ + log $[Ag^+]_0$ obtained by transformation of Eq. (3).

Hence, the free silver ion concentration described by the right branch of the titration curves is

$$[Ag^+] = \frac{K_d}{(n-2)^2[Ag^+]_0}. \quad (2)$$

Taking the logarithm of Eq. (2) gives

$$\log [Ag^+] = \log K_d - 2\log (n-2) - \log [Ag^+]_0. \quad (3)$$

Equation (2) shows that when the initial silver ion concentration is constant, there will be an inverse quadratic decrease in the free silver ion concentration in solution with respect to $n - 2$. Figure 3 shows that the right part of the titration curve falls markedly lower with ammonia than with methylamine. The differences in log $[Ag^+]$ for the right parts of the titration curves approach 0.7, corresponding to an approximately 5-fold difference in the Ag^+ concentrations. The low Ag^+ concentrations in the right part of the titration curve with ammonia apparently prevent silver precipitation in the gel. At the same time, in the steep part where the Ag^+ concentration is high enough for the deposition process, silver staining will be insufficiently reproducible due to the strong dependence of the Ag^+ concentration on unintended variations in the ammonia concentration caused, for example, by its volatility. The methylamine—silver complex has a significant advantage since it retains its staining properties in the right part of the titration curve.

The stronger staining of proteins observed by Marshall and Latner [5] with methylamine—silver complex compared to the ammonia complex and other organic amine complexes must also be accounted for by a higher concentration of free Ag^+ in the solution.

The difference between the Ag^+ concentrations in the right part of the ammonia and methylamine titration curves is due to the considerably higher stability of the silver—ammonia complex compared to the silver—methylamine complex. The logarithms of the dissociation constants can be calculated for these complexes from Eq. (3) using the straight segments of the dependencies of log $[Ag^+]$ on log $(n - 2)$ at constant $[Ag^+]_0$ obtained from the titration curves and the dependencies of

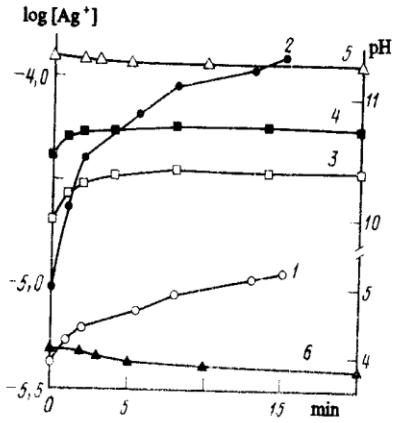

Fig. 4. Kinetics of the establishment of equilibrium of pH and Ag^+ concentration between gel and solutions at different stages of the silver staining procedure. The curves marked with triangles (5, 6), squares (3, 4), and circles (1, 2) are for the treatment of gels with solution of silver–methylamine complex, washing with water, and development with formaldehyde–citric acid solution, respectively. The closed symbols are for the log [Ag^+] and the open symbols are for the pH values. The treatment conditions are given in the section "Staining Procedure with Methylamine".

log [Ag^+] on log [Ag^+] at constant n values obtained by dilution of the complex (see Fig. 3). The log K_d values for the ammonia complex thus obtained are −7.35 and −7.4 and for the methylamine complex −6.6 and −6.7. Three values for log K_d of the ammonia complex are given in [1], −7.7 at 0°C, −7.2 at 25°C, and −6.2 at 52°C. Since our experimental data were taken at 17°C, the calculated values of the constants can be compared. It is seen that the K_d of the ammonia–silver complex is strongly dependent on temperature, so that, for example, changing the temperature from 17 to 52°C changes the value of K_d 10-fold and, therefore, changes the concentration of Ag^+ 10-fold in the presence of excess ammonia in the solution. Therefore, the difference between the Ag^+ concentrations in the solutions of the complexes of silver with ammonia and methylamine in the right part of the titration curves can be compensated by changing the temperature; however, it is necessary to carefully control the temperature during the development.

The break in the ammonia titration curve corresponds to the structure of the ammonia–silver complex, which contains two molecules of ammonia per silver atom. The break in the methylamine titration curve appears to correspond to an equimolar ratio of methylamine and silver, suggesting the inclusion of only one methylamine molecule in the complex. However, the difference in the position of the break in the methylamine titration has another cause. Methylamine is more alkaline (pK_a 10.6) than ammonia (pK_a 9.25), resulting in the formation of AgOH before the formation of $Ag(CH_3NH_2)_2^+$ during the titration with methylamine. This is manifested by the abundant precipitation of Ag_2O and the break in the titration curve corresponding to the equimolar quantity of the silver ions being titrated. In the titration with ammonia AgOH formation is not observed and the equivalence point corresponds to the direct formation of the $Ag(NH_3)_2$ complex. The facts that initial segments of the dependencies of log [Ag^+] on log (n − 2) are parallel (Fig. 3) and that the tangents of their incidence angles α are equal to 2 show that, despite the difference in the procession of the reactions, in both cases a 2:1 complex with silver is finally formed. According to Eq. (3), the value of the tangent indicates the presence of two molecules of ammonia or methylamine in the complex. The facts that the straight lines reflecting the change in log [Ag^+] on dilution of the complexes are parallel and that the tangents of their incidence angles β are equal to 1, which also follows from Eq. (3), serve as a control.

The greater alkalinity of methylamine allows the use of the methylamine–silver complex for the staining of alkaline and neutral proteins in the gel without the addition of NaOH. Exclusion of this component simplifies the staining procedure and improves the reproducibility of the process.

According to the first scheme for staining, the initial stage of gel treatment and treatment with citric acid and formaldehyde are separated by washing of the gels with distilled water. The washing should be performed as rapidly as possible (in practice the procedure takes from 30 s to 1 min). We found that the equilibrium between water and gel relative to both $[Ag^+]$ and pH is established during incubation for 5-8 min (see Fig. 4). Therefore, small errors in the determination of the washing time during the first 2-3 min result in considerable deviations in the concentrations of the solutions in the gels.

Recommendations to reduce the washing time to a minimum are probably intended to minimize the loss of silver from the gels. In fact, some loss of silver is unavoidable. However, it is seen from the experiment (see Fig. 4) that during the equilibrium washing following the first stage of treatment of gels the concentration of free Ag^+ ions in the solution does not decrease but even considerably increases. This apparent paradox follows directly from Eq. (2), according to which a decrease of the initial concentration of silver ions at constant n value (i.e. during dilution of the solution) will cause an inversely proportional increase of the free Ag^+ concentration. The experimental dependencies of log $[Ag^+]$ on log $[Ag^+]_0$ demonstrate especially clearly the increase of Ag^+ concentration on dilution of the amine–silver complexes (see Fig. 3). In full agreement with Eq. (3), these dependencies are described by straight lines with a negative tangent of the incidence angle β for both the ammonia–silver and the methylamine–silver complexes. Proceeding from these facts, one can conclude that increasing the gel washing time up to the time for reaching the equilibrium between solution and gel stabilizes and intensifies staining by increasing the free Ag^+ concentration in the solution. The intensification is clearly confirmed by experiments with prolongation of the washing step.

It should also be noted that the ratio of the gel to water volume during washing step must be kept strictly constant to maintain the ratio between the content of alkali in the gel and acid in the solution at the development step.

Mechanism of Staining According to the Second Scheme. Staining according to the second scheme is very similar to the synthesis of the drug collargolum [2], which is prepared by incubation of a solution of protein and $AgNO_3$ in the presence of NaOH. As a result, soluble colloidal silver forms which has different shades (from yellow and orange to dark blue) depending on the conditions of the preparation. Analogously, particles of colloidal silver seem to form on the protein in the gel and then grow during the reduction of Ag^+ by formaldehyde up to a size at which they absorb light strongly.

However, it remains unclear how this process is retained within the gel under the alkaline conditions without expansion to the border between the gel and solution phases. Certainly, this is favored by the Ag^+ concentration gradient between the gel and the alkaline solution, which is maintained by the precipitation of Ag_2O in the gel immediately after its contact with the NaOH solution. The precipitate thus obtained is observable for the first several minutes of the gel treatment. However, we believe that this explanation is insufficient. More likely, the answer is related to the hydrolysis of the amide group of polyacrylamide during the course of its treatment with the NaOH solution. This hydrolysis can easily be followed by the appearance of a characteristic odor of ammonia or intensive staining of the Nessler reagent on addition of small amounts of alkali to polyacrylamide gel pieces. The formation of ammonia on treatment of gel with alkali suggests that silver staining of proteins according to the second scheme proceeds as follows. The ammonia which is evolved dissolves the Ag_2O precipitate in the gel forming the $Ag(NH_3)_2^+$ complex. If the NaOH solution contains formaldehyde, the latter enhances formation of priming silver microparticles in the gel. Replacement of the NaOH solution by a sodium bicarbonate solution should sharply slow down hydrolysis of polyacrylamide gel by decreasing the pH of the medium and thus prevent the suppression of the formation of silver particles by excess ammonia. Silver reduced by formaldehyde continues to precipitate on the formerly prepared priming. The process of growth of the colloidal silver particles induced by the protein in the presence of NaOH can easily be modeled in a test tube (see the section in "Materials and Methods").

A decrease of the thickness of the reduced silver layer in the gel to the complete inhibition of development as the time of exposure of the gel to 0.75 N NaOH [13] increases serves as an additional confirmation of this mechanism of the development. This phenomenon can be accounted for by an excessive evolution of ammonia. It is easily seen that this effect is similar to the above-mentioned effect of the decrease in the silver layer when the acid concentration in the developing solution is increased. The only difference is that in this case ammonia, which forms a stable complex with silver, is the solubilizing factor rather than acid. The solubility of finely divided silver in ammonia was described as early as in the previous century [3].

A disadvantage of silver staining according to the second scheme is the formation of an intense yellow gel background, which seems to be associated with the appearance of many carboxyl groups on hydrolysis of the polyacrylamide gel by alkali. This conclusion is confirmed by an enhancement of the yellow background on addition of acrylic acid to the gel prior to polymerization.

Kinetic Equation for the Precipitation of Ag on Protein. The kinetics of the precipitation of silver on the proteins can be described proceeding from the following considerations. We assume that the metallic silver accumulation rate dM/dt in an

isolated region of the gel is proportional to the free silver ion concentration, the reducing agent concentration, and the total surface area of the silver microparticles in this region

$$dM/dt = k[Ag^+]rS,$$

where M is the concentration of metallic silver in the gel, t the time, k the temperature-dependent rate constant, $[Ag^+]$ the silver ion concentration, r the reducing agent concentration, and S the total surface area of the silver microparticles.

Assuming that the linear sizes of the microparticles vary proportionally during the growth process, their total surface is

$$S = gNm^{2/3},$$

where g is a constant depending on the density of silver and the form of the particles, N the number of microparticles in the isolated region of the gel, and m the average mass of the microparticle. Taking into account that $m = M/N$, we find

$$S = gN^{1/3}M^{2/3}$$

and

$$dM/dt = kgr[Ag^+]N^{1/3}M^{2/3}. \quad (4)$$

Apparently, the diffusion rate of the silver ions to the reaction site in the gel slab can be expressed as

$$d[Ag^+]/dt = k_d([Ag^+]_s - [Ag^+]), \quad (5)$$

where k_d is a coefficient depending on the gel thickness and density, the concentration of cross-linker, the temperature, etc., and $[Ag^+]_s$ the concentration of silver ions in the solution washing the gel slab.

Proceeding from the fact that in the presence of excess reducing agent the steady state diffusion rate of the silver ions is equal to the precipitation rate, we write

$$dM/dt = d[Ag^+]/dt. \quad (6)$$

Solving the system of Eqs. (4)-(6) for M and t with initial conditions $t = 0$ and $M = 0$, we find

$$M = \frac{k_d kgr[Ag^+]_s N^{1/3} t}{kgrN^{1/3} + 3k_d/M^{2/3}}. \quad (7)$$

Analyzing this formula, we find the expressions for two extreme cases.

The first one corresponds to the situation

$$kgrN^{1/3} \ll 3k_d/M^{2/3},$$

i.e. to a low concentration of the protein in the gel or to the initial step of the reaction, when the amount of silver precipitated is small and k_d does not restrict the approach of Ag^+ to the growing microparticles. Disregarding the term $kgrN^{1/3}$ in Eq. (7) we get

$$M = (1/3kgr[Ag^+]_s t)^3 N. \quad (8)$$

The third power dependence of the amount of silver precipitated on the rate constant k, the reducing agent concentration r, the silver concentration $[Ag^+]$, and the time t accounts for the high sensitivity of the reaction to changes in temperature, pretreatments of the gels with aldehydes [9, 15] and changes of the concentration of the reducing agent [11], the decrease of the free $[Ag^+]$ concentration resulting from an increase of the ammonia concentration in the initial solution, and a long lag-period (up to 15 min and more), which delays the visually observable silver staining of the proteins, respectively. At the same time the linear dependence of the amount of silver precipitated on the number of priming colloidal silver particles N reflects the linear

dependence of the optical density of the silver stained protein spots on the protein concentration, as established by a number of authors [4, 6, 7].

The second case corresponds to the situation

$$kgrN^{1/3} > 3k_d/M^{2/3},$$

i.e. to a high protein concentration in the gel or to the final step of the reaction when the amount of silver precipitated is large and the k_d value limits the approach of Ag^+ to the growing microparticles. Disregarding the $3k_d/M^{2/3}$ in Eq. (7) gives

$$M = k_d[Ag^+]_s t. \tag{9}$$

Thus, the silver precipitation in the gel during the final steps of the reactions has linear dependencies on k_d, the Ag^+ concentration in the solution, and time and does not depend on N. This means that in those regions of the gel, where the N value and the size of silver microparticles are high, their growth rate is restricted to the rate of the Ag^+ diffusion to the microparticles. This accounts completely for the fact that the linear dependence of the amount of silver precipitated on the protein concentration turns into plateau at high protein concentrations [4, 6]. The regions of the gel with the high N reduce the Ag^+ concentration in the neighboring regions with lower N, thereby slowing down the formation of microparticles in these regions in accordance with Eq. (8). A consequence of this is improved contrast of the silver stained protein spots.

This effect of contrast becomes especially apparent in the formation of small light spots on a darkened background in the plane gels. Formation of these spots is due to the presence in their central part of specks of dust which precipitate a lot of metallic silver on their surface. This process leads to a sharp reduction of the Ag^+ concentration around the dust particles and, as a result, to slowing down the microparticle growth in the area of the spot.

When the microparticles sizes become high enough throughout the whole gel surface, Eq. (9) describes the formation of the uniform background in the gel which completely darkens the protein spots which appeared earlier. Because the k_d value depends on the thickness and density of the gel, the concentration of cross-linker, and the temperature, the intensity of staining of the protein spots and the background formation depend on these parameters as well. Particularly, a decrease of the protein staining intensity on an increase of the density in the gradient gel was mentioned by Guevara and coworkers [4]. An increase of the gel density and resulting decrease in k_d can account for the slowing of the rate of protein staining and background formation found by Wray [15] on addition of ethanol to the developing solution, which is known to reduce the linear dimensions of the gel. Sammons [13] found a considerable increase of the staining intensity of the background and spots with a small increase in the temperature of the developing solution.

Equation (7) was obtained for the case of reducing agent in excess. However, it is not difficult to show that, if the reducing agent is the limiting component, this equation does not change significantly; the only change is that r is replaced by $[Ag^+]_s$ in the denominator. Thus, the Eq. (8) will not change and in the other extreme case (Eq. (9)) $[Ag^+]_s$ will be replaced by r, i.e. almost all of the regularities considered above remain valid.

The fact that the change of the Ag^+ concentration in the gel resulting from the silver diffusion into the solution and acidification of the gel was disregarded when Eq. (7) was formulated seems to be insignificant. In fact, it follows from the Fig. 4 that the equilibrium log $[Ag^+]$ value does not decrease as a result of diffusion below its initial level on the gel surface, which is defined by the value of log $[Ag^+]$ after washing with water and, in contrast, even exceeds it. Therefore, despite the decrease of the Ag^+ supply for the reaction from buffer compounds as hydroxide or complexes with amines, an increase of the free Ag^+ concentration resulting from destruction of these complexes in the acidic medium compensates the loss to a significant extent.

In conclusion, attention must be paid to two additional factors which can influence the reproducibility of silver staining.

We found that keeping the gel in 50% ethanol for a long time (up to two weeks and more) increases the staining contrast. A similar effect is achieved by washing the gels for 24 hours in heated 50% ethanol. The changes observed may have two probable causes which are mentioned also in the literature: washing sodium dodecylsulfate out of the protein [4, 10] and modification of the protein by aldehyde contaminations present in ethanol [15].

Another factor, noticed by us and also known from the literature, is illumination, which can significantly influence the reproducibility of silver staining [6]. Therefore, the illumination of the work place must be controlled to achieve reproducible development.

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