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Consequential Alterations in Haemoglobin Structure upon Glycation with Fructose: Prevention by Acetylsalicylic Acid

M. Bakhti[1], M. Habibi-Rezaei[1,*], A.A. Moosavi-Movahedi[2] and M.R. Khazaei[3]

[1]*School of Biology, University College of Science, University of Tehran;* [2]*Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran; and* [3]*International Graduate Research School of Molecular Basis of Dynamic Cellular Processes, Westfaelische Wilhelmes-Universitaet, Muenster, Germany*

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Increased fructose concentration in erythrocytes of diabetic patients subject haemoglobin (Hb) to be glycated by fructose. Haemoglobin glycation results in early and advanced glycation end products which are known as HbA_{1c} and Hb-AGE, respectively. In diabetics the Hb-AGE content raises and the mean parameter multiplication value (PMV) for Hb-AGE has been calculated at 6 against non-diabetics. We are emphasizing on fluorescence descriptive pairs especially of 370/440 nm ($\lambda_{ex}/\lambda_{em}$) and thioflavin T binding process to characterize short-term Hb-AGE formation upon glycation with fructose or fructation. Secondary structure elements were estimated after far-UV circular dichroism spectropolarimetery which reveals up to 11.3% β-content attainment for 20 days incubated Hb with fructose which is concurred with enhanced hydrophobicity of the protein. The later is described through enhanced 1-anilino-8-naphthalene sulphonate (ANS) binding to fructated protein. The preventive effect of acetylsalicylic acid on glycation of methemoglobin and consequently, Hb-AGE formation also has been included.

Key words: fructation, glycation, Hb-AGE, HbA_{1c}, haemoglobin, thioflavin T.

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Protein glycation is a complex cascade of condensations, rearrangements, fragmentations and oxidative modifications which lead to characterized heterogeneous products often with considerable structural alterations at the secondary and tertiary levels and consequently functional properties of the proteins (*1*, *2*). These glycation products are collectively termed Advanced Glycation End products or AGEs. Glycation-induced structural alterations, which are implicated in diabetic circumstances and arise in hyperglycemic conditions (*3*), are believed to be a major cause of spontaneous alteration to the protein, in physiological systems.

Although glucose has been known to play a primary role in the formation of glycation products, physiological concentrations of other monosaccharides have been shown to be sufficient and even act more effectively than glucose to produce glycated variants (*4*, *5*). Among these, fructose has been shown to perform the glycation process at a much faster rate and as a special case, it accumulates in erythrocytes through the metabolic polyol pathway (*5*) and transporter-mediated influx involved by an isoform of the membrane-associated glucose transporter (GLUT), GLUT5. This transporter is one of the six facilitative glucose transporter isoforms characterized in mammalian cells. In spite of its name GLUT5, it acts on fructose and has a Km approximately 10 times lower than the specialized transporter for glucose, GLUT2 (*6*, *7*). The polyol or sorbitol-aldose reductase pathway is important in the progression of diabetic complications (*5*). The amount of fructose has been shown to be 4 times higher in the erythrocytes of diabetic patients, which subjects haemoglobin (Hb) to fructose glycation or fructation (*8*). However the most characterized form of glycated haemoglobin in biological systems is HbA_{1c} and glucose-involved glycation has been emphasized in most cases.

In general glucose and in more extent fructose (*5*) react non-enzymatically to establish reversible Schiff base with ε- amino group(s) from lysyl residue(s), the α-amino group(s) from the N-terminal residue(s) and rarely with arginine and cystein residues (*9*) which are converted into more stable Amadori products by aldimine to ketoamine isomerization (Amadori rearrangement). These products have been recognized as early glycation products and then irreversibly transformed, over time, into advanced glycation end products. It has been observed that α-amino groups of the valine residues at the N-termini of the β-chains of haemoglobin are involved in early glycation product of the Hb or HbA_{1c} formation (*10–13*). Meanwhile it acts as a precursor in the long-term production of the advance glycation end products of Hb (Hb-AGE) (*14*).

Although HbA_{1c} has been attained significant prominence in the modern world of medicinal biology due to its use as an indicator in the long-term control of diabetes mellitus (*15–17*), Hb-AGE is also suggested to be used as a possible index in long-term complications (*14*). Wolffenbuttel *et al*. have implied that long-term modification of haemoglobin and consequently Hb-AGEs would be a better index for long-term glycemia in patients with diabetes (*18*). However a good correlation between HbA_{1c} and Hb-AGE in hyperglycemic patients, especially those with poor glycemic control, has also been reported (*19*).

*To whom correspondence should be addressed. Tel: +98-21-6113214, Fax: +98-21-6640514, Email: mhabibi@khayam.ut.ac.ir

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Structural and functional properties of HbA_{1c} have been studied thoroughly (*20–22*). Glycation induces structural alterations in Hb, such as reduced α-helix content, higher hydrophobic tryptophan surface accessibility, increased thermostability and weaker heme-globin binding (*22*), and consequently higher oxygen affinity (*23*). The effects of protons, 2,3-diphosphoglycerate (2,3-DPG) and some other ligands such as CO_2 and CO on the function of human HbA_{1c} have also been reported (*20*, *21*). In spite of the report dealing with the presence and circulation of 0.42 and 0.75% Hb-AGE in non-diabetic and diabetic patients, respectively (*14*), structural and functional properties of Hb-AGE are poorly understood in comparison with HbA_{1c}. Meanwhile it has been shown that the isoelectric point of AGE haemoglobin is lower than the unglycated form, and a different autofluorescecne property of modified haemoglobin has been reported during recent years (*24*, *25*).

AGE structures are associated with many pathophysiological complications (*26*). Although many attempts dealing with the effective prevention of AGE production have been made, reports on Hb-AGE are limited to a few cases (*14*). Rendell *et al*. reported the inhibitory effect of acetylsalicylic acid (ASA) on the production of HbA_{1c} (*27*), but such preventive effect has not yet been examined on the formation of Hb-AGE. Acetylation of the amino group(s) is generally accepted mechanism for ASA preventive effects on glycation process. Hb-AGE is able to be a valuable indicator in diabetes; therefore its structural elucidation can improve present Hb-AGE applied potential to check up on long-term diabetes complication. The present investigation has undertaken to study the structural alterations of Hb-AGE evolved from methemoglobin in the presence of fructose and indication on the preventive effect of ASA on Hb-AGE formation.

MATERIALS AND METHODS

Materials—Bovine methemoglobin, ASA and 1-anilino-8-naphthalene sulphonate (ANS) were obtained from Sigma Chemical Co. (St Louis, MO). Thioflavin T was purchased from Fluka. All other chemicals were purchased from Merck.

In vitro Preparation of Hb-AGE—A solution of bovine methemoglobin (15 μM) was prepared with 30 mM fructose in a 50 mM phosphate buffer with pH 7.4, containing 0.02% (w/v) sodium azide. The solution was sterilized by filtering through low protein binding filter (Millex.®. -GV 0.22 μm filter unit, Millipore) and was then incubated under sterile conditions at 37°C in the dark. For assessment of its preventive effect on the glycation process 1.5 mM ASA was added to the samples. The control solutions were prepared in the same way, but without fructose. At various intervals 5 ml samples were taken and dialysed extensively against 50 mM phosphate buffer with pH 7.4 at 4°C, to remove the unbounded fructose, or aspirin. After dialysis protein concentrations were estimated by the Bradford method in which bovine methemoglobin was used as standard. The samples were immediately frozen and stored at −70°C until processed.

Hb-AGE-associated Fluorescence Analysis—Fluorescence measurements were performed using a Hitachi F-4500 spectrofluorometer. AGE-related autofluorescence of the sample preparations was monitored by exciting at 370 nm and emission wavelength at 440 nm at 25°C which was determined through wavelength scanning in the range of 400–500 nm. The spectra were corrected with appropriate protein and buffer blanks. Also fluorescence studies at an excitation wavelength of 308 nm and spectra scanning in the wavelength range of 330–450 nm were carried out. For all measurements, the samples contained 0.1 mg/ml protein.

ANS Fluorescence Measurement—Fluorescence probe ANS has been used to investigate the accessible surface-located hydrophobic pockets of the assigned protein samples (*28*). One hundred micromolar ANS was added to the samples with a protein content of 0.1 mg/ml. After exciting at 350 nm, emission spectra in the wavelength range of 450–600 nm were taken. These experiments were performed at 25°C.

Circular Dichroism Spectropolarimetry—Circular dichroism (CD) spectra were collected between 190 and 260 nm, at the far UV region with an Aviv CD spectropolarimeter model 215 (USA) at 25°C. The CD measurement was performed using a 1 mm path length quartz cuvette, at the final protein concentration of 0.2 mg/ml. Two spectra for each sample were recorded, averaged and corrected for buffer background. Deconvolution of the data was performed to estimate the α-helix and β-conformation content of Hb-AGEs using CDNN software (*29*).

Thioflavin T Test—Thioflavin T (ThT) is a benzothiazole dye that exhibits enhanced fluorescence upon binding to amyloid fibrils and is commonly used to diagnose amyloid fibrils, both *ex vivo* and *in vitro* (*30*, *31*). Solutions of 1.5 μM fructated haemoglobin and control samples were incubated at 25°C with 5 μM thioflavin T for an hour. The sample fluorescence was measured through excitation at 435 nm and emission spectra were recorded in the wavelength range of 455–555 nm; band slit was used at 10 nm.

RESULTS

Fluorescence properties of the haemoglobin molecules incubated with fructose were assessed through maximum emission peak at 345 nm after excitation at 308 nm. This emission increased along with incubation time. However in the experiment at the fluorescence descriptive pair of 308/345 nm ($\lambda_{ex}/\lambda_{em}$), a slight increase was also observed for ASA-treated samples, whereas the emission of the controls remained without change (Fig. 1A).

Also fluorescence studies over a 20-day incubation period revealed an increase in the fluorescence intensity at 370/440 nm ($\lambda_{ex}/\lambda_{em}$) for the samples incubated with fructose. AGE-related increase in the intensity of fluorescence was not observed for the controls. The emitted fluorescence intensity values for fructated Hb are plotted as a function of time in Fig. 1B and the inset shows spectra which have been presented in an ascending order of time in the wavelength range of 400–550 nm.

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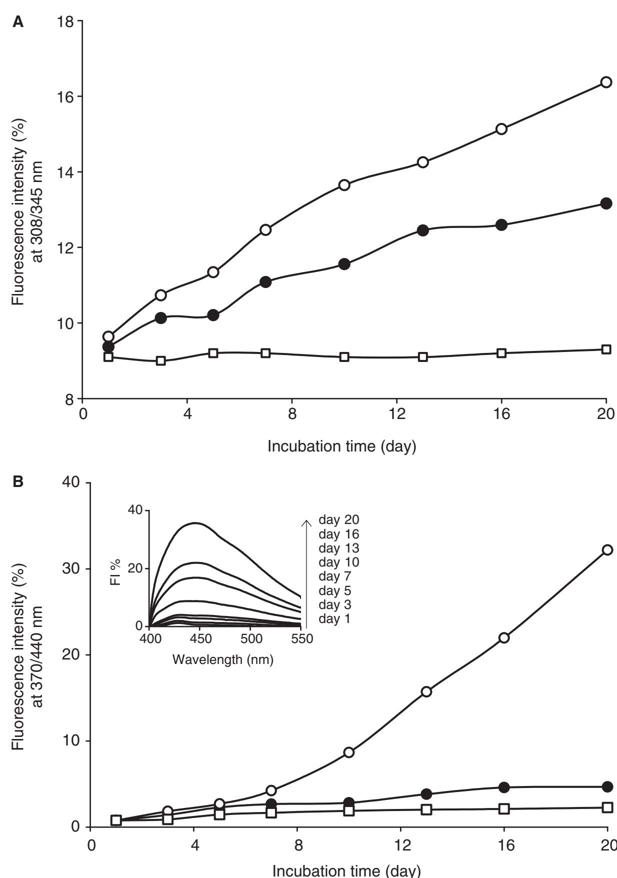

Fig. 1. **The effect of glycation on the formation of Hb-AGE during incubation of 15 μM metHb with 30 mM fructose in the absence (open circle) and presence (closed circle) of acetylsalicylic acid.** (A) Progressive intensification of the fluorescence emission at 345 nm after excitation at 308 nm. (B) The same phenomenon has also been studied in the fluorescence descriptive pair of 370/440 nm ($\lambda_{ex}/\lambda_{em}$) in the absence (open circle) and presence (closed circle) of acetylsalicylic acid. (Inset shows spectra which are presented in ascending order of time in the wavelength range of 400–550 nm.) In the absence of fructose (open square) fluorescence intensity remained almost without change for both representative pair of wavelengths (308/345 and 370/440).

For the samples treated with acetylsalicylic AGE-related fluorescence exhibits little or no change, which reflects the significant preventive effect of this chemical towards the glycation process. Fluorescence intensity was noted to increase non-linearly and the maximum value was revealed to gain even more when the time exceeds 20 days. The control samples did not show any fluorescence emission. For the samples treated with ASA a slight but obvious preventive effect in fluorescence emission was observed.

For the samples under glycation with fructose, ANS fluorescence emission shows a dramatic increase in the fluorescence emission intensity as well as a blue shift of 18 nm (hypsochromic effect) against time of incubation (Fig. 2). In ASA-treated samples, structural alterations remain at an almost constant extent of fluorescence intensity. Meanwhile ANS-binding fluorescence of the control samples did not show any remarkable change.

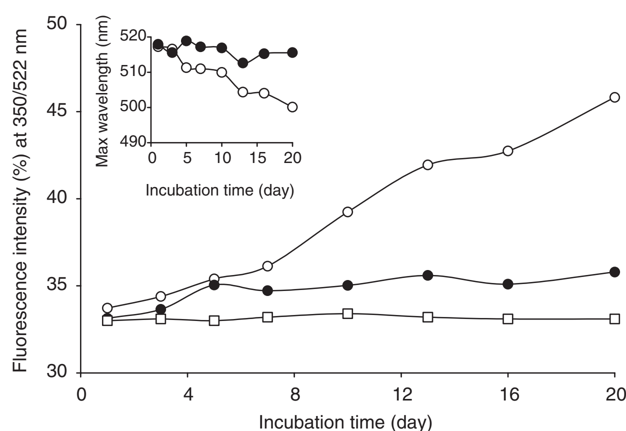

Fig. 2. **The effect of fructation time on AGE-related ANS-binding fluorescence: spectra were obtained after excitation at 350 nm, then plots were constructed at maximum emission wavelengths against fructation time in the absence (open circle) and presence (closed circle) of acetylsalicylic acid [the inset figure shows shift in wavelength of maximum emission (hypsochromic effect) against time of incubation in the absence (open circle) and presence (closed circle) of the inhibitor].** During the same incubation time fluorescence intensity for controls (open square) remained almost without change.

Fructose-induced conformational changes at the secondary structure of Hb were studied by CD. The CD spectra at the far-UV region of Hb-AGE are different from the controls and a significant change in the secondary structure of Hb-AGE was observed by a decrease in negative ellipticity in the wavelength rang of 205–235 nm (Fig. 3). The CD spectrum of the control (non-glycated) sample exhibited two negative minima at 209 and 219 nm and a maximum at 190–195 nm, which were typical of α-helix structure. After incubation with fructose as glycating sugar, spectra for the samples revealed a decrease in minima ellipticity, as a function of incubation time. Meanwhile in progressive glycation with fructose during the examined period of 16–20 days, the negative ellipticity peak at 219 nm continued to decrease monotonically and almost disappeared in day 20. But a large negative minimum began to appear after day 16 and reached its negative minimum extent on day 20. The α-helicity of Hb-AGE decreased dramatically from 61.27 to 42.61% as a function of incubation time, while β-conformation increased from 0 to 11.3%. ASA significantly inhibits the secondary structural changes induced by fructose (Table 1).

Thioflavin T (ThT) is a cationic benzothiazole dye which was introduced by Vassar and Culling (*32*) and exhibits enhanced fluorescence upon binding to amyloid fibrils and is commonly used to diagnose amyloid fibrils, at *ex vivo* and *in vitro*. A tremendous increase in the fluorescence emission of thioflavin T was observed at 492 nm upon binding to the glycated protein. Fructated Hb was exposed to thioflavin T and fluorescence emission was measured in different days. The ThT-related fluorescence intensity increased with increasing the time of incubation, which shows the progressive formation of the amyloid-conformation inflected at day 10, and is in accordance with the binding of ThT (Fig. 4).

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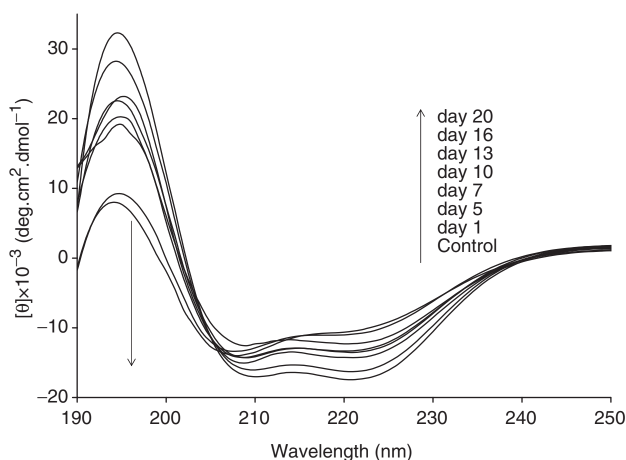

Fig. 3. **Far-ultra-violet CD spectra for the samples of metHb treated with fructose at sterile, dark and 37°C for up to 20 days.** Each sample was taken, then extensively dialysed against buffer, and analysed by CD. Spectra are presented in ascending order of time (0–20 days).

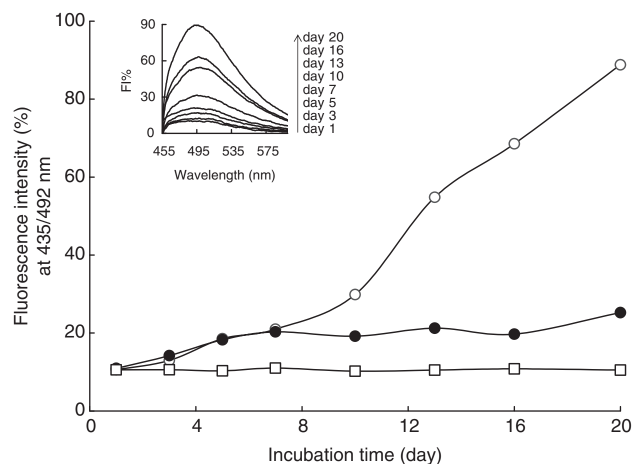

Fig. 4. **ThT-binding function against fructation time effect in which maximum fluorescence emission was measured through excitation at 435 nm and spectra were recoded in the wavelength range of 455–555 nm for control (open square) and test samples in the absence (open circle) and the presence (closed circle) of the acetylsalicylate.** Inset to the figure presents resulting spectra of thioflavin T test.

Table 1. **The percentage of secondary structures of methemoglobin (metHb) in the absence (−) or presence (+) of 30 mM fructose (Fru) and absence (−) or presence (+) of 1.5 mM acetylsalicylic acid (ASA) in 50 mM phosphate buffer with pH 7.4, incubated for different days.**

Days of incubation	metHb			metHb: +Fru			metHb: +Fru,+ASA		
	α-helix (%)	β-sheet (%)	Random coil (%)	α-helix (%)	β-sheet (%)	Random coil (%)	α-helix (%)	β-sheet (%)	Random coil (%)
1	62.45	0	37.55	61.27	0.5	38.23	63.17	0.5	36.33
3	62.12	0.2	37.86	59.83	2.4	37.77	61.45	1	37.55
5	61.80	0.3	37.9	56.03	4.2	39.77	60.02	1.5	38.48
7	61.74	0.5	37.76	53.36	4.2	42.44	59.83	2.1	38.07
10	61.05	0.5	38.45	53.07	5	41.93	59.82	2.5	37.68
13	60.29	0.9	38.81	53.03	6.5	40.47	57.79	2.5	39.71
16	59.94	1.1	38.99	44.94	9.5	45.56	56.36	3.2	40.44
20	59.65	1.3	39.05	42.61	11.3	46.09	56.31	4.3	39.39

ASA significantly prevents ThT binding which has been demonstrated in the same figure.

DISCUSSION

Glycation of haemoglobin (Hb) forms HbA_{1c} which has been described as an Amadori product (*14*) and is distincted from the AGE of the haemoglobin molecule (*26*). Although HbA_{1c} has attained significant prominence in the modern world of medicinal biology due to its use as a diagnostic index in the long-term control of diabetes mellitus (*15–17*) there is an inherent limitation in HbA_{1c} assessment, which has brought about vastly different results based on the assay method (high-performance liquid chromatography, immunoassay, affinity chromatography, etc.) (*33*). However, HbA_{1c} assessment is a valuable glycaemic risk marker (*34*). It has been reported that Hb-AGE content increase significantly from normal to diabetic subjects (*14*). Gopalkrishnapillai *et al*. reported the mean glucose level (mg/dl), HbA_{1c} (%) and Hb-AGE fluorescence intensity (arbitrary units) in patients with type II diabetes and the corresponding values in control subjects (*35*). Based on their reported data, here the 'parameter multiplication value' (PMV) has been estimated by us from normal individuals to diabetic patients, provides raw calculated ranges which have been presented in Table 2. It seems that parameter increment during the progression of disease occurs in higher magnitudes for Hb-AGE than HbA_{1c}. It presumably presents a more suitable potential and resolution for Hb-AGE evaluation which provides a more sensitive long-term assessment of diabetes complications relative to HbA_{1c}. The formation of AGE is now also known to result from the reaction of various metabolites other than glucose which are primarily located intracellularly and participate in the non-enzymatic glycation reaction at a much faster rate. Fructose displays about 8-fold higher reactivity than glucose (*5*). Erythrocytes in diabetic patients accumulate fructose, therefore

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Table 2. **Parameter multiplication values (PMV) from normal to diabetic type II which have been calculated based on data provided by Gopalkrishnapillai *et al.* (*35*).**

Quantified values	Glc (mg/dl)		HbA_{1c} (%)		Hb-AGE (AU)	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
	85.6 ± 10.7	170 ± 40	5.6 ± 0.7	8.2 ± 0.6	1.7 ± 0.2	10.2 ± 5.2
PMV_{Min}.	1.35		1.21		2.63	
PMV_{Max}.	2.81		1.80		10.27	
PMV_{Mean}.	1.99		1.5		6	

intracellular erythrocyte fructose level is 4 times higher in the diabetic patients (*8*) supporting its role as the probable main glycating agent in Hb-AGE producing process.

In this study we are reporting the subsequent structural alterations of methemoglobin due to short-term glycation for 20 days with fructose under extreme conditions. It has been proved that Hb-AGE formed by glucose possesses a characteristic autofluorescecne descriptive pair of 308/345 nm ($\lambda_{ex}/\lambda_{em}$) (*24*, *25*). Present study shows that *in vitro* fructation of Hb produces fluorophore adducts which can fluoresce with a maximum emission at 345 nm upon excitation at 308 nm (Fig. 1A). These findings corroborate with *in vivo* observations on human Hb-AGE (*35*). Moreover Avendano *et al*. (*36*) reported collagen AGE-specific fluorescence by measuring emission at 440 nm after excitation at 370 nm which correlates with glycemia. Similar resultant increase in Hb-AGE species of haemoglobin as a function of incubation time with fructose have been detected through increase in fluorescence intensity at 370/440 nm ($\lambda_{ex}/\lambda_{em}$) (Fig. 1B). A comparative view on intrinsic fluorescence data provided through wavelength pairs of 308/345 and 370/440 (Fig. 1) shows about 6 and more than 30 percent fluorescence intensifications, respectively, at the end of 20 days incubation period with fructose. In spite of slight but meaningful hyperbolic pattern of the fluorescence intensity against time for the study at 308/345 pair, biphasic pattern dealing with low slope followed by high-slope pattern was resulted through study at 370/440 pair. Therefore Hb-AGE assessment can be achieved with more sensitivity through excitation at 370 than 308 nm and studies based on above-mentioned pairs of the wavelengths presents presumably two different evolving fluorescent species which are appeared in progression.

The observed ANS-related intensification upon advancement of glycation along with observed blue shift strongly indicates that ANS is taken up into the haemoglobin structure and less contact with water is established (Fig. 2). Moreover protein conformational changes provide more accessible hydrophobic pockets examined through ANS-binding properties (*37*, *38*). The blue shift of λ_{max} (inset to Fig. 2) can be explained by a decrease in the polarity of the environment of ANS which indicates that the advanced glycation products of Hb shield the ANS probe more effectively from the water than the non- or less-fructated forms of Hb (*28*). ANS-binding sites in intact human oxyhaemoglobin were described by time-resolved laser spectrofluorimetry (*39*) which are different from heme pockets (*40*). ANS binding can be occurred based on electrostatic interactions between the ANS sulphonate group and protein cationic groups known as allosteric effector (2,3-di-phosphoglycerate or DPG) binding site (*41–43*). It is well known that the α-amino groups of the valine residues at the N-terminus of the β-chains of human haemoglobin contribute to form this cluster of eight positive charges. It has been shown that in HbA_{1c} the N-termini of the β-chains of human haemoglobin react non-enzymatically with glucose which consequently reduces the affinity to DPG in HbA_{1c} (*22*). Syakhovich *et al*. (*44*) reported a minor decrease in ANS binding to HbA_{1c} which can be explained through rising of the steric barrier for both DPG interaction and ANS binding to HbA_{1c}. The observed extensive ANS fluorescence as well as the induced blue shift in the λ_{max} from 517 to 500 nm in this study resulted from dramatic conformational state alterations in the haemoglobin structure to enhance hydrophobicity of the protein due to developing of the Hb-AGE species. We presume that during fructation of the protein, distinctive binding sites for ANS evolve to contribute in the observed hyperchromism of the fluorescence intensity.

In the presence of acetylsalicylic acid protein hydrophobicity does not increase significantly as measured by ANS binding, whereas in the control samples it remains absolutely constant (Fig. 2).

Fructation led to increase the transformation of α-helix to β-sheet in metHb confirmed by CD spectropolarimetry. Figure 3 represents the far-UV circular dichroism spectra at different days of incubation with fructose and deconvolution of the CD spectra (see experimental section) yielded percentages that are presented for three groups of experiments (control metHb, metHb incubated with fructose and metHb incubated with fructose in the presence of acetylsalicylate) in Table 1. As expected for the incubated Hb with fructose, β-content increased over time against the control. However the third group is proved to have a $<50\%$ declined increase against second group which can be explained through relative inhibitory effect of acetylsalicylic acid on $\alpha \rightarrow \beta$ transfer during the fructation of the Hb. ThT is used as a diagnostic dye for the amyloid fibrils due to its geometric fitness and therefore binding to amyloid fibrils (*45*, *46*), and Khorana *et al*. (*31*) have calculated the critical micelle concentration (CMC) value of ThT at 3.75–4 μM on the basis of used experimental strategies. So we examined the thioflavin T binding function against incubation time to find out if enhanced β conformations can be gathered to provide amyloid super β structures at ThT

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concentration of 5 μM which is higher than its reported CMC (Fig. 4). Up to about 9 folds enhancement in ThT fluorescence emission was observed upon binding to fructated Hb especially in its advanced states. However protein samples that were incubated with fructose in the presence of acetylsalcylilate failed to present enhanced ThT-binding fluorescence which even is comparable with control samples (Fig. 4). Bi-phasic nature of ThT-related fluorescence emission against time of incubation can be explained through dependency of fructation-induced amyloid-forming on the increase in the β-content of the protein. In the first phase, required β-content is provided to accelerate their gathering to form ThT-binding structures during with the second phase. Hb-AGE product has featured by β conformation enhancement revealed by CD analysis (Fig. 3 and Table 1) followed by amyloid formation which has been shown via ThT-binding properties (Fig. 4). The provided data on the effect of acetylsalicylic acid seems to reflect the higher inhibitory effect of the agent against amyloid formation presented through ThT binding than induced β conformation increase. It can be presumably explained through preventive effect of acetylsalicylic acid to attain least pre-requisite β-content for amyloid formation.

In this article we emphasize on Hb-AGE content as a valuable diabetes criterion. In conclusion, fructose which has been considered as an important natural sweetener is introduced as a putative agent to play an effective role in the glycation-induced formation of Hb-AGE. It seems that fructation proceeds upon a biphasic mode which has been documented through 370/440 fluorescence experiment, thioflavin T-binding properties and enhanced hydrophobicity of the metHb. The above-mentioned structural changes are characteristic of Hb-AGE formation in the presence of fructose, which can be efficiently inhibited by acetylsalicylic acid specially after developing the β conformations.

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