A Comparison of the Impact of Temperature and Glucose Concentration on Percent Glycated Serum Albumin between Chickens and Humans

by

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ABSTRACT

The glycation of plasma proteins leading to the production of advanced glycation end products (AGEs) and subsequent damage is a driving factor in the pathophysiology of diabetic complications. The overall research objective was to elucidate the mechanisms by which birds prevent protein glycation in the presence of naturally high plasma glucose concentrations. This was accomplished through the specific purpose of examining the impact of temperature and glucose concentration on the percent glycation of chicken serum albumin (CSA) in comparison to human serum albumin (HSA). Purified CSA and HSA solutions prepared at four different glucose concentrations (0 mM, 5.56 mM, 11.11 mM, and 22.22 mM) were incubated at three different temperatures (37.0°C, 39.8°C, and 41.4°C) on separate occasions for seven days with aliquots extracted on days 0, 3, and 7. Samples were analyzed by LC-ESI-MS for percent glycation of albumin. The statistically significant interaction between glucose concentration, temperature, albumin type, and time as determined by four-way repeated measures ANOVA ($p = 0.032$) indicated that all independent variables interacted to affect the mean percent glycation of albumin. As glucose concentration increased, the percent glycation of both HSA and CSA increased over time at all temperatures. In addition, HSA was glycated to a greater extent than CSA at the two higher glucose concentrations examined for all temperature conditions. Temperature differentially affected percent glycation of HSA and CSA wherein glycation increased with rising temperatures for HSA but not CSA. The results of this study suggest an inherent difference between the human and chicken albumin that contributes to the observed differences in glycation. Further research is needed to characterize this inherent difference in an effort to elucidate

the mechanism by which birds protect plasma proteins from glycation. Future related work has the potential to lead to the development of novel therapies to prevent or reverse protein glycation prior to the formation of AGEs in humans, thus preventing the development and devastating effects of numerous diabetic complications.

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CHAPTER 1

INTRODUCTION

Background. According to the most recent estimates, 29.1 million people in the United States, or 9.3% of the population, had diabetes in 2012 (Centers for Disease Control and Prevention, 2014). As the prevalence of diabetes continues to increase both in the U.S. and worldwide, so does the economic impact of this disease (Centers for Disease Control and Prevention, 2013). In the U.S. alone, $245 billion in direct and indirect costs were associated with diabetes in 2012 (American Diabetes Association, 2013). More than 1 in 10 health care dollars in the U.S. are spent directly on diabetes and its complications (American Diabetes Association, 2013). The glycation of plasma proteins that leads to the production of advanced glycation end products (AGEs) and subsequent damage is a driving factor in the pathophysiology of diabetic complications such at retinopathy, nephropathy, and neuropathy (Brownlee, Vlassara, & Cerami, 1984). A clear understanding of the factors that affect the formation and destruction of AGEs is therefore essential for the prevention of these life-threatening complications. It is evident that both economic and health benefits would be seen worldwide from prevention of diabetic complications.

In order to further the understanding of diabetes, various animal species have been used as models of this disease. Avian species have proven a useful model because of something that my laboratory calls the “avian paradox”. Simply put, birds far outlive (by up to three times) mammals of comparable body size despite the fact that they exhibit certain biochemical parameters that would suggest the contrary (Braun & Sweazea, 2008; Holmes, Flückiger, & Austad, 2001; Speakman, 2005). The normal plasma glucose

concentrations of many species of birds are 1.5-2 times those of their mammalian counterparts of equivalent body mass (Braun & Sweazea, 2008). Interestingly, birds are also resistant to the glucose-lowering effects of insulin (Braun & Sweazea, 2008) and to protein glycation (Holmes et al., 2001). In fact, birds do not develop any complications that are associated with diabetes. These characteristics of birds suggest that the production of glycated proteins, not the elevated plasma glucose concentration, is what leads to further damage and promotes pathologies. Understanding the protective mechanisms by which birds avoid protein glycation despite high plasma glucose concentrations is crucial in the overall understanding of the avian paradox and the view of diabetes management in general.

Proposed theories to explain the lower levels of protein glycation in avian species are as follows: increased protein turnover (Jaensch, 2013), structural differences of plasma proteins (inability of glucose to bind to lysine on surface of protein because of physical obstruction or shielding due to differential protein folding), decreased abundance of proteins in the plasma (Bartholomew & Dawson, 1954), and the reactant-favored chemical environment of bird plasma (higher plasma pH and body temperature relative to humans, which would reverse protein glycation) (Baumann & Baumann, 1977).

Preliminary studies done *in vitro* have provided evidence to suggest that higher protein turnover rates as well as lower plasma protein concentration are not the factors responsible for decreased protein glycation in birds (Anthony, Sweazea & Braun, 2010). Specifically, it was shown that *in vitro* bovine albumin was more susceptible to protein

glycation than chicken albumin when matched molecule-for-molecule at species-specific physiologic conditions (Anthony et al., 2010).

Other preliminary work has shown that the percent glycated serum albumin measured using top down mass spectrometry was significantly lower in chicken plasma compared to samples obtained from healthy and type 2 diabetic humans (Borges, Unpublished). This finding supports the conclusion that plasma glucose cannot be used to accurately predict protein glycation in avian species. An intriguing theory, based on the reversible exothermic nature of the protein glycation reaction, predicts that the higher body temperature of birds in comparison to mammals may help to prevent protein glycation (Sweazea and Borges, Unpublished). Despite a potential correlation between the higher body temperatures of birds and protein glycation, no study has specifically looked at the reactant-favored chemical environment of bird plasma as a cause for the resistance to protein glycation in birds. Evidence to support the role of body temperature in the determination of percent protein glycation is needed in order to determine the validity of this proposed mechanism.

Validation of the reactant-favored chemical environment mechanism behind the decreased protein glycation in birds has the potential to lead to the development of novel therapies to prevent or reverse protein glycation prior to the formation of AGEs in humans. Thus, the detrimental effects of AGEs, such as the development of oxidative stress and inflammation that promote pathologies and further complications, could be avoided altogether (Brownlee et al., 1984). Therapies of this sort would be of particular interest to humans with diabetes as well as other hyperglycemic conditions as many of the long-term complications associated with these diseases stem from the detrimental

effects of elevated AGEs due to increased protein glycation caused by prolonged exposure to high plasma glucose concentrations.

Purpose of Study. The overall research objective was to elucidate the mechanisms by which birds prevent protein glycation in the presence of naturally high plasma glucose concentrations. This was accomplished through the specific purpose of this study that is to examine the impact of glucose concentration and temperature on the glycation of chicken serum albumin (CSA) in comparison to human serum albumin (HSA).

Research Aim and Hypotheses. The main research aim was to compare the impact of temperature and glucose concentration on percent glycated albumin of purified CSA and HSA samples over time.

H_0 1: The percent glycation of CSA will not significantly differ from the percent glycation of HSA when exposed to the same temperatures and glucose concentrations over seven days.

H_0 2: Variations in incubation temperatures will not differentially affect the percent glycation of CSA compared to the percent glycation of HSA incubated for seven days at the same glucose concentrations.

H_0 3: Variations in glucose concentrations will not differentially affect the percent glycation of CSA compared to the percent glycation of HSA incubated for seven days when the incubation temperature is constant.

Definition of Terms. The following is a list of terms and the author's definition of each term that are commonly used throughout this thesis.

AGEs- Advanced Glycation End Products; compounds irreversibly formed in the body by the rearrangement and further modification of glycated proteins that are deleterious to many normal physiological processes

CSA- chicken serum albumin

GBM- glomerular basement membrane; the basal lamina layer of the glomeruli of the kidneys, which participates in the filtration function of the glomerulus

GFR- glomerular filtration rate; an estimate of the amount of blood that passes through the glomeruli each minute and is used as a measure of kidney function (a lower GFR indicating poor renal function)

Glycation- the non-enzymatic addition of sugars to proteins and nucleic acids

HSA- human serum albumin

In vitro- studies performed with biological cells or molecules outside of the normal biological context, i.e. in a test tube, flask, or petri dish

Delimitations and Limitations. Only purified, pooled CSA and HSA will be used in this experiment, thus the results may not be directly extrapolated to the whole animal or to other species, as there are inherent physiological differences between species. Limitations of this include the inability to account for variables such as age, nutrition status, and disease state of the chickens and humans from which serum albumin was collected for this study. Because of the inherent physiological differences between

mammals and birds, the findings from this study may not directly influence recommendations to the public, but may be used to guide further research and investigation.

CHAPTER 2

REVIEW OF LITERATURE

Diabetes Mellitus. According to the Centers for Disease Control and Prevention, in 2012 there were 29.1 million people in the United States, or 9.3% of the population, living with diabetes (Centers for Disease Control and Prevention, 2014). Diabetes mellitus is reaching pandemic proportions and is currently the seventh leading cause of death in the United States. As the prevalence of diabetes continues to increase both in the U.S. and worldwide, so does the economic impact of this disease (Centers for Disease Control and Prevention, 2013). In the U.S. alone, $245 billion in direct and indirect costs were associated with diabetes in 2012 (American Diabetes Association, 2013). To put this in perspective, the direct spending alone on diabetes and its complications accounted for over 10% of all direct spending health care dollars in the U.S. (American Diabetes Association, 2013).

The pathophysiology of diabetes is dependent upon the type of diabetes with which an individual is diagnosed. There are two main types of diabetes mellitus, type 1 and type 2. In adults, the most common form is type 2 diabetes (American Diabetes Association, 2014). Type 1 diabetes is much less prevalent in the U.S. population (American Diabetes Association, 2014). In addition, there are individuals that exhibit symptoms of diabetes during pregnancy (gestational diabetes) as well as a small number of individuals that have rare types of diabetes caused by genetic conditions or other environmental factors such as surgery, pancreatic injury/disease, medications, or other diseases (American Diabetes Association, 2014). The major differences between type 1 and type 2 diabetes are outlined in Table 1.

Table 1. *Major Differences between Type 1 and Type 2 Diabetes*

	Type 1	Type 2
Pathophysiology	Autoimmune destruction of pancreatic beta cells resulting in little to no insulin production	Insulin resistance; inability of pancreatic beta cells to produce sufficient insulin; or other cause
Endogenous Insulin	Low or absent	Present (can be increased or decreased)
Onset	Sudden	Gradual
Age at Onset	Usually Childhood or Early Adulthood	Generally later in life (adulthood); can occur in childhood
Nutritional Status at onset	Commonly undernourished	Obesity frequently present
Prevalence in general population	about 5-10% of diagnosed diabetics	about 90-95% of diagnosed diabetics

(American Diabetes Association, 2014)

Diabetes diagnosis is based on the positive results of one of three tests: fasting plasma glucose (FPG) greater than 125 mg/dL, hemoglobin A1C greater than 6.5%, or a oral glucose tolerance test (OGTT) indicating a two hour blood glucose concentration greater than 199 mg/dL as presented in Table 2 (American Diabetes Association, 2014). A positive result for diabetes from any of the three tests must be obtained on a second occasion in order to confirm the diagnosis.

Table 2. *Criteria for classification as normal, prediabetes, or diabetes based on results of A1C test, Fasting Plasma Glucose (FPG) test, or Oral Glucose Tolerance Test (OGTT)*

Test	Normal	Prediabetes	Diabetes
A1C, %	<5.7	5.7-6.4	≥6.5
Fasting Plasma Glucose, mg/dl	<100	100-125	≥126
Oral Glucose Tolerance Test, mg/dl	<140	140-199	≥200

(American Diabetes Association, 2014)

No matter the type of diabetes, the hallmark of the disease is the presence of high blood glucose concentrations caused by the lack of insulin production, the inability of insulin to function properly (known as insulin resistance), or a combination of both of these conditions (American Diabetes Association, 2014). Insulin is a regulatory hormone produced by the beta-cells of the pancreas (Saltiel & Kahn, 2001). One of the primary functions of insulin in the body is the regulation of glucose transport via insulin-mediated glucose uptake into adipose tissue, skeletal muscle, and cardiac muscle where it functions to increase glucose uptake (Saltiel & Kahn, 2001). The mechanism of action of insulin-mediated glucose uptake begins with the binding of insulin to the insulin receptor on the surface of the cell (Huang & Czech, 2007; Saltiel & Kahn, 2001). This triggers a cascade of intracellular signaling that leads to the translocation of the glucose transporter isoform 4 (GLUT4) from intracellular vesicles to the surface of the cell (Shepherd & Kahn, 1999). Once at the surface of the cell membrane, GLUT4 is able to transport glucose into the cell. Acute exercise can also stimulate the translocation of GLUT4 to the cell membrane, albeit, through a different signaling pathway than insulin (Shepherd & Kahn, 1999; Huang & Czech, 2007; Saltiel & Kahn, 2001). GLUT4 is a key determinant of glucose homeostasis, thus decreased expression or translocation of GLUT4 will have a significant affect on blood glucose (Huang & Czech, 2007). The absence of insulin production makes the insulin-mediated translocation of GLUT4 impossible, resulting in severe imbalance in glucose homeostasis (i.e. type 1 diabetes). Insulin resistance is the diminished ability of insulin to trigger the translocation of GLUT4 to the cell membrane surface (Reaven, 1995). Insulin resistance disrupts glucose homeostasis resulting in improperly regulated blood glucose concentrations and inefficient nutrient utilization

(Reaven, 1995; Shepherd & Kahn, 1999). The exact cause of insulin resistance is currently being studied. Research indicates that excess weight and physical inactivity are associated with insulin resistance (Reaven, 1995). Also, there are studies that show fat can directly induce insulin resistance by inhibiting the insulin signaling pathway intermediates (Turinsky, O'Sullivan, & Bayly, 1990).

Insulin also serves an important role in the facilitation of metabolic processes in the liver. For example, insulin acts through intracellular signaling within the liver to stimulate glycogen synthesis and cell growth/division, inhibit gluconeogenesis and ketone body production as well as modify lipoprotein metabolism and protein synthesis (Denton & Tavare, 1997). Insulin deficiency consistently opposes these metabolic processes; however, insulin resistance differentially affects these metabolic processes (Wilcox, 2005). Specifically, insulin resistance in the liver further promotes hyperglycemia via increased gluconeogenesis and alters lipoprotein metabolism as in insulin deficiency; however, hyperinsulinaemia present in insulin resistance also further promotes the mitogenic effects of insulin (Denton & Tavare, 1997; Wilcox, 2005).

Of those currently living with diabetes in the U.S., nearly 28% are undiagnosed (Centers for Disease Control and Prevention, 2014). These individuals are not receiving the medical attention necessary to properly treat and manage their disease in order to prevent further complications. When properly managed, individuals with diabetes can live a long, healthy life after diagnosis devoid of the acute complications of the disease. Nonetheless, even patients with very well-controlled diabetes are likely to develop chronic diabetic complications.

Diabetic Complications. Many of the complications associated with diabetes pose a serious risk to health (Zhou et al., 2005). Complications are both acute as well as chronic and include conditions such as diabetic ketoacidosis (DKA), hyperosmolar hyperglycemic nonketotic syndrome (HHNS), gastroparesis, hypoglycemia, hyperglycemia, skin complications, retinopathy, neuropathy, nephropathy, hypertension, stroke, heart disease, and mental health problems (American Diabetes Association, 2014; Pirart, 1977). Research has shown that hyperglycemia as a result of insulin resistance or insulin insufficiency is the driving factor responsible for the development of many of these chronic diabetic complications (Pirart, 1977). Using intensive diabetes management to control blood glucose levels has been shown to delay the onset of diabetic complications; however, it is associated with an increased risk for hypoglycemia (Fullerton et al., 2014). The aim of intensive control of blood glucose is to keep blood glucose levels within a normal range for the majority of the time throughout the day. Generally, this results in a lower average glucose concentration in the blood over time. Less glucose available to interact with proteins in the blood means that there is less opportunity for protein glycation to occur, which results in decreased production of advanced glycation end products (AGEs) (Khan, Rasheed, Khan, & Ali, 2007). A high level of plasma protein glycation and subsequent production of AGEs due to prolonged exposure to high blood glucose levels in individuals with diabetes is one of the primary mechanisms responsible for the development of many diabetic complications (Brownlee et al., 1984; Khan et al., 2007).

Non-Enzymatic Protein Glycation and AGEs. Protein glycation is the non-enzymatic addition of a reducing sugar (i.e. glucose) to an amino acid residue (usually lysine) of a protein resulting in the formation of a Schiff base. The formation of a Schiff base is highly reversible, thus the reaction can be driven backwards (Brownlee et al., 1984; Ulrich & Cerami, 2001). Numerous plasma proteins are subject to protein glycation. Albumin, hemoglobin, and collagen are just a few examples of these plasma proteins with important physiologic functions that are subject to protein glycation in mammals (see Table 3 for additional examples). The Schiff base that forms during the

Table 3. *Partial List of Human Plasma Proteins with Important Physiologic Functions that are Subject to Protein Glycation*

Protein
Albumin
Antithrombin III
Beta-NAc-D-glucosaminidase
Cathepsin B
Collagen
Coronary artery proteins
Endothelial cell membrane
Ferritin
Fibrin
Fibrinogen
Glomerular basement membrane
Hemoglobin
High density lipoproteins
Lens capsule
Lens crystallins
Low density lipoproteins
Myelin
Pancreatic RNase
Red cell membrane
Tubulin

Table 3 adapted from Brownlee et al., (1984).

initial glycation reaction can undergo further rearrangement to form an Amadori Product, a stable sugar-protein adduct (Brownlee et al., 1984; Ulrich et al, 2001). This rearrangement is also chemically reversible. The accumulation of Amadori products from proteins with a slow turnover rate leads to the development of post-Amadori non-enzymatic glycation products through additional rearrangements and dehydration reactions, which are known as advanced glycation end products (AGEs) (Brownlee et al., 1984; Ulrich et al, 2001). While the rate of formation of AGEs is much slower than that of Schiff bases or Amadori products, the formation of AGEs is irreversible thus these products continue to accumulate once formed (Brownlee et al., 1984). A simplified diagram of the formation of AGEs is illustrated in Figure 1.

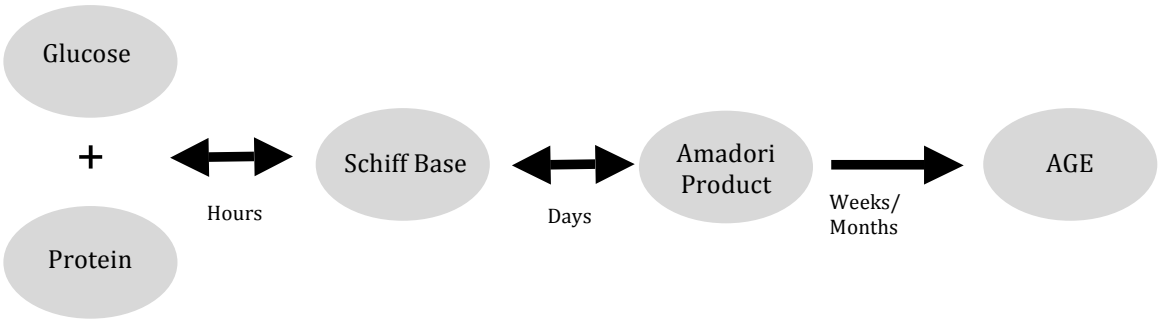


Figure 1. The Formation of Advanced Glycation End Products (AGEs). Adapted from (Tarr, Kaul, Chopra, Kohner, & Chibber, 2013).

The glycation of plasma proteins has many physiological effects. This structural change to the protein can alter its function and thus alter any processes in which the protein is involved (Khan et al., 2007). For example, protein glycation can alter enzymatic activity, binding of regulatory molecules, cross-linking of proteins,

susceptibility to proteolysis, function of nucleic acids, macromolecular recognition and endocytosis, and immunogenicity (Khan et al., 2007). The inability of the glycated protein to perform its physiological function and the new ability of the glycated protein to cause damage to surrounding tissues leads to the development of many diabetic complications (Singh, Bali, Singh & Jaggi, 2014).

Albumin as a Measure of Overall Protein Glycation. All plasma proteins in the body are susceptible to protein glycation. The extent to which different plasma proteins are glycated varies significantly from one protein to another as well as between euglycemic and hyperglycemic states (Austin, Mullins, & Morin, 1987). The differences in the extent of glycation between proteins can mostly be attributed to variations in half-life and glycation susceptibility of the different proteins (Austin et al., 1987). Albumin is the most abundant plasma protein. It is freely circulating, has a relatively long half-life, and has numerous physiological functions in addition to serving as an antioxidant (Kim & Lee, 2012; Singh et al., 2014). Compared to other plasma proteins, albumin exhibits a relatively high proportion of glycation when exposed to the same glycemic conditions for the same length of time (Austin et al., 1987). This characteristic justifies the use of albumin as a marker of overall protein glycation as it will accurately reflect the maximum proportion of overall glycation in a relatively short amount of time. In addition, previous work has shown that the susceptibility of albumin to glycation does not greatly differ across mammalian species as it does for other plasma proteins (i.e. hemoglobin) (Rendell et al., 1985).

Clinically, the measurement of glycated albumin has been used to assess blood glucose control. The use of glycated albumin in place of hemoglobin A1C as a measure

of protein glycation in order to assess blood glucose control has grown in clinical utility (Kim & Lee, 2012). Albumin has a half-life of 12 to 21 days (Rondeau & Bourdon, 2011). This is significantly shorter than the half-life of hemoglobin, which is the plasma protein currently considered the 'gold standard' for assessing protein glycation (Kim & Lee, 2012). Studies have found a strong correlation between the glycation of albumin over a 3-week period and the glycation of hemoglobin over a 3-month period (Kim & Lee, 2012). Therefore, the use of albumin as a marker of protein glycation allows an assessment of blood glucose control over a shorter-period of time (Rondeau & Bourdon, 2011; Kim & Lee, 2012).

Quantification Methods for Determination of Protein Glycation. Methods to accurately quantify the extent of protein glycation within a specific sample are critical when using this measure in research as well as in the clinical setting. The increasing evidence and knowledge regarding the influence that glycated proteins have on biological processes has driven the development of techniques to detect protein glycation with greater sensitivity and higher throughput (Lee et al., 2011; Lapolla, Molin, & Traldi, 2013). Numerous methods to determine the degree of protein glycation present in a particular sample have been described and previously utilized.

The two main approaches to measuring protein glycation are the binding methods and the chemical methods (Schleicher & Wieland, 1989). The binding methods involve the binding of a reagent to the glycated protein. Binding methods are equilibrium reactions and do not chemically alter the glycated protein. Thus, the glycated protein will remain unchanged if the reagent is removed (Schleicher & Wieland, 1989). Chemical methods, on the other hand, result in modification of the glycated protein. This

modification is dependent upon the specific chemical methods used, however, it usually involves hydrolysis or oxidation of the glycated protein (Schleicher & Wieland, 1989).

The exact method of analysis used is determined by the level of detail required in the analysis. Simply detecting the presence of protein glycation is the first step in any analysis and is generally accomplished by either glycan staining/labeling or affinity-based methods (Roth, 2012). The most common staining procedures utilize sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate the proteins based on charge and then stain the gel for glycated proteins (Roth, 2012). The gel is stained using the periodic acid-Schiff (PAS) reaction that results in a magenta color that can be quantified when the aldehyde is formed (Roth, 2012). Affinity-based methods can be enzyme-based or antibody-based and tend to be more specific than staining by allowing the determination of the type of glycation present (Roth, 2012). Once protein glycation has been detected, the exact structure of the glycan, type of glycation and the extent of glycation can be analyzed using more sophisticated techniques (Roth, 2012). Chromatography and mass spectrometry are commonly used for this type of protein glycation analysis. The analysis can be conducted on the glycated protein or after glycoprotein purification (Roth, 2012). Recently, the combined use of liquid chromatography (LC) and mass spectrometry (MS), known as LC-MS has been utilized in the analysis of protein glycation (Borges et al., 2011). The development of this technique has allowed for higher throughput and greater sensitivity in analyses (Wuhrer, Deelder, & Hokke, 2005). In addition, this method has the ability to pinpoint and quantify individual albumin molecules with two (i.e. doubly-glycated), rather than just one, glucose molecules attached. This level of precision in measurement allows for a more

thorough and detailed examination of the extent of protein glycation in a sample that is not available using other methods (Wuhrer et al., 2005).

Overall, there are numerous methods available for measuring and quantifying protein glycation. The technique chosen should be selected based upon the level of analysis required. The level of analysis required will vary based upon the use of data. Research purposes generally require much more detailed analyses in order to properly answer the research question, thus more sophisticated techniques such as LC-MS should be considered. Clinical purposes typically are more time-sensitive and only require the identification of the level of glycation present not a complete structure analysis, thus assays and staining methods are preferable.

The Role of AGEs in the Development of Diabetic Complications. The mechanism by which AGEs promote the development of diabetic complications is unique to each complication. Generally, AGEs cause damage throughout the body by interacting with specific receptors located on the membrane of numerous cells called receptors for advanced glycation end products (RAGEs) (Ramasamy, Yan, & Schmidt, 2011; Szwergold & Miller, 2014; Singh, 2014). When the activation of RAGEs is exacerbated, there is increased deposition of AGEs in tissues and the oxidative stress response that is triggered is amplified (Ramasamy et al., 2011; Szwergold & Miller, 2014; Singh 2014). The specific role of AGEs has been classified for the development of the following microvascular and macrovascular diabetic complications: neuropathy, nephropathy, retinopathy, and macrovascular disease.

Neuropathy in diabetic patients is usually in the form of peripheral neuropathy and presents as numbness or paralysis of the extremities (Brownlee & Cerami, 1981).

Diabetic peripheral neuropathy is the leading cause of non-emergency amputations in the U.S. There is evidence to support the role of AGEs in the development of peripheral neuropathy (Sugimoto, Yasujima, & Yagihashi, 2008). Several structural proteins of nerve, vessel, and extracellular matrix cells of peripheral nerves that have been modified by AGEs have been identified in humans (Wada & Yagihashi, 2005). This interaction of AGEs with structural proteins leaves the peripheral nerve cells susceptible to degeneration (Sugimoto et al., 2008). Specifically, these interactions take many forms depending on the protein involved. For example, segmental demyelination of peripheral nerves can be caused by increased phagocytosis of AGE-modified peripheral nerve myelin; AGE interaction with tubulin, neurofilament, and actin of the axon leads to axonal atrophy and impaired axonal transport; and modification of laminin by AGEs impairs cell regeneration activity (Sugimoto et al., 2008).

Another diabetic complication in which the AGE mechanism is proposed to contribute is nephropathy. This type of renal disease usually seen in long-term diabetic patients is characterized by proteinuria and decreased glomerular filtration rate (GFR). Increased presence of glycated proteins has been linked to the thickening of the glomerular basement membrane (GBM), which is positively correlated with severity of symptoms (Brownlee & Cerami, 1981; McVerry, Fisher, Hopp, & Huehns, 1980). It is the progressive thickening of the GBM that alters the filtering ability of the glomerulus, causes capillary occlusion, and eventually leads to renal failure (Brownlee & Cerami, 1981). Thus, glycated proteins have been implicated as a driving factor in the development of nephropathy in individuals with diabetes.

The increased production of AGEs in individuals with diabetes increases their likelihood to develop retinopathy. Diabetic retinopathy is the leading cause of blindness in adults aged 20 to 74 years (Tarr et al., 2013). It has been shown in experimental trials that increased accumulation of AGEs was accompanied by loss of pericytes, formation of microaneurysms, and formation of acellular capillaries that contribute to retinal damage (Hammes, Martin, Federlin, Geisen, & Brownlee, 1991; Stitt et al., 2002). In addition, research has found a direct correlation with the quantity of AGEs measured in patients and the presence/severity of diabetic retinopathy (McCance et al., 1993).

Diabetes has been shown to be an independent risk factor for certain macrovascular complications including atherosclerosis, cardiovascular disease, cerebrovascular disease, and peripheral artery disease (Cade, 2008). Higher AGE levels in diabetic patients have been correlated with adverse atherosclerotic lipid profiles suggesting a possible role for AGEs in the development of these lesions/plaques and the use of AGE levels as an indicator of atherosclerosis risk (Chang, Chu, Syu, Hsieh, & Hung, 2011). The development of macrovascular complications in individuals is a multifactorial process. Studies have shown that one factor in this process is the ability of AGEs to quench nitric oxide (NO) as well as inhibit NO production leading to an impaired vasodilatory response (Cade, 2008; Linden et al., 2008). In addition, AGEs promote the accelerated development of atherosclerosis through collagen cross-linking, increased susceptibility of LDL to oxidation, and several other identified mechanisms (Basta, Schmidt, & De Caterina, 2004).

The glycation of plasma proteins that leads to the production of AGEs and subsequent damage is a driving factor in the pathophysiology of the diabetic

complications of retinopathy, nephropathy, neuropathy and macrovascular disease (Brownlee et al., 1984). A clear understanding of the factors that affect the formation and destruction of AGEs is essential for the prevention of these life-threatening complications. It is evident that both economic and health benefits would be seen worldwide from prevention of diabetic complications.

Current Therapies to Reduce AGEs. In the clinical setting, current therapies for treating patients with high levels of protein glycation typically focus on lowering blood glucose levels to provide less substrate for glycation or focus on reducing the side effects of glycated proteins as they promote inflammation and oxidative stress (Fullerton et al., 2014). Strictly monitoring blood glucose levels is an effective method to decrease protein glycation thus reducing AGE formation; however, it requires strict adherence and a high level of cooperation from the patient. In addition, it significantly increases the risk of hypoglycemic events (Fullerton et al., 2014). Therapies that focus on reducing the side effects of glycated proteins and AGEs can effectively delay the onset of complications and treat the symptoms, yet they do not address the underlying problem. There is a clear gap in our understanding of protein glycation that leads to the inadequacy of our current therapies. A better understanding of what makes proteins susceptible to glycation has the potential to lead to alternative therapies for decreasing the rate of protein glycation.

Birds as a Model of Type 2 Diabetes. In order to further understand the pathogenesis of diabetic complications, various animal species have been used as models of this disease. Traditional animal models, such as rats and mice, have little promise in the further study of diabetic complications (Szwergold & Miller, 2014). These animals typically do not live long enough to develop the complications seen in humans and the

inherent differences in the biochemistry and physiology of the species make any findings difficult to extrapolate to humans (Szwergold & Miller, 2014). Recently, interest has been placed on the use of birds as a model of type 2 diabetes for several reasons (Braun & Sweazea, 2008; Iqbal, Probert, Alhumadi, & Klandorf, 1999; Klandorf, Rathore, Iqbal, Shi, & Van Dyke, 2001; Szwergold & Miller, 2014). Despite having 1.5-2 times the blood glucose concentrations as a mammal of comparable body mass (Braun & Sweazea, 2008), birds do not exhibit symptoms of diabetes nor develop diabetic complications, which counterintuitively makes them a useful model to study. The interspecies differences between humans and other animal models (rats, mice, etc.) hinder investigation by complicating extrapolation of findings; however, the interspecies differences are in fact an asset in the bird model. The goal of research using bird models is to obtain understanding of these differences in order to apply them to humans.

Currently, only a handful of researchers in the United States use birds as a pathology-free model of type 2 diabetes (Braun & Sweazea, 2008; Iqbal, Probert, Alhumadi, & Klandorf, 1999; Klandorf, Rathore, Iqbal, Shi, & Van Dyke, 2001; Szwergold & Miller, 2014). This research has led to the discovery of several physiological differences between humans and birds that aid in the understanding of avian blood glucose regulation. Of particular interest is the lack of evidence to support the presence of the gene encoding for RAGEs in birds and therefore the presence of RAGEs altogether (As reviewed in: Szwergold & Miller, 2014). The lack of RAGEs in birds could explain why birds with high blood glucose concentrations do not develop the same complications that humans would develop at comparable blood glucose concentrations. Nonetheless, the lack of the RAGE gene and RAGE proteins in birds

does not adequately explain the mechanism by which birds' plasma proteins are protected from protein glycation and have a relatively lower production of AGEs in comparison to humans.

Another reason that avian species have proven a useful model of pathology-free type 2 diabetes is because of something the laboratory of the author's mentor has termed the "avian paradox". Simply put, birds far outlive (by up to three times) mammals of comparable body size despite the fact that they exhibit certain biochemical parameters that would suggest the contrary (Braun & Sweazea, 2008; Holmes et al., 2001; Speakman, 2005). In particular, the higher metabolic rates, higher body temperatures, and higher blood glucose levels that are observed in many avian species should predispose them to more rapid aging according to the current biochemical model of aging in mammals (Speakman, 2005). This is due to the increased production of harmful by-products, such as reactive oxygen species (ROS) and AGEs, caused by these biochemical conditions; however, this is not the case for birds (Holmes et al., 2001). As previously mentioned, the normal plasma glucose concentrations of many species of birds are 1.5-2 times those of their mammalian counterparts of equivalent body mass (Braun & Sweazea, 2008). Fasting plasma glucose concentration in humans is normally 80-100 mg/dL. Humans will begin to experience symptoms of diabetes when fasting plasma glucose concentrations are around 126 mg/dL for an extended period of time. For birds, plasma glucose concentrations are normally at a level that would be considered hyperglycemic for humans (Baumann & Baumann, 1977; Beuchat & Chong, 1998; Braun & Sweazea, 2008). This relationship between plasma glucose concentration and body mass is depicted

in Figure 2 and illustrates the comparatively higher plasma glucose concentrations of birds (Baumann & Baumann, 1977; Beuchat & Chong, 1998; Braun & Sweazea, 2008).

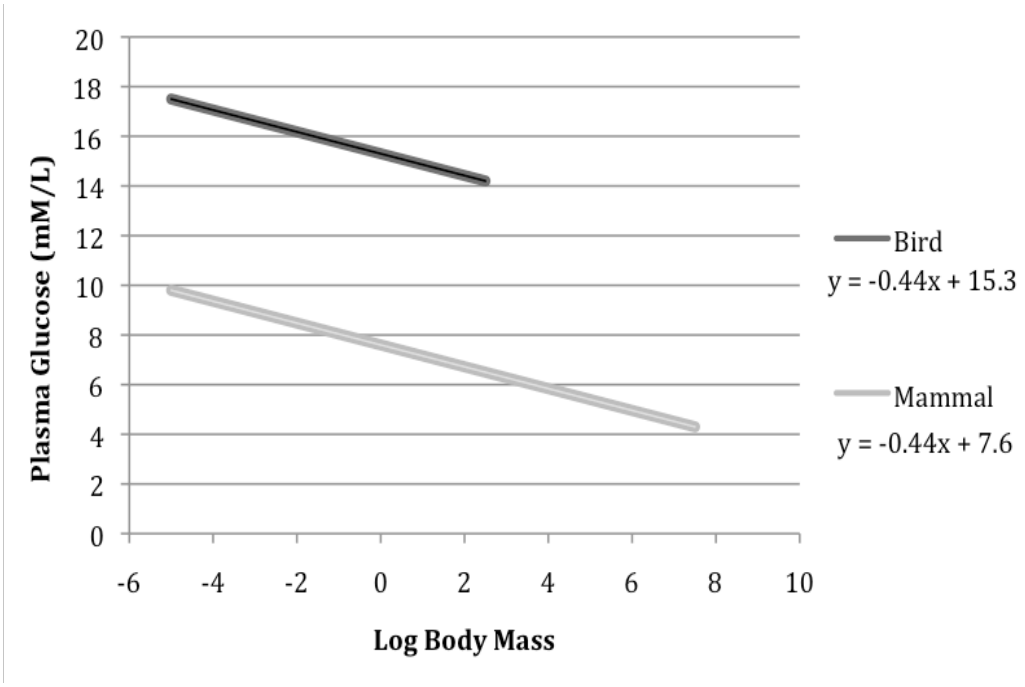


Figure 2. The relationship between plasma glucose concentrations and log body mass (LBM in kg) for birds (n=97) and mammals (n=162). Data were extracted from the International Species Information System (ISIS), Physiological Data Reference Values 2002. Adapted from Braun & Sweazea, 2008.

Interestingly, birds are also resistant to the glucose-lowering effects of insulin (Braun & Sweazea, 2008; Sweazea & Braun 2005; Sweazea, McMurtry & Braun 2006). The GLUT4 transport protein responsible for insulin-mediated uptake of glucose into cells is not present in the skeletal muscle cells of several avian species that have been examined. This lack of GLUT4 transporters in the muscle cells causes a different insulin response in avian species compared to humans. In fact, a very high dose of insulin is required to cause a significant drop in blood glucose concentration in these avian species

(Carver, 2001; Seki, 2003; Sweazea, 2005; Sweazea, 2006a; Sweazea, 2006b). Also, protein glycation has been shown to occur at a significantly lesser degree in several avian species despite higher blood glucose concentrations (Review: Holmes et al., 2001). A study examining hemoglobin glycation found these plasma proteins to be in the range of 0.5-1.0% glycation in three avian species (duck, chicken, and turkey), while mammals fell in the range of 1.7-5.8% glycation (Beuchat, 1998). Hummingbirds, which are known to have among the highest fasting plasma glucose concentrations of all measured vertebrates (roughly 300-756mg/dl in the fasted and fed states, respectively), were found to have glycated hemoglobin levels in the range of 3.7-4.6% glycation. While higher than other avian species, the percent glycation is still significantly lower than observed in diabetic humans (Beuchat & Chong, 1998). Additionally, a lower degree of plasma albumin glycation was observed in budgerigars (parakeets) in comparison to human plasma albumin (Holmes et al., 2001).

The observed protection from protein glycation seen in birds suggests a possible mechanism by which birds are able to maintain high plasma glucose concentrations without detrimental effects that would result in humans at comparable levels. This characteristic of birds also suggests that it is the production of glycated proteins, not the elevated plasma glucose concentration, which leads to further damage and promotes pathologies. While these studies clearly present the disparity in the level of protein glycation, they do not address specifically how birds are able to protect circulating proteins from glycation at high plasma glucose concentrations. Understanding the protective mechanisms by which birds avoid protein glycation despite high plasma

glucose concentrations is crucial in the overall understanding of the avian paradox and the progress towards improved diabetes management in general.

Proposed Mechanisms for Low Protein Glycation in Birds. Proposed theories to explain the lower levels of protein glycation in avian species are as follows: increased protein turnover (Jaensch, 2013), structural differences of plasma proteins (inability of glucose to bind to lysine on surface of protein because of physical obstruction/shield due to differential protein folding- Sweazea unpublished), decreased abundance of proteins in the plasma (Bartholomew & Dawson, 1954), and the reactant-favored chemical environment of bird plasma (higher plasma pH and body temperature relative to humans) (Baumann & Baumann, 1977).

Preliminary studies done *in vitro* have provided evidence to suggest that higher protein turnover rates as well as lower plasma protein concentration are not the factors responsible for decreased protein glycation in birds (Anthony et al., 2010). Specifically, it was shown that *in vitro* bovine albumin was more susceptible to protein glycation than chicken albumin when matched molecule-for-molecule at species-specific physiologic conditions (Anthony et al., 2010). The study was conducted *in* v*itro*, negating the theory of increased protein turnover as the driving mechanism, and albumin molecules were matched molecule-for-molecule, negating the theory of decreased abundance of proteins in the plasma.

Other preliminary work has shown that the percentage of glycated serum albumin measured using top down mass spectrometry was significantly lower in chicken plasma compared to samples obtained from healthy and type 2 diabetic humans (Sweazea and Borges, unpublished). This finding supports the conclusion that plasma glucose cannot be

used to accurately predict protein glycation across species. Even within avian species, plasma glucose cannot accurately be used to predict protein glycation. Based on the exothermic nature of protein glycation, it is reasonable to predict that the higher body temperatures of birds may be a factor that protects them from protein glycation (Sweazea and Borges, unpublished). Despite this suggested correlation, no study has specifically looked at the reactant-favored chemical environment of bird plasma as the cause for the lower level of protein glycation in birds. Further evidence to support the role of temperature in the determination of percent protein glycation is needed in order to determine the validity of this proposed mechanism.

Validation of the reactant-favored chemical environment mechanism behind the decreased protein glycation in birds has the potential to lead to the development of therapies to prevent or reverse protein glycation prior to the formation of AGEs in humans. Thus, the detrimental effects of AGEs, such as the development of oxidative stress and inflammation that promote pathologies and further complications, could be avoided altogether (Brownlee et al., 1984). Therapies of this sort would be of particular interest to humans with diabetes as well as other hyperglycemic conditions as many of the long-term complications associated with these diseases stem from the detrimental effects of elevated AGEs due to increased protein glycation caused by prolonged exposure to high plasma glucose concentrations.

Role of Temperature in Chemical Reactions. The chemical environment in which a reaction is taking place significantly impacts the rate at which the chemical reaction proceeds. Regulation of biochemical reactions is complex and is impacted by many factors. One factor influencing the chemical environment is temperature. In general

chemistry, as the temperature of the chemical environment is increased the rate of the reaction will increase due to the collision theory. The reverse, namely a decrease in temperature results in a decrease in the rate of the reaction, is also generally true. This relationship is not as simple in biochemical reactions. In physiological conditions, enzymes catalyze the majority of biochemical reactions. High temperatures (i.e during a fever) can cause enzymes to denature, thus destroying their ability to function catalytically. Thus, in biochemical conditions an ideal temperature is strictly regulated and maintained to ensure that all biological reactions occur at the desired rate. In physiological conditions, the body temperature is considered the temperature of the chemical environment.

Human and Avian Body Temperature. Humans and birds both tightly regulate internal body temperature through thermoregulation processes. Most animals are not capable of thermoregulation, thus birds and mammals are among the minority of animals capable of effectively controlling internal body temperature (Whittow, 1999). While the thermoregulatory processes serve a similar purpose among birds and mammals, there are differences in the specific mechanisms and behaviors utilized to achieve the temperature stabilization (Whittow, 1999).

Normal human body temperature is considered to be around 37.0°C (Sherwood, 2008). Evidence that the normal body temperature for healthy humans is actually a range of temperatures from 35.5°C to 37.7°C suggests that temperatures of healthy humans can vary from one individual to another (Sherwood, 2008). There are several factors that influence body temperature including age, gender, genetics, disease/infection status, and activity and temperature is known to fluctuate slightly throughout the day (Sherwood,

2008). In addition, some foods and food components such as vitamin C, spices, teas, and caffeine have been shown to elicit changes in metabolism that impact thermogenesis (Johnston, 1990; Johnston, 1989; Westerterp-Plantenga, Diepvens, Joosen, Berube-Parent, & Tremblay, 2006).

Birds have normal body temperatures in the range of 35°C to 43°C dependent upon the species (see Table 4) (Whittow, 1999). Typically, normal body temperatures of birds are around 39-42°C (Whittow, 1999). Therefore, on average, birds have higher normal body temperatures than mammals, such as humans. In addition to having higher normal body temperatures than humans, the body temperature of some avian species can experience greater daily fluctuations than the average human. The body temperature of some smaller avian species (i.e. hummingbirds) can vary by up to 8°C in a single day, while larger birds typically do not fluctuate more than 1°C in a single day (Whittow, 1999). This difference in the amplitude of the normal circadian rhythm of body temperature is likely due to the fact that, as previously mentioned, thermoregulation of

Table 4. *Body Temperatures of Selected Birds at Rest*

Species	Body Temperature (°C)
Domestic turkey (*Meleagris gallapavo)*	41.2
Domestic chicken (*Gallus gallus)*	41.5
Great horned owl (*Bubo virginianus)*	39.9
Rock pigeon (*Comlumbia livia)*	42.2
California quail (*Callipepla californica)*	41.3
Domestic duck (*Anas platyrhnchos*)	42.1
Brown-necked raven (*Corvus corax ruficollis*)	39.9
Tengmalm's owl (*Aegolius funereus*)	39.4
American kestrel (*Falco sparverius*)	39.3
Zebra finch (*Poephila guttata*)	40.3

(Whittow, 1999)

avian species is controlled by different mechanisms than is human thermoregulation (Whittow, 1999).

Body Temperature and Protein Glycation. No previously published studies have specifically examined the *in vivo* relationship between temperature and glucose concentration and protein glycation for either birds or humans. In regards to the effect of temperature on *in vitro* protein glycation, one published study examining this relationship in humans was identified. Urbanowski et al. published results in 1982 from a study examining the non-enzymatic glycosylation of human serum albumin (Urbanowski, Cohenford, & Dain, 1982). For the temperature specific experiments, purified human serum albumin was incubated *in vitro* for 24 hours at various temperatures (5°C, 25°C, 35°C, 45°C, and 55°C) with a constant concentration of D-galactose or D-glucose (Urbanowski et al., 1982). The results showed that as the incubation temperature increased an increase in protein glycation was observed. This increase in protein glycation at higher temperatures was observed for both human serum albumin samples incubated with D-galactose as well as D-glucose (Urbanowski et al., 1982).

The results of this study suggest that increased body temperature will increase protein glycation. Preliminary data from several avian species as presented above suggests that the opposite relationship may be present in avian species as they have higher temperatures than humans along with lower percent glycation, thus there is a discrepancy in the literature in regards to this topic. The study conducted by Urbanowski et al. (1982) was only conducted using human serum albumin, thus these finding cannot be extrapolated to serum albumin from avian species. Additional studies comparing both human serum albumin and avian serum albumin are needed to confirm that the

relationship between temperature and human serum albumin is consistent in avian serum albumin as well. Also, the samples in the Urbanowski et al. (1982) study were only incubated for 24 hours prior to analysis for protein glycation. This incubation period was constant for all samples; however, a longer incubation period may elucidate time-dependent effects of temperature on protein glycation. In addition, the temperatures at which the samples were incubated did not simulate physiological conditions or avian conditions. The incubation temperatures of 35°C and 45°C were the closest to human and avian physiological temperatures, however, these do not adequately reflect the physiological conditions of either humans or most avian species. An analysis of protein glycation at various temperatures within a physiological range in which the albumin protein is functional is necessary to confirm this observed relationship. Finally, these results were obtained *in vitro*. The effect of the physiological environment must be addressed in future research as it could have a significant impact on the rate of protein glycation.

As previously mentioned, some foods and food components such as spices, teas, and caffeine have been shown to elicit changes in metabolism and impact thermogenesis (Westerterp-Plantenga et al., 2006). The resultant increase in body temperature observed in humans after consumption of these foods is short-lived and thermoregulation processes will act to lower body temperature back to the basal level (Westerterp-Plantenga et al., 2006). Supplementation of vitamin C has also been shown to significantly raise body temperature (C. C. S. Johnston, 1990; C. S. Johnston, 1989). The fact that there are identified ways to safely and significantly affect human body temperature suggests that there could be the potential for the development of novel therapies to mildly alter body

temperature in humans to limit postprandial protein glycation if such a relationship is confirmed. In fact, one study has found a decrease in protein glycation among humans that took an oral vitamin C supplement (Davie, Gould, & Yudkin, 1992). In this study, non-diabetic adults consumed an oral dose of 1000mg ascorbic acid daily for three months (Davie et al., 1992). Glycosylated hemoglobin and glycosylated albumin were both measured in all participants using affinity chromatography. Both glycosylated hemoglobin and glycosylated albumin were significantly decreased after three months of ascorbic acid supplementation, from 6.18 ± 0.48% (mean ± SD) to 5.05 ± 0.50% for glycosylated hemoglobin and from 1.56 ± 0.24% (mean ± SD) to 1.04 ± 1.01% for glycosylated albumin (Davie et al., 1992). There was no control group in this study for comparison; this flaw in the study design as well as other limitations of the study (i.e. the inclusions of only healthy, non-diabetic adults) limits the impact of the results. Nonetheless, the findings are compelling and support a potential link between body temperature and protein glycation. No such study has been conducted in avian species. Interestingly, several species of birds are able to synthesize ascorbic acid endogenously and are not reliant upon dietary sources of this compound (Chaudhuri & Chatterjee, 1969). Future studies need to investigate whether this observed relationship between protein glycation and vitamin C supplementation was indeed mediated through an increase in body temperature or if there was another mechanism responsible.

Overall, there has been very little investigation into the effect of physiological temperature change on protein glycation in either humans or avian species. This relationship merits further investigation due to the fact that there is biological plausibility to the proposed theory that increased body temperature in avian species is the primary

mechanism to explain the decreased protein glycation despite significantly increased plasma glucose concentration. Specific, focused studies looking directly at protein glycation of human plasma proteins at various physiologic temperatures compared to avian plasma proteins are needed to elucidate the exact nature of this relationship. No matter the specific results of such studies, the findings will contribute to our understanding of avian glucose regulation and transform the understanding of protein glycation by elucidating the contributions made by plasma glucose concentration and body temperature.

Human Relevance of Unraveling the 'Avian Paradox'. Unraveling the 'avian paradox' will contribute to the understanding of avian glucose regulation and aid in the understanding of the influence of plasma glucose concentration and body temperature on protein glycation in birds as well as humans. Traditional therapies to deal with protein glycation in humans have focused on the management of side effects or on lowering blood glucose levels. Obtaining a full understanding of the 'avian paradox' would offer an alternative approach by studying what makes proteins susceptible to glycation in the first place. Research in this area has the potential to lead to the development of therapies to prevent or reverse protein glycation prior to the formation of irreversible AGEs and thus avoid their detrimental effects. Therapies of this sort would be of particular interest to diabetic humans, as many complications associated with this disease stem from increased protein glycation due to prolonged exposure to high blood glucose concentrations (Brownlee, 1984). Specifically, investigation into the role of temperature and glucose concentration on protein glycation in avian species as compared to humans will identify if interspecies differences exist.

CHAPTER 3

MATERIALS AND METHODS

Materials. Purified chicken serum albumin (Equitech-Bio, Inc., Kerrville, TX; Cat No. CSA62 Lot #CSA62-1254) and human serum albumin (Sigma Aldrich, St. Louis, MO; Cat No. A3782 Lot #SLBD7204V) were purchased from commercially available sources to be used in the *in vitro* manipulations. Chicken serum albumin (CSA) has a reported purity ≥ 96% and human serum albumin has >99.9% purity. HEPES buffered saline solution was prepared in batches of 250 mL by combining 1.963g NaCl (Cat. No. S9888, Sigma-Aldrich, St. Louis, MO), 0.112g KCl (Cat. No. P4504, Sigma, St. Louis, MO), 0.051g $MgCl_2$ (Cat. No. M2670, Sigma-Aldrich, St. Louis, MO), 0.065g $CaCl_2$ (Cat. No. C3881, Sigma-Aldrich, St. Louis, MO), and 0.595g NaHEPES (Cat. No. H3784, Sigma, St. Louis, MO) in a total volume of 250 mL deionized water. D-glucose stock solution (27.78 mM/L) (Cat. No. G8270, Sigma-Aldrich, St. Louis, MO) was prepared in deionized water.

Human and Chicken Serum Albumin Sample Incubation Protocol. Human and chicken serum albumin solutions were prepared with the following glucose concentrations: 0 mM, 5.56 mM (normal human), 11.11 mM (normal avian), and 22.22 mM (Table 5). Human and chicken serum albumins were incubated at various temperatures (37.0°C (normal human), 39.8°C (normal avian), and 41.4°C) on separate occasions. To prepare the solutions for the incubations, HSA or CSA were added to 10 mL of the HEPES-buffered saline solution to create a solution containing 0.47 mM albumin. Both solutions were pH adjusted to 7.4 using HCl and/or NaOH as necessary

using a ROSS electrode (VWR Symphony pH meter, Radnor, PA) and were then sterile-filtered using a syringe filter. Glucose was then added at varying concentrations to the albumin-HEPES solutions as indicated in Table 5.

Table 5. *Preparation of the albumin-HEPES solutions with varying glucose concentrations*

Final Glucose Concentration	Volume of albumin-HEPES solution	Volume of 27.78 mM glucose stock solution
0 mM	500 µL	0 µL
5.56 mM	499 µL	1 µL
11.11 mM	498 µL	2 µL
22.22 mM	496 µL	4 µL

Aliquots (100 µL) of the CSA and HSA-HEPES solutions with varying glucose concentrations were then transferred to PCR tubes in triplicate. The schematic of the incubations is detailed in Figure 3. All samples were incubated in a thermal cycler (MyCycler, Biorad, Hercules, CA) set to run at a constant temperature indefinitely at each specified temperature (37.0°C, 39.8°C, or 41.4°C). Aliquots of 2µl were extracted from all samples at baseline (day 0), day 3, and day 7 and stored at -80°C until analyses (see Appendix A).

Measurement of Percent Glycated Albumin by LC-ESI-MS. All aliquots extracted were diluted in a 30:1 ratio with 0.1% trifluoroacetic acid (TFA) and analyzed intact by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) (Dr. Chad Borges, BioDesign Institute, ASU) on a Dionex Ultimate 3000 HPLC equipped with a 1:100 flow splitter connected to a Bruker Maxis 4G quadrupole-time-of-flight (Q-TOF) mass spectrometer. A trap-and-elute form of LC-MS was carried out in which 15-µL samples were loaded at 10 µl/min in 80/20 water/acetonitrile containing 0.1% formic

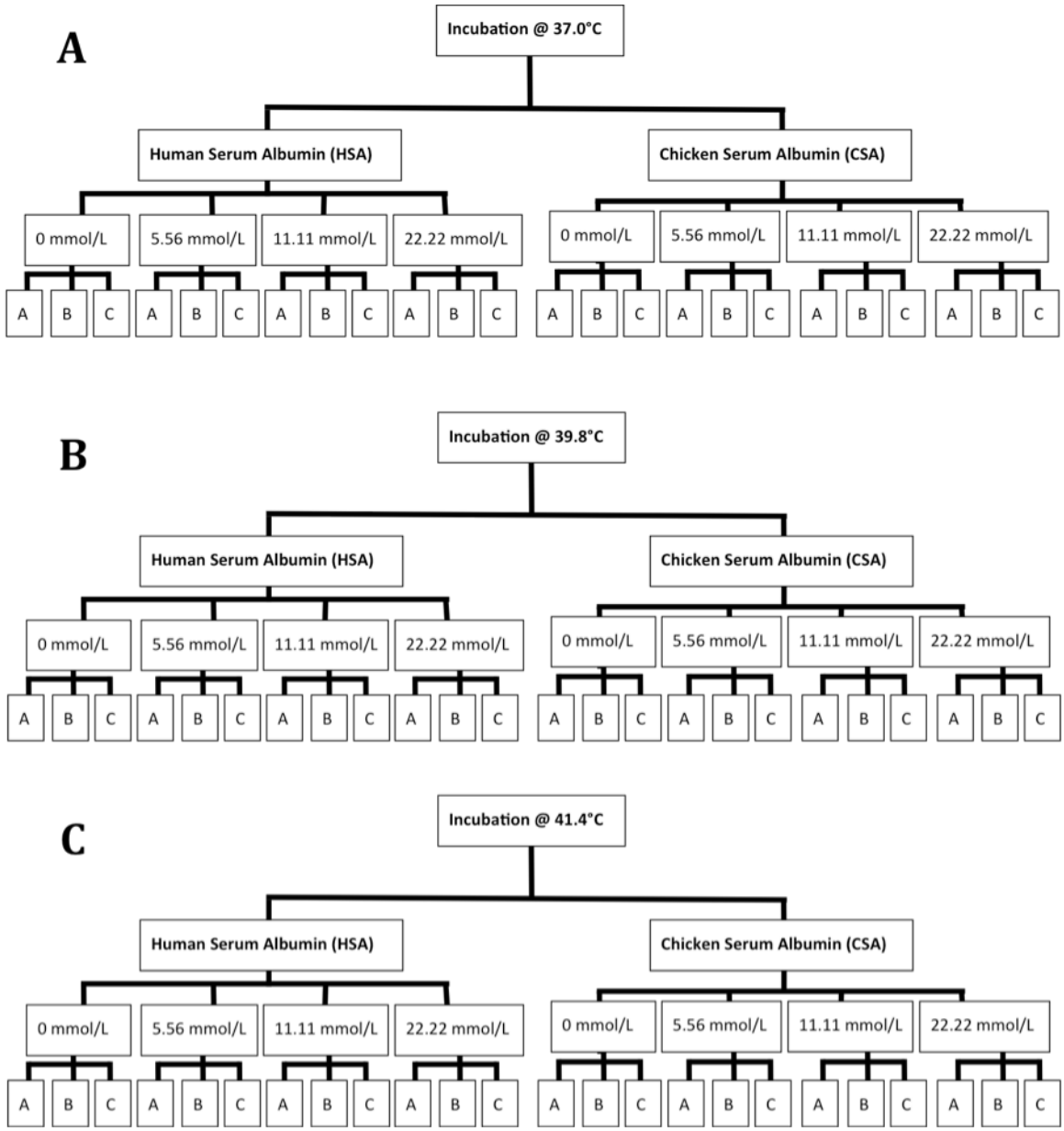


Figure 3. Schematic of the samples incubated at various temperatures. A: 37.0°C; B: 39.8°C; C: 41.4°C.

acid (loading solvent) onto a Bruker-Michrom protein captrap configured for bi-directional flow on a 6-port diverter valve. The flow over the captrap was then switched to the micropump, set at 2 μL/min, and ramped over 5 minutes from 80% water containing 0.1% formic acid (Solvent A) / 20% acetonitrile (Solvent B) to 90% acetonitrile and held for 3 min. The captrap eluent was directed to the mass spectrometer operating in positive ion, TOF-only mode, acquiring spectra in the *m/z* range of 300 to 3000 with a nominal resolving power of ~60,000 m/Δm FWHM. ESI settings for the Agilent G1385A capillary microflow nebulizer ion source were as follows: End plate offset -500 V, capillary -3500 V, nebulizer nitrogen 2 bar, dry gas nitrogen 3.0 L/min at 225 °C. Data were acquired in profile mode at a digitizer sampling rate of 4 GHz. Spectra rate control was by summation at 1 Hz.

HSA eluted over a period of about 1 minute; under the above conditions, HSA ranged in charge state from +32 to +71. Raw mass spectra were averaged across this timeframe, smoothed 0.0482 Da, baseline subtracted 0.85, charge deconvoluted and baseline subtracted 0.85 with Bruker DataAnalysis 4.1 charge deconvolution software to a mass range of 1000 Da on either side of any identified peak.

CSA eluted over a period of about 0.5 minute; under the above conditions, CSA ranged in charge state from +32 to +64. Raw mass spectra were averaged across this timeframe, smoothed 0.0482 Da, baseline subtracted 0.85, charge deconvoluted and baseline subtracted 0.85 with Bruker DataAnalysis 4.1 charge deconvolution software to a mass range of 1000 Da on either side of any identified peak (see Appendix B).

Percent Glycated Albumin Calculation. The mass spectra obtained from the LC-ESI-MS analysis of each sample were used to calculate percent glycated serum albumin.

For HSA samples, the peak counts corresponding to the peaks for S-cysteinylated albumin (~66558 Da), glycated S-cysteinylated albumin (~66720 Da), and doubly glycated S-cysteinylated albumin (~66882 Da) obtained from the mass spectra (see Appendix C) were used in the following formula to calculate percent glycated albumin: *Percent Glycated Human Serum Albumin = {[glycated S-cysteinylated albumin + 2(doubly glycated S-cysteinylated albumin)] / [S-cysteinylated albumin + glycated S-cysteinylated albumin + doubly glycated S-cysteinylated albumin]} * 100*.

For CSA samples, the peak counts corresponding to the peaks for native albumin (~66874 Da), glycated albumin (~67036 Da), and doubly glycated albumin (~67198 Da) obtained from the mass spectra (see Appendix D) were used in the following formula to calculate percent glycated albumin: *Percent Glycated Chicken Serum Albumin = {[glycated albumin + 2(doubly glycated albumin)] / [native albumin + glycated albumin + doubly glycated albumin]} * 100*.

Slightly different formulae were used for the calculation of percent glycation of HSA and CSA. This was due to the fact that CSA does not have a S-cysteinylated form as HSA does. The S-cysteinylated albumin form was used for the percent glycation of HSA calculation instead of the native albumin form because the intensity of the S-cysteinylated peak in the samples tended to be stronger than that of the native peak, thus the S-cysteinylated peaks gave a more accurate reading. The underlying assumption was made that glycation of HSA occurs in equal proportion in native and S-cysteinylated forms, which holds true both in theory as well as in unpublished evidence seen to date.

Statistical Analyses. Percent glycated albumin was measured on a total of 12 unique treatment conditions at three distinct time points for both HSA and CSA. All

statistical analyses were performed using SPSS Statistics version 22. All data are expressed as mean ± SEM. Data was arcsine transformed by taking the arcsine of the decimal value of each data point to achieve normality. Data was normally distributed within each day for all albumin type, temperature, glucose concentration group based on results of the Shapiro-Wilk test of normality. All data was analyzed using 4-way repeated-measures analysis of variance (ANOVA) to examine the change in percent glycation of human and chicken serum albumin over time with each treatment and to identify significant interactions among all independent variables. Two-way repeated measures ANOVA tests were run to explore confirmed interactions and to confirm between group variations within specific time points. A Bonferroni procedure was used for all post hoc analyses. The sphericity assumption of all repeated measures ANOVA tests was met if the Greenhouse-Geisser Epsilon value was greater than or equal to 0.75; if the assumption was not met, then the Greenhouse-Geisser correction factor was used to adjust the degrees of freedom. A p-value ≤ 0.05 (alpha set to 0.05) was considered significant.

CHAPTER 4

RESULTS

Main Effects of Temperature, Glucose Concentration, and Albumin Type on Percent Glycated Albumin Across Time. The results of the four-way repeated measures ANOVA test are summarized below in Table 6. There was not a statistically significant difference between Albumin Type (Human Serum Albumin and Chicken Serum Albumin) group means ($p = 0.095$). There was a statistically significant difference in temperature group means ($p < 0.001$); the mean percent glycated albumin for both chicken and human serum albumin at all glucose concentrations and on all days is significantly higher at 41.4°C than at 37.0°C ($p = 0.001$) and at 39.8°C ($p = 0.038$). In regards to glucose concentration group means, there was a statistically significant difference ($p < 0.001$). Specifically, the mean percent glycated albumin for both chicken and human serum albumin at all temperatures significantly increased as glucose concentration increased ($p < 0.001$ for all comparisons). Also, there was a statistically significant difference in means across time ($p < 0.001$). The mean percent glycated albumin for both chicken and human serum albumin at all temperatures and all glucose concentrations significantly increased across time points ($p < 0.001$ for all comparisons).

Table 6. *Four-Way Repeated Measures ANOVA Summary Table*

Source	Sum of Squares (SS)	Df	Mean Squares (MS)	F	P-value
Between Subjects					
Albumin Type	0.006	1.000	0.006	1.901	0.174
Temperature	0.046	2.000	0.023	7.509	***0.001**
Glucose Concentration	1.835	3.000	0.612	199.338	***<0.001**
Albumin Type x Temperature	0.025	2.000	0.012	4.030	***0.024**
Albumin Type x Glucose Concentration	0.294	3.000	0.098	31.972	***<0.001**
Temperature x Glucose Concentration	0.125	6.000	0.021	6.764	***<0.001**
Albumin Type x Temperature x Glucose Concentration	0.029	6.000	0.005	1.563	0.179
Error	0.147	48.000	0.003		
Within Subjects					
Time	0.231	1.409	0.164	93.382	***<0.001**
Time x Albumin Type	0.397	1.409	0.282	160.786	***<0.001**
Time x Temperature	0.023	2.818	0.008	4.608	***0.006**
Time x Glucose Concentration	1.294	4.227	0.306	174.583	***<0.001**
Time x Albumin Type x Temperature	0.022	2.818	0.008	4.444	***0.008**
Time x Albumin Type x Glucose Concentration	0.223	4.227	0.053	30.080	***<0.001**
Time x Temperature x Glucose Concentration	0.071	8.455	0.008	4.757	***<0.001**
Time x Albumin Type x Temperature x Glucose Concentration	0.039	8.455	0.005	2.600	***0.014**
Error	0.119	67.639	0.002		

Sphericity assumption was not met, thus the Greenhouse-Geisser correction factor was used to obtain the values presented for Within Subjects. Albumin Type, Human or Chicken Serum Albumin. Temperature, 37°C or 39.8°C or 41.4°C. Glucose Concentration, 0mM or 5.56mM or 11.11mM or 22.22mM. Time, Day 0 or Day 3 or Day 7. * $P \leq 0.05$

Interaction Effects of Temperature, Glucose Concentration, and Albumin Type on Percent Glycated Albumin across Time. All possible interactions, except for the interaction of albumin type x temperature x glucose concentration, were significant based on the results of the four-way repeated measures ANOVA test (see Table 6). The statistically significant interaction between glucose concentration, temperature, albumin type, and time ($p = 0.032$) indicates that all independent variables interact to affect the mean percent glycation of albumin. Two-way repeated measures ANOVA were used to characterize these interactions. Figures 4, 5, and 6 directly compare the effect of varying glucose concentrations on the percent glycation of HSA and CSA at 37.0°C, 39.8°C, and 41.4°C, respectively. As shown in the figures, as glucose concentration increased, the percent glycation of HSA and CSA increased over time at all temperatures. In addition, HSA was glycated to a greater extent in comparison to CSA at the two higher glucose concentrations examined for all temperature conditions.

The 0 mM glucose concentration did not differentially affect percent glycation of HSA and CSA at any temperature examined ($p = 0.230$ for 37.0°C, $p = 0.215$ for 39.8°C, and $p = 0.065$ for 41.4°C). Both CSA and HSA decreased in percent glycation over time at all temperatures examined at the 0 mM glucose concentration (see Figure 4A, 5A, and 6A). The percent glycation of HSA and CSA were differentially affected by the 5.56 mM glucose concentration at the two highest temperatures examined ($p = 0.039$ for 39.8°C, and $p = 0.002$ for 41.4°C). At both of these temperatures, CSA decreased in percent glycation over time while the percent glycation of HSA remained constant over time (see Figure 4B, 5B, and 6B). The 11.11 mM glucose concentration differentially affected percent glycation of HSA and CSA at all temperatures ($p < 0.001$ for 37.0°C, $p < 0.001$

for 39.8°C, and p = 0.001 for 41.4°C). At all temperatures examined the percent glycation of CSA remained constant over time, however, the percent glycation of HSA increased moderately over time (see Figure 4C, 5C, and 6C). Finally, the 22.22 mM glucose concentration also differentially affected percent glycation of HSA and CSA at all temperatures (p = 0.001 for 37.0°C, p = 0.004 for 39.8°C, and p = 0.004 for 41.4°C). The percent glycation of HSA and CSA both increased over time at all temperatures, however, the percent glycation of HSA increased to a greater extent (see Figure 4D, 5D, and 6D).

Temperature had a differential effect on the percent glycation of HSA and CSA. The increase in percent glycation of HSA over time at the 11.11 mM glucose concentration and the 22.22 mM glucose concentration was to a significantly greater extent at higher temperatures compared to lower temperatures (see Figures 4C, 5C, and 6C for 11.11 mM glucose and Figures 4D, 5D, and 6D for 22.22 mM glucose). This differential effect of temperature seen at the two highest glucose concentrations examined was not significant for CSA.

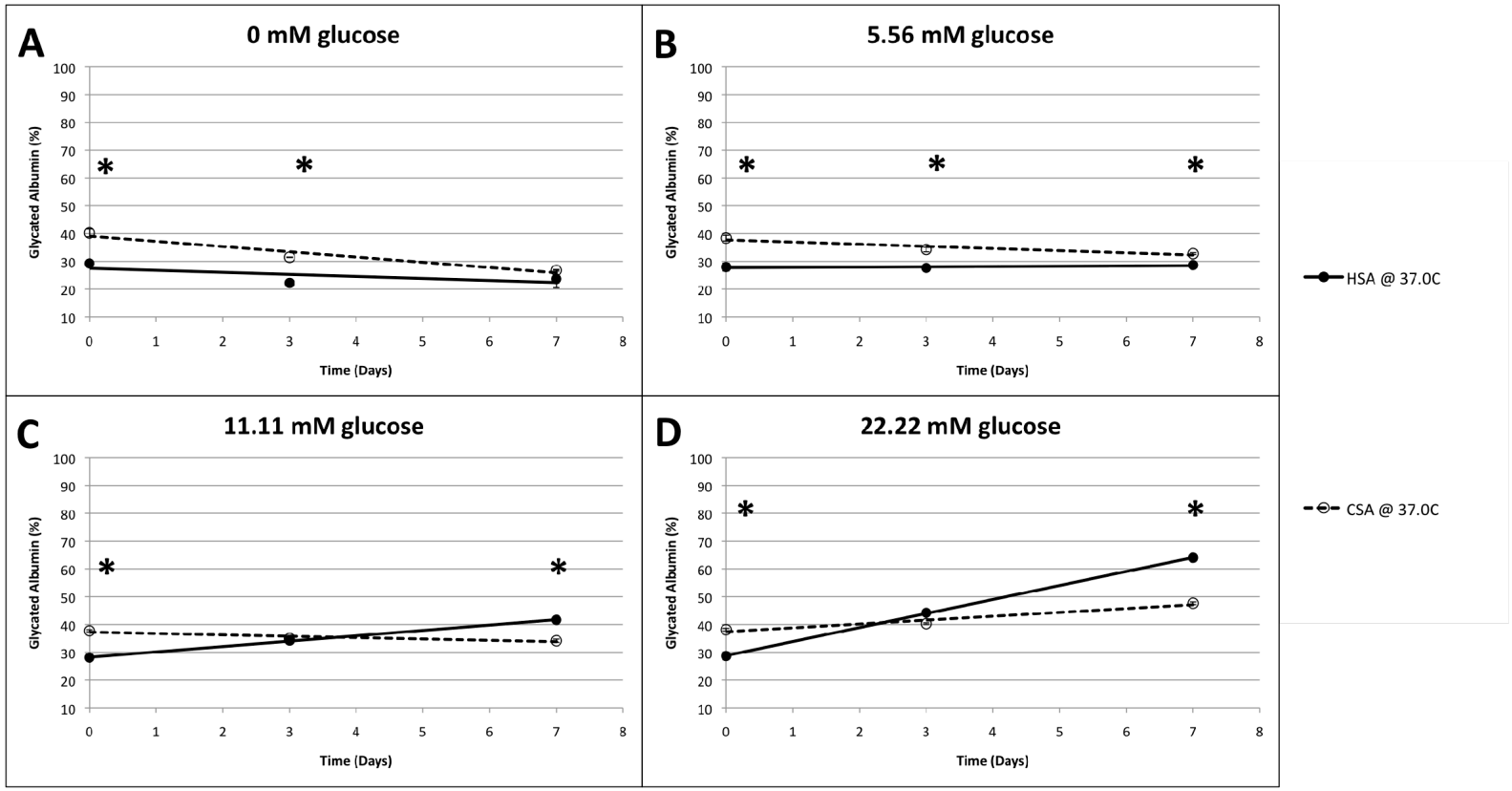


Figure 4. *Percent Glycation in response to varying glucose concentrations at 37°C.* A: Changes in percent glycation of HSA and CSA in the presence of 0mM glucose (Time, $p = 0.013$; Albumin Type, $p = 0.050$; Interaction, $p = 0.230$). *B:* Changes in percent glycation of HSA and CSA in the presence of 5.56mM glucose (Time, $p = 0.135$; Albumin Type, $p = 0.014$; Interaction, $p = 0.092$). *C*: Changes in percent glycation of HSA and CSA in the presence of 11.11mM glucose (Time, $p = 0.010$; Albumin Type, $p = 0.519$; Interaction, $p < 0.001$). *D*: Changes in percent glycation of HSA and CSA in the presence of 22.22mM glucose (Time, $p < 0.001$; Albumin Type, $p = 0.004$; Interaction, $p = 0.001$). Between group variations were confirmed with two-way repeated measures ANOVA and Bonferroni Post Hoc Analyses. * Indicates significant difference between mean percent glycation of HSA and CSA at the time point ($P \leq 0.05$). All data are displayed as mean ± SEM with trendline. n = 3.

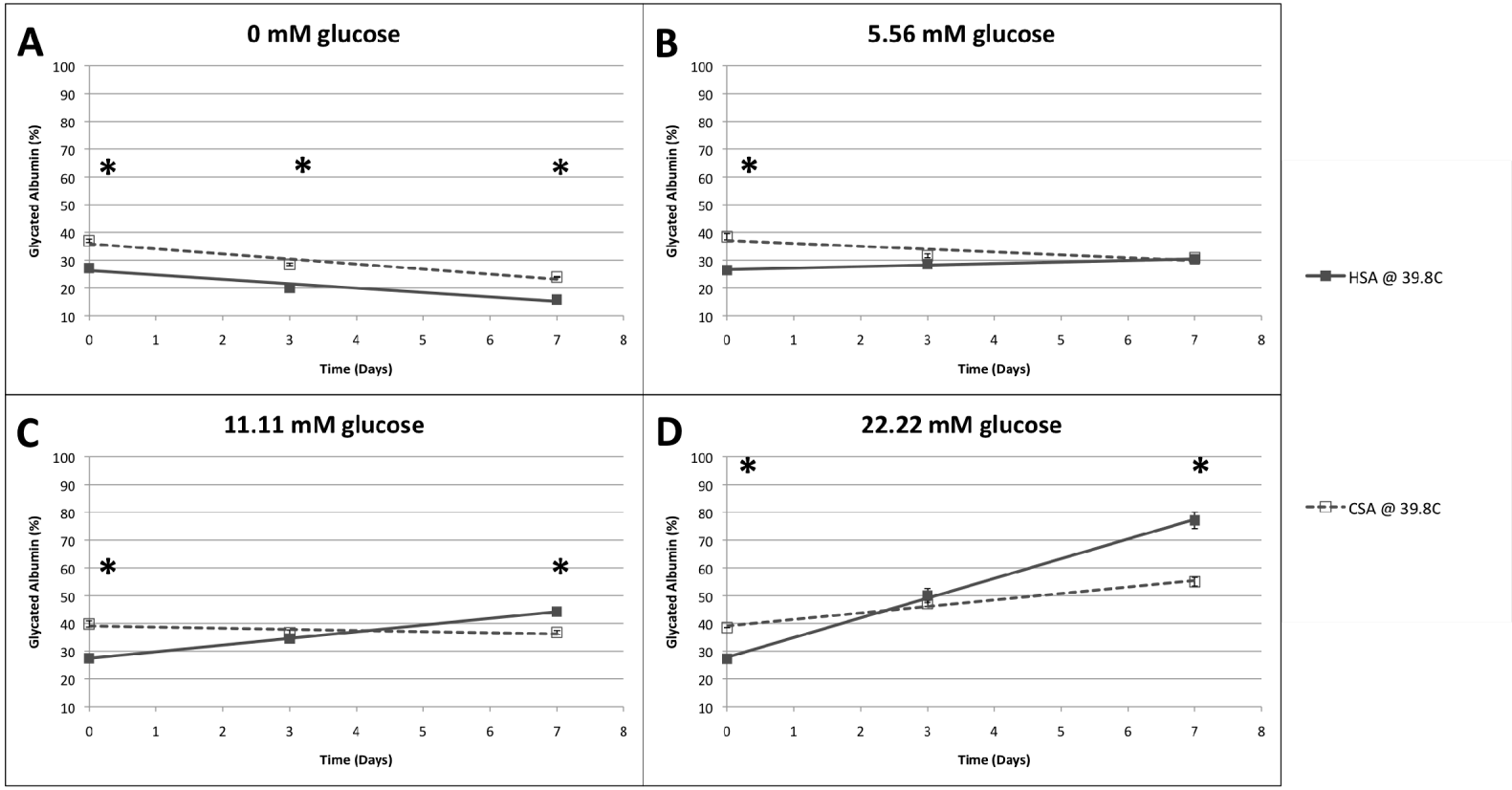


Figure 5. *Percent Glycation in response to varying glucose concentrations at 39.8°C.* A: Changes in percent glycation of HSA and CSA in the presence of 0mM glucose (Time, $p < 0.001$; Albumin Type, $p = 0.002$; Interaction, $p = 0.215$). B: Changes in percent glycation of HSA and CSA in the presence of 5.56mM glucose (Time, $p = 0.294$; Albumin Type, $p = 0.011$; Interaction, $p = 0.039$). C: Changes in percent glycation of HSA and CSA in the presence of 11.11mM glucose (Time, $p = 0.004$; Albumin Type, $p = 0.325$; Interaction, $p < 0.001$). D: Changes in percent glycation of HSA and CSA in the presence of 22.22mM glucose (Time, $p < 0.001$; Albumin Type, $p = 0.226$; Interaction, $p = 0.004$). Between group variations were confirmed with two-way repeated measures ANOVA and Bonferroni Post Hoc Analyses. * Indicates significant difference between mean percent glycation of HSA and CSA at the time point ($P \leq 0.05$). All data are displayed as mean ± SEM with trendline. n = 3.

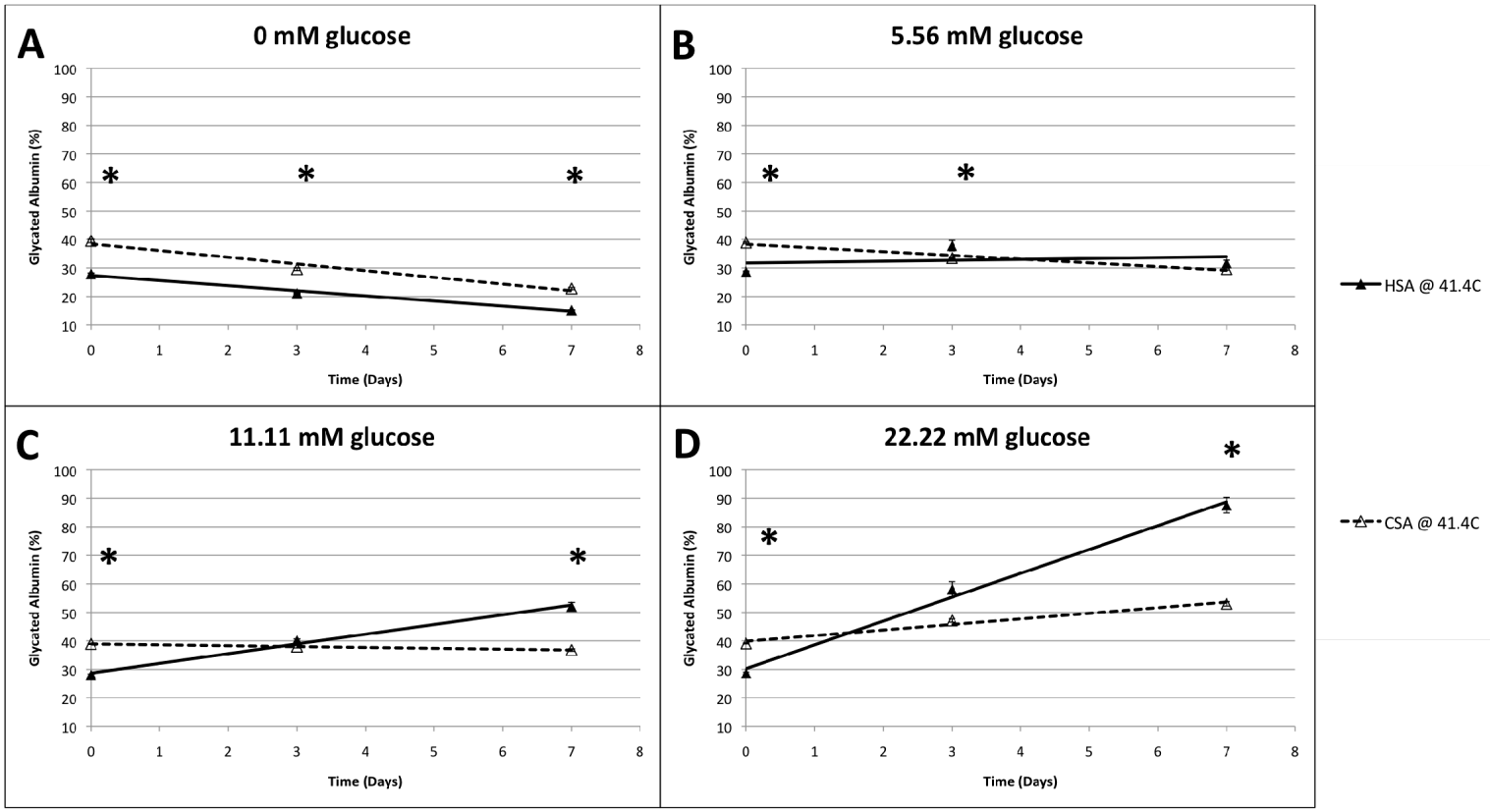


Figure 6. *Percent Glycation in response to varying glucose concentrations at 41.4°C.* A: Changes in percent glycation of HSA and CSA in the presence of 0mM glucose (Time, $p < 0.001$; Albumin Type, $p < 0.001$; Interaction, $p = 0.065$). B: Changes in percent glycation of HSA and CSA in the presence of 5.56mM glucose (Time, $p = 0.026$; Albumin Type, $p = 0.009$; Interaction, $p = 0.002$). C: Changes in percent glycation of HSA and CSA in the presence of 11.11mM glucose (Time, $p = 0.002$; Albumin Type, $p = 0.096$; Interaction, $p = 0.001$). D: Changes in percent glycation of HSA and CSA in the presence of 22.22mM glucose (Time, $p = 0.001$; Albumin Type, $p = 0.029$; Interaction, $p = 0.004$). Between group variations were confirmed with two-way repeated measures ANOVA and Bonferroni Post Hoc Analyses. * Indicates significant difference between mean percent glycation of HSA and CSA at the time point ($P \leq 0.05$). All data are displayed as mean ± SEM with trendline. n = 3.

CHAPTER 5

DISCUSSION

Discussion of Results. Based on the results of the statistical analyses of the data, all three null hypotheses are rejected. The first hypothesis (H_0 1) stating that the percent glycation of CSA will not significantly differ from the percent glycation of HSA when exposed to the same temperatures and glucose concentrations over seven days is rejected as the percent glycation of CSA significantly differed from the percent glycation of HSA under certain conditions. The second hypothesis (H_0 2) stating that variations in incubation temperatures will not differentially affect the percent glycation of CSA compared to the percent glycation of HSA incubated for seven days at the same glucose concentrations is rejected as temperature differentially affected the percent glycation of HSA compared to the percent glycation of CSA at the two highest glucose concentrations examined. Finally, the third hypothesis (H_0 3) stating that variations in glucose concentrations will not differentially affect the percent glycation of CSA compared to the percent glycation of HSA incubated for seven days when the incubation temperature is constant is rejected as glucose concentration differentially affected the percent glycation of CSA compared to the percent glycation of HSA over time at all temperatures examined.

The direct comparison of the effect of temperature and glucose concentration on the percent glycation of HSA and CSA has not previously been examined. The results of this comparative study are unprecedented, thus duplication of this study is warranted in order to confirm these findings. Nonetheless, the examination of the effect of temperature on the percent glycation of HSA has been examined in one previously conducted study

(Urbanowski et al., 1982). The present findings related specifically to HSA are in agreement with the one previously conducted study, which found that the percent glycation of HSA increased over time as temperature increased (Urbanowski et al., 1982).

The assumption from previous work showing that the susceptibility of albumin to glycation does not greatly differ across mammalian species as it does for other plasma proteins (i.e. hemoglobin) (Rendell et al., 1985) needs to be reconsidered given the results of the current study. New, more precise techniques for the quantification of percent glycation of plasma proteins have been developed since the publication of this work. The use of these techniques has the potential to more accurately depict the differences in glycation between mammals and avian species as well as identify previously overlooked interspecies differences in the susceptibility of albumin to glycation.

The present findings showing a differential exists in the percent glycation of HSA and CSA under the same conditions, which is in agreement with previously published studies that have shown other plasma proteins (i.e. hemoglobin) are glycated to a significantly lesser degree in several avian species despite higher blood glucose concentrations (Rendell et al., 1985; Beuchat & Chong, 1998). Fructosamine has been used as a clinical measure of protein glycation, similarly to hemoglobin A1C, as it measures the number of ketoamine linkages resulting from the glycation of plasma proteins (Anguizola, 2013; Beck, 2011). This method is largely a measure of glycated albumin since about 90% of ketoamines are formed from the plasma protein albumin, as it is the most abundant plasma protein (Anguizola, 2013). While a normal range for

fructosamine is known humans (Beck, 2011), fructosamine levels have not been examined in avian species. In fact, only a few researchers have even specifically examined the percent glycation of albumin in avian species. The level of glycated albumin in several avian species examined by Rendell et al. (1985) was found to be higher than levels in humans (Rendell et al., 1985). However, more recent examination of the percent glycation of plasma albumin glycation using more precise measurement techniques was observed to be significantly lower in budgerigars (parakeets) in comparison to human plasma albumin (Holmes et al., 2001). Again, the present findings suggest that the improved methods for quantification of percent glycation more accurately depict the true level of albumin glycation in avian species.

In addition, the findings of this study reinforce our current understanding of protein glycation at physiological conditions in both humans and chickens (Brownlee et al., 1984; Ulrich & Cerami, 2001). When incubated at the condition most physiologically relevant for a human (5.56 mM glucose at 37.0°C), the percent glycation of HSA was at equilibrium, as the percent glycation remained constant over time. The percent glycation of HSA increased over time at glucose concentrations higher than physiological for humans (11.11 mM and 22.22 mM glucose) while the percent glycation of HSA decreased over time at glucose concentrations lower than physiological for humans (0.00 mM glucose). The same was true for the glycation of CSA; however, chickens have different normal physiological conditions than humans. When incubated at the condition most physiologically relevant for a chicken (11.11 mM glucose and 39.8°C), the percent glycation of CSA was at equilibrium, as the percent glycation remained constant over time. The percent glycation of CSA increased over time at glucose concentrations higher

than physiologically normal for chickens (22.22 mM glucose) while the percent glycation of CSA decreased over time at glucose concentrations lower than physiologically normal for chickens (0.00 mM glucose and 5.56 mM glucose).

The observed decrease in percent glycation from day 0 to day 7 at the glucose concentration of 0 mM for both CSA and HSA and at the glucose concentration of 5.56 mM for CSA demonstrates the reversibility of the Schiff base formation reaction (Brownlee et al., 1984; Ulrich & Cerami, 2001). This de-glycation of HSA and CSA occurred at a similar rate at all temperatures examined.

Degradation of the proteins was observed over time during all incubations. All samples were diluted at a 30:1 ratio for the LC-ESI-MS analyses, thus the total count obtained from the mass spectrum for each sample is indicative of the quantity of albumin in the sample. The average total counts for HSA (S-cysteinylated albumin + glycated s-cysteinylated albumin + doubly glycated s-cysteinylated albumin) was 68738 on Day 0, 53797 on Day 3, and 30392 on Day 7. The average total counts for CSA (native albumin + glycated albumin + doubly glycated albumin) was 85295 on Day 0, 56571 on Day 3, and 28888 on Day 7. This decrease in total albumin is not due to loss to the glycated albumin pool as the average total counts for HSA and CSA include the glycated albumin pool. Based on the average total counts obtained from the mass spectrum of each sample, CSA degraded at a faster rate than HSA. This difference in degradation of HSA and CSA has been reported previoulsy in published literature (Rendell et al., 1985). Regardless, the percent glycation is a reflection of the amount of glycated albumin as a ratio of the total albumin present in each sample at each time point. In regard to the disparity in elution time observed between CSA and HSA, this likely has to due with differences in the

relative affinity of the different albumin molecules to the hydrophobic protein captrap that was used; however, this is merely speculation and further research would be necessary to address this difference.

Unlike humans, temperature was not found to have a significant effect on the percent glycation of CSA. This finding indicates that the reactant-favored chemical environment (i.e. higher body temperature of avian species in comparison to humans) is not the mechanism by which birds resist protein glycation. In fact, lack of effect of temperature on the percent glycation of CSA adds to the complexity of the 'avian paradox' as it indicates that birds also resist the increase in protein glycation at higher temperatures than those seen in humans.

Strengths and Limitations. There were several strengths of this study. The *in vitro* manipulations were conducted in a highly controlled environment and thus equalized the background matrix between the two types of albumin. This allowed for the direct comparison of the purified human and chicken albumin molecules at a range of physiologically relevant temperatures and glucose concentrations. The commercially purchased CSA and HSA are pooled samples from numerous individual animals, thus individual variation in the protein molecule is accounted for and a small sample size (n=3) is sufficient. Both the HSA and CSA were highly purified assuring that impurities were not responsible for influencing the data or contaminating the results. In addition, the use of LC-ESI-MS as the method for the quantification and calculation of percent glycation of both HSA and CSA is both precise and reliable. The ability to differentiate between singly and doubly glycated albumin is unique to this method and strengthens the

accuracy of the data as compared to prior studies that only measured singly glycated albumin.

Despite the strengths associated with an *in vitro* experiment, there are inherent limitations as well. The *in vitro* environment does not adequately simulate the *in vivo* conditions. Thus, there could be other factors that play a role in the *in vivo* setting that are not accounted for in an *in vitro* study. For example, there could be other serum factors that impact protein glycation. In addition, the results of this study are not generalizable to the whole animal (either human or chicken) nor are they generalizable to any species not included in this study. Further research is needed to determine if all avian albumin is glycated similarly to chicken albumin. Lastly, there was some degree of oxidation of the CSA and HSA molecules that occurred over time during the incubations, particularly the day 7 samples of both HSA and CSA at the 22.22 mM glucose concentration at all temperatures. Although oxidation increased the variability of the percent glycation data, it was not sufficient to affect the statistical conclusions. Increasing the total volume of the incubation and/or adding 1 mM EDTA to the samples prior to incubation to minimize transition metal redox cycling and generation of reactive oxygen species are two ways that this oxidation can be prevented in future studies. The addition of EDTA was not done in this study in order to simulate physiological conditions as closely as possible.

CHAPTER 6

CONCLUSIONS AND APPLICATIONS

Further research is needed to fully unravel the 'avian paradox'. This study revealed that temperature is not the mechanism by which birds protect against protein glycation. Specifically, temperature did not impact percent glycation of CSA, but temperature did impact percent glycation of HSA at the highest glucose concentrations. It was also shown that as glucose concentration increased, the percent glycation of both HSA and CSA increased. HSA was glycated to a greater extent when compared to CSA in the presence of high glucose concentrations.

The results of this study suggest there is an inherent difference between the purified forms of human and chicken albumin that contributes to the differential glycation. One possible explanation for this inherent difference is a structural variation in the chicken and human albumin proteins. The chicken albumin protein may be folded in such a way that the amino acid residues (i.e. lysine) that glucose binds to are protected or shielded, thus decreasing the susceptibility of the protein to glycation. Future investigations should focus on determining the tertiary structure of avian albumin molecules in order to compare to that of human albumin molecules. Further, this study does not sufficiently rule out the possible role of serum factors or other factors affecting plasma conditions (ie. pH) as contributing to the paradoxical difference in protein glycation seen between birds and humans. Therefore, additional research is recommended to determine the complexity of the mechanism and determine if inherent protein difference is the only factor at play or if there are several factors interacting to result in protection from protein glycation.

There are significant human applications of this research. Specifically, the findings of this study reinforce the importance for diabetic humans to maintain normal blood glucose concentrations, especially during illness/fever, in order to limit protein glycation. Future related work could result in additional human applications. For instance, the identification of the specific mechanism of protection from protein glycation has the potential to lead to the development of novel therapies to help individuals with diabetes prevent protein glycation and therefore limit the production of AGEs. The prevention of AGE formation altogether would drastically reduce the number of diabetic individuals developing severe diabetic complications. If a tertiary structure difference between chicken and human albumin proves true, such therapies could be in the form of a drug or a natural compound found in the diet that acts by blocking amino acid residues to prevent the initial glycation from occurring in humans. In addition, since the possibility of serum factors or other serum conditions as contributing factors has not been ruled out by the present study, then another potential therapy would be a mimetic of specific serum factors found in chickens administered to humans. A natural therapy, such an element found in the diet, might be preferable to a synthetic therapy as it would ease medical expenses in a population that already spends a substantial amount on healthcare annually (i.e. diabetics). If additional investigations ultimately reveal that the innate difference between chicken and human purified albumin cannot be overcome by the development of novel therapies, at the very least the body of scientific knowledge will have grown and further efforts can be shifted in another promising direction.

All in all, the differential effects of temperature and glucose concentration on the glycation of CSA and HSA suggest an inherent difference in these proteins, which is a

significant contributing factor in the mechanism by which birds are protected from protein glycation. The nature of this difference is unknown and the results of this study strongly support the need for further research in order to unravel this mechanism.

REFERENCES

American Diabetes Association. (2013). Economic costs of diabetes in the U.S. in 2012. *Diabetes Care, 36*(4), 1033-1046. doi:10.2337/dc12-2625

American Diabetes Association. (2014). American Diabetes Association. Retrieved from http://www.diabetes.org/

Anguizola, J., Matsuda, R., Barnaby, O.S., Hoy, K.S., Wa, C., DeBolt, E., Koke, M., & Hage, D.S. (2013). Review: Glycation of human serum albumin. *Clinica Chimica Acta.* 425, 64-76.

Anthony, C., Sweazea, K.L., & Braun, E.J. (2010) The analysis of avian plasma for glycated serum albumin. *FASEB J.* 24, 1055-1057.

Austin, G.E., Mullins, R.H., & Morin, L.G. (1987). Non-enzymic glycation of individual plasma proteins in normoglycemic and hyperglycemic patients. *Clinical Chemistry, 33*(12), 2220-2224.

Bartholomew, G.A. Jr., & Dawson, W.R. (1954). Body temperature and water requirements in the mourning dove, zenaidura macroura marginella. *Ecology, 35*(2), 181-187.

Basta, G., Schmidt, A.M., & De Caterina, R. (2004). Advanced glycation end products and vascular inflammation: implication for accelerated atherosclerosis in diabetes. *Cardiovascular Research.* 63(4), 582-592.

Baumann, F.H., & Baumann, R. (1977). A comparative study of the respiratory properties of bird blood. *Respiration Physiology, 31*(3), 333-343.

Beck, R., Steffes, M., Xing, D., Ruedy, K., Mauras, N., Wilson, D.M., & Kollman, C. (2011). The interrelationships of glycemic control measures: HbA1c, glycated albumin, fructosamine, 1,5-anhydroglucitrol, and continuous glucose monitoring. *Pediatr Diabetes.* 12(8):690-695.

Beuchat, C.A., & Chong, C.R. (1998). Hyperglycemia in hummingbirds and its consequences for hemoglobin glycation. *Comparative Biochemistry and Physiology.Part A, Molecular & Integrative Physiology, 120*(3), 409-416.

Borges, C.R., Oran, P.E., Buddi, S., Jarvis, J.W., Schaab, M.R., Rehder, D.S., & Nelson, R.W. (2011). Building multidimensional biomarker views of type 2 diabetes on the basis of protein microheterogeneity. *Clinical Chemistry, 57*(5), 719-728. doi:10.1373/clinchem.2010.156976

Braun, E.J., & Sweazea, K.L. (2008). Glucose regulation in birds. *Comparative Biochemistry and Physiology.Part B, Biochemistry & Molecular Biology, 151*(1), 1-9. doi:10.1016/j.cbpb.2008.05.007

Brownlee, M., & Cerami, A. (1981). The biochemistry of the complications of diabetes mellitus. *Annual Review of Biochemistry, 50*, 385-432. doi:10.1146/annurev.bi.50.070181.002125

Brownlee, M., Vlassara, H., & Cerami, A. (1984). Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Annals of Internal Medicine, 101*(4), 527-537.

Cade, W.T. (2008). Diabetes-related microvascular and macrovascular diseases in the physical therapy setting. *Physical Therapy, 88*(11), 1322-1335. doi:10.2522/ptj.20080008

Carver, F.M. (2001). Differential expression of glucose transporters during chick embryogenesis. *Cellular and Molecular Life Sciences: CMLS, 58*(4), 645-652.

Centers for Disease Control and Prevention. (2013). Diabetes public health resource. Retrieved from http://www.cdc.gov/diabetes/statistics/prev/national/figpersons.htm

Centers for Disease Control and Prevention. (2014). *National diabetes statistics report: Estimates of diabetes and its burden in the United States, 2014*. Atlanta, GA: U.S. Department of Health and Human Services.

Chang, J., Chu, N., Syu, J., Hsieh, A., & Hung, Y. (2011). Advanced glycation end products (AGEs) in relation to atherosclerotic lipid profiles in middle-aged and elderly diabetic patients. *Lipids in Health and Disease*. 10, 228.

Chaudhuri, C.R., & Chatterjee, I.B. (1969). L-ascorbic acid synthesis in birds: Phylogenetic trend. *Science (New York, N.Y.), 164*(3878), 435-436.

Davie, S.J., Gould, B.J., & Yudkin, J.S. (1992). Effect of vitamin C on glycosylation of proteins. *Diabetes, 41*(2), 167-173.

Denton, R.M., & Tavaré, J.M. (1997). Molecular basis of insulin action on intracellular metabolism. In: Alberti KGMM, Zimmet P, Defronzo RA, Keen H (Hon), editors. International Textbook of Diabetes Mellitus (2nd ed) John Wiley & Sons, New York. p. 469–88.

Fullerton, B., Jeitler, K., Seitz, M., Horvath, K., Berghold, A., & Siebenhofer, A. (2014). Intensive glucose control versus conventional glucose control for type 1 diabetes mellitus. *The Cochrane Database of Systematic Reviews, 2*, CD009122. doi:10.1002/14651858.CD009122.pub2

Hammes, H.P., Martin, S., Federlin, K., Geisen, K., & Brownlee, M. (1991). Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. *Proceedings of the National Academy of Sciences of the United States of America, 88*(24), 11555-11558.

Holmes, D.J., Flückiger, R., & Austad, S.N. (2001). Comparative biology of aging in birds: An update. *Experimental Gerontology, 36*(4–6), 869-883. http://dx.doi.org.ezproxy1.lib.asu.edu/10.1016/S0531-5565(00)00247-3

Iqbal, M., Probert, L.L., Alhumadi, N.H., & Klandorf, H. (1999). Protein glycosylation and advanced glycosylated endproducts (AGEs) accumulation: An avian solution? *The Journals of Gerontology.Series A, Biological Sciences and Medical Sciences, 54*(4), B171-6; discussion B177-8.

Jaensch, S. (2013). Veterinary hematology and clinical chemistry - edited by MA thrall, G weiser, RW allison and T campbell. *Australian Veterinary Journal, 91*(9), 365.

Johnston, C.S. (1990). Effect of a single oral dose of ascorbic acid on body temperature and trace mineral fluxes in healthy men and women. *Journal of the American College of Nutrition, 9*(2), 150-154.

Johnston, C.S. (1989). Effect of single oral doses of ascorbic acid on body temperature in healthy guinea pigs. *The Journal of Nutrition, 119*(3), 425-427.

Khan, M.W., Rasheed, Z., Khan, W.A., & Ali, R. (2007). Biochemical, biophysical, and thermodynamic analysis of in vitro glycated human serum albumin. *Biochemistry.Biokhimiia, 72*(2), 146-152. doi:BCM72020175

Kim, K.J., & Lee, B.W. (2012). The roles of glycated albumin as intermediate glycation index and pathogenic protein. *Diabetes & Metabolism Journal, 36*(2), 98-107. doi:10.4093/dmj.2012.36.2.98

Klandorf, H., Rathore, D.S., Iqbal, M., Shi, X., & Van Dyke, K. (2001). Accelerated tissue aging and increased oxidative stress in broiler chickens fed allopurinol. *Comparative Biochemistry and Physiology.Toxicology & Pharmacology : CBP, 129*(2), 93-104. doi:S1532-0456(01)00186-7

Lapolla, A., Molin, L., & Traldi, P. (2013). Protein glycation in diabetes as determined by mass spectrometry. *International Journal of Endocrinology, 2013*, 412103. doi:10.1155/2013/412103

Lee, B.S., Jayathilaka, G.D., Huang, J.S., Vida, L.N., Honig, G.R., & Gupta, S. (2011). Analyses of in vitro nonenzymatic glycation of normal and variant hemoglobins by MALDI-TOF mass spectrometry. *Journal of Biomolecular Techniques : JBT, 22*(3), 90-94.

Linden, E., Cai, W., He, J.C., Xue, C., Li, Z. Winston J, & Uribarri J. (2008). Endothelial dysfunction in patients with chronic kidney disease results from advanced glycation end products (AGE)-mediated inhibition of endothelial nitric oxide synthase through RAGE activation. *Clinical Journal of the American Society of Nephrology : CJASN, 3*(3), 691-698. doi:10.2215/CJN.04291007

McCance, D.R., Dyer, D.G., Dunn, J.A., Bailie, K.E., Thorpe, S.R., Baynes, J.W., & Lyons, T.J. (1993). Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. *The Journal of Clinical Investigation, 91*(6), 2470-2478. doi:10.1172/JCI116482

McVerry, B.A., Fisher, C., Hopp, A., & Huehns, E.R. (1980). Production of pseudodiabetic renal glomerular changes in mice after repeated injections of glucosylated proteins. *Lancet, 1*(8171), 738-740. doi:S0140-6736(80)91234-9

Pirart, J. (1977). Diabetes mellitus and its degenerative complications: A prospective study of 4,400 patients observed between 1947 and 1973 (author's transl). *Diabete & Metabolisme, 3*(2), 97-107.

Ramasamy, R., Yan, S.F., & Schmidt, A.M. (2011). Receptor for AGE (RAGE): Signaling mechanisms in the pathogenesis of diabetes and its complications. *Annals of the New York Academy of Sciences, 1243*, 88-102. doi:10.1111/j.1749-6632.2011.06320.x

Rendell, M., Stephen, P.M., Paulsen, R., Valentine, J.L., Rasbold, K., Hestorff, T., & Shint, D.C. (1985). An interspecies comparison of normal levels of glycosylated hemoglobin and glycosylated albumin. *Comparative Biochemistry and Physiology.B, Comparative Biochemistry, 81*(4), 819-822.

Rondeau, P., & Bourdon, E. (2011). The glycation of albumin: Structural and functional impacts. *Biochimie, 93*(4), 645-658. doi:10.1016/j.biochi.2010.12.003

Roth, Z. (2012). Identification and quantification of protein glycosylation. *Carbohydrate Research, 131*(2), 1-10.

Schleicher, E., & Wieland, O. (1989). Protein glycation: Measurement and clinical relevance. *J Clin Chem Clin Biochem, 27*(9), 577-587.

Seki, Y. (2003). Broiler chickens (ross strain) lack insulin-responsive glucose transporter GLUT4 and have GLUT8 cDNA. *General and Comparative Endocrinology, 133*(1), 80-87.

Shepherd, P.R., & Kahn, B. (1999). Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus. *The New England Journal of Medicine, 341*(4), 248-257. doi:10.1056/NEJM199907223410406

Sherwood, L. (2008). *Human physiology: From cells to systems* Cengage Learning.

Singh, V.P., Bali, A., Singh, N., & Jaggi, A.S. (2014). Advanced glycation end products and diabetic complications. *The Korean Journal of Physiology & Pharmacology: Official Journal of the Korean Physiological Society and the Korean Society of Pharmacology, 18*(1), 1-14. doi:10.4196/kjpp.2014.18.1.1

Speakman, J.R. (2005). Body size, energy metabolism and lifespan. *The Journal of Experimental Biology, 208*(Pt 9), 1717-1730. doi:208/9/1717

Stitt, A., Gardiner, T.A., Alderson, N.L., Canning, P., Frizzell, N., Duffy, N., & Thorpe, S.R. (2002). The AGE inhibitor pyridoxamine inhibits development of retinopathy in experimental diabetes. *Diabetes, 51*(9), 2826-2832.

Sugimoto, K., Yasujima, M., & Yagihashi, S. (2008). Role of advanced glycation end products in diabetic neuropathy. *Current Pharmaceutical Design, 14*(10), 953-961.

Sweazea, K.L., Braun, E.J. (2005) Glucose transport by English sparrow (*Passer domesticus*) skeletal muscle. *Journal of Experimental Zoology*. 303A: 143-153.

Sweazea, K.L. (2006). Glucose transporter expression in english sparrows (passer domesticus). *Comparative Biochemistry and Physiology.Biochemistry & Molecular Biology (2000), 144*(3), 263-270.

Sweazea, K.L., McMurtry, J.P., Braun, E.J. (2006) Inhibition of lipolysis does not affect insulin sensitivity to glucose uptake in the mourning dove. *Comparative Biochemistry and Physiology*. 144B(3): 387-394.

Szwergold, B.S., & Miller, C.B. (2014). Potential of birds to serve as a pathology-free model of type 2 diabetes, part 1: Is the apparent absence of the rage gene a factor in the resistance of avian organisms to chronic hyperglycemia? *Rejuvenation Research, 17*(1), 54-61. doi:10.1089/rej.2013.1498

Tarr, J.M., Kaul, K., Chopra, M., Kohner, E.M., & Chibber, R. (2013). Pathophysiology of diabetic retinopathy. *ISRN Ophthalmology, 2013*, 343560. doi:10.1155/2013/343560

Turinsky, J., O'Sullivan, D.M., & Bayly, B.P. (1990). 1,2-Diacylglycerol and ceramide levels in insulin-resistant tissues of the rat in vivo. The Journal of biological chemistry, 265, 16880–16885.

Ulrich, P., & Cerami, A. (2001). Protein glycation, diabetes, and aging. *Recent Progress in Hormone Research, 56*, 1-21.

Urbanowski, J.C., Cohenford, M.A. & Dain JA. (1982). Nonenzymatic galactosylation of human serum albumin. in vitro preparation. *The Journal of Biological Chemistry, 257*(1), 111-115.

Wada, R., & Yagihashi, S. (2005). Role of advanced glycation end products and their receptors in development of diabetic neuropathy. *Annals of the New York Academy of Sciences, 1043*, 598-604. doi:1043/1/598

Westerterp-Plantenga, M., Diepvens, K., Joosen, A.M., Berube-Parent, S., & Tremblay, A. (2006). Metabolic effects of spices, teas, and caffeine. *Physiology & Behavior, 89*(1), 85-91. doi:S0031-9384(06)00054-0

Whittow, G.C. (1999). *Sturkie's avian physiology.* Academic press.

Wilcox, G. (2005). Insulin and Insulin Resistance. *Clin Biochem Rev.* 26(2), 19-39.

Wuhrer, M., Deelder, A.M, & Hokke, C.H. (2005). Protein glycosylation analysis by liquid chromatography–mass spectrometry. *Journal of Chromatography B, 825*(2), 124-133.

Zhou, H., Isaman, D.J., Messinger, S., Brown, M.B., Klein, R., Brandle, M., & Herman, W.H. (2005). A computer simulation model of diabetes progression, quality of life, and cost. *Diabetes Care, 28*(12), 2856-2863. doi:28/12/2856

APPENDIX A

PROTOCOL FOR HSA/CSA SAMPLE INCUBATION PREPARATION

Methods

Materials:

- Purified Human Serum Albumin
- Purified Chicken Serum Albumin
- HEPES Buffered saline:
  - 134.4 mM NaCl
  - 6 mM KCl
  - 1 mM $MgCl_2$
  - 1.8 mM $CaCl_2$
  - 10 mM NaHEPES
- D-glucose
- DI water

Sample Preparation:

1. Prepare 1 mL of a 500mg/mL glucose stock solution in DI water (final concentration 0.5mg/uL).
2. Make 250mL of HEPES buffer (fill to volume and do not adjust pH at this point)
3. Make 10mL of 8.4mg/mL (84 mg in 10 mL) human serum albumin (HSA) or chicken serum albumin (CSA) dissolved in this high pH HEPES (fill to volume).
4. Adjust pH to 7.4 using the ROSS electrode using HCl and/or NaOH as necessary.
5. Sterile filter solution.
6. Make one 1-mL solutions for each of the samples 1-8 below. Take out three final 150uL aliquots for incubation in the Thermocycler.
   - Sample 1: 0mM glucose HSA
     (1000uL HEPES with HSA)
   - Sample 2: 5.56mM glucose HSA
     (2uL 500mg/mL glucose + 998 HEPES with HSA)
   - Sample 3: 11.11mM glucose HSA
     (4uL 500mg/mL glucose + 996 HEPES with HSA)
   - Sample 4: 22.22mM glucose HSA
     (8uL 500mg/mL glucose + 992 HEPES with HSA)
   - Sample 5: 0mM glucose CSA
     (1000uL HEPES with CSA)
   - Sample 6: 5.56mM glucose CSA
     (2uL 500mg/mL glucose + 998 HEPES with CSA)
   - Sample 7: 11.11mM glucose CSA
     (4uL 500mg/mL glucose + 996 HEPES with CSA)
   - Sample 8: 22.22mM glucose CSA
     (8uL 500mg/mL glucose + 992 HEPES with CSA)

7. Incubate at 37.0, 39.8, or 41.4°C for seven days, taking a 2 uL aliquot off each sample at baseline, end of day 3, and end of day 7.

APPENDIX B

PROTOCOL FOR LC-ESI-MS ANALYSIS OF HSA/CSA SAMPLES FOR PERCENT GLYCATION

Machine Preparation:

1. Turn MS from 'stand-by' mode to 'operate' mode
2. Change loading flow from 3µl/min to 10µl/min and change micro flow from 0.105µl/min to 3µl/min
3. Screw in the connection between the LC and MS
4. Clean source using methanol and water
5. Clean needle using methanol and water

Sample Preparation:

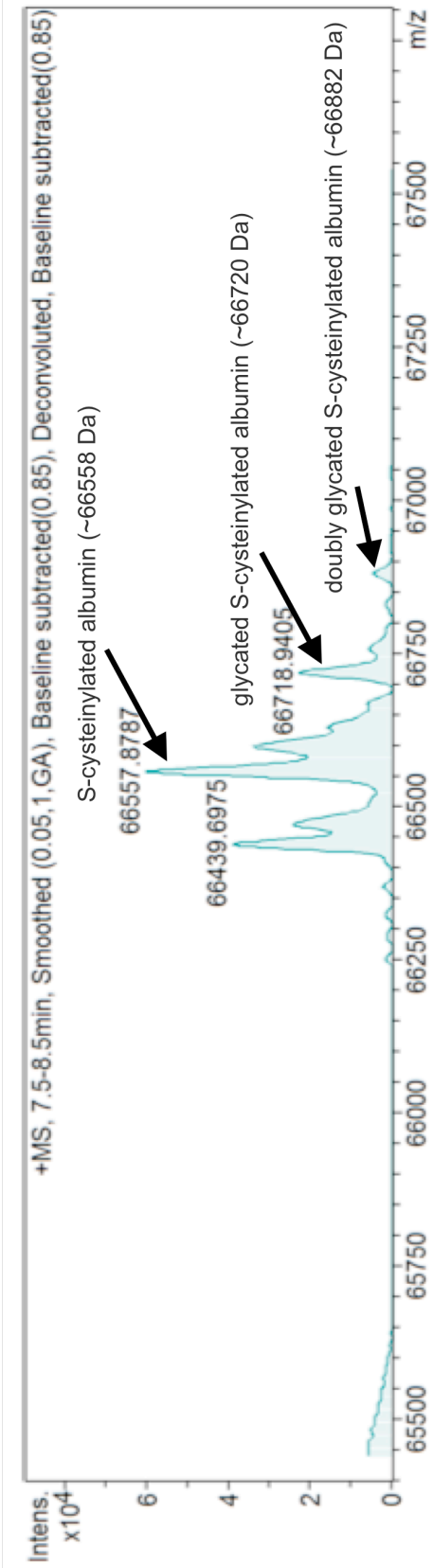
8. Prepare 5ml 0.1% TFA by combining 4.95ml distilled H_2O with 50µl 10% TFA stock solution.
9. Remove sample from -80°C and place on ice.
10. Prepare 30:1 dilution of HSA/CSA sample by combining 15µl 0.1% TFA with 0.5µl sample. Vortex diluted sample.
11. Load 15µl of the diluted sample into one well of the 96-well plate.
12. Load 40µl of 0.1% TFA into a separate well of the 96-well plate.
13. Load 96-well plate onto the plate-holder of the LC-MS.
14. Acquisition the two wells as follows:
    a. Sample well-
        i. Injections: 1
        ii. Amount: 5.000µl
        iii. LC Method Part: crb_protein trap and ramp
        iv. Autosampler Method Part: Standard
        v. MS Acquisition Method Part: 150126 protein
    b. Solvent well-
        i. Injections: 1
        ii. Amount: 10.000µl
        iii. LC Method Part: crb_protein trap and ramp
        iv. Autosampler Method Part: Standard
        v. MS Acquisition Method Part: 150126 protein

Spectra Analysis:

1. Open Spectra in Compass DataAnalysis
2. Average raw spectra across the approximate 1 min (HSA) or 0.5 min (CSA) timeframe of sample elution
3. Copy to compound spectra
4. Smooth spectra (0.0492 Da)
5. Baseline subtraction (0.85)
6. Charge Deconvolution
7. Baseline Subtraction (0.85)
8. Record the mass (m/z) and counts for the relevant peaks for HSA samples (native albumin, S-cysteinylated albumin, glycated albumin, s-cysteinylated + glycated albumin, and s-cysteinylated + doubly glycated albumin) or for CSA samples (native albumin, glycated albumin, doubly glycated albumin)
9. Calculate Percent Glycated Albumin for each spectrum using the appropriate counts.
10. Save spectra

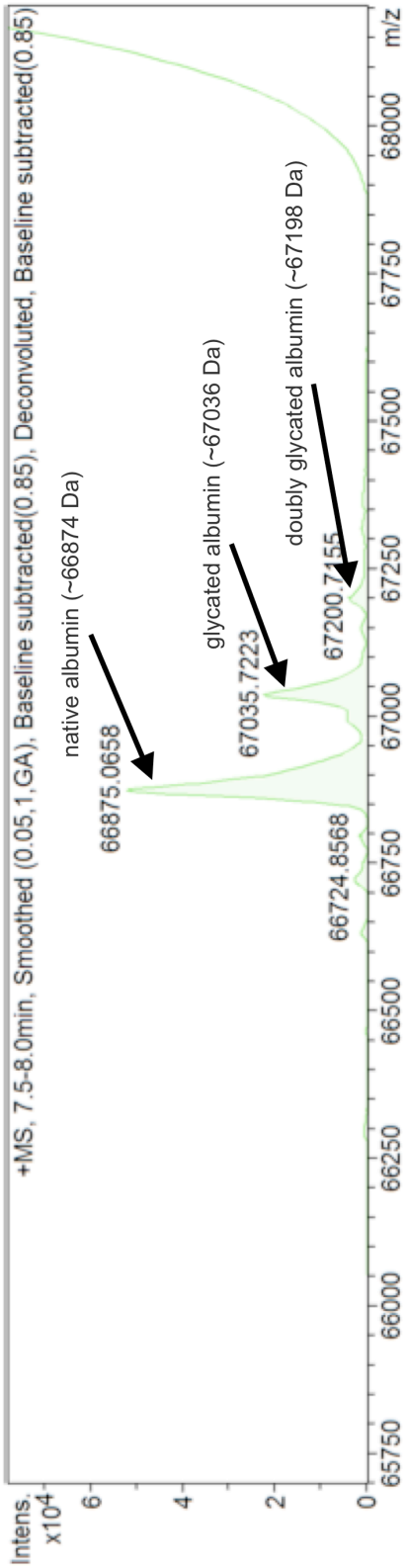
APPENDIX C

EXAMPLE OF HUMAN SERUM ALBUMIN MASS SPECTRUM FROM A SAMPLE INCUBATED AT 39.8°C AT 11.11mM GLUCOSE EXTRACTED ON DAY 3


+MS, 7.5-8.5min, Smoothed (0.05,1,GA), Baseline subtracted(0.85), Deconvoluted, Baseline subtracted(0.85)
Intens.
x10^4
6
4
2
0
65500
65750
66000
66250
66500
66750
67000
67250
67500
m/z
66557.8787
66439.6975
66718.9405
S-cysteinylated albumin (~66558 Da)
glycated S-cysteinylated albumin (~66720 Da)
doubly glycated S-cysteinylated albumin (~66882 Da)

APPENDIX D

EXAMPLE OF CHICKEN SERUM ALBUMIN MASS SPECTRUM FROM A SAMPLE INCUBATED AT 39.8°C AT 11.11mM GLUCOSE EXTRACTED ON DAY 3


+MS, 7.5-8.0min, Smoothed (0.05,1,GA), Baseline subtracted(0.85), Deconvoluted, Baseline subtracted(0.85)
Intens.
x10^4
6
4
2
0
65750
66000
66250
66500
66750
67000
67250
67500
67750
68000
m/z
native albumin (~66874 Da)
66875.0658
66724.8568
glycated albumin (~67036 Da)
67035.7223
doubly glycated albumin (~67198 Da)
67200.7155