ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2008, Vol. 34, No. 6, pp. 683–690. © Pleiades Publishing, Ltd., 2008.
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Carbon Dioxide of Air Inhibits the Formation of Silver Nanoparticles Initiated by Proteins in Polyacrylamide Gel and in Solution

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Received July 24, 2007; in final form, April 1, 2008

Abstract—It was shown that the staining of proteins in polyacrylamide gel by silver is inhibited by contact with air of the ammonia complex with silver ions used at the first stage of detection. It was proved by experiments on the reduction of silver by ethanolamine from a complex with ethanolamine and by formaldehyde from a complex with ammonia that the formation of silver nanoparticles initiated by proteins is inhibited by air carbon dioxide. The participation of carbon dioxide in this process is discussed. It was found that even the breathing of an experimenter can induce variations in carbon dioxide concentration sufficient to adversely affect the reproducibility of the silver staining techniques. It was concluded that, for stable staining of proteins by silver in polyacrylamide gel, it is necessary to maintain a low concentration of carbon dioxide in air over the detection solutions.

Key words: silver, staining, colloids, proteins, polyacrylamide gel, carbon dioxide, nanoparticles

DOI: 10.1134/S106816200806006X

INTRODUCTION

The methods of staining the biological preparations by silver, borrowed from histochemistry, are widely used for the detection of protein zones in PAGE. Because PAGE of proteins is the major method of proteomic investigations [1], the use of silver staining techniques is a topic of particular interest. The advantage of silver staining technique is a high sensitivity, which is comparable with the sensitivity of radioactive labeling methods. However, the development by silver has one grave drawback. It is not always reproducible, which substantially retards its application.

The instability of the results of development by silver is due to the fact that all essential parameters that affect the formation of silver nanoparticles and must be controlled for the steady run of the process have not yet been determined [2]. At the same time, the reproducibility of the development of proteins is obligatory for the unambiguous estimation of their quantities in detailed studies of physiological and metabolic processes. The most commonly used reagent in the methods of protein development by silver is a complex of silver ions with ammonia ($[Ag(NH_3)_2]^+$) [3-5]. In our previous study, we have substituted ethanolamine for ammonia in the complex $[Ag(NH_3)_2]^+$ [6] to eliminate the fluctuations of ammonia concentration in the system (due to the volatility of ammonia) as a possible reason for the poor reproducibility of the results. An additional result of this substitution was that ethanolamine acted not only as a complex-forming element but also as a reducer, which substantially simplified the system.

It was assumed that the main reason for the nonreproducibility of the results with the use of a complex of silver ions with nonvolatile ethanolamine would be fluctuations in illumination intensity, which can change tens of times during the day and be invisible for eye. However, it appeared that the main factor responsible for the nonreproducibility is the fluctuations of carbon dioxide (CO_2) concentration in air. In particular, it was found that even insignificant concentrations of CO_2 substantially inhibit the reduction of silver ions from the complex with ethanolamine both in the gel and solution.

Preliminary experiments showed that the inhibitory action of CO_2 also extends to the reduction of silver ions from the complex with ammonia. Therefore, the practice of using the $[Ag(NH_3)_2]^+$ complex to develop proteins in the gel also requires a study of the effect of CO_2 on the reproducibility of the results.

This work is devoted to a methodical study of the effect of low CO_2 concentrations on the sensitivity of protein detection in PAGE or in solution with the use of the $[Ag(NH_3)_2]^+$ complex as a source of silver and of formaldehyde as a reducer.

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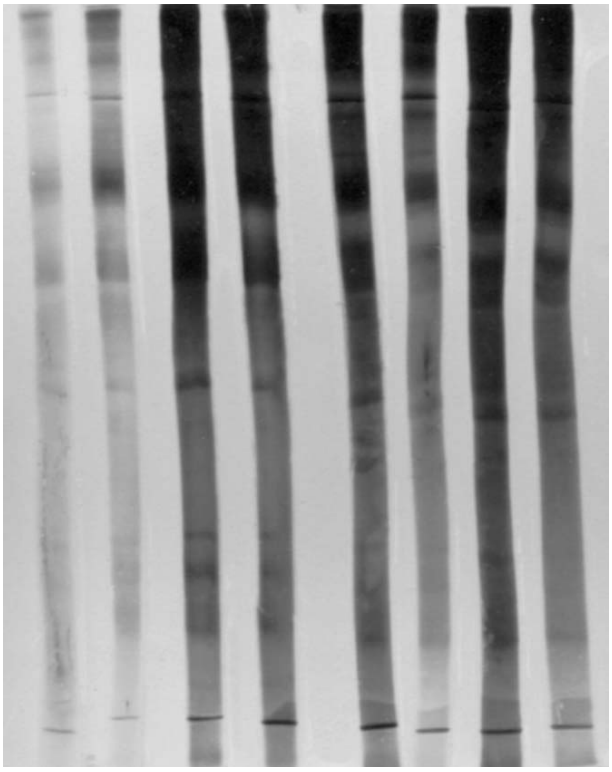

Fig. 1. Staining of blood serum proteins in PAG bands using a complex of silver with ammonia. *1*, *2*, cuvettes are open only at the first stage of the process (contact with air inhibits staining); *3*, *4*, cuvettes are covered with glass plates at all stages; *5*, *6*, cuvettes are open only at the second stage (contact with air does not affect the final result); *7*, *8*, cuvettes are covered with glass plates at all stages.

RESULTS AND DISCUSSION

Inhibition of Development of Protein Zones in PAGE by Silver as a Result of Contact of Staining Solutions with CO_2 from Air

To determine at which of three stages in the method of developing the proteins in PAGE with the use of the $[Ag(NH_3)_2]^+$complex the contact with air leads to a decrease in the intensity of gel staining, experiments were initiated in which two cuvettes were left open only at the staining stage being examined, whereas at the other two stages they were covered by glass plates. Two control cuvettes remained closed at all stages.

The results in Fig. 1 show that the first stage, the saturation of gels with $[Ag(NH_3)_2]^+$, was most sensitive to the contact with air. Treating the gels in open cuvettes at this stage led to a decrease in staining intensity, which was observed at the last stage. The sensitivity of the second stage (washing with water) showed up only as a slight delay in the development at the last stage, with the staining at the end of the process being leveled off. The contact with air at the third stage (treatment with formaldehyde and citric acid) had no effect either on the course of the assay or the final result. The experiments were carried out at 20°C. Similar results were obtained in experiments with an air thermostat at an elevated temperature (32–34°C). The only difference was an increase in the development rate at the last stage (data not shown).

These and all subsequent experiments were carried out at a CO_2 concentration in air of 0.06-0.08% (v/v). It follows from the results obtained that, if gels are treated under the conditions of free contact with air containing low concentrations of CO_2, an inhibition of the reduction of silver ions from the $[Ag(NH_3)_2]^+$ complex occurs. This effect is similar to what happens with the use of a complex of silver ions with ethanolamine [6]. Thus, the effect does not depend on the nature of the amine involved in the complex.

Inhibition of the Formation of Silver Nanoparticles in a Homogeneous System Being in Contact with Air Containing CO_2

Because the study of the inhibitory action of CO_2 on silver reduction in a heterogeneous gel-solution system presents a problem, the phenomenon was further explored in a one-phase homogeneous system. Gel bands were replaced by an aqueous protein solution. The method of detecting the proteins in a homogeneous system was first refined using a complex of silver ions with ethanolamine. Because the reduction of silver by ethanolamine proceeds at a low rate, the results of the experiment were observed 24 h after the termination of the experiment or even later. Owing to the low reaction rate, the advanced penetration of CO_2 from the gas phase into the liquid phase and its uniform distribution throughout the volume of the liquid occurred. The homogeneity of the reaction conditions was confirmed by special experiments with the reduction of silver in horizontally positioned long (30 cm) test tubes at high and low concentrations of CO_2 in the gas phase. The absence of the gradient of optical density of colloidal silver along the length of test tubes with both high and low CO_2 concentration in the course of the reaction indicated an equal concentration of CO_2 in the volume of the test tubes.

Parallel experiments in glass and polystyrene test tubes (Fig. 2) showed that silver is not deposited on the walls of polystyrene test tubes. Figure 2a presents the results of experiments with albumin. In closed glass test tubes (*1*, *2*), silver was deposited on the walls, whereas, under conditions of free access of air (*3*, *4*), silver was not deposited on the walls although the solution was slightly stained. In closed polystyrene test tubes (*5*, *6*), the solution turned deep brown without the deposition of silver walls. With free access of air to polystyrene test tubes (*7*, *8*), silver was not deposited on walls either, and the solution was only slightly stained.

Experiments with hexokinase gave similar results (2b) with the difference that colloidal silver nanoparticles in closed test tubes were less intensively stained

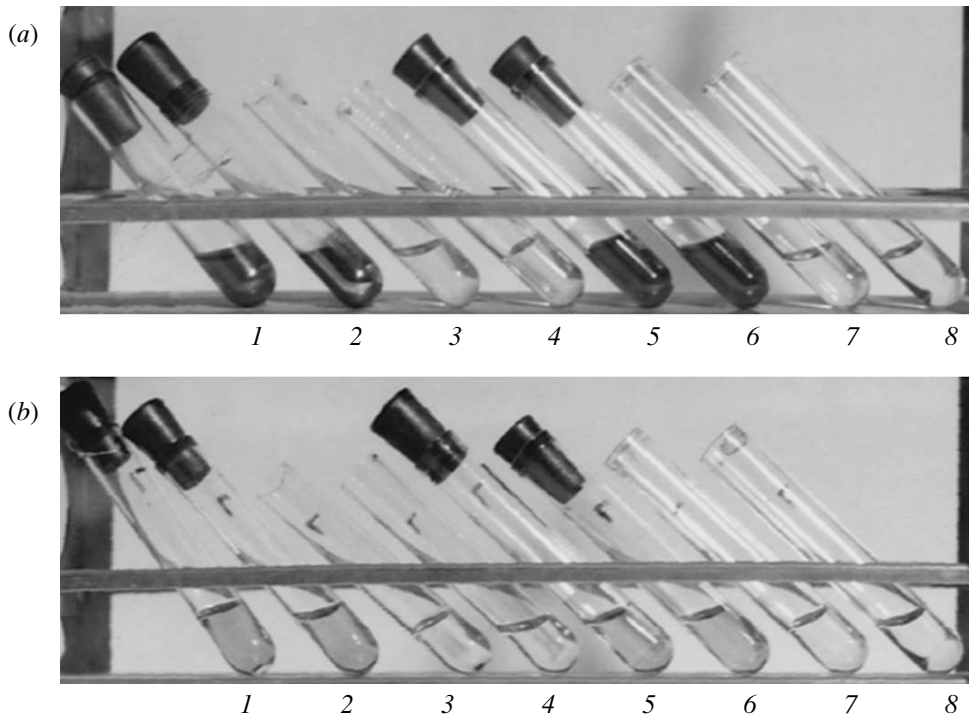

Fig. 2. Effect of contact with air on the reduction of silver ions from the complex with ethanolamine 24 h after the incubation in glass (*1–4*) or polystyrene (*5–8*) test tubes in the presence of (a) albumin and (b) hexokinase. *1*, *2*, *5*, *6,* test tubes are closed; *3*, *4, 7, 8*, test tubes are open; *1*, *2,* silver is deposited on the walls; *3, 4*, the reaction is inhibited; *5*, *6,* silver transformed into stained colloids; *7*, *8*, the reaction is inhibited.

than in the experiment with albumin; in open test tubes, the stain was almost invisible.

To examine the effect of CO_2 on the reduction of silver ions from the $[Ag(NH_3)_2]^+$ complex, ethanolamine was replaced in subsequent experiments by ammonia, which is formed in a homogeneous system from ammonium sulfate in an alkaline medium. Formaldehyde was used as a reducing agent. The experiments with albumin, hexokinase, glucosoxidase, and hyaluronidase showed that the solution in open polystyrene test tubes remains colorless, whereas in closed test tubes, it is stained by colloidal silver nanoparticles. Figure 3 demonstrates the reduction of silver ions after a 48-h incubation of a reaction mixture containing glucosoxidase. In closed test tubes, colloidal silver nanoparticles were formed, and in open test tubes, the reaction was inhibited.

Thus, we found conditions for staining proteins in a homogeneous system, which simplify the study of the effect of carbon dioxide on the reduction of silver ions from the $[Ag(NH_3)_2]^+$ complex.

Evidence for the Inhibition of the Reduction of Silver Ions from the $[Ag(NH_3)_2]^+$ Complex by Low Concentrations of CO_2

In previous experiments we showed only the fact of the inhibition of the reduction of silver ions from the $[Ag(NH_3)_2]^+$ complex when the reaction mixture is in contact with the atmospheric air containing low concentrations of CO_2. To convincingly prove that the inhibition of the reaction in a homogeneous system is caused by CO_2 contained in air, experiments were performed to estimate the amount of CO_2 that produces the inhibitory action. For this, the above-described homogeneous system containing the $[Ag(NH_3)_2]^+$ complex in

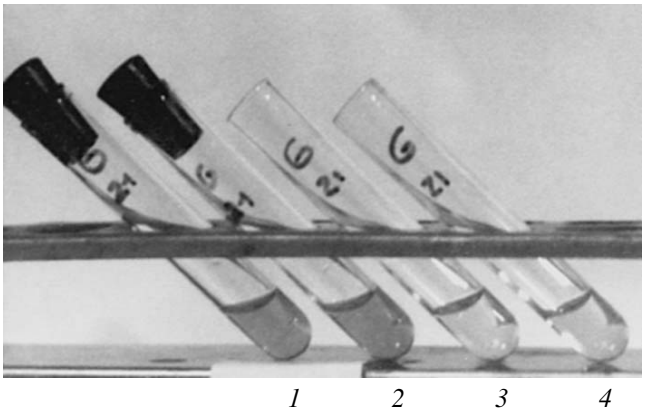

Fig. 3. Reduction of silver ions from the$[Ag(NH_3)_2]^+$ complex in the presence of glucosoxidase in (*1*, *2*) closed and (*3*, *4*) open polystyrene test tubes. After 48 h, stained silver colloids formed in test tubes *1* and *2*; in test tubes *3* and *4*, the reaction was inhibited.

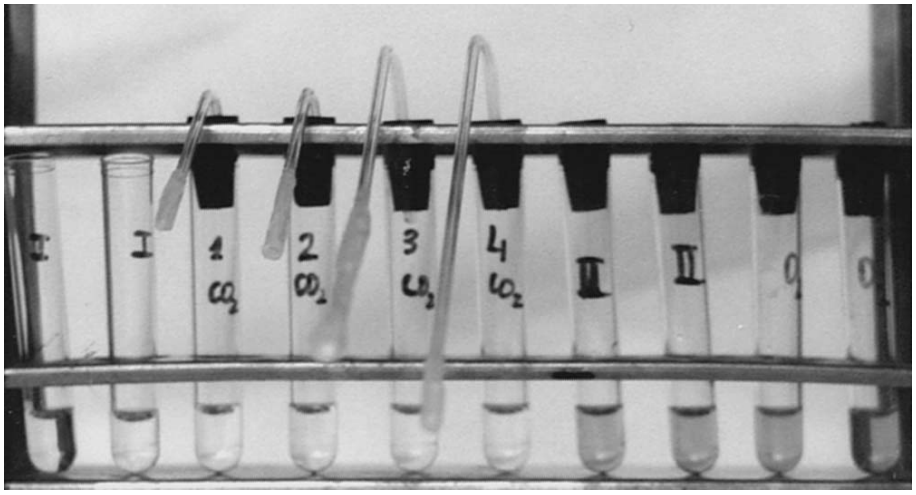

Fig. 4. Direct evidence for the involvement of CO_2 in the inhibition of silver reduction from the $[Ag(NH_3)_2]^+$ complex in the presence of hexokinase. After 24 h, the reaction in open test tubes (*1*, *2*) was inhibited. In closed test tubes with capillaries of volume of 60 (*3*, *4*) and 120 μl (*5*, *6*) filled with CO_2, the reaction is inhibited; in test tubes filled with air (*7*, *8*), silver transformed into stained colloids; in test tubes filled with O_2 purified from CO_2 impurity (*9, 10*), silver transformed into stained colloids.

polystyrene test tubes was used. One pair of test tubes was left open, and the second and third pairs were plugged with stoppers with the inserted glass capillaries filled with CO_2, short capillaries (60 μl) for the second pair and long capillaries (120 μl) for the third pair; the fourth pair was plugged with stoppers, and the fifth pair was filled with oxygen purified from a CO_2 contamination and also plugged with stoppers.

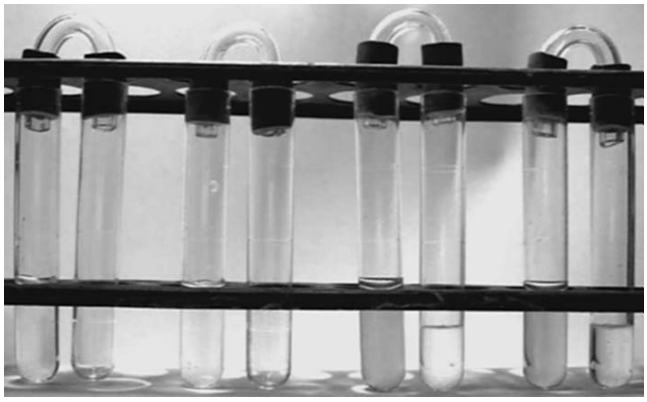

Fig. 5. Inhibitory influence of small amounts of air CO_2 on the reduction of silver from the $[Ag(NH_3)_2]^+$ complex in the presence of hexokinase. After 16 h of incubation, the formation of stained silver colloids in test tubes *1* and *2*, connected with test tubes without $Ba(OH)_2$ is hardly visible. In test tubes *3* and *4*, connected with test tubes containing 1 ml of 0.08 M $Ba(OH)_2$ solution, the formation of stained colloids is well pronounced. The initial concentration of CO_2 in the test tubes is 0.08 volume percent. The volume of the reaction mixture was doubled compared with the volume given in the Experimental section to bring it in correspondence with the total volume of air in connected test tubes.

Experiments with hexokinase as an initiating protein (Fig. 4) showed that, in the first, second, and third pairs of test tubes where the solutions were in contact with sufficient amount of CO_2, the formation of stained colloid silver nanoparticles was blocked (test tubes *1*–*6*). The inhibition of the reaction in the first pair of the test tubes is due to the fact that CO_2 entered the tubes from air without limit. At a concentration of CO_2 in air of 0.07% (by volume), its content in closed test tubes was ≈0.11 μmol. Because this value is insignificant, the total content of CO_2 in test tubes with capillaries was determined by the capillary volume (2.68 and 5.36 μmol of CO_2 in capillaries of volume 60 and 120 μl, respectively). In closed test tubes filled with air (*7*, *8*) or oxygen purified from CO_2 (*9*, *10*), silver ions were reduced to stained silver colloids. The amount of CO_2 contained in the volume of air of closed test tubes had no marked effect under the given experimental conditions. Similar results were obtained with glucosoxidase as a protein. The facts that oxygen purified from CO_2 does not inhibit the formation of colloidal silver from the $[Ag(NH_3)_2]^+$ complex in solutions, and that different amounts of CO_2 in test tubes inhibit the reaction to a variable degree indicate unambiguously that, with ammonia substituted in the complex for ethanolamine, the effect is also caused by to CO_2.

To prove that still lower CO_2 concentrations inhibit the reduction of silver ions from $[Ag(NH_3)_2]^+$ in the homogeneous system, the following experiment with hexokinase or glucosoxidase was performed (Fig. 5). Two test tubes (*1* and *2*), each containing 1.5 μl of reaction mixture, were connected in pairs by U-shaped tubes with two empty test tubes. Other two test tubes (*3*, *4*) containing the same mixture were connected in pairs in a similar way with test tubes containing 0.08 M

$Ba(OH)_2$ (1 ml in each), which absorbed CO_2 of air. The amount of silver in the solution was 0.54 μmol, and the amount of sodium carbonate (Na_2CO_3) and sodium hydrocarbonate ($NaHCO_3$), as calculated for carbonate ions (CO_3^{2-}), was 2.1 μmol, whereas the initial content of CO_2 in communicating test tubes was about 0.2 μmol, i. e., 2.5 times less in the molar ratio than that of silver and about ten times less than that of Na_2CO_3.

The results showed that the solution in test tubes communicating through U-shaped tubes with empty test tubes remained almost colorless within 16 h after the incubation; in test tubes communicating with test tubes containing $Ba(OH)_2$, stained colloidal silver nanoparticles were formed due to the removal of air CO_2. Consequently, not only relatively large amounts of CO_2 that either accumulate due to free absorption from air or are introduced through glass capillaries, but also very small amounts of CO_2, determined by the volume of test tubes, markedly inhibit the reduction of silver from $[Ag(NH_3)_2]^+$ at CO_2 concentrations of 0.08 volume percent.

It is seen from Fig. 6 that the absorption spectra of reaction products with the complex of Ag^+ with both ethanolamine and ammonia as a source of silver have a similar bell-shaped form in the visual region with an absorption maximum at a frequency of 22×10^3 cm^{-1} and, correspondingly, at a wavelength of about 450 nm. The data on the absorption in the UV-region of the spectrum were omitted due to a high level of absorption of the initial reaction mixture in this region and a low informativity.

Figure 7 shows the results of measuring the CO_2 concentration in air at different distances from the experimenter's face. It is easily seen that the CO_2 concentration in air at distances from 0.3 to 0.5 m changes two and five times, respectively, as compared with the background concentration.

CONCLUSIONS

The fact that CO_2 of air equally inhibits the silver deposition in gels, on the walls of glass test tubes, and in solutions in polystyrene test tubes suggests that the sites initiating the formation of silver mirror and stained silver nanoparticles are of similar origin.

As for the molecular mechanism of the inhibition of silver reduction by carbon dioxide, the inhibition in the above-described experiments might be explained at first glance by the acidification of the reaction mixture and the action of CO_3^{2-} or HCO_3^-, formed from CO_2 by the dissociation of carbonic acid. However, it has been found previously that the amount of CO_2 (μmol) capable of inhibiting the reduction of silver from the complex with ethanolamine is 40 times smaller than the amount of this complex and therefore cannot induce any marked shift of the acid-base equilibrium in the reaction mixture [6]. It has also been shown that

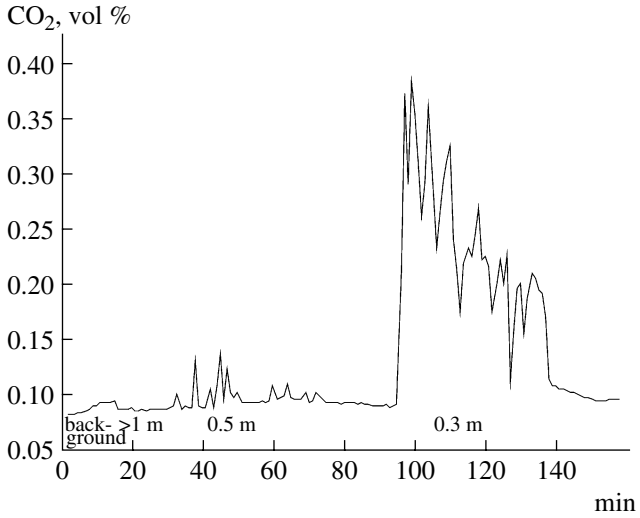

Fig. 7. Results of measurements of CO_2 concentration at the working table at a distance of 50 and 30 cm from the experimenter's face. The background concentration of CO_2 in the room was 0.08-0.09 volume percent.

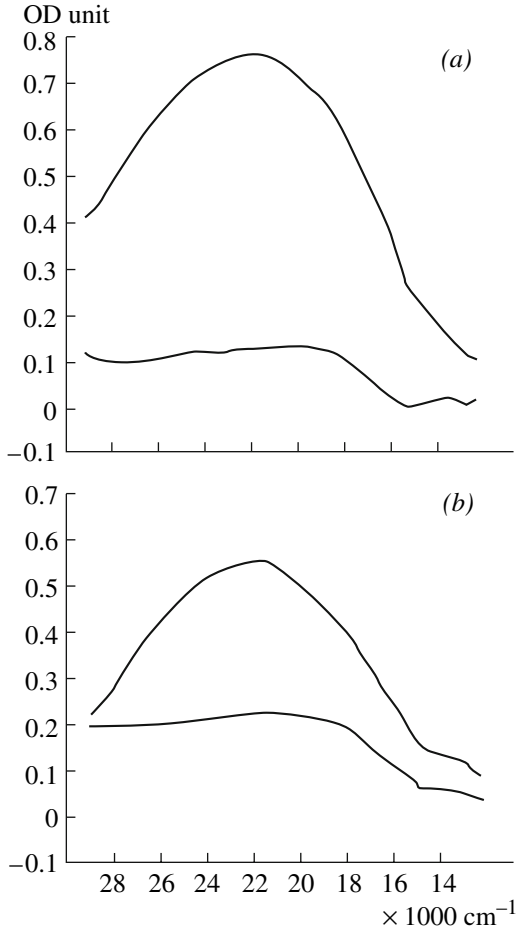

Fig. 6. Absorption spectra of the reaction medium in the visible region ($30–14 \times 1000$ cm^{-1}) after incubation under conditions of free contact with air CO_2 (lower curves) and in closed test tubes (upper curves): (*a*) with the complex of silver with ethanolamine for the development and (*b*) with the complex of silver with ammonia.

Na_2CO_3 added in the amounts exceeding the amount equimolar relative to the amount of silver ions in solution does not inhibit its deposition on the walls of glass test tubes [6]. It follows from the numerical data reported in the literature that the molar concentration of the intermediate ion HCO_3^- in the pH range from 8 to 10 changes insignificantly compared with changes in the concentration of hydrogen ions, namely, from 95 to 76%, whereas the CO_2 concentration in solution decreases from 4.25 to 0.034%, and the concentration of CO_3^{2-} increases from 0.30 to 24% relative to the sum of molar concentrations of these compounds [7].

Taken together, these data allow the conclusion to be drawn that CO_2 inhibits the reduction of silver ions from the complex with ethanolamine not due to the acidification of the reaction mixture by carbonic acid or the formation of ions HCO_3^- and CO_3^{2-} as a result of its dissociation. This is also consistent with the fact that sodium carbonate is successfully used instead of alkali in some methods of protein detection in PAGE by silver [8, 9], and potassium carbonate impurities do not substantially affect the results in the technology of mirror manufacture [10].

The use of Na_2CO_3 for maintaining the alkaline medium in experiments involving the reduction of silver ions from the $[Ag(NH_3)_2]^+$ complex (Figs. 3–5) also indicates that CO_3^{2-} ions are not implicated in the inhibition of this reaction. At the same time, the difference in the Na_2CO_3 concentration values optimal for the initiation of the reduction of silver ions by different proteins can be attributed to different buffer capacity of their solutions. On the other hand, a slowed down conversion of CO_2 to carbonic acid in water even under alkaline conditions indicates that CO_2 inhibits the reduction of silver immediately in the molecular form. The initial stage of inhibition can be represented as the reversal of the reduction of silver ions by the carbon dioxide ion radical ($C\dot{O}_2^-$) , described by radiochemists [11].

$$Ag^+ + CO_2^- \longrightarrow Ag^0 + CO_2.$$

This reaction opens a cascade of reactions that lead to the formation of Ag_2^+, Ag_3^+, Ag_4^{2+}, Ag_8^{2+} clusters, and subsequently of silver nanoparticles [12]. The difference between the electrochemical potentials of the reduction of Ag^+ and CO_2 during the formation of clusters is as little as 0.1 V; therefore, the reaction can be considered as an equilibrium one. Consequently, an excess of CO_2 in the system should lead to the trapping of Ag^0, a reversal of the reaction, and as a consequence, the inhibition of the cascade of reactions of silver nanoparticle formation. This mechanism is consistent with the fact established in our study that the stage of the incubation of gels in a solution of the $[Ag(NH_3)_2]^+$ complex was most sensitive to the inhibitory action of contact with air on the development by silver. In other words, at the first stage of the process, CO_2 contained in air prevents the formation of clusters initiating the silver deposition. In this process, ammonia, by binding free silver ions, facilitates the reversal of the reaction of their reduction by carbon dioxide anion radical, thereby contributing to the transformation of the reaction product, CO_2, into its inhibitor.

A delay in the staining at the stage of reduction by formaldehyde after the contact with air at the stage of washing the gels with water can be explained by the inhibition of the growth of clusters themselves by the action of CO_2. This is consistent with the data indicating the involvement of $CO_2^{\bullet-}$ anion radical in the reduction of positively charged silver clusters [11]. One way or the other, further studies are needed to solve the question concerning the mechanism of the participation of CO_2 in the inhibition of silver reduction.

As is known, the concentration of CO_2 in a closed room can change by the action of human breathing by two orders of magnitude (from 0.03 to 3%). The determinations of CO_2 concentration in air indicate that the CO_2 concentration in the zone of experiment can change several times depending on the position of the experimenter's face (Fig. 7). Considering that the sites of initiation of silver deposition are inhibited by low CO_2 concentrations, one can come to the conclusion that the fluctuations of CO_2 concentration in air are the major uncontrolled parameter that affects the reproducibility of the results obtained by the methods of staining the histological preparations by silver and the development of proteins by silver in PAGE. Therefore, a stable development of proteins by silver can be achieved by maintaining the low values of CO_2 concentration in ambient air.

EXPERIMENTAL

Reagents. The following preparations were used: acrylamide, sodium dodecylsulfate (Bio-Rad, United States); bisacrylamide, Tris, glucosoxidase (Serva, FRG); glycine, *N*,*N*,*N*,*N*-tetramethylethylenediamine (TEMED), hyaluronidase, bromophenol blue (Reanal, Hungary); lysozyme B, ethanolamine (Reakhim) twice distilled in a vacuum; rectified ethyl ester; human blood serum containing the major fractions: prealbumin, albumin, α_L, α_1', α_2', $F\alpha_2$, transferrin, β_2', β_γ, $S_{\alpha 2}$, S_β; hexokinase (Fluka, Switzerland); albumin (Calbiochem, UK); glycerol (analytical grade); citric acid (special purity grade); formaldehyde, acetic acid, and inorganic reagents were chemical purity grade preparations.

Electrophoresis of blood serum in PAG. PAGE of proteins was carried out by a modified method of Laemmli [1, 13] (0.1% SDS; concentration of PAG in the slap 10%).

Initial solutions: 39% acrylamide with 1% bisacrylamide; buffer pH 8.8 (1.5 M Tris-HCl); buffer pH 6.8 (0.5 M Tris-HCl); 10% SDS; 10% ammonium persulfate.

Solution for the separating gel. A solution of acrylamide with bisacrylamide (15 ml) was mixed with Tris buffer pH 8.8 (12.5 ml) and water (22 ml). The solution was filtered through a nitrocellulose filter after which TEMED (0.01 ml) and a 10% ammonium persulfate solution (0.3 ml) were added.

Solution for the concentrating gel. A solution of acrylamide with bisacrylamide (1.3 ml) was mixed with Tris buffer pH 6.8 (2.5 ml) and water (5.1 ml). The solution was filtered through a nitrocellulose filter after which 10% SDS (1 ml), TEMED (0.003 ml), and a 10% ammonium persulfate solution (0.1 ml) were added.

Electrode buffer of a tenfold concentration. Tris (3 g), glycerol (14.3 g), and SDS (1 g) were dissolved in water. The volume of the solution was brought to 100 ml.

Buffer for dissolving the protein sample. A 10% SDS solution (1 ml) was mixed with glycerol (0.8 ml), dithiothreitol (24 mg), and water (3 ml).

Preparation of protein samples for PAGE. A solution of 0.1% lysozyme (20 μl) was mixed with a standard blood serum (10 μl), dissolved in buffer for dissolving the proteins, and a 0.2% bromophenol blue solution (10 μl) was added

Formation of a gel slab and electrophoresis of proteins. A slab of separating gel ($150 \times 140 \times 1.5$ mm) was formed in a device for electrophoresis. The upper boundary of the gel-forming solution that was poured between two glass planes was isolated from oxygen of air by a water layer (3–5 mm). After the termination of polymerization, water was decanted, and a solution for the concentrating gel was layered onto the horizontal boundary. In the concentrating gel, a pocket over the entire width of the slab was formed using a plexiglass insertion piece. After the formation of the gel slab, the electrode chambers were filled with electrode buffer preliminarily diluted ten times. Then a protein sample preheated in water for 1 min at 90–95°C and containing glycerol with bromophenol blue was layered onto the surface of the concentrating gel. Electrophoresis was carried out at a constant current strength of 40 mA. The process was terminated when the band of bromophenol blue approached the lower boundary of the gel. Then the gel slab was placed in a 50% ethyl alcohol solution.

Development of protein zones in PAGE by silver. In order to compare the reproducibility of the results of protein development in identical gel bands, the process was performed parallel in four glass cuvettes ($235 \times 23 \times 23$ mm): in two cuvettes proteins were developed under changed conditions, and the other two served as a control. Gel bands of size $150 \times 9 \times 1.5$ mm were obtained by cutting a PAG slab in which the proteins contained in 20 μl of human blood serum were preliminarily electrophoresed. Before the cutting, the slab was fixed for 24 h in 50% ethanol.

The development was carried out by the method of Wray [5] with minor modifications. After washing the gel bands from alcohol in bidistilled water for 30 min, the procedure involved three stages: (1) saturation of gel for 1 h with a solution containing 3 mM $AgNO_3$, 9 mM ammonia, and 2.4 mM NaOH; (2) washing with bidistilled water for 20 min; and (3) treatment with a solution of 72 mM formaldehyde and 0.17 mM citric acid for about 10 min. The volume of solutions at all stages was 25 ml.

The concentration of CO_2 in air was measured either directly by the previously developed chemical method of determining the absolute content of CO_2 in air [14] or by a PKU-4 CO_2 gas analyzer (OAO Praktik SC, Russia) preliminarily calibrated according to this method. The carbon dioxide concentration in the air of the room where the experiment was carried out was 0.06–0.08 volume percent.

Reduction of silver ions from a complex with ethanolamine in solution. A complex of silver with ethanolamine was obtained by adding 50% ethanolamine (230 μl) to a 0.1 M $AgNO_3$ solution (8 ml). At first, a brown precipitate of silver oxide formed, which gradually dissolved until the solution became colorless. Then a solution of a protein (albumin or hexokinase) (0.4 ml) at a concentration of 1 mg/ml was added. The resulting solution was poured into glass tubes, 1 ml to each. The contact of the solution with external air was excluded by plugging the test tubes with rubber stoppers. The volume of the air above the solution in closed test tubes was approximately 3.3 ml. The formation of stained silver colloidal particles in test tubes as a result of the reduction of silver ions by ethanolamine from the complex with ethanolamine occurred usually within 24 h.

Reduction of silver ions from a complex with ammonia in solution. Silver was reduced from the $[Ag(NH_3)_2]^+$ complex by formaldehyde. The reaction rate within the specified limits was maintained through the regulation of the alkaline medium by changing the concentration of the equimolar mixture of Na_2CO_3 and $NaHCO_3$. For a rigorous control of ammonia concentration in reaction medium, an ammonium sulfate $[(NH_4)_2SO_4]$ solution was used as an ammonia source. In an alkaline medium, free ammonia was evolved from this solution to form a complex with Ag^+ by the reaction:

$$2\,NH_4^+ + 2OH^- + Ag^+ = [Ag(NH_3)_2]^+ + 2H_2O.$$

The concentrations of the components of the solution were so chosen that the molar ratios of $AgNO_3$ and $(NH_4)_2SO_4$ corresponded to the stoichiometry of the reaction of complex formation between silver and ammonia.

The final mixture was prepared as follows. A mixture (105 μl) containing 40 mM $AgNO_3$ and 40 mM $(NH_4)_2SO_4$ was added to an aqueous solution of a pro-

tein (7.35 ml) at a concentration of 1 mg/ml. The solution was stirred and poured into polystyrene or glass test tubes (480 μl). The experiments were performed with the proteins: albumin, glucosoxidase, and hyaluronidase both in open and closed polystyrene test tubes. The concentration of the Na_2CO_3–$NaHCO_3$ mixture for each of these proteins was so chosen that the rate of silver reduction by formaldehyde was comparable with the rate of reduction by ethanolamine. As a result, it was found that an optimal volume of a mixture of 25 mM Na_2CO_3 and 25 mM $NaHCO_3$ is 21 μl for albumin, 35 μl for hexokinase, 28 μl for hyaluronidase, and 21 μl for glucosoxidase. The reaction was initiated by adding a freshly prepared 7.3 mM formaldehyde solution, 250 μl to each test tube. The final concentrations of the reagents in the system per one test tube were: protein 0.06%; 0.36 mM $AgNO_3$; 0.36 mM $(NH_4)_2 SO_4$; 0.6–1.15 mM of each component of the Na_2CO_3–$NaHCO_3$ mixture; and 2.4 mM formaldehyde. The total volume of the reaction mixture in test tubes was 751–765 μl. The contents of the test tubes were isolated from contact with external air by rubber stoppers. The volume of the air over the solution in closed test tubes was approximately 3.5 ml. The amount of CO_2 in test tubes was regulated by means of glass capillaries of the known volume, filled with CO_2 and closed at the external end by parafilm. The capillaries were inserted through resin stoppers plugging the test tubes. In the case of open test tubes, the CO_2 concentration in the air was controlled as described in the preceding section.

Carbon dioxide was obtained by the decomposition of sodium hydrocarbonate by sulfuric acid immediately in a gasometer [15].

Oxygen was obtained by the decomposition of dry $KMnO_4$ by heating in a Wurtz flask. For the removal of CO_2 impurities, the oxygen obtained was stored in a gasometer over a potassium hydroxide solution [15].

Absorption spectra in the visible region were recorded on a Specord UV VIS spectrophotometer.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 08-04-00864).

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