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Air Carbon Dioxide Prevents Proteins from Being Developed by Silver Staining in Polyacrylamide Gel

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Staining with colloid metal silver is widely used for developing of protein zones produced in polyacrylamide gel electrophoresis (PAGE). Because PAGE is the main method of protein assay in proteomics, a new branch of biological research, the high sensitivity of the method of protein development (visualization) with silver makes it particularly important. However, the poor reproducibility is a substantial disadvantage inherent in all modifications of this method described in the literature thus far. This is due to the fact that the process of protein-induced precipitation of metal silver is insufficiently understood to provide effective control of its essential parameters [1]. On the other hand, the reproducibility of protein development is a necessary condition of exact quantitative assessment of the protein concentration in studies of physiological and biochemical processes. To meet this condition, we studied uncontrollable parameters affecting the process of protein development through silver staining.

Conventional procedures of protein development in polyacrylamide gel by silver staining include the stages of gel impregnation with a solution of a silver–amine complex in the presence of sodium hydroxide and development with a solution containing low concentrations of formaldehyde and citric acid. In most procedures, protein is developed using the silver complex with ammonia $[Ag(NH_3)_2]^+$ [2–4].

To provide comparative assessment of the reproducibility of the results of development of identical electrophoretic gel bands, the process of development was simultaneously run in four glass cuvettes: two cuvettes were control, whereas the development conditions in the other two cuvettes were modified. Gel strips ($150 \times 9 \times 1.5$ mm) were cut out of polyacrylamide gel plates after the plates had been used for protein electrophoresis. In accordance with the method of Laemmli [5], a 20-μl sample of human blood plasma was spread over the whole width of the electrophoresis plate (in the presence of 0.1% sodium dodecylsulfate and at a polyacrylamide gel concentration of 10% in the plate). Before cutting into strips, the plate was fixed for one day in 50% ethanol.

The volatility of ammonia was originally suggested to be a main cause of the poor reproducibility of the results of silver-mediated development of electrophoretic gel bands and insufficiently precise measurement of the concentration of silver complex with ammonia, $[Ag(NH_3)_2]^+$, in the solution. To overcome this disadvantage, ammonia was replaced by its nonvolatile analogue (ethanolamine). Although this replacement did not cause significant improvement of the efficiency of the method, it allowed one of the possible causes of poor reproducibility of this process to be avoided *a priori.* When the gel strips had been washed for 30 min in double distilled water, they were developed as follows: (1) the stage of gel impregnation for 1 h with a solution containing 3 mM $AgNO_3$, 24 mM ethanolamine, and 2.4 mM NaOH; (2) the stage of washing in double distilled water for 20 min; and (3) the stage of development for about 10 min with a solution containing 72 mM formaldehyde and 0.17 mM citric acid. The solution volume at all stages was 25 ml.

An unexpected result was obtained in studies of the effect of light on the reproducibility of the development of electrophoretic gel bands. Shielding of the cuvettes not only prevented experimental samples from being exposed to light, but also decreased their contact with air. The staining intensity of developed gels in the shielded cuvettes was significantly higher than in the cuvettes freely contacting with ambient air. This experiment was repeated many times using glass plates for insulating the cuvettes from ambient air, and the results obtained earlier using nontransparent shields were confirmed with a 100% reproducibility (Fig. 1).

To identify the stage of the procedure sensitive to the contact with air (i.e., to determine at which of three stages the contact with air causes a decrease in the intensity of gel staining with silver), we used experimental settings at which the cuvettes containing samples were selectively exposed to air only during the development stage of interest, whereas during the other stages, the cuvettes were covered with glass plates. These experiments demonstrated that the first stage of

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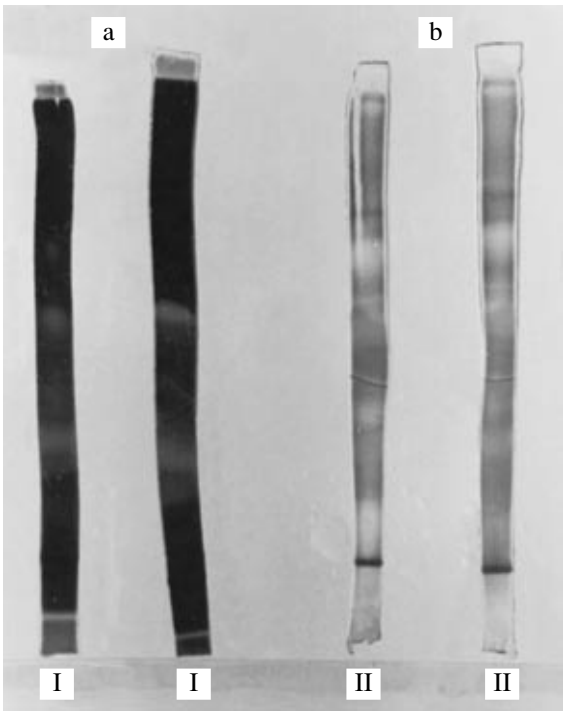

Fig. 1. The results of development of blood serum proteins in polyacrylamide gel strips using the ethanolamine complex with silver in (a) cuvettes covered with glass plates and (b) open cuvettes.

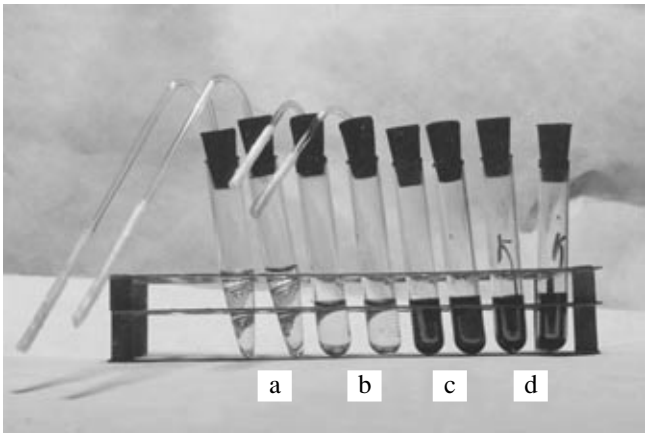

Fig. 2. The formation of a mirror layer on the inner surface of glass test tubes containing a 0.1 mM solution of the ethanolamine complex with silver after one day of incubation at room temperature: inhibition of silver precipitation as a result of CO_2 diffusion to the test tube from (a) a 120-μl capillary and (b) a 60-μl capillary; (c) silver precipitation in test tubes filled with CO_2-free oxygen; (d) silver precipitation in control test tubes filled with atmospheric air.

the procedure (gel saturation with the ethanolamine–silver complex) was the most sensitive to contact with air. The sensitivity of the second stage (washing with water) was expressed only as an insignificant delay of the onset of the third stage. Neither the process of development nor its final result was affected by the sample contact with air during the third stage of the procedure (development with a solution containing formaldehyde and citric acid). Similar experiments were performed using ammonia instead of ethanolamine. Because silver is more strongly bound in complexes with ammonia than in complexes with ethanolamine, the concentration of ammonia in the impregnation solution (9 μM) was lower than the corresponding concentration of ethanolamine. Development with ammonia was accompanied by the same effects as development with ethanolamine.

It was concluded on the basis of these results that exposure to air during the first stage of the procedure prevents the formation of invisible nucleation centers of silver precipitation. Contact with air during the second stage of the procedure decreases the size of the nucleation centers, thereby delaying the development at the last stage.

It was necessary to determine which of the two active components of air (oxygen or carbon dioxide) inhibited the formation of nucleation centers at the first stage of the development procedure. The effect of air-induced inhibition of silver-mirror formation on the walls of glass test tubes containing 1 ml of a 0.1 mM solution of the ethanolamine complex with silver ion that we found earlier was used to answer this question. This effect was observed in open test tubes, but not in the test tubes sealed with Parafilm. Within one day of incubation at room temperature, the walls of the test tubes sealed with Parafilm were coated with a dense layer of metal silver. Obviously, this phenomenon was similar to the effect of inhibition of silver precipitation in gels, but nucleation centers appeared on glass walls, and ethanolamine itself rather than formaldehyde played the role of reducer.

The fact that the Parafilm membrane was not drawn into test tube during incubation of the silver complex with ethanolamine can be regarded as evidence that this reaction is mediated by carbon dioxide (concentration in the atmosphere, 0.03%) rather than oxygen (concentration in the atmosphere, 21%).

The following experiment was performed to test this suggestion and to assess the amount of carbon dioxide required to inhibit the silver-mirror formation. Using glass capillaries filled with carbon dioxide gas, 120-μl samples of CO_2 were added to two test tubes (Fig. 2a) and 60-μl samples of CO_2 were added to other two test tubes (Fig. 2b). At the outer end, the capillaries were sealed with the Parafilm film, whereas the other end of each capillary was inserted in a rubber plug. Two more test tubes containing the solution were filled with oxygen preliminarily purified from carbon dioxide by treatment with potassium hydroxide (Fig. 2c). Finally, two test tubes filled with air served as a control (Fig. 2d). In the first case, there was a complete inhibition of the silver-mirror formation on the test-tube walls, whereas in the second case, there was a faintly visible thin silver

coating on the test-tube walls (not seen in Fig. 2). The results of the calculation of the rate of carbon dioxide diffusion from capillary showed that the amount of carbon dioxide diffusing from the capillary to the test tube in the second case (60 μl) was 40 times lower than the content of the silver complex with ethanolamine and 27 times higher than the content of carbon dioxide in the test tube. Such a large molar ratio of the silver–ethanolamine complex to the carbon dioxide concentration capable of preventing metal silver from being precipitated on the test-tube walls supports the conclusion that carbon dioxide inhibits formation of the nucleation centers for silver precipitation, rather than inhibits the process of silver precipitation itself. On the other hand, no symptoms of inhibition of the silver-mirror formation were observed in test tubes containing oxygen.

To determine whether carbon dioxide, in the form of carbonate ions, is involved in the inhibition of the formation of the nucleation centers for silver precipitation, samples of 60, 120, and 240 μl of 0.5 M sodium hydrocarbonate were added to test tubes containing 1 ml of 0.1 mM solution of the ethanolamine complex with silver. It was found that silver precipitation on test-tube walls was inhibited only in the case of addition of 240 μl of 0.5 M sodium hydrocarbonate. The amount of sodium hydrocarbonate added in this case was 1.2 times larger than the equimolar amount of the silver–ethanolamine complex. The addition of identical concentrations of sodium carbonate did not inhibit the reaction of the silver-mirror formation on test-tube walls. The results of these experiments suggest that the formation of nucleation centers for silver precipitation is inhibited by carbon dioxide in the form of CO_2 rather than carbonate ions. This suggestion is consistent with the literature data indicating that sodium carbonate was successfully used to substitute alkali in some PAGE methods for protein development by silver [6, 7], whereas potassium carbonate impurities had almost no effect on the quality of the mirror manufacturing technology [8].

It is well known that the concentration of carbon dioxide in a closed room as a result of vital activities of the human body may change by two orders of magnitude (from 0.03 to 3%). Taking into account this fact, as well as the dependence of the effect of inhibition of silver precipitation nucleation centers on carbon dioxide concentration, it may be concluded that variation of carbon dioxide concentration in air is the main uncontrollable parameter that affects the reproducibility of protein development by silver in polyacrylamide gel.

REFERENCES

1. Malygin, A.G., Dorokhina, N.I., and Gantserova, I.N., *Biokhimiya*, 1992, vol. 57, pp. 517–530.
2. Oakley, B., Kirsh, D.R., and Morris, D., *Anal. Biochem.*, 1980, vol. 105, pp. 361–363.
3. Poehling, H.-M. and Neuhoff, V., *Electrophoresis*, 1981, vol. 2, pp. 141–147.
4. Wray, W., *Anal. Biochem.*, 1981, vol. 118, pp. 197–203.
5. Laemmli, U.K., *Nature*, 1970, vol. 227, pp. 680–685.
6. Merril, C.R., Goldman, D., and Van Keuren, M., *Electrophoresis*, 1982, vol. 3, pp. 17–23.
7. Sammons, D.W., Adams, L.D., and Nishizawa, E.E., *Electrophoresis*, 1981, vol. 2, pp. 135–141.
8. Vinokurov, V.M., *Khimicheskie metody serebreniya zerkal* (Chemical Methods of Mirror Silver-Plating), Moscow, 1950.