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EFFECTS OF NONENZYMATIC GLYCOSYLATION AND FATTY ACIDS ON TRYPTOPHAN BINDING TO HUMAN SERUM ALBUMIN

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Abstract—The effects of bound fatty acids and nonenzymatic glycosylation (NEG) on tryptophan binding to human serum albumin (HSA) were examined utilizing a rate of dialysis technique. HSA with 0, 1, 2, 3, or 5 mol of palmitate bound per mol of HSA was glycosylated *in vitro* to a level exceeding that seen in diabetes. NEG was not inhibited by fatty acids, suggesting that Lys-525, the primary site for NEG, is not an essential component of the principal sites for long-chain fatty acid binding to HSA. Scatchard analysis of binding data showed an expected fatty acid dependent decrease in the number of available tryptophan binding sites, but showed that fatty acids did not affect tryptophan affinity. The binding data failed to show an effect of NEG on tryptophan binding. The lack of inhibition of tryptophan binding by NEG suggests that drug-binding Site II, the indole/benzodiazepine site, is resistant to both NEG and to any conformational changes in HSA which may occur with NEG. These data suggest that elevated plasma free tryptophan and the resulting altered serotonin metabolism seen in diabetes are independent of increased NEG and likely result from diabetic hyperlipidemia.

Human serum albumin (HSA†) is the most abundant plasma protein, and contains 585 amino acid residues in a single polypeptide chain of known sequence [1]. HSA displays the property of conformational adaptability which allows the protein to transport an enormous variety of organic ligands including bilirubin, fatty acids, many drugs, and tryptophan [1].

In humans, about 80% of the total plasma tryptophan is bound to HSA [2] at a single high-affinity site: Site II, the indole/benzodiazepine binding site [1, 3, 4]. The binding of tryptophan to HSA and the effects of fatty acids on tryptophan binding have been investigated [5–9], and the evidence for the location of the indole-binding site has been reviewed [1, 3, 10]. At least one lysine residue has been implicated as a requirement for tryptophan binding. Results from studies using affinity labels suggest that Lys-414 has a low pK and is accessible at or near the surface of the HSA molecule. In addition, Lys-414 is found in one of four Lys-Lys sequences in albumin, a sequence suggested to facilitate the Amadori rearrangement resulting in enhanced nonenzymatic glycosylation [11].

Nonenzymatic glycosylation (NEG) of HSA by free glucose in the blood occurs at specific lysine residues and appears to affect the ligand binding of bilirubin, fatty acids [12], and some drugs [13, 14]. NEG of HSA and several other proteins is elevated 2- to 3-fold in diabetes [15] and substantial evidence suggests that the end-products of advanced NEG may be responsible for many of the long-term complications associated with the disease [16, 17]. Because of the enhanced NEG and hyperlipidemia which accompany diabetes [16], we have investigated the relationships among protein-bound fatty acids, NEG, and ligand binding at Site II using a rate of dialysis technique [18] to measure tryptophan binding to HSA nonenzymatically glycosylated *in vitro*. NEG was performed in the presence of various molar ratios of bound fatty acids in order to mimic diabetic hyperlipidemia.

MATERIALS AND METHODS

Human serum albumin (Cohn Fraction V) was obtained from the American Red Cross. [^{3}H]L-Tryptophan (34 mCi/mmol) was obtained from the New England Nuclear Corp. (Boston, MA). Chemicals and biochemicals were of the purest grade available (Sigma Chemical Co., St. Louis, MO, or Fisher Scientific, Cincinnati, OH). Scintillation counting supplies were from Research Products International (Mt. Prospect, IL). Rate of dialysis cells were constructed from Lucite and employed dialysis membranes having a molecular weight cut-off of 14,000. Human serum albumin and tryptophan were quantitated by absorbance at 280 nm; HSA, specific absorptivity = 0.53 $mg^{-1} \cdot mL^{-1} \cdot cm^{-1}$ [19]; tryptophan, molar absorptivity = 5650 $M^{-1} \cdot cm^{-1}$ [5]. Phosphate-buffered saline (PBS), pH 7.4, contained sodium chloride, 8.0 g/L; potassium chloride, 0.2 g/L; sodium phosphate diabasic, 1.15 g/L; potassium phosphate, 0.2 g/L; and sodium azide, 0.2 g/L.

Albumin purification. Human serum albumin was further purified by affinity chromatography on Affi-Gel Blue (Bio-Rad, Richmond, CA) in order to

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† Abbreviations: BSA, bovine serum albumin; HSA, human serum albumin; glc-HSA, nonenzymatically glycosylated human serum albumin; NEG, nonenzymatic glycosylation; and PBS, phosphate-buffered saline.

remove contaminating globulins [20]. Only HSA was detected by immunoelectrophoretic analysis versus rabbit anti-human serum antiserum. Polyacrylamide gel electrophoresis, nondenaturing and in the presence of sodium dodecyl sulfate [21], indicated that HSA preparations contained less than 5% polymeric HSA (data not shown).

Albumin defatting and refatting. HSA was defatted using the procedure of Chitpatima and Feldhoff [22]. Briefly, HSA at 10 mg/mL was incubated with 10 mg/mL dextran-coated charcoal in 0.25 M ammonium formate, pH 3.0, for 1 hr at 25°. Charcoal was removed by centrifugation for 15 min at 25,000 *g* and filtration through 0.22 μm filters. The ammonium formate was replaced with PBS by ultrafiltration. Aliquots of palmitic acid were dissolved in ethanol and evaporated onto the inside surfaces of Corex test tubes by rotating the tubes in a stream of dry nitrogen. Defatted HSA was added to the tubes and stirred with a magnetic stir-bar for 2 hr at room temperature and overnight at 4°. HSA samples were filtered through 0.22 μm filters before NEG *in vitro*. We determined in separate experiments that filtration did not remove bound fatty acids. Briefly, [^{14}C]-palmitic acid (New England Nuclear) was bound to HSA samples in 1:1 and 5:1 molar ratios. The protein samples were filtered through five consecutive 0.22 μm filters, and an aliquot of each filtered sample was removed and counted in a scintillation counter. Since the protein-bound radioactivity remained constant after each filtration with a 2.4% coefficient of variation, it was concluded that the filtration did not remove protein-bound fatty acids.

In vitro *nonenzymatic glycosylation.* HSA (30 mg/mL) containing bound fatty acids was incubated in sterile screw-cap culture tubes with 100 mM glucose in PBS, pH 7.4, in a dark incubator at 37° for 8 days. The mixtures were sterilized by filtration prior to incubation. After removal of free glucose by ultrafiltration, NEG was quantified using a modified thiobarbituric acid assay [23]. Control samples of HSA with bound fatty acids were incubated identically, but in the absence of glucose.

Binding studies. Radiolabeled tryptophan was purified by affinity chromatography using the procedure of Stewart and Doherty [24]. Rate of dialysis experiments followed the procedure of Colowick and Womack [18]. Binding experiments were initiated by transferring 2.0 mL of HSA (20 mg/mL) in PBS, pH 7.4, containing 9×10^6 dpm of [^{3}H]-tryptophan to the top chamber. Additions of unlabeled tryptophan in PBS (10 μL) were made after 20 mL of buffer had flowed through the bottom chamber. This allowed for the attainment of a steady-state rate of dialysis of free ligand to the bottom chamber. The final addition was 100 μL in order to ensure a considerable excess of ligand to protein. PBS was pumped through the bottom chamber at a rate of 1 mL/min and 2-mL fractions of the bottom chamber effluent were collected. An aliquot (0.4 mL) of each 2-mL fraction was mixed with 4 mL of Budget-Solve scintillation fluid and counted in a Beckman liquid scintillation counter at 35% efficiency.

Data analysis. Binding parameters were determined by the method of Scatchard [25]. The binding affinity constant and the number of tryptophan binding sites were determined from the slope and y-intercept of the line determined by linear regression through the number of data points shown in Table 1. The statistical significance of the effects of NEG on tryptophan binding was determined using Student's *t*-test (two-tailed) for common slope and elevation [26] and considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Approximately 80% of the [^{3}H]L-tryptophan was recovered as a single peak when purified by affinity chromatography on an HSA-agarose column [24]. The use of affinity-purified tryptophan eliminated non-specific binding by radioactive contaminants and/or breakdown products. NEG of HSA was performed *in vitro* in the presence of absence of bound fatty acids. There was no effect of fatty acids on NEG at fatty acid:HSA ratios of 1:1 and 2:1, and only a 5% decrease in NEG was observed at fatty acid:HSA ratios of 3:1 and 5:1 (Table 1).

The fractions of bound and free tryptophan

Table 1. Binding constants for tryptophan in HSA

	Nonglycosylated				Glycosylated			
Pal:HSA	%NEG	$10^{-3} \times K_a$ (M^{-1})	n	(N;X)	%NEG	$10^{-3} \times K_a$ (M^{-1})	n	(N;X)
0	[15.6]	9.9	0.69	(30;5)	[80.0]	9.1	0.62	(32;5)
1	[15.2]	9.0	0.61	(12;2)	[82.0]	9.3	0.59	(14;2)
2	[15.4]	7.6	0.45	(12;2)	[76.0]	8.7	0.48	(12;2)
3	[15.8]	9.6	0.22	(5;1)	[75.0]	8.1	0.30	(5;1)
5	[15.0]	*	*		[75.0]	*	*	

HSA was glycosylated *in vitro* at the indicated molar ratios of palmitate (Pal) to HSA. The apparent affinity constant (K_a) and the mol tryptophan binding sites/mol HSA (n) were determined from Scatchard analysis in Fig. 3. Results are reported as the mean determined from linear regression through the total number of data points "N" obtained from "X" experiments (N;X). Percent nonenzymatic glycosylation (%NEG, mol ketoamine/mol HSA × 100%) is given in brackets.

* No binding detected.

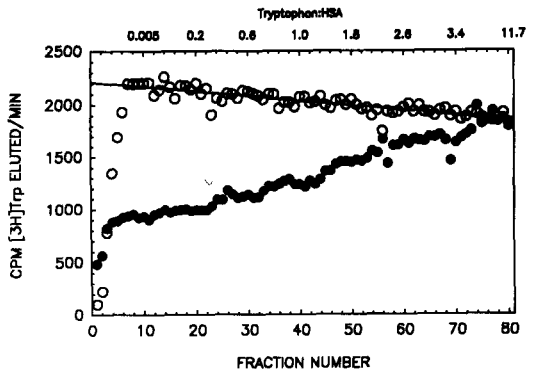

Fig. 1. Rate of dialysis of [^{3}H]L-tryptophan in the presence (●) and absence (○) of defatted HSA. Radiolabeled tryptophan (3×10^6 cpm, 24.4 Ci/mmol) was mixed with defatted HSA (20 mg/mL) in a final volume of 2.2 mL PBS, pH 7.4, 25°. At time zero, 2.0 mL of the albumin mixture was transferred to the empty top chamber of the dialysis apparatus and fractions were collected. An aliquot of unlabeled tryptophan (10 μL) was added to the top chamber during the collection of fraction 1. Subsequent 10-μL additions were made at the start of fractions 11, 21, 31, 41, 51, and 61 and a 100-μL addition was made at fraction 71 to obtain the indicated Trp:HSA ratios at the steady state. The fraction of free ligand was calculated as the steady-state radioactivity in the presence of HSA (●) divided by the steady-state radioactivity in the absence of HSA (○).

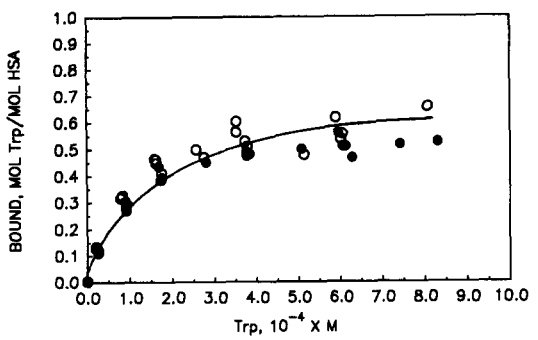

Fig. 2. Saturation of L-tryptophan binding in defatted HSA (○) and defatted HSA glycosylated *in vitro* (●). The concentration of bound Trp was determined from the difference in total and free Trp. The concentration of free Trp was determined from the data in Fig. 1.

obtained from rate of dialysis experiments were determined from data plots similar to that shown in Fig. 1. The data were first plotted to show saturation of the tryptophan binding sites (Fig. 2), and then plotted using the method of Scatchard [25] for the determination of binding constants (Fig. 3). In glycosylated as well as nonglycosylated HSA there was a decrease in the number of binding sites for tryptophan when the fatty acid:HSA ratio was 2:1 or greater. There appeared to be no difference in binding affinity for tryptophan up to a fatty acid:HSA ratio of 5:1, at which point binding was below the limits of detection. Based on statistical analyses of the binding constants and the number of binding sites determined from the data in Fig. 3 (summarized in Table 1), there was no significant difference in tryptophan binding between HSA and glc-HSA glycosylated *in vitro* ($P > 0.05$).

This study was undertaken to investigate for the first time the interrelationships among NEG, fatty acids, and tryptophan binding, and to gain new information on NEG site-specificity in HSA. Other researchers have examined the effects of bound fatty acids on tryptophan binding to HSA [5, 7, 9]; however, this study is the first to examine the effects of NEG in the presence and absence of fatty acids on tryptophan binding. These data enhance our ability to interpret the causes and effects of altered tryptophan metabolism in diabetes, and they further our understanding of the effects of hyperglycemia on HSA drug binding.

The affinity constants for tryptophan binding to defatted HSA which we obtained by rate of dialysis are in very good agreement with results determined by equilibrium dialysis [5–8] and by ultrafiltration [9]. Scatchard analysis of our data consistently indicated less than 1 mol of high-affinity binding sites per mol of HSA at saturation (Fig. 3). Others have obtained less than one binding site for tryptophan [9] and for medium chain fatty acids [27], and this is attributed to the heterogeneity of the protein source and to the microheterogeneity of the albumin molecule [28].

As indicated in Table 1, addition of 1 mol palmitate/mol HSA had little effect on the affinity or the number of available binding sites for tryptophan. These findings are similar to those of Cunningham *et al.* [9], and confirm that the primary binding site for long-chain fatty acids is distinct from the tryptophan binding site [1]. When a molar ratio of palmitate:HSA of 2:1 or greater was attained, a pronounced decrease in the number of available binding sites was observed. Furthermore, no binding was detected when 5 mol palmitate were bound per mol of HSA. Since we did not observe a significant effect of fatty acids on the binding affinity for tryptophan, our results are consistent with a mechanism in which access to the tryptophan binding site becomes progressively blocked when two or more fatty acids are bound to HSA. The loss of binding sites is consistent with the step-wise equilibrium model of fatty acid binding to HSA [29].

In the step-wise equilibrium model, binding constants are not sufficiently different to direct all fatty acid molecules to a single site on HSA. Rather, when fatty acids are present in a 1:1 molar ratio with HSA, they are spread over two or three binding sites of similar affinity. A further prediction of the model is that not all of the HSA molecules in the solution contain one fatty acid. The effect of reduced tryptophan sites at fatty acid:HSA ratios of 2:1 or greater is consistent with a portion of the HSA molecules having bound fatty acids blocking the tryptophan site.

Nonenzymatic glycosylation of HSA was per-

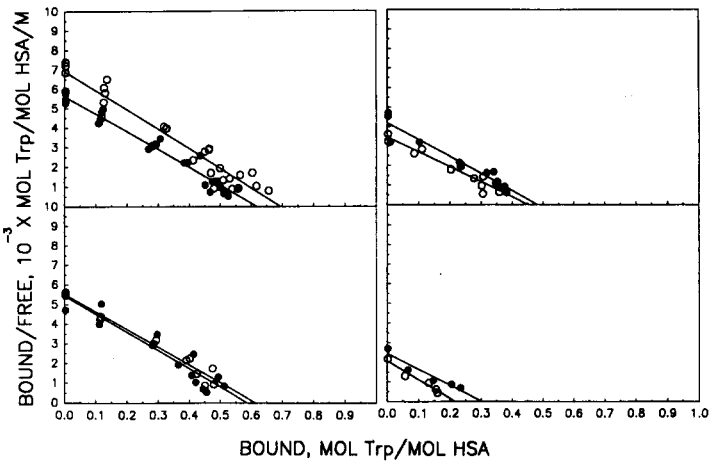

Fig. 3. Scatchard analysis of L-tryptophan binding to HSA. Binding to glycosylated (●) and nonglycosylated (○) HSA was measured at the indicated molar ratios of palmitate:HSA. Top left panel, 0:1; lower left panel, 1:1; upper right panel, 2:1; and lower right panel, 3:1. The experiment was also performed at five fatty acids/HSA with no binding detected. The binding affinity constant, K_a, and the number of binding sites, n, were determined from the slope and y-intercept of the line determined by linear regression.

formed *in vitro* in the presence of variable fatty acid:HSA ratios, and total NEG was decreased only slightly even at fatty acid:HSA ratios of 5:1 (Table 1). These results provide indirect evidence suggesting that Lys-525, the major site of NEG in HSA [11, 12, 30], is not an integral part of the primary long-chain fatty acid binding site. According to the step-wise equilibrium model [29], a reduction in palmitate binding at its primary binding site would result in a greater distribution of palmitate to the tryptophan binding site. However, we observed no effect of NEG on tryptophan binding at any fatty acid:HSA ratio, further suggesting that Lys-525 is not a required residue at the primary and secondary long-chain fatty acid binding sites. While this is in disagreement with Shaklai *et al.* [12] who reported a 20-fold reduction in the binding affinity of glc-HSA for *cis*-parinaric acid, a fluorescent fatty acid analog, our results support the conclusions of Murtiashaw and Winterhalter [31] who found no effect of NEG on palmitate binding.

There was no apparent effect of NEG on tryptophan binding. This suggests that Lys-414, which appears to be required for tryptophan binding [1], is not nonenzymatically glycosylated. Lack of appreciable NEG at Lys-414 is surprising since dansylation studies in HSA [32] and BSA [33] suggest that Lys-414 of HSA should be available for condensation with glucose to form a Schiff base. Ketoamine formation would be expected if catalysis of the Amadori rearrangement were facilitated by a Lys-Lys sequence as has been suggested [11]. With the exception of Lys-525, no other lysine residues in either of the three other Lys-Lys sequences of HSA have been shown to be nonenzymatically glycosylated by peptide mapping studies with radiolabeled sodium borohydride [11] or with radiolabeled glucose [30]; however, not all glycosylated peptides of HSA have been sequenced. By showing no effect of NEG on Trp binding, this study suggests that the Amadori rearrangement is influenced by factors other than location in a Lys-Lys sequence.

Elevated plasma fatty acids [34, 35] and free tryptophan [36] occur in poorly-controlled diabetes, and elevated free tryptophan directly increases brain serotonin levels [36–38]. Increased serotonin has been suggested to be responsible for altered consciousness seen in ketoacidosis, and victims of fatal diabetic coma were found to have elevated brain serotonin levels upon post-mortem examination [36]. Until the present study, the effects of increased NEG in diabetes could not be separated from the effects of elevated fatty acids on tryptophan binding, plasma free tryptophan, and brain serotonin concentration. Our results support a mechanism in which elevated fatty acids and not NEG are responsible for increased free tryptophan as a consequence of fatty acid inhibition of tryptophan binding to HSA. This information should facilitate a better understanding of the mechanisms of altered tryptophan and serotonin metabolism in diabetes.

Altered drug binding to plasma proteins in diabetes is well-established, and both NEG and elevated fatty acids provide mechanisms for decreased binding to HSA [35]. Nonenzymatic glycosylation of HSA inhibits binding of bilirubin [12], phenytoin [14], tolbutamide and glibenclamide [13]; and fatty acids inhibit the binding of thyroxine [1], diazepam and warfarin [39]. Two distinct drug binding sites for different drug classes have been identified on HSA [1, 10]. The oral hypoglycemic drugs tolbutamide and glibenclamide appear to bind at both Site I and Site II [40]. An effect of NEG of HSA on the binding of oral hypoglycemic drugs is important information

since they are used in the treatment of non-insulin-dependent diabetes.

Tsuchiya *et al.* [13] demonstrated an approximately 50% reduction in the binding capacity of nonenzymatically glycosylated HSA for both tolbutamide and glibenclamide using an equilibrium gel filtration assay. However, Kearns *et al.* [41] have reported no effect of NEG on glibenclamide binding in preliminary studies using equilibrium dialysis. The contrasting data obtained in these studies likely reflect differences in experimental approach and design. The gel filtration studies [13] were performed using a glibenclamide:HSA ratio which was more than two orders of magnitude greater than that seen in the treatment of diabetes [42]. Therefore, some care should be exercised in extrapolating the applicability of the results to patients with diabetes mellitus. The studies of Kearns *et al.* [41] utilized a clinically therapeutic range of glibenclamide concentrations [42] such that glibenclamide:HSA ratios were consistent with those seen in the treatment of hyperglycemia. While their studies appear to have clinical relevance, an effect of NEG on glibenclamide binding to HSA at molar ratios approaching 1:1 cannot be excluded.

Although the effects of NEG on sulfonylurea binding to HSA have not been fully elucidated, our results suggest that ligand binding to Site II, the tryptophan/benzodiazepine binding site [1], is not affected by NEG. This is further supported by a report of no effect of NEG on the binding of diazepam [43], a Site II drug which competes with tryptophan for binding to HSA [1]. Site II appears to be resistant to NEG and to the conformational changes in HSA which may occur as a consequence of NEG [12].

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