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# Nonenzymatic Glycosylation of Human Serum Albumin: Fluorescence and Chemiluminescence Behavior

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In the present study the progress of the cross-linking reaction between glyceraldehyde (GCA) and human serum albumin (HSA) was followed by monitoring the development of new absorption bands characteristic of the cross-linked product. Generation of electronically excited species (EES) during horseradish peroxidase (HRP)-catalyzed oxidation of glycosylated HSA was followed by measuring direct chemiluminescence. It was found that adducts of HSA with GCA in the presence of HRP are capable of forming EES. The mechanism of EES generation is discussed further on the basis of fluorescence and chemiluminescence measurements. Formation of EES in studied systems thus could be a possible source of free radicals generated *in vivo* during age- and diabetes-related complications.

**KEY WORDS:** Nonenzymatic glycosylation; human serum albumin; glyceraldehyde; horseradish peroxidase; chemiluminescence; electronically excited species; diabetes; aging.

## INTRODUCTION

Nonenzymatic binding of saccharides, especially of glucose, to proteins is intensively studied as an important posttranslational modification of biopolymers. This process, called nonenzymatic glycosylation, involves the nonenzymatic binding of glucose to reactive amino groups located on lysine side chains and N-terminal amino acid residues of protein molecules. The reaction of glyceraldehyde (GCA) as an aldotriose with human serum albumin (HSA) is analogous to the nonenzymatic glycosylation of the protein with glucose in that the initial reversible Schiff base adduct—aldimine—undergoes a slow Amadori rearrangement to the more stable ketoamine adduct [1]. The reaction of HSA with GCA thus presents an ideal model system for studying the mechanistic details of the nonenzymatic glycosylation reaction.

The Amadori rearrangement of the Schiff base adducts with α-hydroxyaldehydes provides a new aldehyde function, an aldoamine, which is generated *in situ* and is capable of forming Schiff base linkages with another adjacent amino group leading to the covalent cross-linking of proteins.

Recently, it has been shown that horseradish peroxidase (HRP), acting as a dioxygenase, is capable of catalyzing the aerobic oxidation of Schiff base adducts of proteins with glycolaldehyde. This oxidation is accompanied by light emission that is attributed to the generation of triplet species [2].

Nonenzymatic glycosylation is studied very intensively with respect to aging and diabetes mellitus-related complications [3]. Products of nonenzymatic glycosylation were studied as a possible source of free radicals which could be responsible for observed complications. Unfortunately the mechanism of corresponding processes remains unknown [4].

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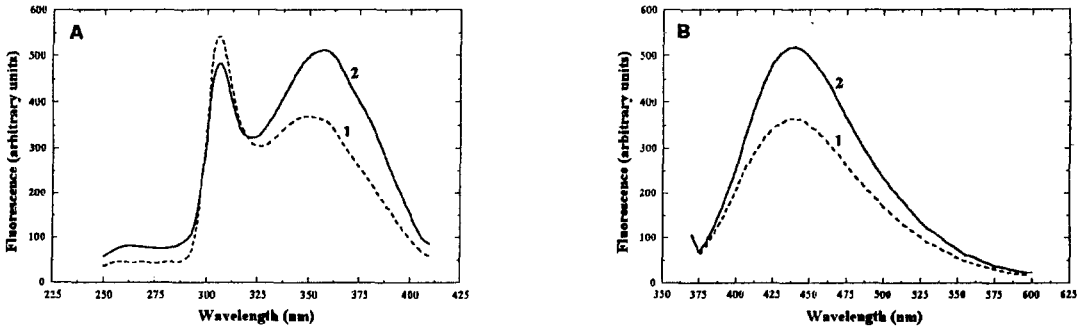



**Fig. 1.** Excitation (A) and emission (B) spectra of the HSA ($2 \cdot 10^{-4}$ *M*)–GCA ($8 \cdot 10^{-4}$ *M*) adduct in phosphate buffer, pH 7.4, at 20°C ($\lambda_{exc} = 359$ nm, $\lambda_{em} = 433$ nm): (1) immediately after mixing; (2) after 78.75 h (4725 min).

## MATERIALS AND METHODS

HSA was a product of Imuna (Šarišské Michal'any, Slovak Republic). DL-glyceraldehyde (GCA) was obtained from Sigma (St. Louis, MO). HRP was used as purchased from Serva (Heidelberg, FRG).

Chemiluminescence was measured as integrated emission intensity as counts per minute (cpm) using a detection equipment of scintillation counter Beckman LS 6000 SE. The reaction vessel was placed in the measuring apparatus immediately after mixing all the components needed for the chemiluminescence reaction. The fluorescence measurements were performed on a Perkin-Elmer LS-5B spectrofluorimeter. All fluorescence measurements are presented uncorrected. All experiments were carried out in phosphate buffer, pH 7.4, at 20°C.

## RESULTS AND DISCUSSION

The fluorescence excitation spectrum (uncorrected) of the HSA/GCA system exhibits two maxima ($\lambda_{exc1}$ = 306 nm, $\lambda_{exc2}$ = 360 nm) immediately after mixing of the reaction components (Fig. 1A, *curve 1*). The emission spectrum (uncorrected) of the same system exhibits a maximum at $\lambda_{em}$ = 440 nm (Fig. 1B, *curve 1*). Both spectra were time dependent. At the excitation wavelength $\lambda_{exc}$ = 306 nm the tryptophan residue (Trp-214) of HSA was selectively irradiated. Changes in fluorescence intensity thus apparently reflect structural changes in the surrounding area of Trp-214. The excitation maximum at 360 nm and the emission maximum at 440 nm are attributed to a certain chromophore on the surface of HSA macromolecule. This chromophore is formed during HSA/GCA interaction. Time dependence of the fluorescence of the HSA/GCA system is presented in Figs. 2–4 (*curves 1*). Time dependence of the fluorescence of HSA/GCA system was also observed in the presence of HRP (Figs. 2–4; *curves 2*). It is evident that there are great differences in fluorescence behavior between the HSA/GCA and the HSA/GCA/HRP systems. In the systems studied HRP acts as a dioxygenase and catalyzes the oxidation of products formed in the course of the protein–aldehyde interaction.

As shown in Fig. 2 the quenching of fluorescence at 306 nm occurred after ca. 1000 min of interaction. This could be explained by cross-linking reactions in the surrounding of Trp-214, which is buried in modified protein macromolecule. In the presence of HRP the quenching of fluorescence occurred immediately after the mixing of the reaction components. Fluorescence quenching acceleration observed in the presence of HRP is obviously due to the oxidation of products formed in the reaction between HSA and GCA.

As follows from the time dependence of fluorescence at 360 and 440 nm (Figs. 3 and 4; *curves 2*) HRP causes

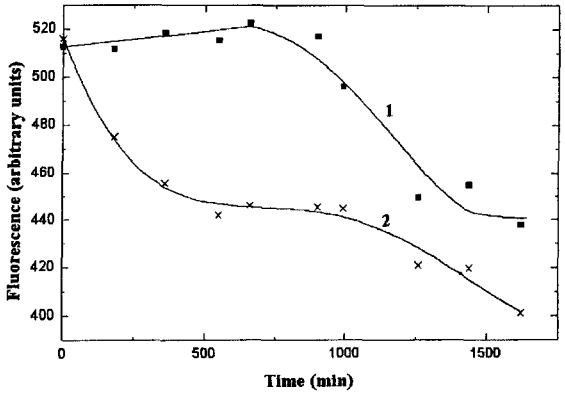



**Fig. 2.** Time course of fluorescence at 306 nm. GCA ($8 \cdot 10^{-4}$ mol dm$^{-3}$); HSA ($2 \cdot 10^{-4}$ mol dm$^{-3}$), and HRP ($1 \cdot 10^{-6}$ mol dm$^{-3}$): (1) system GCA/HSA; (2) system GCA/HSA/HRP.

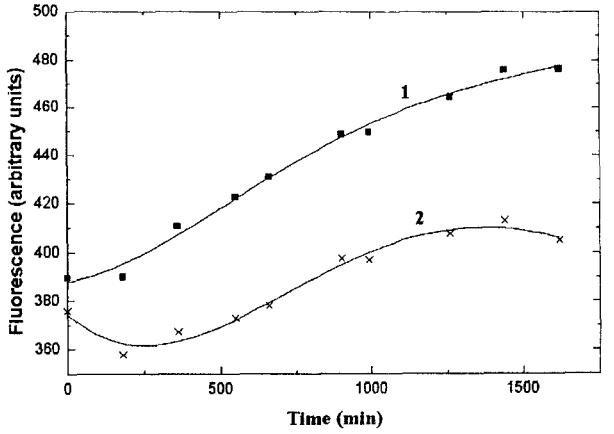



**Fig. 3.** Time course of fluorescence at 360 nm. GCA ($8 \cdot 10^{-4}$ mol $dm^{-3}$), HSA ($2 \cdot 10^{-4}$ mol $dm^{-3}$), and HRP ($1 \cdot 10^{-6}$ mol $dm^{-3}$): (1) system GCA/HSA; (2) system GCCA/HSA/HRP.

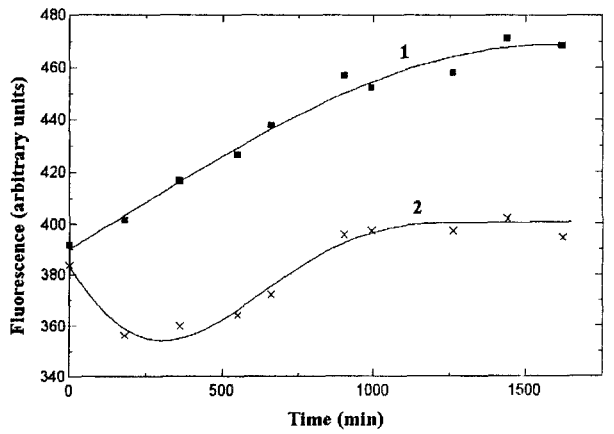



**Fig. 4.** Time course of fluorescence at 440 nm. GCA ($8 \cdot 10^{-4}$ mol $dm^{-3}$), HSA ($2 \cdot 10^{-4}$ mol $dm^{-3}$), and HRP ($1 \cdot 10^{-6}$ mol $dm^{-3}$): (1) system GCA/HSA; (2) system GCA/HSA/HRP.

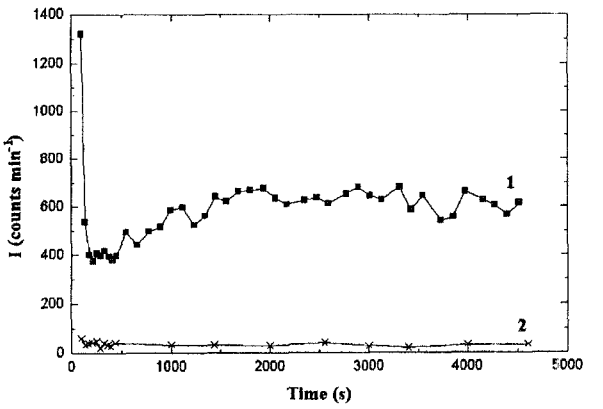



**Fig. 5.** Time course of chemiluminiscence intensity. GCA ($16 \cdot 10^{-4}$ mol $dm^{-3}$), HSA ($2 \cdot 10^{-4}$ mol $dm^{-3}$), and HRP ($1 \cdot 10^{-6}$ mol $dm^{-3}$): (1) system GCA/HSA/HRP; (2) system GCA/HRP.

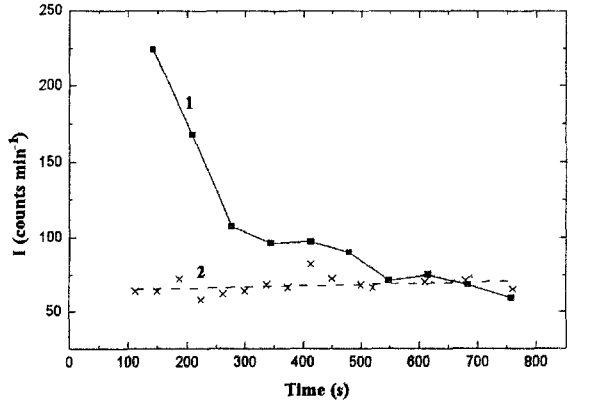


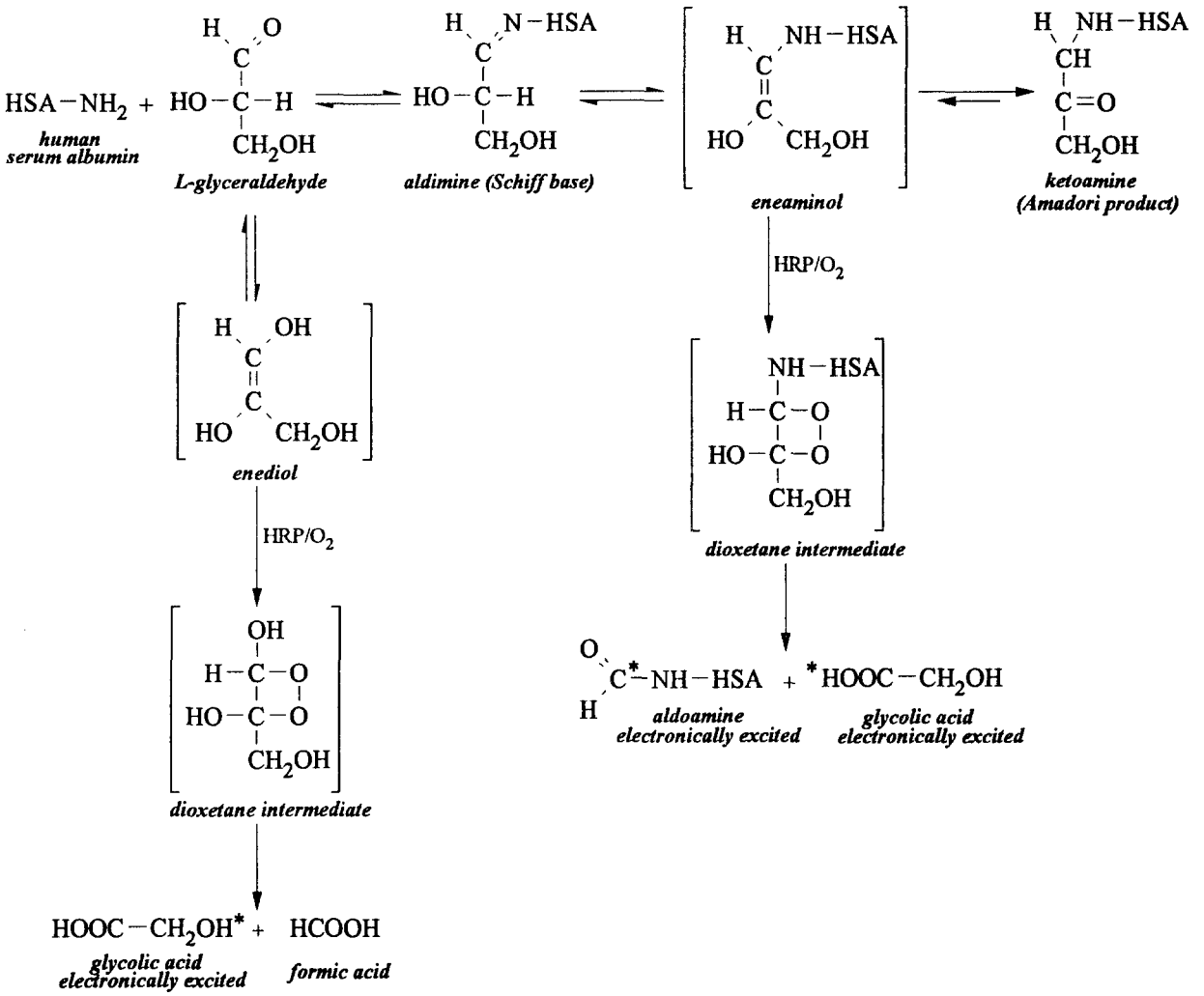

**Fig. 6.** Time course of chemiluminiscence intensity. GCA (0.1 mol $dm^{-3}$) and HRP ($1 \cdot 10^{-6}$ mol $dm^{-3}$): (1) system GCA/HRP; (2) phosphate buffer, pH 7.4.

slower development of chromophores arising from the HSA/GCA interaction. As can be seen from Figs. 3 and 4 (*curves 1*), fluorescence measurements thus present a convenient method for studying protein–aldehyde interaction.

The oxidation of HSA/GCA adducts by $O_2$ catalyzed by HRP acting as a dioxygenase is accompanied by light emission, which appears immediately after mixing of the reaction components (Fig. 5). These results suggest that the generation of electronically excited states (EES) is responsible for the observed chemiluminescence. In experiments with a large excess of GCA the chemiluminescence of GCA/HRP system was observed even in the absence of HSA (Fig. 6). This finding represents a new insight into the enzymatic generation of EES in systems containing biologically important substances. In previous studies it was believed that the oxidation of Schiff bases (aldoimines) was responsible for the generation of EES and the corresponding chemiluminescence [2]. We can conclude that the real substrate for the enzymatic generation of EES is not a Schiff-type adduct but the reactive intermediate formed during the Amadori rearrangement—an eneaminol. Scheme 1 presents possible reaction pathways of the HSA/GCA interaction. This scheme also proposes the possible reaction mechanism in the presence of HRP with respect to the generation of EES.

## CONCLUSIONS

1. Measurement of the time course of fluorescence during nonenzymatic glycosylation of proteins provides valuable data about studied interaction.




**Scheme I.** Possible reaction pathways of GCA/HSA interaction.

2. The presence of HRP substantially influences the time course of fluorescence by catalyzing the oxygenation reaction of products formed during nonenzymatic glycosylation.

3. The generation of EES and the corresponding chemiluminescence were observed in the oxygenation reaction.

4. From the results obtained, we propose that the true substrate for enzymatic oxygenation and subsequent generation of EES is likely an eneaminol, representing a mesomeric form of Schiff base formed during Amadori rearrangement.

5. The suggested relation between nonenzymatic glycosylation of proteins and generation of EES may play an important role in the elucidation of the molecular mechanism of aging and several free radical diseases (e.g., atherosclerosis, diabetes mellitus, Alzheimer's disease) [3].

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