[Chem. Pharm. Bull. 32(3)1219—1223(1984)]

Influence of Blood Proteins on Biomedical Analysis. VI.[1] Effect of Bovine Serum Albumin on the Color Reaction of Xanthurenic Acid with 4-Aminoantipyrine—Inhibition of Antipyrine Red Production by Bovine Serum Albumin

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(Received June 18, 1983)

In order to characterize the amplifying effect of bovine serum albumin (BSA) on the coloration of xanthurenic acid (XA) with 4-aminoantipyrine (4-AAP) and an oxidizing agent (potassium ferricyanide), the effects of amount of BSA, time of BSA addition to the reaction mixture, and the side reaction producing antipyrine red were studied. The BSA-induced recovery of the coloration was 100% when BSA was added to the reaction mixture within 3 min after the initiation of the color reaction, though after 5 min it was not more than 50%. A lower temperature, a lower concentration of potassium ferricyanide and a higher concentration of 4-AAP in the reaction mixture before the BSA addition enhanced the BSA-induced recovery effect on the coloration. The production of antipyrine red was inhibited by BSA in the reaction mixture without XA. In conclusion, the present results suggest that the BSA-induced amplifying effect on the color reaction of XA with 4-AAP and potassium ferricyanide is based on the inhibition of the side reaction which produces antipyrine red by the bimolecular condensation of 4-AAP.

Keywords——xanthurenic acid; 4-aminoantipyrine; antipyrine red; bovine serum albumin; oxidative condensation; bovine serum albumin-induced inhibition of antipyrine red production

Xanthurenic acid (XA, 4,8-dihydroquinoline-2-carboxylic acid) is a metabolite of tryptophan produced in metabolically disordered rat or human.[2] It has been suggested that diabetic symptoms are induced by the binding of XA to blood insulin, leading to inactivation of the hormone, or by the interaction of pancreatic islets of Langerhans with XA.[3] Conventionally, the XA level in urine or blood has been determined by a colorimetric method using 4-aminoantipyrine (4-AAP) and an oxidizing agent.[4] Previously, we reported that the addition of bovine serum albumin (BSA) to the reaction mixture depressed the decrease of optical density, and even enhanced the optical density in the coloration for the determination of XA using 4-AAP.[5] Although XA is able to bind with BSA, and it has been suggested that XA–BSA binding may induce the amplifying effect on the coloration,[6] we could not find any correlation between XA–BSA binding and the coloration in our previous study on the BSA binding with XA and XA analogs.[7]

In the present study, we examined the relationship between the amplifying and recovery effects, and the time of BSA addition to the reaction mixture. Moreover, the inhibiting effect of BSA on the production of antipyrine red,[8] which is a side reaction, was studied in the reaction system without XA using 4-AAP and an oxidizing agent.

Experimental

Materials——All reagents used for experiments were of reagent grade. Xanthurenic acid, 4-aminoantipyrine and

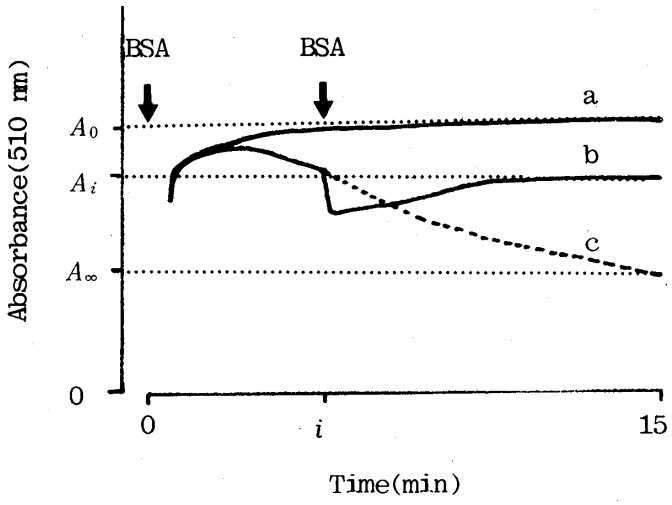

Fig. 1. Effect of Time of BSA Addition on the Time Course of Coloration for XA with 4-AAP (Schematic Profiles)

a, 1.2 mM BSA solution was added, followed by addition of XA solution; b, 1.2 mM BSA was added at i min after the initiation of the reaction; c, the color reaction was carried out without the addition of BSA solution. The symbols A_0, A_i and A_∞ indicate the absorbances (at 510 nm) of the reaction mixture at 15 min after the initiation of the reaction in experimental systems a, b and c, respectively.

potassium ferricyanide were obtained from Wako Pure Chemical Industries Ltd., Japan. Bovine serum albumin (fraction V) was purchased from the Armour Laboratories Ltd., U.S.A. All other chemicals were products of Wako Pure Chemical Industries Ltd., Japan. Solutions of 100 μg/ml XA, 1.2 mM BSA, 2.4% 4-AAP and 2.4% potassium ferricyanide were prepared with 0.1 M carbonate buffer (pH 10.0).

Measurements——The color reaction was carried out in a test tube or glass spectrophotometer cuvette at room temperature (25—26 °C). The optical density of the reaction mixture was measured at 510 nm with a double-beam spectrophotometer (Hitachi 124, Hitachi Ltd., Japan) and the time course of coloration was monitored with a recorder (Hitachi QPD_{54}, Hitachi Ltd., Japan).

Effects of Time of BSA Addition on Coloration——The color reaction was performed as follows; 0.5 ml each of buffer (0.1 M carbonate buffer, pH 10.0) and XA solution (100 μg/ml) were mixed in a glass spectrophotometer cuvette, and then 0.5 ml each of 2.4% 4-AAP solution and 2.4% potassium ferricyanide solution were added in that order. The monitoring of the coloration was started at 1 min after the initiation of the color reaction. Further, 0.1 ml of 1.2 mM BSA solution was added to the reaction mixture at various times (0—13 min) after the initiation of the color reaction. Each reference solution was prepared by replacing the XA solution with an equivalent volume of the buffer. The overall color reaction was carried out for 15 min at room temperature (25—26 °C). Figure 1 shows schematic profiles of the time course of coloration. The recovery effect of BSA addition on the coloration was calculated by means of Eq. (1).

$$\text{recovery } (\%) = (A_i - A_\infty / A_0 - A_\infty) \times 100 \quad (1)$$

Effects of Temperature and Reagent Concentration in Preincubation on the BSA-Induced Effect on Coloration——1) The reaction mixture was preincubated for 5 min at various temperatures (0—60 °C), then 0.1 ml of 1.2 mM BSA solution was added. The optical density (A_{15}) was measured after a 10-min standing period at room temperature and the effect of temperature on the recovery of coloration was calculated according to Eq. (1). 2) The reaction mixture containing 4-AAP and potassium ferricyanide at various concentrations was preincubated for 5 min, and then 0.1 ml of 1.2 mM BSA solution was added. The effects of 4-AAP and potassium ferricyanide concentrations were obtained by the same procedure as described in 1).

Effect of BSA on the Production of Antipyrine Red——One ml each of 2.4% 4-AAP solution and 2.4% potassium ferricyanide solution was added to a mixture of 1.0 ml of the buffer and 0.1 ml of BSA solution at various concentrations in that order. The optical density of the reaction mixture was monitored at 510 nm from 1 min after the potassium ferricyanide addition. Each reference solution was prepared by replacing 2.4% 4-AAp solution with an equivalent volume of the buffer. The inhibitory effect of BSA on the coloration was represented as the ratio of optical density (A_{15}/A_1) at 1 min and 15 min after the initiation of the reaction.

Results and Discussion

Time-course profiles of coloration when BSA was added to the reaction mixture at various times (0—13 min) after the initiation of the color reaction are shown in Fig. 2A. BSA-induced recovery (%) was calculated from the results in Fig. 2A according to Eq. (1). Figure 2B shows the relationship between the BSA-induced recovery of the coloration of XA with 4-AAP and the time of BSA addition. The BSA-induced recovery of the coloration was 100% when BSA was added to the reaction mixture within 3 min after the initiation of the color reaction, but then the recovery effect decreased in proportion to the delay of the BSA addition. At 13 min after the initiation, BSA addition was no longer effective. The present result is in accordance with our previous data,[6] which showed that the coloration depended on the order of reagent addition. Thus, the BSA solution should be added to the reaction mixture prior to initiation of the oxidative reaction by potassium ferricyanide for the best

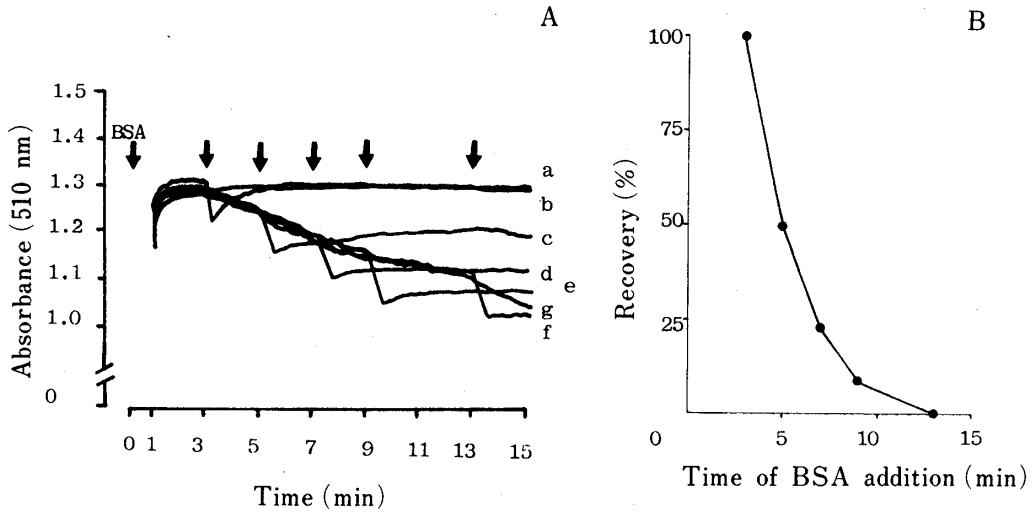

Fig. 2A. Effect of Time of BSA Addition on the Time Course of Coloration of XA with 4-AAP

Arrows show time of BSA addition: a, 0; b, 3; c, 5; d, 7; e, 9; f, 13 min; g, no addition.

Fig. 2B. Effect of Time of BSA Addition on the Recovery Effect on Coloration of XA with 4-AAP

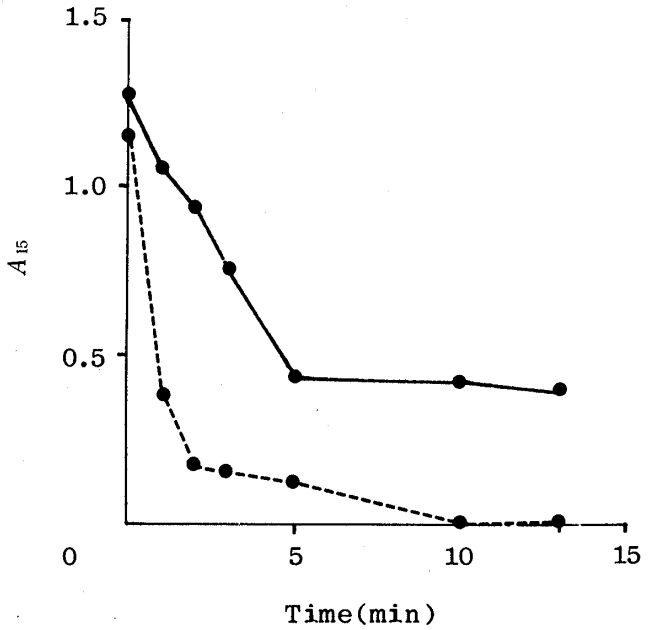

Fig. 3. Effect of Time of XA Addition on Coloration of XA with 4-AAP

First, 0.1 ml of 1.2 mM BSA (—●—) or buffer (---●---), and 0.5 ml each of 4-AAP and potassium ferricyanide were mixed in that order, then 0.5 ml of XA solution (100 μg/ml) was added at various times after the addition of 2.4% potassium ferricyanide solution. The optical density of the reaction mixture (A_{15}) was measured at 510 nm at 15 min after the addition of 2.4% potassium ferricyanide solution.

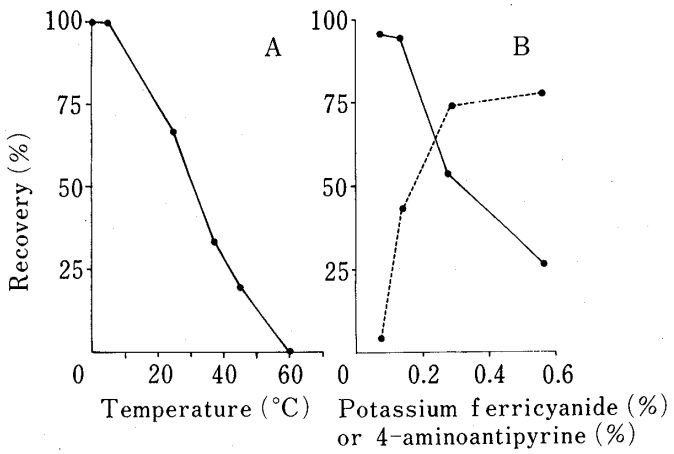

Fig. 4. Effects of Temperature and Concentrations of Potassium Ferricyanide and 4-AAP in Preincubation on the Coloration of XA with 4-AAP

—●—, potassium ferricyanide; ---●---, 4-AAP.

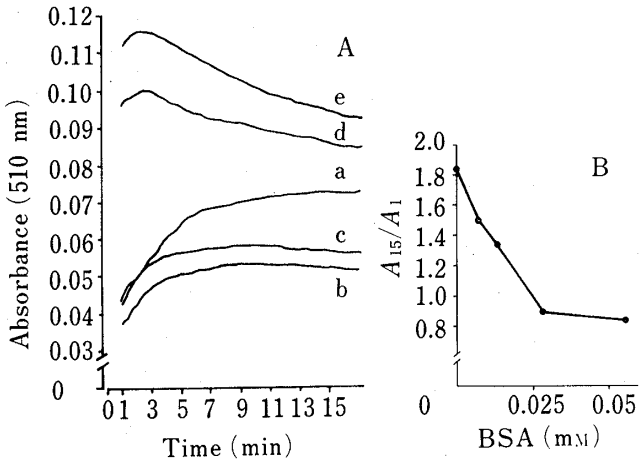

Fig. 5A. Effect of BSA Concentration on Coloration Profiles Based on Antipyrine Red Production

BSA concentration in the medium was as follows; a, 0; b, 0.007; c, 0.014; d, 0.028; e, 0.056 mM.

Fig. 5B. Inhibitory Effect of BSA on Coloration Based on Antipyrine Red Production

coloration.

Solutions of 1.2 mM BSA, 4-AAP and potassium ferricyanide were mixed, followed by addition of XA solution. When XA solution was added within 5 min after the potassium ferricyanide addition, the coloration was reduced in proportion to the delay of XA addition, then it remained constant at around 0.4 or A_{15}. On the other hand, in the absence of BSA, the coloration decreased rapidly within 2 min, then gradually decreased until 10 min (Fig. 3). Figure 4A shows the relationship between the BSA-induced recovery and the temperature during a 5 min preincubation of the reaction mixture before the BSA addition. The recovery was 100% at temperatures within 0 to 5 °C, whereas it decreased linearly at temperatures from 5 to 60 °C. The BSA-induced recovery was maintained at a higher level with decreasing concentration of oxidizing agent (potassium ferricyanide) (Fig. 4B). In contrast a lower concentration of 4-AAP decreased the coloration (Fig. 4B).

It is known that 4-AAP produces an unstable blue-red dye (antipyrine red) by oxidative

bimolecular condensation in the presence of an oxidizing agent.[8] The effect of BSA on the production of antipyrine red was examined in reaction systems containing 4-AAP and oxidizing agent without XA. Figure 5A shows the time course of the coloration based on antipyrine red production in the reaction systems without and with BSA at various concentrations. The coloration was increased by 1.8 times at 15 min as compared with that at 1 min after the initiation of the reaction in the absence of BSA. When 0.007—0.014 mM BSA was contained in the reaction mixture, the coloration reached a plateau around 5 min after the initiation, while at BSA concentrations above 0.028 mM it reached a peak at about 2 min after the initiation, then gradually decreased. Figure 5B shows the relationship between the ratio of optical density (A_{15}/A_1) and the added BSA concentration, based on the results in Fig. 5A. The ratio of optical density (A_{15}/A_1) was reduced to around 0.8 at BSA concentrations above 0.028 mM, suggesting the inhibition of antipyrine red production.

In conclusion, the present results show that the BSA-induced recovery effect on the coloration was enhanced under all conditions which delay the oxidative reaction, such as a lower temperature or a lower concentration of oxidizing agent. Therefore, these results suggest that BSA selectively inhibits the bimolecular condensation of 4-AAP to produce antipyrine red, rather than the condensation of 4-AAP with XA, thus reducing the amount of 4-AAP lost in the side reaction, leading to the enhanced coloration.

References and Notes

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