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INSULIN RESISTANCE IN DIABETES MELLITUS:
FORMATION OF ADVANCED GLYCOSYLAION ENDPRODUCTS (AGEs) ON
INSULIN RECEPTORS IN 3T3-L1 ADIPOCYTES:
A NOVEL MODEL OR MERIE PHENOMENON?

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in the Graduate School of
The Ohio State University

by

David A. Smith, M.S, D.C.

*****

The Ohio State University
1997

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ABSTRACT

Diabetes mellitus is a disease characterized by chronic fasting hyperglycemia. Hyperglycemia is widely believed to be the primary pathophysiological mechanism underlying the complications that comprise a significant portion of the morbidity, mortality and healthcare expenses associated with diabetes.

One effect of long term hyperglycemia is the formation of irreversible adducts, commonly referred to as advanced glycosylation endproducts (AGEs), via the nonenzymatic Maillard reaction. The pathological consequences associated with AGE formation include alterations in the physical and chemical properties of many molecules that are important in cellular communication, membrane integrity, and metabolic pathways.

Insulin resistance, or a diminished tissue sensitivity to insulin, is a hallmark feature seen in non-insulin dependent diabetes mellitus (NIDDM) or type II diabetes. The relative degree of insulin resistance appears to be a function of the level of hyperglycemia. In Insulin resistance the ability of insulin to stimulate target cells through the receptor is greatly impaired. The specific mechanism of this impairment has not been clearly described.
The global hypothesis for this dissertation is that the formation of advanced glycosylation end product (AGEs) adducts on insulin receptors inhibit insulin receptor binding by altering the physical and chemical properties of the insulin receptor. This alteration in the insulin receptor is demonstrated clinically as insulin resistance. The specific aims of this dissertation were to glycate insulin receptors in 3T3-L1 adipocytes, measure the functional, biochemical and physical properties of the insulin receptors as a function of the amount of glycation and to monitor AGE formation in human erythrocytes as a demonstration of the clinical applications of AGE formation.

I found a significant, yet reversible increase in insulin receptor-ligand binding affinity directly related to glycation at physiological levels. There was also a significant increase in the amount of cross-linking within the holoreceptor and significant changes in the isoelectric point directly related to glycation. Functional changes were also noted. There was a significant decrease in the amount of glycogen deposition related to glycation. However, the ability of the adipocyte to store glycogen was not totally abolished. There was no significant effect on
activation of hexokinase activity related to the amount of glycation. Phosphorylation of tyrosine kinase was also impaired. The results obtained indicate that there is impairment within the insulin receptor physical and chemical properties related to the amount of non-enzymatic glycation. These altered physical and chemical properties appear to support the concept of a novel model for the development of insulin resistance based on the formation of AGEs in insulin receptors.
"The road to failure is the path of least persistance."

- Ziggy -

For my wife Dee Dee,

You allowed me to maintain a sense of reality through all the insanity.

For my children, Kelsey and Erik,

Every day you reminded me of what is really important in life.
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I want to thank my co-advisors, Dr. Karla Roehrig and Dr. Kory Ward. They both saw the potential that I possessed, and took the chance to help me fully develop that potential.
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Ed; ed by Kaplan & Pesche

of medians and "average of Normals" of patients' data for
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(gHb) testing reliability. Clinical Chemistry; 41(6):S198

FIELDS OF STUDY

Major Fields:
Diabetic complications attributed to AGE formation.
Clinical laboratory medicine.

Minor Fields:
Effect of AGE formation in non-A variant hemoglobin on
glycohemoglobin testing.
Point of Care Testing.

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DEFINITIONS

Glycation - A non-enzymatic condensation of carbohydrate and protein.

Glycosylation - A post translational modification of protein by addition of a carbohydrate moiety.
ABBREVIATIONS

AGE...........Advanced Glycosylation End Products
GHb..........Total Glycated Hemoglobin
gHb..........Glycohemoglobin
HbA1c.......Hemoglobin A1c
CML.........Carboxymethyl lysine
FFA..........Free Fatty Acids
DCCT.........Diabetes Complications and Control Trial
IRS-1......Insulin Receptor Substrate 1
CHAPTER 1

INTRODUCTION

Diabetes mellitus is a disease characterized by a chronic fasting hyperglycemia that affects sixteen million Americans and accounts for approximately 12 to 15% of the annual total healthcare expenditures in the United States. (ADA Vital Statistics 1996, Rubin et al 1992, Raskin 1994, Geiss 1995) A significant portion of the morbidity, mortality and healthcare expense associated with diabetes is attributable to the development of complications such as nephropathy, retinopathy, and cardiovascular disease. (Brownlee et al 1988, Hunt et al 1990, Beisswenger et al 1993a, Deininger 1993, Vlassara 1994) Hyperglycemia is widely believed to be the primary pathophysiological mechanism underlying all of these complications. (Brownlee et al 1988, Vlassara 1994)

Long term hyperglycemia has either a direct toxic effect on susceptible tissue, or results in the formation of irreversible adducts referred to as advanced glycosylation endproducts (AGEs). (Njorge and Monnier 1989, Bucala et al 1995a, Strowig and Raskin 1995) The pathological
consequences associated with the formation of AGEs include alterations in the physical and chemical properties of receptors, regulatory and structural proteins, nucleic acids and other biological compounds. (Vlassara 1994, Bucala et al 1995a) Physical alterations in these compounds have been associated with altered receptor functioning, decreased tissue friability, increased mutation rates, and altered rates of mRNA production. (Hunt et al 1990, Beisswenger et al 1993a, Deininger 1993) Specific complications related to AGE formation seen in diabetes mellitus include cross-linking of collagen with thickening of the renal tubular basement membranes, leading to nephropathy; activation of immune responses in the vascular intimal layers leading to formation of atherosclerotic plaque; alterations in binding capacity of low density lipoprotein receptors leading to dyslipidemia and accelerated atherogenesis; and alterations in the retinal membranes leading to retinopathy. (Brownlee et al 1988, Hunt et al 1990, Deininger 1993, Vlassara 1994)

A diminished tissue sensitivity to insulin, known as insulin resistance is a hallmark feature seen in non-insulin dependent diabetes mellitus (NIDDM) or type II diabetic individuals (Raskin 1994, Hagino et al 1994) Insulin resistance is one of the predominant clinical signs in individuals who are unable to control their hyperglycemia. (Hagino et al 1994, Gugliucci 1994, White and Kahn 1994) The relative degree of insulin resistance appears to be a
function of the level of hyperglycemia. (Schade and Boyle
1992) In individuals with insulin resistance there is
apparently adequate insulin available, indeed they commonly
have a hyperinsulinemia. (Raskin 1994) However, the ability
of insulin to stimulate target cells through the receptor is
greatly impaired. (Haring et al 1994, Raskin 1994) Some
researchers believe that the glucose fatty-acid cycle
proposed by Randle et al (1963) provides a mechanism for the
development of glucose intolerance, insulin resistance, and
type II diabetes. However, the specific mechanisms leading
to insulin resistance have not been clearly described either
with in vivo or in vitro experiments.

Insulin resistance can be defined on both a systemic
and a cellular level. Systemically, insulin resistance can
best be described as a defect in the ability of the
individual to provide adequate glucose to those tissues that
are insulin responsive. Insulin action in these tissues
initiates characteristic metabolic and mitogenic actions.
At the cellular level insulin resistance is best
characterized by a decrease in the ability of the insulin
receptor to stimulate the uptake of glucose into the cell.
(Schade and Boyle 1992) Glucose is required by those
insulin responsive tissues for such metabolic functions as
glycolysis in hepatocytes, triglyceride production in
adipocytes, and ATP production in myocytes. At this level
insulin resistance could result from a defect at any point

3
in the insulin signaling pathway. This defect is demonstrated by inappropriate binding kinetics of the ligand-receptor interaction, or inability to generate an appropriate signal within the target cells, or inability of that signal to effect the subsequent metabolism of glucose within the cell (post receptor defect).

**Hypothesis**

Formation of advanced glycosylation end product (AGEs) adducts on insulin receptors inhibits insulin receptor binding by altering the physical and chemical properties of the insulin receptor.

**Objective**

This research focused on the effects commonly seen in type II diabetes. The specific model system for this research is designed to approximate the hyperglycemic conditions which might be found in type II diabetes. The objective of this project is to demonstrate that formation of advanced glycosylation end products (AGEs) on the insulin receptor alters the physical and chemical properties of the insulin receptor. AGE adduct formation and the subsequent alteration in the physical and chemical properties of the insulin receptor was tested as a potential mechanism for the development of insulin resistance.

**Specific Aims**

The specific aims of this study include glycation of insulin receptors obtained from 3T3-L1 adipocytes;
measurement of the functional, biochemical and physical properties of the insulin receptors as a function of amount of glycation; and evaluation of the rate of AGE formation in human erythrocytes.

The initial component of this dissertation is to demonstrate that glycation occurs in adipocytes. This is accomplished by incubation of adipocytes in physiologically relevant levels of glucose. Then the amount of glycation was measured as a function of formaldehyde release via a periodate oxidation. (Ahmed and Firth 1991) The principle model system is the Swiss mouse embryonic fibroblast, 3T3-L1 cell line. These cells undergo a terminal differentiation to adipocytes commonly found in white adipose tissue under appropriate conditions. (Green and Meuth 1974, Greene and Kehinde 1974, 1975) Fully differentiated 3T3-L1 cells are insulin sensitive, and are commonly used to study the effects of insulin resistant states.

Following glycation and AGE formation the effects of in vitro AGE formation on the physical and chemical characteristics of the insulin receptor were tested. This includes an analysis of the receptors' isoelectric point, extent of dimerization, potential cross-linking and molecular weight as determined by isoelectric focusing, non-denaturing gel electrophoresis, and denaturing gel electrophoresis. In addition an analysis of the binding affinity between insulin and the insulin receptor was
determined by Scatchard analysis. Effects of both dose and time was determined for AGE formation on the insulin receptor in vitro. The ability of insulin to dissociate from the receptor, as well as the number of cryptic receptors was determined by affinity binding studies.

Next the concept that AGE formation on the insulin receptor is a potential mechanism for increasing insulin resistance seen in type II diabetic individuals was tested. In this model increased serum glucose levels result in an increase in the amount of AGE formation on proteins, including receptor proteins. In insulin receptors, AGE formation on or near the insulin binding sites alters the binding site such that insulin is not bound appropriately. Inappropriate ligand binding is expressed as a decrease in metabolic activity of the cell. Clinically a decrease in metabolic activity at the cellular level is described as a decrease in insulin sensitivity, or insulin resistance. This phase of the project explored the effect that AGE-receptor adducts have upon the ability of the insulin receptor to generate a signal through it's intrinsic tyrosine kinase activity, as well as the ability to influence glucose metabolism within the cell.

A study of in vitro hemoglobin glycation provides a bridge between experimental and clinical applications of AGE formation. This portion of the study used an in vitro human erythrocyte model. Glycated hemoglobin (gHb) is a clinical
measure of glycemic control. Current methods measure either the A1c fraction of hemoglobin or the total glycated hemoglobin. In either case, current gHb measures are semi-quantitative and do not adequately consider the rate of glycation. Whole blood samples from non-diabetic individuals were incubated in glucose to determine a dose and time dependent rate of glycation.
CHAPTER 2

LITERATURE REVIEW

DIABETES MELLITUS

Diabetes mellitus is a syndrome characterized by chronic fasting hyperglycemia, glucose intolerance and disturbances of carbohydrate, fat and protein metabolism associated with a relative or absolute deficiency in insulin secretion or action. (ADA Vital Statistics 1996, Bennett 1994, Raskin 1994) It is a chronic and potentially disabling disease. Diabetes mellitus affects nearly 16 million Americans and accounts for approximately 12 to 15% of the total health care expenditures, making it one of the major public health concerns in this country today. (Rubin et al 1992, Songer 1992) While we often think of diabetes solely as a clinical problem in western society, it is in fact a chronic disease of growing significance in many economically developing countries worldwide. (Songer 1992)

The National Diabetes Data Group (NDDG) of the National Institutes of Health established guidelines or classifications for the diagnosis and treatment of diabetes
in the United States in 1979. (Bennett 1994) The World Health Organization (WHO) adopted these classifications in 1980 under the broad category of glucose intolerance. (Bennett 1994) These classifications include insulin-dependent diabetes mellitus (IDDM) or Type I diabetes, non-insulin-dependent diabetes Mellitus (NIDDM) or type II diabetes, gestational diabetes mellitus, and secondary diabetes mellitus. (ADA Vital Statistics 1996, Bennett 1994)

Insulin-dependent Type I diabetes mellitus (IDDM) is defined clinically by the presence of classical symptoms of polyphagia, polydipsia, polyuria, muscle wasting and ketoacidosis. (Bennett 1994) Insulin treatment is required because insulin production is either absent or insufficient. (ADA Vital Statistics 1996, Bennett 1994) This type of diabetes comprises approximately 5-10% of the known cases of diabetes in the United States. (ADA Vital Statistics 1996, Raskin 1994) Prior to the NDDG classification system this type of diabetes was often labeled juvenile onset. Most cases of IDDM are initially diagnosed in individuals under 30 years old, and nearly all individuals less than 20 years old at the time of diagnosis have IDDM. (ADA Vital Statistics 1996)

Type II diabetes comprises approximately 90-95% of the total cases of diabetes in the United States. (Raskin 1994) Patients with type II diabetes are characteristically asymptomatic until middle age or when complications of
diabetes become clinically apparent. They may or may not have the classical symptoms of polyphagia, polydipsia and polyuria and generally do not demonstrate muscle wasting or ketoacidosis except under severe stress. (Bennett 1994, Raskin 1994) Age, obesity and hyperinsulinemia are the principal clinical characteristics that distinguish NIDDM from IDDM. (ADA Vital Statistics 1996) These patients are usually over the age of 30 at the time of diagnosis and commonly have a strong family history of type II diabetes. (Bennett 1994, Raskin 1994) Obesity, or a history of obesity is seen in approximately 80% of patients with type II diabetes. (Raskin 1994) Hyperinsulinemia at the time of diagnosis is common, and is thought to result from a decreased sensitivity or responsiveness to insulin. (ADA Vital Statistics 1996) This decreased insulin sensitivity is termed insulin resistance. While obesity will exacerbate insulin resistance and may precipitate the hyperglycemia commonly seen in type II diabetes, it is not necessary for the development of type II diabetes. (Bennett 1994)

Patients with type II diabetes are often able to control the hyperglycemia with a combination of appropriate dietary and exercise regimens. Oral antihyperglycemic medications are commonly used when dietary and exercise regimens do not provide the level of glycemic control necessary. However, obesity often diminishes the effectiveness of these drugs. Insulin is not always
required in patients with type II diabetes, however some patients will require exogenous insulin to control their hyperglycemia. (Raskin 1994) Patients with type II diabetes commonly demonstrate insulin resistance and may develop hypoinsulinemia as the disease progresses. (ADA Vital Statistics 1996) In either case there is a reduction in insulin activity necessitating increased exogenous insulin to fully control the hyperglycemia.

Type II diabetes can be further categorized into types of defects in insulin action (intracellular defect, receptor defects, abnormal insulin structure); defects in insulin secretion (defects in signaling, destruction of β-cell mass); unknown pathogenesis (type II diabetes secondary to another disease); unclassified (underlying abnormality is uncertain). (Bennett 1994) The key concept in type II diabetes is that the disease is in reality a consequence of a deficiency in insulin action that normally occurs at the level of the target tissue or cell as opposed to a frank defect or absence in insulin itself. (ADA Vital Statistics 1996, Bennett 1994)

Epidemiology

Individuals with type II diabetes often demonstrate some classic patient demographics. The average type II diabetic individuals are over 50 years old, with a strong family history of type II diabetes, and tend to be obese (body weight greater than 20% of ideal), or have a history
of obesity. (Rubin et al 1992, Songer 1992) Other risk factors include African, Hispanic, or Native American ancestry, previously identified glucose intolerance, hypertension, hyperlipidemia (cholesterol ≥240 mg/dL or triglycerides ≥250 mg/dL), and a history of gestational diabetes or delivery of an infant greater than nine pounds. (ADA Vital Statistics 1996)

Type II diabetes is apparently rising faster in developing nations of the world, suggesting that as cultures become more "Westernized" or affluent their populations become more obese and more susceptible to developing diabetes. (Songer 1992)

Type II diabetes mellitus is the most common form of diabetes. There are approximately 625,000 new cases of type II diabetes and approximately 13,200 new cases of type I diabetes in the USA each year. (ADA Vital Statistics 1996) Type II diabetes comprises nearly 85% of all cases of diabetes mellitus world wide. (Bennett 1994, Warram et al 1994) Approximately 49% of all new cases occurs in individuals over 55 years. (ADA Vital Statistics 1996)

The average annual incidence rate (1990-1992) in the US is 2.4 per 1,000 people. The incidence varies with age, ranging from 1.8 per 1,000 at age 24-44, to 8.6 per 1,000 at 65-74. (ADA Vital Statistics 1996)

The overall incidence of type II diabetes has steadily increased from a rate of 0.4 per 1,000 in 1935 to 2.4 per
1,000 in 1990-1992. (ADA Vital Statistics 1996) Further, the incidence of diabetes appears to be increasing faster in the minority segments of the population. (ADA Vital Statistics 1996, Songer 1992) It is not yet clear whether this increase in the incidence of type II diabetes among the minority populations is real or artifactual. (Warram et al 1994) It is thought that genetic factors, increased caloric intake, or adaptation to western society may all play a role in the increase in incidence seen with minority populations in the US. (Hamman 1992, Levitt et al 1993, ADA Vital Statistics 1996) However, it may be a situation where improved screening and access to more modern health care facilities provide a more accurate assessment of the incidence of type II diabetes within minority populations. (Munetta et al 1993, Sekikawa et al 1993, Levitt et al 1993)

While the number of new cases, or incidence, of type II diabetes within the United States is considerable, the prevalence represents a significant public health concern. Approximately 7.8 million individuals in the United States reported having a diagnosis of diabetes in 1993. (ADA Vital Statistics 1996, Rubin et al 1992, Raskin 1994) This represents approximately 3.1% of the total population, and includes all types of diabetes. The actual prevalence rate for diabetes in the general population is probably considerably higher because type II diabetes has a long asymptomatic period. The long asymptomatic period leads to
a situation where a significant number of adult cases of type II diabetes are undiagnosed and therefore go unreported. In fact, the prevalence of undiagnosed diabetes may be as high as that for individuals with a confirmed diagnoses of diabetes. This means that as many as 16 million Americans or 6% of the population are diabetic. (ADA Vital Statistics 1996, Rubin et al 1992, Raskin 1994)

Prevalence rates also vary according to ethnic background, age and environmental influences. (ADA Vital Statistics 1996, Warram et al 1994). Pima Indians, African Americans and Hispanics have the highest number of total cases of type II diabetes in the United States, while European and Asian populations all have significantly fewer numbers of diagnosed type II diabetic individuals. (Warram et al 1994)

The highest prevalence is among Pima Indians over the age of 45 (50%). This is followed by African Americans over age 65 (19.9%), Hispanics over the age of 45 (14.3%) and non hispanic whites over the age of 65 (10.2%). With all races combined, the highest prevalence for type II diabetes is in the over 65 year old group (10.7%).(ADA Vital Statistics 1996)

The overall prevalence of diabetes has increased steadily since 1935. A part of the increase results from the aging of the US population, a decrease in the mortality of individuals with diabetes, and improved diagnostic and screening procedures. (ADA Vital Statistics 1996) Much of
the increase in diabetes is probably real and owing to increases in risk factors such as obesity and physical inactivity. (ADA Vital Statistics 1996) There have been a wide range of epidemiological and cross-sectional studies that have provided both direct and indirect evidence supporting the concept that physical inactivity increases the risk for the development of type II diabetes. (Kriska and Bennett 1992) Many of these studies have followed individuals that emigrate from a culture where strenuous daily physical activity is the norm, to a more "westernized" culture where physical inactivity is the norm. In nearly all of the studies the incidence of type II diabetes is inversely related to physical activity. (O'Dea 1992, Kriska and Bennett 1992) Closely associated with physical activity is obesity. Body composition changes with age. Muscle mass diminishes and adiposity increases with age. (Sowers 1992) Changes in body composition are due to both natural aging process, and lifestyle changes. Older individuals tend to be less active and eat more than their younger counterparts. This is more conducive to increasing adiposity than to maintaining or increasing muscle mass. (Hamman 1992, Kriska and Bennett 1992) Insulin sensitivity is inversely related to adiposity, especially central or "android" obesity commonly seen with type II diabetes. Hypertrophic adipocytes, the predomint cell type in centralized obesity, demonstrate a high degree of insulin resistance. (Sowers 1992)
Economic Cost of Diabetes

The total cost of diabetes mellitus has steadily increased over the past 15 years at a rate that exceeds all other health care costs. In 1969 the Statistical Bureau of the Metropolitan Life Insurance Company estimated the economic impact of diabetes at $2.6 billion. (Songer 1992) By 1984 the total cost of diabetes based on a conventional prevalence-based cost-of-illness technique had risen to $14 billion. (Entmacher et al 1985) The total economic burden attributable to diabetes was estimated at $91.8 billion in 1991. (Geiss 1995) While different studies have measured the total costs in different ways, it is generally accepted that direct costs, or actual health care expenditures, have risen much faster than indirect costs, or costs related to lost productivity associated with diabetes. (Songer 1992) Direct costs rose from $7.4-12.0 billion in 1985 to $45.2 billion in 1991. (Entmacher et al 1985, Geiss 1995) In 1995, it was estimated that approximately one in every seven health care dollars is spent on diabetes care. (Rubin et al 1992) This means that confirmed diabetics incur health costs 4.28-fold greater than nondiabetics and that nearly 15% of the total health care expenditures in the United States were incurred by less than 5% of the population. (Rubin et al 1992) The total health care costs incurred by diabetic individuals includes estimated costs associated with inpatient hospital care, outpatient care, professional
office visits, emergency room care, dental care, prescription drugs, home care, and durable medical equipment. This does not include costs for nursing home care or indirect costs associated with foregone productivity and premature death, nor for undiagnosed diabetes. (Entmacher et al 1985, Rubin et al 1992) A significant proportion of these costs are due to chronic complications, increased use of health care services and excess mortality. (Geiss 1995) In fact, while hospitalization costs accounted for 41% of the total costs and approximately 80% of direct costs, 30% of hospitalization costs involved chronic complications of diabetes or comorbidities. (Geiss 1995)

Pathophysiology of Diabetes

Diabetes is a heterogenous disorder where genetic and environmental factors are important to both its development and progression. (Raskin 1994) Even though the specific etiology of type II diabetes is still unclear, genetic and environmental influences are readily apparent. (Raskin 1994) A concordance rate among monozygotic twins of 58-75% provides strong evidence for a genetic component. (Raskin 1994) A strong correlation between obesity and the onset of type II diabetes indicates a genetic component with a strong environmental relationship. (Raskin 1994) Recent work with the ob-gene product leptin points to a genetic predisposition towards obesity and insulin resistance. Kieffer et al (1996) proposed the concept that leptin is an
integral component of a feedback loop between adipose tissue and the endocrine pancreas. The expression of leptin, and hence appropriate operation of the feedback loop, is affected by environmental influences such as food intake and cold exposure. (Hardie et al 1996, Meier 1996)

Type II diabetes mellitus may be asymptomatic and is commonly discovered as a result of a routine blood or urine glucose test. (Bennett 1994) Classically type II diabetic patients are generally over 40 years of age, have a strong family history of type II diabetes mellitus, are markedly obese (greater than 20% over ideal body weight), with a history of hypertension and/or hyperlipidemia, especially hypertriglyceridemia. (Bennett 1994, Raskin 1994) Patients may or may not present with the classic symptoms of polyuria, polyphagia, and polydipsia that are characteristic of type I diabetes. (Bennett 1994) Increasingly, individuals with type II diabetes come from a minority racial background such as American Indian, Hispanic, or African American. (Bennett 1994, Raskin 1994) The NDDG and WHO have established guidelines for the diagnosis of diabetes. These guidelines confirm a diagnosis of diabetes in susceptible individuals when the random serum glucose concentration is greater than 200 mg/dL or the fasting serum glucose concentration is greater than 140 mg/dL on at least two occasions. (Raskin 1994) The differential diagnosis between type I and type II diabetes is commonly made by
patient demographics and a determination of the bioavailability of insulin.

A diagnosis of insulin resistance is often indicated when the insulin dosage regimen is not responsive and the patient is following an appropriate dietary and exercise regimen while decreasing their weight. Insulin resistance may be seen in individuals with overt diabetes, impaired post prandial glucose tolerance or euglycemia. The overall degree of glucose tolerance is determined by the response of the β-cell to glucose and the magnitude of the peripheral insulin resistance. (Goldstein 1994) Insulin resistance can be confirmed by administration of a glucose clamp test. The glucose clamp test involves infusion of a fixed dose of glucose (100 μU/mL) into a patient and then determining the amount of exogenous insulin required to maintain normoglycemia. (Boden et al 1993, Kohrt et al 1993) A non-insulin resistant adult should require approximately 100-200 mg·m⁻²·min⁻¹, while individuals with insulin infusion rates greater than 150 mg·m⁻²·min⁻¹ (1.5-2.0 U/kg body weight) are considered insulin resistant. (Schade and Boyle 1992, Goldstein 1994) This test is not commonly performed in a clinical setting because the patient must be truly following a dietary and drug regimen that is appropriate to their glycemia. (Goldstein 1994)
Complications of Diabetes

Individuals with type II diabetes are at an increased risk for development of complications related to ophthalmic, renal, neurological, cerebrovascular, cardiovascular, and peripheral vascular diseases. (Rubin et al 1992, Raskin 1994) These complications mean that type II diabetics are more likely to have heart attacks, strokes, amputations, kidney failure, and blindness than the general population. (Rubin et al 1992) In fact the Diabetes Complications and Control Trial (DCCT) determined that nearly all individuals suffering from diabetes will develop tissue damaging complications. (DCCT 1993) The importance of complications and their relationship to hyperglycemia was underscored by the DCCT which determined that a reduction in mean daily blood glucose concentration of 50 mg/dL, as estimated by glycated hemoglobin, resulted in a 60% reduction in the severity of diabetic complications. (DCCT 1993) While the DCCT only considered individuals with type I diabetes mellitus, there is sufficient evidence to suggest that the findings also apply to type II diabetics. (Crofford 1995, Turner and Holman 1995, Colwell 1996, Tattersall 1995, Carey 1995) Many of these complications have been associated with the non-enzymatic glycosylation of structural, functional and regulatory proteins, especially collagen, hemoglobin, and lipoproteins. (Brownlee et al 1988) Individuals with diabetes mellitus classically exhibit elevated levels of
circulating protein and lipid bound AGEs. (Bucala et al 1995a) While many of these compounds are formed via the non-enzymatic condensation described below, a significant portion arise from the catabolism of AGE-modified tissue proteins. (Makita et al 1991, 1994, Bucala et al 1995a, 1995b)

Glycemic control, as measured by glycated hemoglobin, has a close positive association with AGE formation. (Beisswenger et al 1993a, 1993b) Beisswenger et al (1993a) observed that the level of glycemic control over a one year period is highly predictive of AGE levels. On the other hand, Tilton (1993) describes a scenario where diabetic complications may not be due to AGE formation, but rather due to nitric-oxide toxicity. Nitric-oxide toxicity results in increased formation of oxidized metabolites, eg lipid peroxides, that have been closely related to cellular injury. (Gross and Wolin 1995, Tilton et al 1993) This concept is partially supported by the observation that glucose generates hydroxyl free radicals under conditions conducive to protein glycosylation. (Hunt et al 1993) Free radicals formed under these conditions do lead to significant structural alterations in proteins that include fragmentation of the protein. (Hunt et al 1993) However, the glucose concentrations (25 mM) required for this effect are not commonly seen in physiological conditions.
Complications seen in diabetes can be divided into three major areas: vascular disease, nephropathy and neuropathy. (Santiago 1986, Haas 1993) Vascular disease is further divided into macrovascular complications and microvascular complications, which includes retinopathy. Microvascular disease involves the smaller blood vessels (eg arterioles, capillaries and venules), and results in a decrease in blood flow to the target organs that often results in ischemia which leads to visual loss, kidney failure and multiple neurological signs and symptoms. Macrovascular disease involves the larger vessels and is primarily due to atherosclerosis of blood vessels with complications of angina, heart attacks, strokes and amputations resulting from ischemia. (Steffes 1986, Greene and Lattimer 1986) The collagen in vascular structures is the presumed site of pathology in diabetes because the accumulation of AGEs on collagen and other proteins produce functional and structural changes in vascular tissues (Beisswenger 1993, Brownlee et al 1988) The precise mechanism is still unclear because tissue remodeling and receptor-turnover processes are variable for different tissues, and because it is not clear which of the many possible AGE forms plays the most important role in development of vascular sequelae. (Beisswenger et al 1993b) Atherosclerosis in diabetes has been linked to oxidative damage from AGE-modified low density lipoprotein (LDL)
particles. (Hunt et al 1993, Bucala et al 1994, 1995b) This damage initiates an immunological reaction mediated by monocytes and tissue macrophages. (Bucala et al 1994, Schmidt et al 1994) AGE formation also impairs the ability of the LDL receptor to recognize, bind and initiate uptake of AGE-modified LDL particles. (Bucala et al 1995b) This impairment is both dose and time dependent, with a decreasing binding affinity seen with increasing time of exposure to hyperglycemic conditions. (Bucala et al 1995b) Some LDL particles (eg apo B) appear to accumulate more AGE products because they possess a high content of basic amino acids (eg lysine, arginine, histidine) including numerous sites of contiguous, basic residue repeats. (Bucala et al 1995b)

Ophthalmic complications include proliferative retinopathy, cataract formation and increased intraocular pressure. Hyperglycemia alters the osmotic balance of the vitreous, increasing intraocular pressure and contributing to vision loss. Non-enzymatic glycosylation of the lens proteins of the eye contribute to vision loss via cataract formation. There is also a direct relationship between hyperglycemia and the development of diabetic retinopathy. (Beisswenger et al 1993a) Retinopathy is a direct result of microvascular complications leading to vascular proliferation and vision loss. The presence of hyperglycemia appears to be the major predictor of the early
development of proliferative retinopathy. (Krolewski et al 1992, 1986) The DCCT and other studies have shown that the severity of the hyperglycemia rather than the type of diabetes predicts the progression of background retinopathy that has a potent imprinting effect that is difficult to erase in less than four years. In actual numbers this translates to any increase in mean blood glucose greater than 30 mg/dL for more than four years, increases the risk of proliferative retinopathy by 30-50%. (Santiago 1993, DCCT 1993) In addition, diabetic individuals with the highest risk of developing proliferative retinopathy during the second decade of diabetes had the highest hyperglycemia during the first 15 yrs of diabetes. (Krolewski et al 1986) In a study of young persons with diabetes, those individuals who maintained a normoglycemia had neither evidence of retinopathy nor increased albuminuria. (Vlassara et al 1986) While those individuals that had hyperglycemia 1.5 times the upper limit of normal had retinopathy (37%) and increased albuminuria (29%). (Vlassara et al 1986)

Nephropathy is due to both microvascular impairment of the blood flow to the kidney and thickening of the basement membrane, each can eventually lead to kidney failure. The mechanism for impaired blood flow to the kidney is the same as seen in microvascular and macrovascular disease. Cross-linking of the collagen and other protein components of the basement membrane contribute to the development of
nephropathy. As was seen in other complications of diabetes, there is a direct relationship between hyperglycemia and nephropathy. (Beisswenger et al 1993a)

The primary neurological complication is neuropathy. Neuropathy can be caused by microvascular impairment of the nutritive supply to nerves, schwann cells and oligodendrocytes, as well as non-enzymatic glycosylation of the neuronal membranes. Nerve conduction deficits are the most notable signs with peripheral paraesthesia the most common symptoms. (Santiago 1986, Steffes et al 1986, Vinik et al 1992) Accumulation of maillard-like products in the polyol pathway has also been proposed as a possible mechanism for neuropathy. There is considerable evidence to support both theories, and the real picture is likely to include aspects of both mechanisms. Central nervous system involvement in the form of deficits in spatial and behavioral functions may also be involved in advanced diabetic neuropathy. (Belush and Reid 1991, Kern et al 1994, Ziegler et al 1994) However, the specific mechanisms of diabetic induced neuropathy are still unclear.

**Ligand-Receptor Interactions**

Insulin is a multifunctional hormone with both mitogenic and metabolic properties. Insulin is considered a mitogen because it acts on the DNA to promote increased protein production and increased cellular proliferation which commonly results in hyperplasia or hypertrophy in the
target tissue. The metabolic properties of insulin principally deal with the control of blood glucose concentration and hence energy partitioning. This control is accomplished by stimulating glucose influx in insulin responsive tissues while inhibiting hepatic gluconeogenesis. (White and Kahn 1994) Insulin also stimulates the uptake of amino acids. Under normal physiological conditions these amino acids are commonly used in mitogenic activity which is also stimulated by insulin. However, under certain physiological conditions, such as fasting or severe stress, the amino acids may also be utilized as sources of energy.

The insulin receptor is the primary effector of insulin action. (White and Kahn 1994, Roth et al 1994) The specific characteristics of this transmembrane receptor will be discussed in greater detail later. Briefly, the insulin receptor is a glycoprotein with intrinsic tyrosine kinase activity. The specific mechanism of signal generation is still unclear, however it appears to be mediated through an autophosphorylation of tyrosine residues in the catalytic site or by phosphorylation of other second messengers such as the insulin receptor substrate 1 (IRS-1). (White and Kahn 1994, Exton 1991, Roth et al 1994) Ligand binding to the receptor activates the associated tyrosine kinases which appear to be essential for biologic activity. (White and Kahn 1994, Roth et al 1994)
Insulin secretion in patients with type II diabetes is generally normal or may be elevated. The principal problem in type II diabetics appears to be an inappropriate response to appropriate levels of insulin secretion leading to the hallmark of type II diabetes, insulin resistance. (Raskin 1994)

**Insulin Resistance**

Insulin resistance can be simply defined as a physiologic setting in which the target tissue demonstrates an abnormal response to ordinary levels of insulin. (Schade and Boyle 1992, White and Kahn 1994) In fact, the pathophysiological hallmark of type II diabetes is a diminished tissue sensitivity to insulin, as opposed to a frank insulin deficiency. (Raskin 1994, White and Kahn 1994, Schade and Boyle 1992) Insulin resistance can be pathologically classified into excessive glucose production (hepatic resistance) and inadequate glucose utilization (muscle or adipose resistance). (Schade and Boyle 1992) Even though insulin resistance is a very common pathological condition, it is also a common, and desirable feature in several clinical conditions. (Schade and Boyle 1992) Conditions frequently associated with clinical insulin resistance include certain normal physiologic states, such as pregnancy and puberty. (Goldstein 1994, Müller-Wielund et al 1993, Schade and Boyle 1992) In all of these conditions, the insulin resistance is considered to be a part of the
normal physiological regulatory response since it is both acquired and reversible. (Goldstein 1994, Schade and Boyle 1992) In some pathological conditions insulin resistance is necessary and desirable. These states include fasting, stress, and severe illness, such as sepsis. In the fasting state, energy substrate mobilization from peripheral tissues demands a peripheral insulin resistance allowing the adipocytes to release additional nonesterified fatty acids. (Schade and Boyle 1992) In stress, such as acute myocardial infarction or surgery, food intake is commonly decreased and metabolic needs are increased. (Schade and Boyle 1992) Insulin resistance is harmful and hence undesirable only if it occurs chronically or is present in an individual with other underlying pathology, such as hypertension or diabetes. (Schade and Boyle 1992) There is a strong association between hyperinsulinemia, and obesity, and hypertension, atherosclerosis and dyslipoproteinemia. (Goldstein 1994, Müller-Wielund et al 1993, Schade and Boyle 1992, Rakugi et al 1996) Hyperinsulinemia is a key factor accounting for the clustering of obesity, hypertension, and diabetes mellitus. (Sowers 1992, Rakugi et al 1996) Hyperinsulinemia does cause an increase in mitogenic activity which results in increased proliferation of smooth muscle cells. This is a similar mechanism as seen in proliferative retinopathy. An increase in smooth muscle proliferation in the peripheral vasculature results in an
increased peripheral vascular resistance, and hence hypertension. However, hyperinsulinemia itself does not necessarily cause obesity or hypertension. In fact, short term hyperinsulinemia, in an otherwise uncompromised individual, will result in a hypotensive state. (Sowers 1992) The key factor relating hyperinsulinemia and hypertension, obesity, atherosclerosis, and dyslipoproteinemia is a net decrease in insulin action. Decreased insulin action is most commonly the result of increased peripheral insulin resistance. A frank defect in insulin action will also cause decreased insulin action, however, this is usually only seen in type I diabetic individuals. Body composition is a key factor in the development of peripheral insulin resistance. There is an inverse relationship between adiposity and insulin resistance (Sowers 1992) There may be other factors that contribute to the development of hypertension and atherosclerosis. An example is the observation that a link between insulin resistance and hypertension may be a requirement for optimal intracellular levels of calcium to effect insulin action. (Sowers 1992)

In order to provide an adequate description of insulin resistance, it must be defined on both a systemic and a cellular level. Systemic insulin resistance is best described as a defect in the ability of the individual to provide adequate glucose to insulin responsive tissues.
There are several proposed mechanisms for systemic insulin resistance. Systemic insulin resistance is highly correlated with obesity, however it is not clear whether percent body fat is a cause or an effect of the insulin resistance. (Boden et al 1993, Kohrt et al 1993) There is a direct positive correlation between changes in obesity and changes in insulin resistance, with little or no correlation between obesity and insulin secretion. (Schade and Boyle 1992, Goldstein 1994) Randle et al (1963) proposed that insulin resistance is caused by an elevation in plasma non-esterified fatty acids, or free fatty acids (FFA). The key concept here is based on the fact that nearly all diabetic individuals demonstrate a central obesity. Because of this there is a greater number of lipolytically active intra-abdominal adipocytes and thus increased levels of circulating FFA. (Flier 1995, Elks 1990) Increased levels of free fatty acids inhibit the action of insulin in muscle, liver and perhaps adipose tissue which is seen clinically as insulin resistance. (Randle and Smith 1958a, 1958b, 1963, Flier 1995, Elks 1990) This is commonly referred to as the glucose-fatty acid cycle of Randle, or the Randle Hypothesis of Insulin Resistance, and is considered by many to be a major cause of insulin resistance in type II diabetic individuals. (Milburn 1995, Schade and Boyle 1992) The hypothesis states that if tissues "prefer" to oxidize FFA and they are readily available, then FFA will be oxidized
for energy and carbohydrate oxidation correspondingly decreases. (Elks 1990) Support for this hypothesis stems from several findings. First there is evidence that excess availability of FFA impairs muscle glucose oxidation and storage. (Elks 1990) Non-esterified fatty acids are a primary energy source for muscles, and enhance hepatic glucose release by augmenting gluconeogenesis and inhibiting glucose uptake in peripheral tissues. (Schade and Boyle 1992, Randle and Smith 1958a, 1958b, 1963) Secondly, there is evidence that FFA alter release of insulin and glucagon, tilting the insulin-glucagon ratio toward the side of increased glucagon action on the liver. (Elks 1990) Elevations in the plasma FFA concentration as a result of their antilipolytic effect may play a role in the hyperinsulinemia seen in obese individuals with normal glucose tolerance. (Milburn 1995) This hypothesis has been tested in several animal models and may be operative in humans in some metabolic conditions. One of the principle problems that appears in all of these experiments is the fact that few studies have actually been able to demonstrate the Randle Effect in vivo. (Rigalleau et al 1994, Bonadonna et al 1994, Coppack et al 1994) Because of the difficulties in demonstrating in vivo operation of the Randle Effect, it is unclear whether FFA's are elevated in response to insulin resistance, or a cause of the insulin resistance. (Schade and Boyle 1992, Milburn 1995)
Cellular insulin-resistance is best characterized by a decrease in the ability of the insulin receptor to stimulate the uptake of glucose into the cell. (Haring et al 1994, Schade and Boyle 1992) Insulin resistance results from an impairment in the signalling pathway between the initial binding of the insulin molecule to the insulin receptor and activation of the glucose transport system. (Haring et al et al 1994) In fact, most investigators have shown that insulin resistance is at or beyond the level of the insulin receptor. (Schade and Boyle 1992) Much of the work in this area involves the study of syndromes of extreme insulin resistance caused by naturally occurring genetic defects in the insulin action pathway. (Goldstein 1994) Among these defects are a variety of insulin receptor structural defects that often exhibit a marked alteration in insulin receptor binding or abnormal autophosphorylation of the insulin receptor. (Goldstein 1994)

The concept that insulin resistance is limited to the insulin signalling pathway is supported by the observation that adipocytes in primary culture develop a glucose-dose-dependent insulin resistance without affecting the total number of glucose transporters or insulin receptors. (Garvey 1987) This seems to indicate that the defect is probably not at the DNA level, but rather in the actual processing of the signal.
Defining cellular insulin resistance in terms of a defect in the signalling pathway does not identify all of the possible steps involved in the signaling process because the signalling pathway has not yet been fully described. (Butler et al 1990, Haring et al et al 1994) Because it is still not clear, insulin resistance on both the systemic and cellular level is experimentally described in terms of glucose metabolism. (Schade and Boyle 1992) A common means to quantify systemic glucose metabolism, and hence insulin resistance, in a clinical setting is by use of the glucose clamp, discussed earlier.

On the cellular level there are several measures of insulin resistance based upon what is known about the insulin signalling pathway. The principal measures of cellular insulin resistance involve the intrinsic tyrosine kinase activity, and glucose transport. (Roth et al 1994, Haring et al et al 1994) Tyrosine kinase activity has perhaps the clearest mechanistic relationship to insulin resistance. Roth et al (1994) and Haring et al et al (1994) have both demonstrated a reversible decrease in tyrosine kinase activity possibly due to a covalent modification of the insulin receptor in insulin resistant tissues. The fact that the insulin resistance is reversible is another indication that the defect is not at the DNA level, but rather in the signalling pathway. (Roth et al 1994, Haring et al et al 1994) The importance of the tyrosine kinase
activity of the insulin receptor in the development of insulin resistance is strengthened by several different lines of evidence. First is the finding that solubilized insulin receptors isolated from insulin resistant tissues clearly demonstrate a defect in the tyrosine kinase activity. Second, individuals that have severe insulin resistant states, such as Leprechaunism, commonly have a mutation in the tyrosine kinase domain of the \( \beta \)-subunit. (Goldstein 1994) Finally, prediabetic individuals demonstrate an impaired activation of tyrosine kinase in insulin receptors. (Haring et al et al 1994) While this last observation has not been fully elucidated, it does strengthen the argument that a defect in the tyrosine kinase activation component of the insulin signalling system may play an important role in the development of insulin resistance in type II diabetic individuals.

Glucose uptake is the one measure of insulin receptor functioning that is most closely related to the metabolic properties of insulin. The ability of a target tissue to increase glucose uptake in the presence of insulin is clearly demonstrative of an appropriate cellular response. Any defect in the insulin signalling system will result in a decreased glucose uptake. Muller-Wielund demonstrated that reduced insulin mediated glucose uptake is a major cause of hyperglycemia in insulin resistant diabetes mellitus and is due to a defect in the hormone action at the cellular level.
The problem with utilizing glucose uptake as a measure of insulin resistance is the fact that glucose uptake is mediated by several different factors. These factors include glucose transporters, as well as the metabolic needs of the cell. As discussed earlier, there are thought to be one or more second messengers that mediate and/or perhaps modulate the insulin signaling system. Not all of the signals will initiate glucose uptake as evidenced by the mitogenic effects of insulin such as amino acid uptake.

**Advanced Glycosylation End-products (AGEs)**

The French food chemist Maillard described the non-enzymatic condensation between a protein and a carbohydrate as the mechanism for the browning of foods during processing. (Monnier 1989) This reaction, which is commonly referred to as a Maillard reaction, involves the condensation of any reducing sugar and any primary or secondary amine, (Whistler 1985, Njorge et al 1989) In addition to browning reactions in foodstuffs, numerous Maillard reactions have been shown to occur in the human body and to correlate with a wide range of disease processes as well as the aging process. (Glomb and Monnier 1995, Bucala et al 1995a, Baynes et al 1989, Yang 1994, Lee and Cerami 1989) Perhaps the most commonly studied Maillard reaction seen in diabetes and occurs in hemoglobin with the
formation of glycated hemoglobin. This parameter is widely used to determine the long term glycemic control in diabetic patients. As described earlier, Maillard reactions are also associated with a number of diabetic complications. (Brownlee et al 1988, Bucala et al 1995a, Strowig and Raskin 1995)

Briefly, the Maillard reaction is a non-enzymatic condensation between a carbohydrate (ketose or aldose) and a free amino group of a protein followed by two different rearrangements with the final formation of irreversible adducts termed Maillard products or advanced glycosylation end products (AGEs). (Glomb and Monnier 1995, Kennedy and Lyons 1989, Vlassara 1994) The entire reaction is primarily dependent on pH, temperature, the relative concentrations of proteins and carbohydrates and exposure time. (Glomb and Monnier 1995, Whistler 1985) Interactions between sugars and amino acids vary with the composition of the amino acid. The relative intrinsic acidity of the reactive sites within a protein or amino acid facilitates AGE formation. (Njorge et al 1989) Hence, areas of proteins that contain several contiguous basic amino acid residues will be prone to AGE formation regardless of the pH of external environment.

Since the in vivo protein concentration, and temperature are held relatively constant in the body, in vivo AGE formation is primarily dependent on carbohydrate concentration as reflected in the blood glucose concentration and the time that the protein and glucose are
in contact with each other. (Glomb and Monnier 1995, Vlassara 1994, Lee and Cerami 1989) The rate of formation is clearly time-dependent and glucose concentration-dependent. Glomb (1995) reported obtaining a maximum formation of AGE products at 0.5 hours with a glucose concentration of 20 mM. This peak was extended to 120 h with a glucose concentration of 0.28 mM. (Glomb and Monnier 1995) These findings correlate with an increased rate of protein cross-linking, a key element in AGE formation, with time as shown on SDS-polyacrylamide gel electrophoresis. (Glomb and Monnier 1995) In vivo pH, temperature and protein concentrations are all maintained within very narrow limits under non-pathological conditions. This is significant because the relative serum glucose concentration and the time of exposure become the driving forces behind in vivo AGE formation.

The Maillard reaction can be divided into four basic steps; condensation of an amine with a reducing sugar; rearrangement to a labile intermediate; further progression to irreversible intermediates; finally development of AGE adducts. (Whistler 1985, Njorge et al 1989)

The initial step (See Figure 1) involves a condensation between the carbonyl group of glucose and either the N-terminal or an ε-amino group of an amino acid residue. (Njorge et al 1989, Vlassara 1994, Bunn and Forget 1986) The amine acts as both a nucleophile and a base in this
Figure 1: Maillard Reaction

The glycosylamine undergoes a reversible dehydration and enolization with ring closure through an aldose-ketose (Amadori) rearrangement to a more stable ketose-amine, commonly referred to as an Amadori product. (Glomb and Monnier 1995, Whistler 1985, Baynes et al 1989, Ledl et al 1989, O'Brien and Labuza 1994) These compounds are commonly referred to as the "labile fraction" in clinical literature because of their relative transient nature.

The Amadori product undergoes a relatively rapid irreversible degradation into one of four possible intermediates. (Ledl et al 1989) These intermediates include 1, 3 or 4 deoxyglucosones and methyl-α-dicarbonyl, commonly known as amino-deoxy ketoses (Kato et al 1989). The deoxysones that are not bound to the protein can react
with another protein or can be removed from the system. (Ledl et al 1989) One example of this is the condensation of secondary reaction products with hydroxy methyl groups in pyrroles leading to a cross-linking of proteins (Ledl et al 1989). The dehydration and rearrangement reactions can be enhanced by anhydrous and lipophilic environmental changes such as pH, temperature etc. (Bucala et al 1994) The ability to remove deoxysones and changes in the internal biochemical environment may both influence tissue specific accumulation of AGEs. Nearly all intermediates are removed from the system, react with other proteins or are further degraded to advanced glycosylation endproduct (AGE) adducts. (Whistler 1985, Njorge et al 1989)

Unlike enzymatic glycosylation or many in vitro glycation reactions, non-enzymatic glycation generally does not result in a single or a few well defined products. Rather, the reaction proceeds through any one of several complex pathways resulting in a large number of structures (Glomb and Monnier 1995, Ledl et al 1989). So far, few structures besides the Amadori product have been successfully identified in vivo. Pentosidine and carboxymethyl lysine occur in vivo. (Grandee and Monnier 1991, McCance et al 1993, Beisswenger et al 1993b) In fact, it appears that the amadori product may be a necessary intermediate in pentosidine synthesis. (Glomb and Monnier 1995) Pyrraline has also been found in tissue matrix by
immunochemical and chromatographic methods (Miyata and Monnier 1992, Portero-Otin et al 1995), but its existence in vivo is controversial. (Vlassara 1994) One major difficulty in identifying AGEs in vivo is the fact that all of the sources, ie sugar, amino acid, amadori product and Shiff's base, are all present at nearly the same time, and are all in a dynamic equilibrium with each other. 

AGES can be further degraded to other compounds such as furfuraldehyde and pyranone or can react further with amino groups to form pyrroles, pyradines and pyrrolinone reductones. (Njorge et al 1989, Baynes et al 1989, Vlassara 1994) Pyrrole compounds represent AGEs with a functional group capable of reacting with other amino groups to give rise to cross-linked compounds. (Njorge et al 1989) In addition AGEs can go on to form \( \alpha \)-dicarbonyl compounds and melandoin pigments. (Njorge et al 1989, Baynes et al 1989, Whistler 1985) It is important to note that many of the structural aspects of AGEs have been identified through in vitro experiments, few in vivo studies have elucidated the precise structural nature of protein bound AGEs. (Njorge et al 1989)

Another important fact is the observation that some proteins are more reactive with glucose than others in forming AGEs. More specifically, there are highly reactive sites within each protein. (Baynes et al 1989) It is important to note here that the conformation of the sugar in
the amadori product is not sensitive to the pKₐ or the local protein environment. (Baynes et al 1989) Even though some researchers have identified different "reactivities" of lysines in apolipoprotein B based on differences in their pKₐ, in vivo studies clearly demonstrate that pKₐ values alone appear to be an insufficient determinant of lysine reactivity. (Shapiro et al 1980, Iberg and Fluckiger 1986, Lund-Kats et al 1988) These sites become reactive because their proximity to areas of a lower pKₐ or higher nucleophilicity favor the condensation reaction. For example, AGE formation is favored in areas with contiguous, basic residues. A high local charge found in these areas would suppress protonation thus increasing the nucleophilicity and reactivity of any reactive residues in the area. (Iberg and Fluckiger 1986) Basic amino acids are generally more reactive because of presence of the relatively basic nitrogen in the side chain and a low pKₐ. (Whistler 1985) These amino acids include lysine, arginine, and histidine. However, in vivo a low pKa is not significant in determining the degree of reactivity of an active site because the in vivo pH and temperature are held within very narrow tolerances. (Baynes et al 1989) In fact, the overall three dimensional structure of the protein provides a greater predictor of the reactive amino acid residues than the pKa of the individual residues. A protein configuration that partially shields a lysine residue will
prevent that lysine from participating in the Maillard reaction. (Baynes et al 1989) In addition, vicinal acidic or basic amino acid residues modulate the reactivity of lysines with the more reactive lysines being located near more acidic amino acids in the three dimensional protein structure. (Baynes et al 1989) This observation suggests that properly oriented vicinal carboxylate groups act to catalyze the progression of the Maillard reaction for specific lysine residues as well as lys-lys. (Baynes et al 1989) Some of the amino acid sequences that appear to be highly reactive are lysine-histidine, lysine-lysine-lysine and lysine-histidine-lysine. (Baynes et al 1989) Finally, the relative rate of AGE formation may be determined by the equilibrium distribution of the Schiff base adducts and the specific rate of Amadori product formation. (Baynes et al 1989)

Under steady state conditions the non-enzymatic condensation of a protein and a carbohydrate to a Schiff base takes only a few days, (Wu 1993) while the conversion to the Amadori product may take up to 28 days to reach equilibrium in a non-diabetic individual or in a diabetic individual who is in good glycemic control. The Schiff base condensation is a reversible reaction that can be directly affected by the daily blood glucose concentration. (Bunn and Forget 1986) The Amadori rearrangement is more
representative of the mean of the daily blood glucose concentration over the period required to reach equilibrium.

The rate of glycation is pH dependent, and occurs at a characteristically slow rate throughout the protein's life span. (Tara and Shima 1993) The pH dependency relates to the fact that the amino groups at the reactive sites are not protonated. The rate of condensation also depends on the equilibrium between open chain and ring forms of the carbohydrates, and the relative concentrations of aldoses and ketoses. Aldoses tend to be more reactive than ketoses because they are more prone to open chain formation.

Glycation is a continuous process throughout a protein's life span. The rate of glycation is dependent on the specific protein and the tissue involved. The extent of glycation in human lens proteins remains essentially constant between age 5 and 80. (Baynes et al 1989) Goldstein has calculated that the total glycated hemoglobin represents the mean glucose concentration over the 4 month life span of the erythrocyte. (Goldstein et al 1986) It does appear that a steady state level requires four months to achieve. Approximately 50% of the glycosylation is determined by glucose concentration in the one month immediately prior to sample collection. Of the remaining hemoglobin that will undergo glycosylation, 25% will be formed two months prior to collection and 25% in the initial two months of the erythrocytes life. (Tara and Shima 1993)
This indicates that the glycosylated hemoglobin level does not simply reflect the mean glucose concentration in the preceding two to three months but, rather reflects the weighted mean glucose concentration in the preceding four months.

The initial maillard reaction is characterized by a colorless solution, but as the reaction progresses the products become visible. (Whistler 1985) The AGEs demonstrate a characteristic yellow-brown color with an internal fluorescence that can be quantitatively determined by excitation in the 350-370 nm range and emission in the 420 to 440 nm range. (Gugliucci 1994, Whistler 1985) Other less precise but direct measures of AGEs are chromatography and analysis by ultraviolet and infrared spectra. An indirect determination of Maillard products can also be obtained by carbon dioxide evolution. (Whistler 1985) Detection of AGEs in vivo by utilizing both polyclonal and monoclonal antibodies to AGEs is gaining wider application. Currently these procedures are primarily limited to immunohistochemical staining of tissue preparations. However, antibody use for in vitro analysis of cellular components is increasing. (Schmidt et al 1994 Nakayama et al 1993) One of the principal difficulties in using antibodies to detect AGE formation is variability of AGE products that can be obtained. (Nakayama et al 1993)
The principal pathophysiological complications of AGE formation are due to the impact on the function of a wide variety of proteins. In vivo AGE formation adversely modifies proteins such as hemoglobin, lens crystallins, collagen, lipoproteins, extracellular matrix, and intracellular proteins. (Njorge et al 1989, Brownlee et al 1988) Glycation is not always harmful; it apparently does not affect the biological functioning of albumin, bilirubin, transferrin, fibrinogen, α-2-macroglobulin, and many of the immunoglobulins. (Baynes et al 1989) In diabetes it has been proposed that the most important effects of AGE formation are on structural proteins such as collagen, basement membranes, lens crystallins, and the extracellular matrix. (Brownlee et al 1988) It has been proposed that there may be relatively high levels of circulating reactive AGE-peptides under normoglycemic states especially if plasma filtration is slightly impaired. (Bucala et al 1995a) Glycation and subsequent AGE formation in these proteins could lead to conditions such as retinopathy, nephropathy, microvascular disease, and neuropathy. (Brownlee et al 1988)

Clinically, the most commonly useful AGEs are the glycated serum proteins, specifically hemoglobin and albumin. These proteins have provided critical insight into the process of glycation and the implications for the pathogenesis of AGE formation as well as providing an easy way to measure long term serum glucose levels. As an
example the rate of in vitro AGE formation for hemoglobin is approximately 0.0008%/mM glucose/day and 0.0037%/mM glucose/day for serum albumin. (Baynes et al 1989) The rate of AGE formation becomes more significant for proteins that are not cleared as rapidly as hemoglobin or albumin. Clearance studies with AGE albumin infusion demonstrate that AGE-albumin is cleared more rapidly than native non-AGE-albumin; however there is a significant accumulation of AGE-albumin in liver, lung, kidney, intestine and heart tissues. (Schmidt et al 1994)

The principal pathological condition seen in retinopathy is AGE formation in the lens crystallins of the eye. This is thought to be the result of AGE formation on the enzyme aldose reductase. (Srivastava et al 1989) Glycosylation of aldose reductase inhibits the action of the aldose reductase regulatory proteins, allowing a build up of sorbitol within the lens. This condition leads to cataract development commonly associated with long-term diabetes or aging. (Njorge et al 1989)

Diabetic nephropathy associated AGE pathophysiology is most commonly associated with alterations in the structural proteins of the basement membrane. Most proteins contain less than one mole of glucose per mole of protein. (Baynes et al 1989) This is dramatically increased with glycation leading to AGE formation occurring within weeks to months after the onset of diabetes in experimental animals. (Baynes
et al 1989) There is a strong correlation between carboxymethyl lysine formation, the appearance of pentosidine and early development of nephropathy as diagnosed by abnormal 24 hour albumin excretion rates. (McCance et al 1993, Beisswenger et al 1993b) In spite of this, kidneys from diabetic individuals demonstrate a variable extent of AGE deposition, likely due to the variability in glycemic control. (Schmidt et al 1994)

The potential effects of AGE formation are much greater in structural proteins such as collagen because of the long half lives of these proteins. (Baynes et al 1989, Schmidt et al 1994) There is a direct, dose and time dependent correlation between glucose concentration, appearance of carboxymethyl lysine and decreased tissue friability in collagen. (Bailey and Kent 1989) Dyer et al (1993) showed a fivefold increase in the appearance of carboxymethyl lysine in collagen in non-diabetic patients between the ages of 20 and 80 years. There was an even greater increase (three fold) in diabetic individuals over the same age range. This decrease in friability suggests an increase in the cross-linking of the collagen proteins and thus tissue stiffening. The changes in collagen as a result of AGE formation has led some researchers to characterize diabetes on the biochemical level as a disease of accelerated aging of collagen. (Dyer et al 1993)
Complications due to microvascular disease are likely the result of at least two processes. One is the development of AGEs in the connective tissue of the vessels. This process is basically the same as seen in diabetic nephropathy. A second potential cause of diabetic microvascular disease relating to AGE is the effect that AGEs have on the immune response and the subsequent development of atherosclerotic plaque.

Soluble AGEs induce monocytes to migrate down a concentration gradient. When the cells reach AGEs that have been immobilized, the rate of migration is diminished allowing them to bind to the AGEs and become activated. (Schmidt et al. 1994, Shaw and Crabbe 1993) The concept here is similar to the action of lymphokines in an inflammatory reaction. The interaction of soluble AGEs with the monocytes is mediated by a receptor that appears to be specific for AGEs, termed receptor for AGE or RAGE. (Schmidt et al. 1994) Binding of RAGE by an AGE induces activation of various cytokines and growth factors, including PDGF, IGF-1, IL-1 and TNF-α. (Schmidt et al. 1994) In addition to solubilized AGEs, immobilized AGEs found in basement membranes also act to slow monocyte migration, and may play a role in their activation as tissue macrophages. (Schmidt et al. 1994)

Dyslipidemia associated with microvascular disease may be a direct result of AGE formation. Lipid particles such
as LDL and apolipoprotein B are extensively modified by AGE formation in a dose and time dependent manner in diabetic patients. (Bucala et al 1994) Apolipoprotein B contains a high content of basic amino acid residues, including numerous sites of contiguous, basic residue repeats making it a prime target for AGE modification. (Bucala et al 1995b) In-vitro and in-vivo studies of AGE modified LDL and apolipoprotein B clearly demonstrate a decrease in ligand receptor binding that is readily ameliorated by the AGE inhibitor aminoguanidine. (Bucala et al 1994) This leads to the concept that an increase in AGE modification decreases the ability of the receptors to identify, bind and ultimately remove lipid particles, a situation that clearly results in a dyslipidemia commonly associated with diabetes.

The Maillard reaction itself may play a role in the development of microvascular disease aside from actual AGE formation. Many of the intermediates in the Maillard reaction generate highly reactive oxygen free radicals. (Schmidt et al 1994) Glucose itself may induce formation of lipid peroxides and monoaldehydes which can then cause a cascade of chemical modifications to the protein that is not directly dependent on glucose. (Baynes et al 1989) It has been proposed that this whole process leads to a condition where there is greater oxidative stress on the vessel walls as a result of the hyperglycemia seen in diabetes. (Schmidt et al 1994)
Another area where the effects of AGE formation have been implicated in pathogenesis, but not fully elucidated is the effect of AGE formation on the coagulation system. Glycation of antithrombin III (AT III) may interfere with the inhibitory activity of AT III toward thrombin. (Baynes et al 1989) The effect on AT III is exacerbated by the observation that extensive glycation adversely affects the ability of fibronectin and laminin to interact appropriately with heparin and collagen. (Baynes et al 1989) Exposure of endothelial cells AGES also suppresses the expression of thrombomodulin, increases diffusional transit of macromolecular solutes across the endothelium and leads to low levels of procoagulant tissue factor and enhanced endothelial cell response to TNF. (Schmidt et al 1994) All of this contributes to the hypercoagulable state seen in diabetes, and exacerbates the other pathophysiological mechanisms of diabetic microvascular disease.

**Insulin Receptor**

Insulin is critical for the regulation of mitogenic and metabolic processes in nearly all vertebrates. (Czech 1985) On the cellular level, the initial effector for most of the known mitogenic and metabolic properties of insulin is the insulin receptor. Insulin receptors are present in nearly all vertebrate cells with concentrations ranging from 2,000 molecules per cell in erythrocytes to over 200,000 molecules
per cell in adipocytes and hepatocytes. (Shoelson et al 1993, White and Kahn 1994)


The insulin receptor is an integral membrane protein that can be divided into three functional segments, extracellular, transmembrane and cytoplasmic. The extracellular segment of the receptor is formed by the α-subunit and a small portion of the β-subunit. The transmembrane segment is 23 amino acids long with predominantly neutral nonpolar side chains. (Frattali 1991) Functionally the transmembrane domain has a primary of role of anchoring the receptor in the phospholipid bilayer; it allows for proper processing and transport of the receptor; and most importantly provides a link to the tyrosine kinase domain of the β-subunit. (White and Kahn 1994, 1988, Frattali 1991, Tornqvist et al 1988) The cytoplasmic segment consists of the remainder of the β-subunit which includes a tyrosine-specific protein kinase domain. (Kasuga et al 1982, Ullrich et al 1985, 1986, Ebina et al 1985, White and Kahn 1994)

The gene for the insulin receptor protein is located on the short arm of chromosome 19. It is 150 kb long with 22 exons and encodes a 4.2 kb cDNA. (Seino et al 1989, White and Kahn 1994) Both of the subunits are synthesized in a single precursor form and cleaved by proteolytic processing at a cleavage site consisting of four basic amino acids. (White and Kahn 1994, Schumacher et al 1993) There are two
alternative splicing sites which give rise to two isoforms, each differing by 122 amino acids near the carboxy terminus of the α-subunit. (White and Kahn 1994, Haring et al 1994) The expression of the two isoforms appears to have a tissue specific pattern, that may be related to the development of type II diabetes. (Haring et al 1994) However these isoforms have no demonstrable difference in either signaling nor recycling. In fact, their differential presence in diabetic and non-diabetic skeletal muscle has not been confirmed on PCR, although non-diabetic individuals may have variable amounts of both isoforms with a possible shift to higher level of one isoform in NIDDM. (Haring et al 1994) Mutations in the insulin receptor have been identified in type II diabetic individuals, as well as individuals with syndromes of severe insulin resistance such as Leprechaunism. (Barbetti et al 1992, Cocozza et al 1992) These mutations affect the production, processing, half life and function of the insulin receptor. (Barbetti et al 1992) While the majority of the mutations are substitution type point mutations involving the tyrosine kinase portion of the β-subunit catalytic loop, (Cocozza et al 1992) several have been identified in the α-subunit. (Barbetti et al 1992) Some of these mutations include a substitution of a lysine or arginine residue. (Barbetti et al 1992)

The insulin receptor is a member of the receptor/tyrosine kinase family of transmembrane signaling
proteins. (Lee et al 1993) This receptor family includes the insulin receptor, insulin-like growth factor (IGF-1) receptor and the insulin receptor related receptor which has no known ligand. (White and Kahn 1994) The insulin receptor family has 80% sequence homology in the tyrosine kinase domain of the β-subunit but little homology in the extracellular domain. (White and Kahn 1994)

The insulin receptor is activated only upon ligand binding. (Frattali 1991) Studies on insulin binding indicate that under physiological concentrations of insulin, the stoichiometry of the insulin-insulin receptor binding is between one and two insulin molecules per receptor. (Shoelson et al 1993, White and Kahn 1994) This is supported by four lines of evidence.

1) Insulin binding to cells and membranes shows negative cooperativity as determined by Scatchard analysis. In other words the binding of one insulin molecule to the receptor makes the binding of the second insulin molecule more difficult. (De Meyts 1973)

2) Purified receptors, with reduced disulfide bonds, demonstrate low affinity binding with a stoichiometry of one insulin molecule per receptor, whereas purified holoreceptors exhibit negative cooperativity and only one high affinity insulin-binding site. (Boni-Schnetzler 1987; Sweet et al 1987) This difference between the holoenzyme and the reduced enzyme confirms
the concept that a dimer is necessary for proper insulin receptor action. (Massague et al 1980, Ullrich et al 1985, Ebina et al 1985)

3) Shoelson et al (1993) used a gel shift assay in cross-linking studies to confirm a lower binding affinity of the second insulin-insulin receptor interaction. They noted that if two insulins were bound in similar orientations then at saturating concentrations two insulins would cross-link per holo receptor, which clearly does not occur. (Shoelson et al 1993)

4) A double probe analysis using two different insulin analogues showed that only one analogue will bind to the receptor with high affinity at any one time. (Pang et al 1983).

Negative cooperativity between insulin and the insulin receptor is an integral, functional part of the insulin receptor that can be used to explain the accelerated dissociation of the ligand from the receptor. (Liu et al 1995) The importance of negative cooperativity has been demonstrated by analyzing naturally occurring mutants and substitution of amino acids at key regulatory areas, such as lysine 121, tyrosine 960 and lysine 460. (Liu et al 1995)

Alteration in negative cooperativity does not alter the steady-state insulin binding data (Scatchard plot) and the
kinetic data that measure insulin dissociation. One would expect a reduction in negative cooperativity to diminish the curvilinear property of the Scatchard plot. However, very little if any difference exists between the plots for the mutant receptor, which displays a reduced negative cooperativity, and the wild-type receptor, which displays normal cooperativity. This lack of agreement may arise from a greater sensitivity provided by dissociation studies for determining receptor binding affinity or from an inability of negative cooperativity to contribute significantly to the curvilinearity of Scatchard plots (Liu et al 1995).

cysteine rich region may be in close proximity to the ligand binding region. (Fabry et al 1992) These residues are felt to be necessary but not sufficient to bind insulin and generate a signal. (Kjeldsen 1991, Waugh et al 1989, De Meyts 1990, Schumacher et al 1991, Zhang and Roth 1991) Both the N-terminal and C-terminal appear to be required and be within proximity to each other for signal generation to occur. (Fabry et al 1992) In other words, the key features of the receptor required for signal generation appear to involve specific amino acid sequences, their relationship to each other, and the overall confirmation of the α-subunit in relation to its ligand. Ligand-receptor interactions could be either directly between side chains of amino acids within these key areas and insulin or purely as a result of alterations in receptor conformation in response to ligand binding. (Andersen et al 1992) This is confirmed by experiments in which point mutations and insulin cross-linking in these areas cause alterations in subunit configuration and either an abolition or decreased ligand binding affinity. (Andersen et al 1992, Fabry et al 1992, Schumacher et al 1991, De Meyts 1990) Other residues that do not directly contact the insulin binding site, such as lysine 121 and lysine 460, regulate insulin receptor conformation, influencing negative cooperativity interactions with insulin and thereby modulate signal transduction. (Liu et al 1995)
The binding affinity of the insulin receptor for insulin is influenced by several factors. The most obvious is the requirement for the ligand binding domain as described above. Other factors include the tertiary structure of the membrane-bound receptor, a cysteine rich domain, the carboxy-terminal of the $\alpha$-subunit, and possibly other non-specific amino acid residues. (Schumacher et al 1991) There are also properties of insulin such as separation of the carboxy terminus of the insulin $\beta$-chain from the rest of the ligand, that influence ligand receptor interactions. (Shoelson et al 1993) In addition there are apparently tissue specific characteristics that may impact on ligand-receptor binding. For example, there is a clustering of insulin receptors in adipocytes that is not found in hepatocytes or myocytes. (Jarrett et al 1980) High affinity insulin binding by the insulin receptor requires an interplay between all of these factors. The most important factor is the amino acid residues in the N-terminal of the $\alpha$-subunit. (Schumacher et al 1991)

The N-terminal amino acid residues recognize and bind the insulin receptor in a ligand binding pocket. The ligand binding pocket is an integral part of the tertiary structure of the insulin receptor. Even minor changes in this region, such as seen with solubilization of the receptor protein, appear to cause at least partial misfolding of the ligand binding pocket, which results in a variety of functional
defects. (Schumacher et al 1993) These functional defects are clearly demonstrated by binding affinity studies. Solubilized insulin receptors demonstrate an approximately ten fold decrease in binding affinity when compared to membrane-bound receptors. (Andersen et al 1990, Frattali et al 1991) Frattali et al (1991) suggested that the possible reason for this is that the solubilized receptors have a more relaxed tertiary configuration than membrane-bound receptors. A more constrained tertiary structure may play a significant role in either direct receptor-ligand interaction or in stabilization of the ligand binding pocket architecture.

A third factor in insulin binding affinity is the carboxy terminus of the α-subunit. In the membrane-bound insulin receptor, the carboxyl-terminal α-subunit sequences appear to be crucial for the formation of the insulin binding pocket. (Schumacher et al 1991). This region interacts with the ligand binding domain to bind and hold the insulin receptor. (Schumacher et al 1993) In all probability, these two regions are brought into close proximity by the tertiary folding of the α-subunit when the ligand binding pocket is formed.

The cysteine-rich region may also play an important, yet probably minor role in insulin binding. (Schumacher et al 1991, 1993, Andersen et al 1990, Kjeldsen 1991, Zhang and Roth 1991, Yip 1988) Some reports have proposed a major
role for the cysteine-rich domain in insulin binding (Yip 1988, Gustafson and Rutter 1990) There are other as yet unidentified amino acid residues located near, but not in the ligand binding domain that are apparently required for high insulin binding affinity. (Schumacher et al 1993) The exact importance of these residues, as well as their specific locations is still unclear.

**Insulin Signal Generation**

The basic mechanism for signal generation by the insulin receptor is widely accepted to be an autophosphorylation of the tyrosine kinase domain of the β-subunit. The initiating signalling event involves intramolecular interactions between receptor subunits within a single receptor rather than between receptors. (Wilden et al 1989, Sweet et al 1987, Morrison et al 1988, Boni-Schnetzler 1986, 1988, Feener et al 1993)

In this model, insulin binding to the α-subunit affects a conformational change in the α-β-subunit interface resulting in an autophosphorylation of the tyrosine kinases on the cytoplasmic face of the opposite β-subunit. (Frattali et al 1991, 1992, Treadway et al 1991, Roth 1994, Waugh et al 1989, Bargmann and Weinberg 1988) Autophosphorylation of the insulin receptor is an essential component in the activation of its protein tyrosine kinase activity. (Lee et al 1993) When insulin occupies the ligand binding domain of either α-subunit, autophosphorylation of the opposite β-


The mechanism involved in the autophosphorylation of the β-subunit when insulin binds the receptor is largely unknown. There are, however, several clues to the mechanism. Intramolecular interactions between α-subunits and β-subunits are necessary for insulin-dependent activation of the protein kinase domain. (Wilden et al 1989) This concept is supported by the observation that unoccupied α-subunits inhibit tyrosine kinase activity in the β-subunit. (White and Kahn 1994)

The β-subunit contains three major autophosphorylation sites in the regulatory region and two sites in the C terminus. These sites have been identified by reverse-phase HPLC analysis, radiosequencing, direct sequencing and two-dimensional tryptic peptide mapping (Tornqvist et al
At high insulin concentrations (17 nM), the regulatory region is mainly bis-phosphorylated while tris-phosphorylation predominates at physiological insulin levels. (Feener et al 1993) The juxtamembrane region contains most of the basal phosphorylation before insulin stimulation and is the most sensitive site of insulin-stimulated phosphorylation. (Feener et al 1993) Phosphorylation of the juxtamembrane region accounts for 15% of the autophosphorylation of partially purified insulin receptor and involves primarily phosphoserine and to a lesser extent phosphotyrosine. (Feener et al 1993)

Work with various mutant insulin receptors have demonstrated a strong correlation between the ability of ligands to induce an autophosphorylation of the tyrosine kinase domain and the mitogenic and metabolic effects seen with insulin stimulation. (Kishimoto et al 1994, Chou 1987, Tornqvist et al 1988, Feener et al 1993). This means that the direct coupling of ligand binding affinity and the capacity to phosphorylate the tyrosine kinase domain is an essential component of the cellular signaling process. (Schumacher et al 1993) In fact, several naturally occurring mutations in the tyrosine kinase segments of the β-subunit have been identified in several pathological conditions, most notably Leprachaunism which is a condition that is characterized by severe insulin resistance. (White and Kahn 1994) In addition, induced deletions and
truncations in the β-subunit that result in the loss of insulin stimulated kinase activity may decrease hexose transport. (Exton 1991)

The specific mechanism of tyrosine autophosphorylation appears to occur through a trans mechanism in which insulin binding to the α-subunit of one α-β dimer stimulates phosphorylation of all of the tyrosine residues in the tyrosine kinase domain of the adjacent covalently linked β-subunit (White and Kahn 1994) This autophosphorylation stimulates kinase activity 10-20 fold. (White and Kahn 1994)

Phosphorylation of several additional cytoplasmic polypeptides occurs in response to the autophosphorylation of the β-subunit tyrosine kinase domain. (Schumacher et al 1993, Exton 1991) Activated tyrosine kinases will in turn transfer phosphate groups from ATP to several cytoplasmic proteins. (Müller-Wielund et al 1993) Many of these substrates are involved in signal transduction cascades, and may be involved in the specific insulin signalling system. Some of these substrates include phosphatidylinositol 3-kinase. (Exton 1991) While many of these substrates have been identified in vitro, such as insulin receptor substrate-1 (IRS-1) (White et al 1985, Sun et al 1991, Backer et al 1992, Feener et al 1993), few have actually been shown to undergo an in vivo activation. (Exton 1991) The key difference between insulin phosphorylation of these substrates and other tyrosine kinase receptors (eg platelet
derived growth factor and epidermal growth factor) is the fact that there is no direct communication between the $\beta$-subunit and the individual substrates. (White and Kahn 1994)

The tyrosine kinase domain of the insulin receptor appears to act through an intermediate messenger, probably IRS-1. (White and Kahn 1994)

A more subtle phosphorylation than the tyrosine kinase domain, is the activation of serine and or threonine kinases. (Roth 1994) Insulin stimulates extensive serine and threonine phosphorylation in the $\beta$-subunit which activates serine threonine kinases (eg ATP citrate lyase, acetyl Co A CBX, S6 ribosomal protein and cAMP phosphodiesterase isozymes and protein kinase C). (Exton 1991, Roth 1994) Activation of the serine (ser) and threonine (thr) kinases by insulin and other ligands are generally more stable and have been extensively reported in insulin resistance. (Roth 1994, White and Kahn 1994)

Insulin binding and signal generation can be summarized by the following sequence of events:

1. One to two insulin molecules bind to the ligand binding region of the $\alpha$-subunits. This binding requires multiple, specific contacts between the ligand and the receptor. (Shoelson et al 1993, Lee 1994) The ligand receptor contact consists of a predominant interaction with one receptor half and less complete contacts with the other half receptor. (Boni-Schnetzler
1987, Sweet et al 1987) This results in an asymmetrical binding that acts to prevent a functional binding of the receptor by a second insulin molecule. (Shoelson et al 1993)

2. Ligand binding triggers autophosphorylation of tyrosine residues, most probably by a "trans" mechanism, within the catalytic region on the cytoplasmic face of the other β-subunit in an asymmetric fashion activating the receptors intrinsic tyrosine kinase activity. (Lee et al 1993, Frattali et al 1992) In addition there is an allosteric regulation of this process; the phosphorylated receptor half is kinase inactive. (Boni-Schnetzler 1988, Frattali et al 1992)

3. Autophosphorylation of the tyrosine residues then triggers phosphorylation of serine (Ser) and threonine (Thr) residues in the catalytic loop of the β-subunit as well as phosphorylation of other proteins with in the signalling pathway. It appears that regulatory domain tyrosines may have an important role in insulin-stimulated Ser/Thr phosphorylation. (Feener et al 1993)

4. Autophosphorylation of the insulin receptor triggers internalization of the ligand-receptor complex via a rapid, saturable coated pit-dependent pathway. (Backer et al 1991, Feener et al 1993) While internalization of the ligand-receptor complex may not be a part of the
initial signal response, it does play a role in the availability of unbound receptors, and may contribute to secondary signaling mechanisms.

This combination of ligand binding, tyrosine autophosphorylation, serine and threonine phosphorylation provides three levels of control that are sensitive to extracellular and intracellular events (White and Kahn 1994).

The cause of insulin resistant states may be a defect that lies in the signalling pathway between ligand binding and activation of the cellular metabolic components. It is clear that an impaired response of the insulin receptor tyrosine kinase is an effective measure of insulin resistance on the cellular level. Impaired insulin receptor kinase responses are commonly found in clinical cases of type II diabetes and contribute significantly to the pathogenesis of the disease. (Haring et al 1994) Maegawa (1995) and Müller (1991) demonstrated a significant decrease in β-subunit autophosphorylation and cytosolic phosphotyrosine phosphatase (PTPase) activity with chronic glucose administration. These adverse effects are seen at a physiologically relevant dose dependent glucose range (5.5 to 16 mM) in vitro and in skeletal muscles of type II diabetic patients with hyperinsulinemia and clinical insulin resistance. (Maegawa et al 1995)
In vitro insulin resistant states have been induced in adipose tissue by administration of tissue necrosis factor-α (TNF-α), which inhibits autophosphorylation and tyrosine kinase activity. (White and Kahn 1994) Receptor half life is one aspect of the insulin receptor that is outside the usual signaling pathway that may impact the pathogenesis of insulin resistance. The insulin receptor plays an important mediating role in the internalization and degradation of insulin. (White and Kahn 1994) Bound insulin is internalized by one of two mechanisms. The first is a coated pit-mediated mechanism that requires functional insulin receptor tyrosine kinase domains. (White and Kahn 1994) The other mechanism is a constitutive or nonsaturable internalization pathway that does not require receptor autophosphorylation. (White and Kahn 1994) In both mechanisms a continuation of the insulin signal in response to glucose levels depends on proper insulin, insulin receptor interactions. If an impairment in the insulin receptor prohibits autophosphorylation of the tyrosine kinase domains then the receptor may not be internalized. Prolonged stimulation of the receptor, as seen with delayed or inappropriate dissociation of the receptor may result in receptor down regulation and attenuation of the insulin signal. (White and Kahn 1994)
Model System

The model system for this project is based on a murine embryonic fibroblast cell line, swiss mouse embryonic fibroblasts (3T3-L1), which differentiate to an adipocyte-like phenotype that ultimately appears as white adipose tissue. (Green and Kehinde 1974, Frost and Lane 1985, Sadowski et al 1992) The maturation of the 3T3-L1 adipocytes is a terminal differentiation with the cells becoming more spherical, more self associating, and less spread out. (Green and Kehinde 1974, Kosaki and Webster 1993) Mature 3T3-L1 adipocytes synthesize and store triglyceride, demonstrating significant lipid droplets upon staining with Oil Red O. (Green and Kehinde 1974) Adipocyte differentiation results in a marked induction in insulin receptor expression with increased insulin sensitivity. (Kosaki and Webster 1993) Fully differentiated adipocytes demonstrate an insulin sensitivity that is paralleled by increased glucose transport in response to insulin stimulation. (Schurmann 1994, Rosen et al 1983) Full maturation with terminal differentiation can be obtained solely by administration of fetal bovine serum. (Green and Kehinde 1974) However, administration of dexamethasone results in an increase in the number of low affinity insulin receptors, as well as an increase in the number of lipid droplets with in each cell. (Kosaki and Webster 1993)
Induction of 3T3-L1 fibroblasts to adipocytes is accomplished by exposing confluent cells to media containing Dulbecco’s Modified Eagles Media (DMEM) containing high glucose (450 mg/dL) and 10% bovine calf serum (BCS) supplemented with corticosterone, dexamethasone, insulin and the cAMP-PDE inhibitor methylisobutylxanthine for 48 hours, followed by a three day incubation with unsupplemented media, and an additional three days with media containing DMEM with low glucose and fetal bovine serum (FBS) (See Methods and Materials for specific formulations used in this project). (Green and Kehinde 1974, Sadowski et al 1992, Frattali and Pessin 1993)

Glycohemoglobin

Long term glycemic control in diabetic individuals is vital to maintaining an optimal state of health and preventing many complications. The Diabetes Complications and Control Trials (DCCT) determined that by maintaining tight control of individual’s glycemic state, the risk of developing complications such as retinopathy, neuropathy, nephropathy and vascular disease could be significantly reduced. In fact, the researchers found that in insulin dependent diabetic (IDDM) individuals, the risk of long-term complications was reduced by as much as 60% when the mean blood glucose was decreased to and maintained at 150 mg/dL. (DCCT 1993)
Presently the most useful marker of the mean blood glucose concentration is the percentage of glycohemoglobin (gHb). Hemoglobin, like other proteins, becomes glycosylated via the non-enzymatic condensation first described by Maillard discussed earlier in this review. (Monnier 1989) Later, Rahbar described an abnormal, fast moving hemoglobin fraction in patients suffering from diabetes mellitus, with otherwise unremarkable hematological findings. (Rahbar 1968) This hemoglobin moved more rapidly toward the anode in electrophoresis and appeared in front of the A band to the same position of hemoglobin F. (Rahbar 1968) This abnormal "fast hemoglobin" described by Rahbar has since been identified as a glycosylated hemoglobin (gHb) fraction which has become an exceedingly important parameter for clinicians to evaluate the glycemic control of a diabetic individual over an extended period of time.

Erythrocytes are freely permeable to glucose, which allows for the formation of gHb at a rate proportional to the ambient glucose concentration. This means that measurement of gHb products provide a reliable index of the mean blood glucose concentration over the life span of the erythrocyte, approximately three to four months. (Leese 1992, Gabbay et al 1977, Lodewick 1992) In a report by the Expert Committee on Glycosylated Hemoglobin of the National Diabetes Data Group, the clinical application of glycohemoglobin products, specifically the A\textsubscript{1c} fraction,
allows for the assessment of metabolic status of patients with diabetes mellitus. As will be discussed later in this review, hemoglobin A$_{ic}$ is a subfraction of hemoglobin A that commonly undergoes a non-enzymatic condensation with glucose and other carbohydrates. The expert committee further stated that gHb products reflect the mean blood glucose concentration over a six to eight week period. (NDDG 1984) Little and Goldstein (1992) also described the glycohemoglobin test as providing the only accurate, objective measurement of long-term glycemic status. They reasoned that traditional methods of assessing glycemic control such as medical history, physical examination and analysis of urine and blood glucose concentration have limited value as indices of long-term glycemic control.

The DCCT determined that a reduction in gHb products (A$_{ic}$ fraction) from 9% to 7.2% was equivalent to reducing the mean blood glucose concentration 50 mg/dL and resulted in a 60% reduction in the severity of diabetic complications.

Because of the significant decrease in diabetic complications seen with tight control of a diabetic individual's glycemic state, periodic measurement of gHb products are rapidly becoming a standard of care for diabetic patients. However, there is still a great deal of confusion in the interpretation of gHb results. This confusion is caused by three basic problems. First is
puzzlement over some of the terminology. Rapid developments in methodologies and differences in what is actually measured in various assay procedures have led to a bewildering array of approaches and methodologies that measure different components and utilize different nomenclature to describe those components. Second, a lack of a standardized reporting method for gHb products have impaired the full utilization of gHb product measurement for long-term monitoring. Interlaboratory standardization is difficult because there is little agreement on which glycohemoglobin product to measure. (Bruns 1992) The DCCT measured only the $A_1c$ subfraction of hemoglobin A. This is the most stable gHb product and is present in most individuals. However, because of the incidence of hemoglobin variants and the possibility of abnormal hemoglobins, standardization to a method specific for the $A_1c$ fraction may be inappropriate. Finally there is disagreement over which gHb products are the most sensitive indicators of glycemic state. Those with the least interferences and variability generally should best reflect the patients mean blood glucose concentration over the last three months.

The arguments over the specific hemoglobin fraction to measure have been met with far less success. One of the major stumbling blocks is the heterogeneous nature of hemoglobin. Hemoglobin is really a heterogeneous group of
proteins with a significant number of variants in the population. (Winter 1986) In addition, hemoglobin has the ability to undergo nonenzymatic glycosylation at a variety of sites that may be different for each hemoglobin variant, therefore having an impact upon the determination of gHb products. (Shaprio 1980)

HbA1c is the subfraction measured in the DCCT to reflect the average blood glucose concentration over a three month period. (DCCT 1993, NDG 1984) In the DCCT a high gHb (>7.2%) indicated poor glycemic control. Initially HbA1c was used interchangeably with gHb, however with the realization that hemoglobin variant chains and abnormal hemoglobins contain glucose ketoamine linkages to lysine residues of α-chain and β-chain N-terminal valines and ε-amino groups of lysine residues the two terms are no longer interchangeable.

Summary

It is clear from the literature review presented above that cellular insulin resistance is a dysfunction of the insulin receptor at a point preceding signal generation. This indicates that the problem lies in ligand-receptor binding. It is also clear that insulin resistance, and many of the other complications of diabetes are related to hyperglycemia. Many of the complications of diabetes have been shown to be directly related to hyperglycemia induced
AGE formation. It is reasonable to expect that AGE formation on the insulin receptor would adversely affect ligand-receptor binding, and that this impaired binding would be manifested as insulin resistance. This project will test that hypothesis that AGE formation on insulin receptors alters the physical, chemical and functional characteristics of the insulin receptor to determine if AGE formation on the insulin receptor is indeed related to insulin resistant states.
CHAPTER 3

ANALYTICAL METHODS

The Model System

Mouse embryo fibroblasts (NIH 3T3-L1) from American Tissue Culture Collection (ATCC) were grown to confluence (Appendix A) in high glucose growth media, consisting of Dulbecco's Modified Eagles Media (DMEM) (Gibco BRL) with 10% Bovine Calf Serum (BCS) (Gibco BRL) and 16.7 mM (450 mg/dL) glucose with 1 mg/mL bacitracin and 1 mg/mL ampicillin. Differentiation from fibroblasts to adipocytes was initiated at two days post confluence by incubating for forty eight hours in differentiation media consisting of high glucose growth media supplemented with dexamethasone (1 µM), methylisobutylxanthine (0.5 mM), and insulin (10.0 µg/mL) (Sadowski 1992, Kowsaki 1993). Following initiation of differentiation, the media was changed to a modified growth, low glucose media consisting of DMEM with 20% FBS and 5.5 mM (100 mg/dL) glucose. This media was changed every 48 hours until 80% to 90% of the cells produced visible lipid. Media composition is shown in detail in Appendix B.
Differentiation of 3T3-L1 fibroblasts to adipocytes was confirmed by Oil-Red O lipid stain (Sadowski 1992). This process consists of rinsing the cell monolayers two times with iced phosphate buffered saline (PBS), then fixing the cells for one hour at 22°C with PBS containing 3.7% formaldehyde and finally staining the cells neutral lipid with Oil-Red O (Sigma Chemical Co.) lipid stain. After removal of stain, the cells were rinsed with 60% isopropanol and several washes of distilled deionized water (DDI).

Receptor Isolation and Purification

Confluent differentiated 3T3-L1 adipocytes to be solubilized were incubated in $10^{-8}$ M insulin for 30 minutes at 37°C. Media was then replaced with iced PBS. Cells were scraped from the flask and transferred to a siliconized centrifuge tube. The cells were centrifuged for 10 minutes at 2000 rpm. The supernatant was decanted and discarded. The cells were solubilized by immersion in 6 mL of iced solubilization solution consisting of 50 mM Hepes (pH 7.6), 1 mM phenylmethylsulfonyl fluoride (PMSF), 150 mM NaCl and 50 μg/mL of polyglycine for five minutes. This was followed by sonication on ice for ten seconds. Cells were then incubated on ice for an additional five minutes. This extract was ready for further purification.

Solubilized insulin receptors were purified by wheat germ agglutinin (WGA) agarose chromatography. A wheat germ-
Sepharose column was poured, washed and equilibrated with equilibration buffer (see Appendix B). The solubilized receptor solution (from the initial supernatant and the initial pellet) was diluted two-fold in Hepes (50 mM, pH 7.6) and added to the wheat germ column; eluant was recycled four times at 27 °C (Appendix A). The final eluate was retained. After the solubilized receptor solution was passed through the column four times, the columns were washed with a wash buffer. The bound fraction was eluted from the column with elution buffer. The final eluate was concentrated to four mL on an Amicon PM-10 membrane under nitrogen at 18 PSI. The presence of insulin receptor protein was confirmed by the Bradford method. (Bradford 1976) Figure 2 shows a standard curve for BSA.

The molecular weights of the insulin receptor subunits were determined by denaturing gel electrophoresis utilizing sodium dodecyl sulfate, and an 5% polyacrylamide gels (SDS-PAGE). Vertical, SDS mini-gels (Bio-Rad) were prepared as shown in detail in Appendix A. Insulin receptor aliquots were boiled for two minutes in dithiothreitol and 1% SDS. Samples and high molecular weight markers (Bio-Rad) containing small amounts of sucrose were added to the gel sample wells. Gels were electrophoresed at 90 mV (constant voltage) until the dye front reached the bottom of the gel. Completed gels were either utilized for western blotting or were stained by coomassie blue stain for protein. Following
Figure 2: Standard curve for bovine serum albumin (BSA).
electrophoresis proteins were transferred from the polyacrylamide gel to nitrocellulose (NC) paper by either non-electrophoretic or electrophoretic blotting.

A non-electrophoretic transfer method was utilized to detect the holo-receptor molecule. Briefly, filter paper "wicks" in transfer buffer were placed under the gel. A piece of nitrocellulose (NC) paper (Bio-Rad) was then placed on top of the gel. A second piece of filter paper was placed on top of the NC-paper followed by a 2½ inch layer of clean, dry brown paper towels. A weight was then placed on top of the brown paper towels. The transfer buffer reservoir was filled with transfer buffer to a level such that the "wicks" were barely touching the top of the transfer buffer. The level of the transfer buffer was maintained by addition of buffer throughout the transfer.

An electrophoretic transfer method was used to detect receptor protein electrophoresed in a denaturing gel. This allowed for a more rapid and controlled transfer of protein. A transfer apparatus was set up and the gel was placed in the apparatus (See Appendix A). A magnetic stirrer was placed in the bottom of the transfer buffer reservoir with an ice pack. The cover was then placed on top of the transfer buffer reservoir. The entire apparatus was placed into an iced water bath and set on top of a stir plate. Transfer was accomplished under constant power at a density of 0.8-1.0 mA/cm² for 0.5 hours.
Isoelectric focusing was used to determine changes in the isoelectric point of the insulin receptor with glycation. Precast gels (Pharmacia) with a pH range of 4 - 6.5 were used to separate insulin receptor proteins by their isoelectric point.

A Resolve IEF unit (Isolab) was used. The cooling surface of the IEF was cleaned with DDI water. Approximately 2 mL of distilled/deionized water was evenly distributed on the cooling plate. The gel was placed carefully on top of the cooling surface so as to not to trap air bubbles between the gel/gel support film and the cooling plate. Excess water was thoroughly blotted from the gel periphery with a clean dry paper towel. The gel was centered on the cooling plate. An electrode wick was evenly saturated with anolyte solution (0.1 M glutamic acid in 0.5 M H$_3$PO$_4$) and then placed on the anode (+) side of the gel. The cathode wick was prepared and placed in the same manner using the catholyte solution (0.1 M β-alanine). Pieces of Wattman #1 filter paper (4 mm x 8 mm) were soaked in sample and placed near the cathodic wick. An additional 5 μL of sample was added to each sample "well". The IEF unit was cooled to 15°C and the unit was set to 15 watts (constant power) and focused for eight hours. Low pI (pH 2.5 - 6.0) calibrators were run alongside samples to provide the pH gradient. Confirmation of pH gradient was accomplished by removing 1 cm sections of the gel after isoelectric focusing.
and placing them in DDI water overnight. The pH of the water containing the gel sections was then determined using a pH meter calibrated to pH 7.0 and pH 4.0. Proteins were fixed at their isoelectric points by immersing the gel in fixing solution (see Appendix B) for one hour. Gels were then washed for five minutes by immersion in a destaining solution. Gels were then either stained for the presence of protein by coomassie blue stain as described in Appendix A or reacted with antibody as described previously. Gels were preserved by first washing in distilled deionized water for one hour then by immersion in a preserving solution (see Appendix B) for one hour and drying overnight.

Measures of Cellular Insulin Activity

Receptor-ligand binding was measured by a $^{125}$I-Insulin binding radioimmunoassay (Wilden et al 1991a, 1992b, Schumacher et al 1991, Frattali et al 1993).

Undifferentiated cells were grown in standard growth media (see above) to confluence in T-75 flasks to a final concentration of $7.5 \times 10^5$ cells/flask. Cells were then differentiated to adipocytes and glycated as described above. Cells were washed with iced PBS and then incubated in 10 mL low glucose media containing 0.5 μCi $^{125}$I-insulin for 24 hours at 2 - 4 °C. Media was then replaced with low glucose media containing increasing concentrations of unlabeled insulin (from 0, $10^{-10}$, $10^{-9}$ M, $10^{-8}$ M and $10^{-10}$ M).
for 4 hr at 2 - 4 °C. Media was then removed an the cells lysed with 1.0 ml of 1 M NaOH. An insulin assay (Binax) was then done according to manufacturers instructions. Activity was counted in a gamma counter (Isodata) for 1 minute. Scatchard analysis of insulin binding data was carried out as described previously. (Murakami and Rosen 1991, Scatchard 1949).

Tyrosine kinase activity was measured according to the method of Roehrig et al (1993). Briefly, differentiated 3T3-L1 adipocytes were transferred to 12 well plates and allowed to adhere for 24 hours in low glucose growth media. Cells were then subjected to a glycation procedure for 96 hours. Glycated and non-glycated adipocytes were incubated in media containing varying levels of glucose (25 mM and 5.5 mM) and insulin (0 M, 10^{-10} M, and 10^{-9} M) for variable time intervals (0, 2, 5, and 10 minutes) at 37°C. Media was then removed and the cells were washed with normal (0.9%) saline. The cells were removed from the plates by scraping and transferred to glass test tubes. Cells were then sonicated for five seconds and boiled in gel loading buffer for 2 minutes. Samples were then frozen at -20°C for further analysis by western blot analysis.

Protein visualization was accomplished by use of a alkaline phosphatase-labeled antibody. Aliquots (40 uL) of each sample were dot blotted on NC-paper using a 96 well manifold (Biorad). NC-paper containing the transferred
protein was washed with 20 mL of Tris buffered saline plus
tween-20 (TBST) plus 1% gelatin for four hours at room
temperature to block non-specific binding. This was
followed by two additional wash steps with TBST without
gelatin for ten minutes each. The primary antibody was
added to the NC-paper containing the transferred protein and
incubated for 60 minutes at room temperature with gentle
shaking. Antiphosphotyrosine antibody was obtained from
Zymed and diluted 1:3000 with TBST plus 1% gelatin. The
primary antibody was collected and the NC-paper was washed
two times for 10 minutes each in TBST without gelatin. A
second antibody was then added and incubated with gentle
shaking for 60 minutes at room temperature. The secondary
antibody was a rat-anti-mouse IgG coupled to alkaline
phosphatase (Bio-Rad) and was diluted 1:1000 in TBST with 1%
gelatin. Following incubation the secondary antibody was
collected and the NC-paper was washed three times for ten
minutes each in TBST without gelatin. A final wash with
TBS without tween-20 for five minutes was done before the
detection reagent was added. Detection reagent (see
Appendix B for contents) was added to the NC-paper and
incubated in complete darkness for 15 minutes or until
adequate color development was seen.

Glycogen deposition was measured according to the
method of Roehrig and Allred (1974) Flasks of confluent,
differentiated 3T3-L1 adipocytes were separated into 3
experimental groups. Media in each group was changed to the treatment growth media as previously described. One treatment group (No Insulin) was incubated for 96 hours without any further treatment. A second treatment group (Glucose with Insulin) received $10^{-9}$ M insulin when the media was changed on day 1 and was incubated for 96 hours. The third treatment group (Glucose Delayed Insulin) was incubated for 96 hours before receiving $10^{-9}$ M insulin and was incubated for an additional 96 hours. Media was then removed from all the flasks, and the cells were washed with iced normal (0.9%) saline five times to remove any residual media. The cells were then scraped from the plates, placed in a plastic centrifuge tube and centrifuged at 2,000 rpm for 10 minutes. The supernatant was removed and the cells were resuspended in 4 mL of iced normal (0.9%) saline. The cells were then sonicated and perchloric acid was added to a final concentration of 7% to denature enzymes. The cellular extract was then neutralized to pH 6-7 by addition of sodium hydroxide. Amyloglucosidase was then added to the cellular extract and the mixture was incubated at 55°C for 10 minutes. Glucose concentration was then determined by glucose oxidase method (Sigma). A standard curve for both glycogen (Figure 3) and glucose (Figure 4) was developed, and the relative amounts of glycogen within each sample was determined by comparison to the standard curve.
Figure 3: Standard curve for glucose.
Figure 4: Standard curve for glycogen. Glycogen concentration is in glucose equivalents.
An adaption of the method of Hohorst (1959) was used to measure hexokinase activity. Flasks of confluent, differentiated cells were incubated in four different levels of glucose as described earlier. Cells were washed, centrifuged, scraped and sonicated as described in the glycogen assay above. Following sonication the cellular extracts were placed on ice. Briefly an aliquot (0.05 mL) of the cellular extract was mixed with a reaction mix containing ATP, MgCl₂, NADP⁺, glucose, and glucose-6-phosphate dehydrogenase. The absorbance was then measured spectrophotometrically at 340 nm at 0.5 second intervals, for two minutes. Aliquots (0.010 mL, 0.005 mL, and 0.003 mL) of purified hexokinase (Sigma) were assayed to validate the method. (See Figure 5) The concentration of glucose phosphorylated per unit time was determined from the absorbance using Beer's Law and a molar extinction coefficient for NADPH of 6.22 x 10³. Phosphorylation rates were determined by calculating the NADPH (mM) production per minute per unit volume (uL) reaction mixture added to the aliquot. The initial rate of the reaction was used as an indication of hexokinase activity.

Glycation of receptors in intact cells was accomplished by incubating the cells in low glucose growth media (DMEM with 10% BCS and 5.5 mM (100 mg/dL) glucose) without antibiotics and supplemented with varying concentrations of glucose (See Appendix A) for 96 hours at 37°C.
Figure 5: Hexokinase Activity
Glycated cells were either analyzed directly or subjected to receptor solubilization and purification.

The degree of glycation was determined in triplicate by periodate oxidation according to the method of Ahmed and Firth (1991). This method oxidizes compounds containing cis-diol groups to formaldehyde and then measures the amount of glycation as a function of formaldehyde release. Oxidation of the samples was initiated by the addition of 0.1 M HCl and 0.05 M NaIO₄ to each aliquot which was then incubated for 30 minutes at room temperature. Oxidation was terminated by cooling samples in an ice water bath for 10 minutes. Periodate was precipitated by adding 15% ZnSO₄ and 0.7 M NaOH while vortexing aliquots throughout addition. Aliquots were then centrifuged for 10 min at 9000 g to remove precipitated zinc periodate. The supernatant was then transferred to 96 well flat bottomed microtiter plate wells. Freshly prepared formaldehyde detection reagent (acetylacetone in 3.3 M ammonium acetate) was added to each well and mixed thoroughly. The detection was allowed to develop by incubating for an hour at 37°C. The extent of glycation was determined by the quantity of formaldehyde released during the periodate oxidation as measured by fluorescence following excitation at 410 nm using a Biotek 311 microplate reader.

Bovine serum albumin (BSA) was used to determine a relative rate of glycation. BSA (50 μg/mL) was incubated at
37 °C with three different levels of glucose (5.5 mM, 16.7 mM, and 55 mM) dissolved in PBS. Samples were taken at intervals up to 108 hours. The results shown in Figure 6 clearly demonstrate a time and dose dependent rate of glycation, with a maximum level of glycation at 96 hours. The degree of glycation remained constant with 5.5 mM glucose. Controls included PBS without protein or glucose, PBS without protein, and PBS without glucose. Control values demonstrated no glycation.

Glucose concentration was confirmed by glucose oxidase assay. (Sigma 1995) Briefly, a combined enzyme-color reagent solution was prepared (See Appendix A) and added to sample aliquots in 13 x 100 mm test tubes. This mixture was incubated for 30 minutes at 37 °C and the absorbance read on a spectrophotometer (Beckman Instruments, Brea, CA) at 500 nm. Fresh standards were made up for each determination.

**Glycohemoglobin**

Fresh venous samples were randomly obtained from five healthy non-diabetic volunteers. All samples were processed within two hours after the blood was drawn. Samples were centrifuged at 2000 rpm for 10 minutes, the plasma was removed and the cells were washed three times in iced PBS. Aliquots of each sample were taken to determine baseline erythrocyte count and degree of glycation. Aliquots (1 mL) of packed erythrocytes were then transferred to sterile
Figure 6: Glycation in bovine serum albumin (50 μg/mL) following incubation with 5.5 mM (a); 16.7 mM (b) and 55.5 mM (c) glucose
plastic 50 mL centrifuge tubes containing 10 mL of Earl's basic salts solution supplemented with one of four different levels of glucose (5.5 mM, 11.1 mM, 16.7 mM, or 22.2 mM). All samples were incubated in a Dubanoff shaking incubator at 37 °C with 5% CO₂. Cell counts and degree of glycation was determined on 0.1 mL aliquots taken from each sample every 48 hours. Cell counts were determined on a Coulter Counter. Degree of glycation was determined by periodate oxidation method described above.

**Statistical Analysis**

Analysis of Variance (ANOVA) and Tukey's multiple comparison test (HSD) was used to determine significant differences at each glucose treatment level for each parameter measured. Correlation analysis were used to test the relationship with the effect seen and the degree of glycation. Unless otherwise noted, all data were obtained from pooled samples of seven flasks with 1.5 x 10⁷ cells per flask. The critical value for statistical significance of data analysis was determined *a priori* at *p* < 0.05. Statistics were either calculated by hand according to Kuzma (1992) or with the SYSTAT PC-based program. Figures were created with Microsoft Excell and Microsoft Paint programs.
CHAPTER 4

RESULTS

This study used a murine adipocyte model to test the hypothesis that formation of advanced glycosylation end product (AGEs) adducts on insulin receptors inhibits insulin receptor binding by altering the physical and chemical properties of the insulin receptor. Specifically 3T3-L1 fibroblasts were differentiated to adipocytes. Formation of AGE was initiated by incubating the cells in growth media supplemented with four different, physiologically relevant, levels of glucose for 96 hours. The functional, biochemical and physical properties of the insulin receptors were then determined. To provide a link to clinical applications of AGE formation, whole blood samples from non-diabetic individuals were then incubated in glucose to determine a dose and time dependent rate of glycation.

Induction of differentiation of 3T3-L1 fibroblasts to adipocytes was confirmed by the appearance of lipid droplets within the cytoplasm of the cells after staining with Oil-Red-O (Sigma) and giemsa (Sigma). Figures 7, 8 and 9 demonstrate the difference between undifferentiated 3T3-L1
Figure 7: Undifferentiated 3T3-L1 Fibroblasts Stained with Oil Red-O and counter stained with Giemsa. Cells photographed with Fuji Film using AO Scientific Instruments Microstat microscope under 400x magnification.
Figure 8: Differentiated Adipocytes - Unstained. Cells photographed with Fuji Film using AO Scientific Instruments Microstat microscope under 400x magnification.
Figure 9: Differentiated Adipocytes - Stained with Oil Red-O and counter stained with giemsa. Lipid droplets appear reddish-orange. Cells photographed with Fuji Film using AO Scientific Instruments Microstat microscope under 400x magnification.
fibroblasts and differentiated stained and unstained adipocytes. Triglyceride droplets within the cell appeared red and are refractile. Cells lose the thin spindle-like shape with three to four arms that is characteristic of fibroblasts when they differentiate to adipocytes. The number of cells expressing lipid droplets significantly increased \((p < 0.05)\), while the relative size of the droplets remained constant when the cells were subjected to increased levels of glucose in the glycation experiments. (See Table 1) Figures 10 and 11 demonstrate the morphological differences in the amount of lipid deposition with increasing glucose in the growth media. Lipid formation was quantified by image analysis. Adipocytes were grown in 3 mL chamber slides (Falcon) to confluence, differentiated and glycated as described above. Cells were then stained for lipid with Oil-Red-O and counter stained with giemsa. Six random photomicrographs (Kodak color print film) were taken of each slide at 100 x magnification using a Nikon Microscope. The total number of distinct lipid droplets per photomicrograph was determined by visual count and the average number of droplets per photomicrograph determined. The average diameter of the twenty largest lipid droplets on each photomicrograph was determined by use of a micrometer. Table 1 \((p < 0.05)\) gives the average number and size of distinct lipid droplets per photomicrograph.
Figure 10: Microscopic appearance (100x) of adipocytes after incubation with 5.5 mM (A) and 11.1 mM (B) glucose supplemented growth media. Photomicrograph taken on Kodak film using a Nikon Optiphot-2 microscope.
Figure 11: Microscopic appearance (100x) of adipocytes after incubation with 16.7 mM (A) and 22.0 mM (B) glucose supplemented growth media. Photomicrograph taken on Kodak film using a Nikon Optiphot-2 microscope.
Table 1: Average number and size of lipid droplets. Six random photomicrographs (Kodak color print film) were taken of each slide at 100 x magnification using a Nikon Microscope. The total number of distinct lipid droplets per photomicrograph was determined by visual count and the average number of droplets per photomicrograph determined. The average diameter of the twenty largest lipid droplets on each photomicrograph was determined by use of a micrometer.

<table>
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<th>SD</th>
<th>Ave Size (μm)</th>
<th>SD</th>
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<tr>
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<td>3.7</td>
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Receptors were glycated by incubating differentiated cells in DMEM supplemented with 20% FBS and either 5.5 mM, 11.1 mM, 16.7 mM or 22 mM glucose for 96 hours. The time required to achieve a maximum level of glycation was based on glycation experiments with albumin described earlier. Cell viability following incubation was determined by staining the cells with Trypan Blue vital stain. There was less than one Trypan Blue stained cell per high power field (500 x magnification) for all four levels of glucose incubation. The higher magnification was used to more clearly identify those cells that take up the Trypan Blue stain. Total variability between 100 x and 500 x magnification was less than 1%.

The degree of glycation for each level of glucose supplementation was determined by periodate oxidation as described earlier. Figure 12 shows the relative amounts of total glycation for each level of glucose supplementation. The amount of glycation represents the total glycation for the adipocyte. Glycation at the 5.5 mM level is base line measure of both enzymatic glycosylation and non-enzymatic glycation. There is a slight, yet significant (p < 0.05), dose dependent increase (r² = 0.98) in the amount of glycation relative to the glucose dose.
Figure 12 - Total glycation for cells incubated in glucose supplemented DMEM for 96 hours. Cells were grown to confluence at a density of $1.5 \times 10^5$ cells/flask. Glycation measured as a function of formaldehyde release, where there are 2 moles of formaldehyde released for each mole of glucose.
Receptor Purification

Insulin receptors were solubilized by a standardized protocol described in Chapter 5 and in more detail in Appendix A. Solubilized receptors were then purified by wheat germ agarose (WGA) column chromatography. Raw cellular extract WGA purified receptors and purified concentrated receptors were compared on 5% SDS-PAGE gels.

Aliquots from the WGA eluates were also subjected to isoelectric focusing as described earlier. Confirmation of pH gradient was accomplished by measuring the pH of 1 cm sections of the gel after isoelectric focusing for eight hours. A regression line of the pH vs distance was calculated from the raw data and a "distance map" for the gel was developed. Albumin was used as a marker protein because it has an isoelectric point near 4.2, which is close to the published range of 4.8 for the insulin receptor and within the range of the IEF gels used. Six albumin replicates were run on three different gels to establish the variance for this procedure. For this study the pI for albumin was 4.15 ± 0.19 (S.D.). The RF values for each sample was determined by comparing the distance of migration of the protein band and the dye front. The isoelectric point was then calculated by comparing the RF value for the insulin receptor to the experimentally determined pH for the gel. Table 2 shows the RF, calculated pI, and relative amount of glycation for each protein band. When the RF for
each insulin receptor is calculated and the pI determined, there is a significant change (p < 0.05) in the isoelectric point that is strongly correlated (r = 0.994) with the degree of glycation.

Aliquots from WGA isolates for each treatment level were boiled in gel loading buffer for two minutes to partially denature the protein. Samples were then electrophoresed on a 5% SDS-PAGE gel as described earlier. An RF was calculated for each band, and the molecular weight for each band determined from the RF as described previously. The gel was analyzed by a scanning densitometer (Pharmacia Biotech, San Francisco CA) and the relative quantity of each protein band determined as a function of the area scanned. There was a significant increase in the amount of protein in the 300 kDa band in the 16.7 mM and 22.2 mM treatment levels that was not detected in the 5.5 mM and 11.1 mM treatment levels. Table 3 shows the relative increase in the amount of protein in the 300 kD band with increasing levels of glucose in the incubation media.

Ligand-Receptor Binding

Ligand receptor binding was tested by $^{125}$I-Insulin radioimmunoassay. Growth media was removed from confluent monolayers (7.5 x $10^5$ cells/flask) of differentiated 3T3-L1 adipocytes and cells were incubated with low glucose media containing 0.5 μCi $^{125}$I-insulin (Binax) per flask for 24
Table 2: RF, pI and relative amounts of glycation. Precast SDS-PAGE gels (Pharmacia, San Francisco CA) with a pH range of 4.0 to 6.5 were used. The anolyte solution was 0.1 M glutamic acid in 0.5 M H$_3$PO$_4$. The catholyte solution was 0.1 M β-alanine. Whatman #1 filter paper was soaked in sample with an additional 5 μL of sample added. The gel was focused for eight hours at 15 watts (constant power). Confirmation of pH gradient was accomplished with low pI (pH 2.5 - 6.0) calibrators and as described in Chapter 3, Methods. Glycation for each sample was measured by periodate oxidation as described in Chapter 3, Methods. Glycation is given as micromoles of formaldehyde released.
hours at 2-4 °C. Media containing labeled ligand was then removed and cells were incubated for 4 hours at 2-4 °C in low glucose media containing unlabeled insulin (0, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-6} M). Media was then removed and the cells were washed with PBS 1 time, then lysed with 1 N NaOH. Total counts were determined by counting for one minute on a gamma counter. The bound and free fractions were determined, the association constant calculated and a Scatchard analysis was performed. Figure 13 shows the relative relationships between the Scatchard plots for each of the treatment groups. Figure 14 shows Scatchard plots for the 5.5 mM and 22.0 mM treatment groups. Receptor-ligand binding for cells incubated in 11.1 mM and 22.0 mM glucose supplemented media is essentially the same. Receptor-ligand binding for cells incubated in 5.5 mM and 16.7 mM glucose supplemented media is essentially the same. There is, however a significant (p < 0.05) and reversible right shift in the receptor-ligand binding curve for cells incubated in 11.1 mM/22.0 glucose supplemented media compared to cells incubated in 5.5 mM and 16.7 mM glucose supplemented media.

Tyrosine phosphorylation

Effects of AGE formation on signal generation was determined by tyrosine phosphorylation as described earlier. Briefly cells incubated with variable levels of glucose for
<table>
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<th>SD</th>
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Table 3: Relative amounts of protein in the 300 kD band as determined by scanning densitometer. Densitometry performed on an LKB Ultrascan XL (Pharmacia Biotech, San Francisco CA). The molecular weights of the insulin receptor subunits were determined by denaturing gel electrophoresis utilizing sodium dodecyl sulfate, and an 5% polyacrylamide gels (SDS-PAGE) vertical, mini-gels (Bio-Rad). Gels were electrophoresed at 90 mV (constant voltage) until the dye front reached the bottom of the gel.
Figure 13: Relative relationships between the Scatchard plots for cells incubated in 5.5 mM (a), 11.1 mM (b), 16.7 mM (c) and 22.0 mM (d) glucose.
Figure 14: Scatchard plots for 5.5 mM (a) and 22.0 mM (b) treatment groups
96 hours were stimulated with two levels of glucose (5.5 mM and 25.0 mM) in the presence of variable levels of insulin (0, 1 x 10^{-10} M, and 1 x 10^{-9} M) for predetermined time periods (0, 2, 5, and 10 minutes). At the specified time periods, cells were lysed by sonication and aliquots were boiled for two minutes in gel loading buffer. Dot blots of cell lysates were treated with antiphosphotryosine antibody (Zymed). The bound antibody was detected with an alkaline phosphatase detection system. The relative densities of the individual dot blots were determined on a scanning densitometer, and reported as function of the area under the curve of the scan. Preliminary dot blot analysis of non-glycated cells incubated in 5.5 mM glucose indicated maximal phosphorylation at 2 minutes at both 1x10^{-9} M and 1x10^{-10} M insulin stimulation. Statistical analysis using a two way ANOVA demonstrates that cells incubated in 22.0 mM glucose for 96 hours exhibited a significant decrease (p < 0.05) in the level of tyrosine phosphorylation. The decrease in tyrosine phosphorylation was greatest when comparing cells incubated in physiologically normal (5.5 mM and 11.1 mM) glucose and physiologically high (16.7 mM and 22.0 mM) glucose. (See Figures 15 through 17) There was a slight difference in tyrosine phosphorylation between cells incubated in 5.5 mM and 11.1 mM glucose, however the difference was not statistically significant. There was no significant difference in tyrosine phosphorylation between
Figure 15: Average activity of tyrosine phosphorylation in cells incubated in 5.5 mM and 11.1 mM glucose. Activity represents the relative area of dot blots following incubation with 5.5 mM (Blue) and 25.0 mM (Yellow) glucose and in the absence of insulin (●) and in the presence of 10⁻¹⁰ M (□), or 10⁻⁹ M (○) insulin.
Figure 16: Average activity of tyrosine phosphorylation in cells incubated in 16.7 mM and 22.0 mM glucose. Activity represents the relative area of dot blots following incubation with 5.5 mM (Green) and 25.0 mM (Red) glucose and in the absence of insulin (□) and in the presence of $10^{-10}$ M (○) or $10^{-9}$ M (●) insulin.
Figure 17: Combined average activity of tyrosine phosphorylation in cells incubated in 5.5 mM and 11.1 mM glucose (Blue) compared to cells incubated in 16.7 mM and 22.0 mM glucose (Red). Activity represents the average relative area of dot blots following incubation with 5.5 mM (○) and 25.0 mM (●) glucose for all levels of insulin stimulation.
cells incubated in 16.7 mM and 22.0 mM glucose. The data demonstrates a dose dependent change in tyrosine phosphorylation with increasing glycation.

**Glycogen Deposition**

The effect of glycation on glycogen deposition was determined by a modification of the method of Roehrig and Allred (1974) described earlier. This method measures the amount of glycogen deposition in response to insulin stimulation and in the presence of glucose. Confluent differentiated adipocytes (1.5 x 10^7 cells per flask) were incubated in high (25 mM) glucose supplemented media and in the presence or absence of insulin stimulation. One group of cells received no insulin stimulation. This was to measure the basal level of glycogen deposition present in the cells. Insulin was added either with the glucose load (glucose with insulin group) or 24 hours after the glucose load (glucose delayed insulin group). Following incubation, cells were removed from the plates, washed with PBS - no glucose 2 times, and lysed by sonification. The amount of glycogen was measured following digestion of cell lysates with α-amylase and subsequent measurement of free glucose in the sample. As expected, glycogen deposition was significantly increased (p < 0.05) with insulin stimulation. However, the magnitude of the glycogen deposition varied with the timing of the insulin stimulation and the amount of...
glucose in the incubation media. Glycogen deposition for the first three treatment groups rose slightly, and significantly \((p < 0.05)\) in cells that received no insulin stimulation, and when glucose and insulin were added at the same time. The peak increase was seen in the 16.7 mM treatment group. The amount of glycogen deposition then fell significantly in the 22.0 mM glucose treatment group. The greatest increase in glycogen deposition occurred when insulin stimulation followed incubation with 5.5 mM and 11.1 mM glucose for 96 hours. See Figure 18. Glycogen deposition was significantly decreased \((p < 0.05)\) at both the 16.7 mM and 22.2 mM treatment levels, when compared to the 5.5 mM and 11.1 mM treatment levels. However, there was no significant difference between the 16.7 mM and 22.0 mM treatment levels for those cells that received insulin stimulation after incubation with glucose for 96 hours. The most significant drop in glycogen deposition was in those cells that received no insulin stimulation or received insulin at the same time as the glucose supplementation. Glycogen deposition in these two treatment groups was reduced to nominal levels.

**Hexokinase Activity**

Hexokinase activity was determined by the method of Hohorst (1959). This procedure measures the activity of the glucose phosphorylating enzyme, hexokinase in response to...
Figure 18: Glycogen Deposition. Cells were incubated in 25.0 mM glucose supplemented growth media without (A) insulin stimulation; with (B) insulin stimulation concurrent with glucose (25.0 mM); and with (C) insulin stimulation 24 hours post glucose supplementation.
insulin stimulation. There is no significant difference in
the activity of hexokinase across the four different
treatment groups. (Figure 19) Glycation had no apparent
impact on hexokinase activity.

Glycohemoglobin

For the in-vivo rate of AGE formation in hemoglobin,
freshly obtained erythrocytes were incubated in growth media
for set periods and the amount of glycation was measured by
periodate oxidation as described in Chapter 3. Erythrocyte
viability was measured by counting the number of viable
cells in a hemocytometer. The amount of glycation per
erthrocyte are given in Figure 20. There was a significant
amount of cellular destruction associated with increasing
glucose supplementation in the growth media. (Figures 21
through 23) In erythrocytes incubated in 16.7 mM glucose,
(Figure 23) there are more crenated cells, more cellular
debri, and fewer viable cells that seen in erythrocytes
incubated in 5.5 mM (Figure 21) or 11.1 mM (Figure 22)
glucose supplemented media. Using Tukeys Honestly
Significantly Different, there is a significant increase in
the amount of glycation that is both time dependent and
glucose dose dependent. There is also a significant
decrease in the total cell count. All cell counts are
derived from packed red cells which will account for an
apparently higher than normal count.
Figure 11: Hexokinase Activity. The relative amounts of hexokinase activity for cells incubated in 5.5 mM (A), 11.1 mM (B), 16.7 mM (C) and 22.0 mM (D) glucose supplemented media.
Figure 20: Glycation per erythrocyte for cells incubated in 5.5 mM (A), 11.1 mM (B), and 16.7 mM (C) glucose supplemented media.
Figure 21: Photomicrograph (400x) of erythrocytes incubated in 5.5 mM glucose media for 72 hours. Photographed with Fuji film using an Olympus microscope.
Figure 22: Photomicrograph (400x) of erythrocytes incubated in 11.1 mM glucose media for 72 hours. Photographed with Fuji film using an Olympus microscope.
Figure 23: Photomicrograph (400x) of erythrocytes incubated in 16.7 mM glucose media for 72 hours. Photographed with Fuji film using an Olympus microscope.
CHAPTER 5

Discussion

The purpose of study was to test the hypothesis that formation of AGEs on insulin receptors alters the physical chemical and functional characteristics of the insulin receptor. The basic concept was to evaluate whether AGE formation could be a viable model for the development of cellular insulin resistance commonly seen in type II diabetes.

This study utilized a murine adipocyte model, swiss mouse embryonic fibroblasts (3T3-L1). Embryonic fibroblasts were differentiated to adipocytes by incubation with supplemented growth media. (see Chapter 5 and Appendix A) Differentiated fibroblasts and clearly demonstrated morphological and metabolic characteristics common to adipocytes. These characteristics included a spherical shape and an ability to convert glucose to triglyceride as evidenced by a glucose dose dependent increased lipid formation. Full differentiation was complete within five to seven days after initiation. These findings agree with other studies that have used this model to study insulin-

Glycation was presumed to reached a maximum level after 96 hours incubation at 37 °C. This is based on a time course study with albumin, and agrees with previous studies that report maximum AGE formation occurring between 0.5 and 120 hours depending on the glucose concentration. (Glomb and Monnier 1995)

Cell viability following incubation was determined by staining with the vital stain Trypan Blue. Trypan Blue stains the nuclei of non-viable cells because it is taken up by osmotic action through damaged cellular membranes. There was less than one Trypan Blue stained cell per high power field (50x magnification) for all four levels of glucose incubation. This is a good indication that not only was there nominal to no toxic effect of glucose on the cells, but also that any nominal effect was consistent across all four treatment levels.

The principal limitation of this study is the fact that the degree of glycation is indicative of the amount of both enzymatic glycosylation and non-enzymatic glycation present in the adipocyte. Periodate oxidation cannot distinguish non-enzymatic glycation and enzymatic glycosylation. It is the change in the degree of glycation with respect to glucose dose that is significant, not the absolute amount of glycation.
The rate of AGE formation was clearly time-dependent and glucose concentration-dependent. Glomb (1995) reported obtaining a maximum formation of AGE products at 0.5 hours with a glucose concentration of 20 mM. This peak was extended to 120 h with a glucose concentration of 0.28 mM. (Glomb and Monnier 1995) These findings correlate with an increased rate of protein cross-linking, a key element in AGE formation, with time as shown on SDS-polyacrylamide gel electrophoresis. (Glomb and Monnier 1995)

**Molecular Weight**

Denaturing gel electrophoresis provided strong evidence for an increase in cross-linking of the insulin holoreceptor protein. There was a significant and dose dependent increase in the amount of holoreceptor protein with glycation. In fact there was almost a ten fold increase in the amount of holoreceptor with the 16.7 mM glucose treatment level when compared to the 5.5 mM glucose treatment level. At the same time there was an apparent decrease in the appearance of the α and β subunits at the higher glucose concentrations. The change in the molecular weight of the holoreceptor protein argues for an increase in the cross-linking. The relative decrease in the appearance of the receptor subunits, combined with the apparent increase in cross-linking indicates that the cross-linking
may increase the resistance of the protein to the
denaturation conditions used.

The insulin receptor is a symmetrical, disulfide-linked
heterotrimeric dimer, consisting of two identical α-
subunits, and two identical β-subunits. (Lee et al 1993,
Class I disulfide bonds maintain the dimeric structure of
the receptor in the absence of bound ligand. (White 1994,
Massague and Czech 1982; Boni-Schnetzler 1986; Sweet et al
1987) Binding affinity of the receptor is greatly
diminished when the disulfide bonds are reduced. Purified
holoreceptors with intact disulfide bonds exhibit a negative
cooperativity and only one high affinity insulin-binding
site. (Boni-Schnetzler et al 1987; Sweet et al 1987) This
difference between the holoenzyme and the reduced enzyme
confirms the concept that a dimer is necessary for proper
insulin receptor action. (Massague et al 1980; Ullrich et al
1985; Ebina 1985) The dose dependent increase in the amount
of holoreceptor seen here indicates that the receptors are
less susceptible to reduction of the disulfide bonds. While
the evidence presented here strongly indicates either an
increase in the number of disulfide bonds or some protective
action on the disulfide bonds, there was no direct
indication that AGE formation provides a protective
mechanism for the disulfide bonds. Increased holoreceptor

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may be due to the increased crosslinking of the protein that is commonly associated with AGE formation. (Glomb and Monnier 1995) In either case the fact that the holoreceptor appeared to be resistant to denaturation argues for an alteration in the receptors structure that should be reflected by changes in other characteristics such as the binding affinity of the ligand-receptor interaction.

**Isoelectric Focusing**

There was a significant change in the isoelectric point of the protein as a function of glucose dose and the degree of glycation. The isoelectric point reflects the net charge on the protein, and may be an indirect indicator of the relative acidity of the protein and or the ligand binding site. (Allen et al 1984) Alterations in the isoelectric point may impact upon the binding affinity of the receptor when the binding sites have a pH dependence. The relative intrinsic acidity of the reactive sites within a protein or amino acid facilitates AGE formation. (Njorge and Monnier 1989) This could result in increased protein cross-linking or could initiate AGE formation on other proteins. (Glomb and Monnier 1995) Increased protein crosslinking is one possible explanation for the increased resistance to denaturation seen above.
Ligand Binding

There was a definite right shift in the receptor-ligand binding curve when the cells were incubated with both 11.1 mM and 22.0 glucose supplemented media. This shift was both significant (p < 0.05) and reversible. Reversibility was demonstrated by incubation with 10% polyethylene glycol. The amount of ligand bound returned to a level near to that seen with those cells incubated in 5.5 mM glucose. However treatment of cells incubated with 22.0 mM glucose with polyethylene glycol did not decrease the amount of bound ligand to the same level as seen when cells incubated in 5.5 mM glucose were treated with the same quantity of polyethylene glycol.

There was no significant difference when the receptor-ligand binding curves for cells incubated in 11.1 mM and 22.0 mM glucose supplemented media are compared to each other. There was no apparent change in the receptor-ligand binding curve when cells are incubated in 16.7 mM glucose supplemented media. Cells incubated with 16.7 mM and 22.0 mM glucose media are less firmly attached to the flasks than cells incubated in 5.5 mM glucose media. This was possibly due to the increased lipid content of the cells. Trypsin was utilized to free cells incubated in 5.5 mM and 11.1 mM glucose media. Use of trypsin in a bioassay of insulin binding may be problematic when the bioassay involves binding of insulin to an inert support such as agarose.
beads. Some of the initial evidence that indicated insulin receptors are localized at the cell surface was obtained using agarose beads. The concept was to attach insulin to the agarose beads at the $\alpha$-amino groups of the $\alpha$ and $\beta$ chains or the $\varepsilon$-$\text{NH}_2$ group of lysine in the $\beta$ chain. In these experiments trypsin was added to the media as a control measure. The action of trypsin is to break peptide bonds at lysine groups. Trypsin treated insulin-agarose should have lower activity because trypsin will cleave insulin off of the agarose support thereby making it unavailable for biological action. (Cuatrecasas and Hollenberg 1976, Cuatrecasas 1973)

A curvilinear Scatchard plot is common in most ligand-receptor binding studies. (Nattras and Dodds 1987) Shifts in the curvilinear Scatchard plot are indicative of alterations in ligand-receptor interactions. A shift to the right may be indicative of a receptor defect, assuming conditions of maximal biological effect. (Kahn 1985) As noted earlier, insulin resistance is often described in terms of a defect in glucose metabolism as a result of some impairment in the insulin-receptor binding interaction. Insulin resistance can potentially result at two different cellular levels; at the binding site and at a point distal to the binding site. If the site of the defect lies distal to the binding site then a maximal response cannot be achieved. (Nattras and Dodds 1987) The shift in the
insulin-receptor binding curve seen in these experiments is indicative of a defect in the ligand-binding site.

A competitive binding assay was used to determine insulin receptor binding. This assay is based on application of the law of mass action, wherein a reaction is controlled by the opposing interactions, association of the reactants and dissociation of the products. The law of mass action applies only when ligand binding to the receptor is bimolecular, reversible, and when there is no other interaction between the binding sites. Since insulin has been shown to interact directly with the insulin receptor, and is required for activation of the insulin receptor, (Shoelson et al 1993, White and Kahn 1994, Frattali et al 1991, Cuatrecasas and Hollenberg 1976) the assumption that insulin receptor-ligand binding is bimolecular is valid. If insulin receptor-ligand binding is considered to be simply bimolecular, then the law of mass action predicts that at equilibrium: (Nattras and Dodds 1987)

\[ \text{Insulin} + \text{Receptor} \rightleftharpoons \text{Insulin-Receptor} \]

In a competitive binding assay, receptor binding by a radioactively labeled ligand (\(^{125}\text{I-Insulin}\)) followed by saturation of the remaining specific and non-specific binding sites with excess unlabeled ligand is allowed to occur simultaneously until a mass equilibrium is reached.
between the reactants and the products. (Zettner 1973, Nattras and Dodds 1987) This is commonly referred to as an equilibrium saturation assay. (Zettner 1973) Quantitation of ligand-receptor binding is based on the degree of saturation of the receptor by the unlabeled ligand, and extent of competition with the labeled ligand for specific and non-specific binding sites. (Zettner 1973) Achieving an equilibrium between the ligand and receptor is the critical step and is based on the laws of mass action. (Zettner 1973) This equilibrium can be expressed by the reaction:

\[
[P+P^*]+[Q]^{k_a=k_d}[PQ+P^*Q]
\]

Where:
- \(P\) = test substance or unlabeled ligand.
- \(P^*\) = labeled ligand.
- \(Q\) = specific binding agent.
- \([P + P^*]\) = Combined concentration of the mixture of labeled and unlabeled ligand.
- \([Q]\) = Molar concentration of the receptor.
- \([PQ + P^*Q]\) = Combined molar concentration of the ligand-receptor complexes.
- \(k_a\) = Association constant.
- \(k_d\) = Dissociation constant.

The affinity constant (K) is the ratio of the association and dissociation constants. The affinity constant can either be calculated or estimated. The affinity constant can be calculated by the equation:

\[
K = \frac{K_a}{K_d} = \frac{Insulin-Receptor}{Insulin+Receptor}
\]

(Nattras and Dodds 1987)
The $k_d$ for insulin receptor binding has been estimated by bioassay. Cuatrecasas and Hollenberg (1976) elicited a biological response in adipocytes at approximately 50% of the maximal potential biological response ($ED_{50}$) with addition of $3 \times 10^{-11}$ M insulin. In experiments reported in this dissertation the dissociation constants ($k_d$) were calculated based on the assumption that total receptor concentration within the cell remained constant at $10^4$ insulin receptors per cell over the 96 hour incubation period. (Cuatrecasas and Hollenberg 1976) The calculated $k_d$ ranged between 0.13 and 0.80 nM. Which matches other reported $k_d$ for insulin receptors between 0.25 and 0.72 nM. (Murakami and Rosen 1991, Ellis et al 1986)

Scatchard analysis is the most widely used method to estimate the affinity constant as well as to describe the interactions between receptors and their ligands, and to determine the affinity of a receptor for its ligand. A Scatchard plot is the ratio of bound to free plotted against concentration of bound insulin. (Scatchard 1947, Nattras and Dodds 1987) This analysis presumes that different reaction sites on a protein are sufficiently spaced so as to be able act independently of each other. With this assumption the law of mass action can be applied to binding interactions as though each group were on a separate molecule. (Scatchard 1947) The strength of binding can be expressed as a single constant. (Scatchard 1947) The presumption of independent
action between different binding groups on the same protein is critical to use of Scatchard analysis for receptor-ligand binding interactions. In the original publication Scatchard stresses that "independent action between adjacent groups is based on the concept that there is an interaction between a protein (eg hemoglobin, albumin or ovalbumin) and a small molecule (eg iron, ammonia or KOH)". (Scatchard 1947) Based upon this concept the slope of a Scatchard plot is the negative of the association constant and the abscissa intercept gives the concentration of binding sites. (Nattras and Dodds 1987) However, when reaction groups come closer together, as occurs when the ligands are relatively large proteins (eg insulin), it becomes increasingly difficult to distinguish between the intrinsic properties of the ligands/receptors, and the interaction among the groups. (Scatchard 1947) When the reaction groups are close together the probability of reaction may not be the same for all points because the initial reaction may make reactions at the adjacent point more or less difficult depending on the type of interactions. (Scatchard 1947) This commonly results in a curvilinear as opposed to a linear plot. Indeed it is this curvilinear appearance that makes it difficult to interpret a Scatchard plot of ligand-receptor binding. (Nattras and Dodds 1987, Plumbridge et al 1978) The curvilinear Scatchard plot may be indicative of technical difficulties in the assay. It is also compatible
with two physiological interpretations; negative cooperativity and two different classes of receptors. (Nattras and Dodds 1987)

Non-specific binding is one of the principal technical difficulties that will result in a curvilinear Scatchard plot. (Kermode 1989, Feldman 1983) Non-specific binding is commonly defined as binding to non-biological materials and binding to sites other than receptors on biological materials. (Kermode 1989) Binding to non-biological materials can be measured with accuracy, and thus can be corrected. Binding to biological, non-receptor sites can only be estimated, and hence present a potential problem in ligand-receptor studies. Even low non-specific binding, that is not adequately accounted for, can dramatically influence the Scatchard plot by underestimating the degree of non-specific binding, and hence introduce noticeable artifactual curvature. (Kermode 1989) Non-specific binding is countered by adding relative large amounts of unlabeled insulin. (Nattras and Dodds 1987) Use of 100 to 1000 fold excess of unlabeled ligand is acceptable only when labeled ligand is used in saturating concentrations. (Kermode 1989) This solution to non-specific binding can cause problems with low-affinity receptors, because they are easily saturated. In fact the definition of non-specific binding given above, more accurately describes non-saturable binding because the components tend to saturate as the ligand
concentration is increased. Studies with non-target tissues and with ligands not directed towards the receptor have demonstrated that "non-specific binding" is saturable. (Kermode 1989) Because of this, Feldman (1983) notes that the level of non-specific or saturable binding should be included among the fitted parameters rather than applied as a putatively error-free correction to raw data.

Insulin receptor-insulin binding interactions are thought of as bimolecular interactions because there is only one (maybe two) insulin molecules bound for each insulin receptor. However, because of the aggregation of receptors that is commonly seen, especially in adipocytes (Jarrett et al 1980), there may be an interaction between "adjacent" receptor binding sites. Cooperativity is the effect that adjacent receptors have on ligand-receptor interactions. Cooperativity modifies the binding process to permit the specificity and selectivity required to respond to changes in the physiochemical environment, eg nutrient demands, transport etc. (Cuatrecasas and Hollenberg 1976) The net result of this effect determines whether the cooperativity has a negative or positive effect. Negative cooperativity within a single class of receptors is when affinity decreases with an increase in binding. It is a commonly accepted explanation for the curvilinear Scatchard plot because it is supported by observations that increased
dissociation of tracer is prompted by excess insulin. 
(Faguet and Beebe 1986, DeMeyts 1973)

**Signaling**

The cause of insulin resistant states may be a defect that lies in the signalling pathway between ligand binding and activation of the cellular metabolic components. In fact, most investigators have shown that insulin resistance is at or beyond the level of the insulin receptor. (Schade and Boyle 1992) The impact of AGE formation on signal generation was measured by phosphorylation of the tyrosine kinases, activation of hexokinase, and glycogen deposition.

**Tyrosine phosphorylation**

The effects of AGE formation on tyrosine phosphorylation was measured by stimulating adipocytes with insulin in the presence of glucose for predetermined time periods. Dot blots of cell lysates were then treated with an antiphosphotryosine antibody followed by an alkaline phosphatase detection system. The relative densities of the individual dot blots were determined on a scanning densitometer, and reported as function of the area under the curve of the scan. Statistical analysis using a two way ANOVA demonstrated a significant dose dependent decrease in tyrosine phosphorylation, with increasing glycation.
It is clear that an impaired response of the insulin receptor tyrosine kinase is an effective measure of insulin resistance on the cellular level. (Haring et al 1994, Maegawa et al 1995, Müller-Wielund et al 1991, 1995) The clearest mechanistic relationship to insulin resistance are reversible decreases in tyrosine kinase activity possibly due to covalent modification of the insulin receptor in insulin resistant tissues. (Roth et al 1994, Haring et al 1994)

The basic mechanism for signal generation by the insulin receptor is widely accepted to be an autophosphorylation of the tyrosine kinase domain of the β-subunit. In this model, insulin binding to the α-subunit affects a conformational change in the α-β-subunit interface resulting in an autophosphorylation of the tyrosine kinases on the cytoplasmic face of the opposite β-subunit. (Frattali et al 1991, 1992, Treadway et al 1991, Roth et al 1994, Waugh et al 1989, Bargmann and Weinberg 1988) This means that the direct coupling of ligand binding affinity and the capacity to phosphorylate the tyrosine kinase domain is an essential component of the cellular signalling process. (Schumacher et al 1991)

The principle limitation in this study was that total cellular phosphorylation in response to insulin stimulation was measured. Phosphorylation of several additional cytoplasmic polypeptides occurs in response to the
autophosphorylation of the β-subunit tyrosine kinase domain. (Schumacher et al 1993, Exton 1991) Activated tyrosine kinases will in turn transfer phosphate groups from ATP to several cytoplasmic proteins. (Muller-Wielund 1993) Many of these substrates (eg phosphatidylinositol 3-kinase) are involved in signal transduction cascades, and may be involved in the specific insulin signalling system as well as other signalling systems.

**Glycogen Deposition**

Glycogen deposition was determined by a method that measures the amount of glycogen deposition in response to insulin stimulation in the presence of glucose. As expected, glycogen deposition was significantly increased (p < 0.05) with insulin stimulation. However, the magnitude of the glycogen deposition varied with the timing of the insulin stimulation and the amount of glucose in the incubation media. The peak increase in glycogen deposition was seen in cells incubated with 11.1 mM glucose, receiving insulin stimulation 96 hours after glucose supplementation. Glycogen deposition then fell significantly in those cells incubated in 22.0 mM glucose. The most significant drop in glycogen deposition was in those cells that received no insulin stimulation or received insulin at the same time as the glucose supplementation. Glycogen deposition in these two treatment groups was reduced to nominal levels.
A significant increase in glycogen deposition with insulin stimulation is consistent with previous studies and is considered to be a part of the normal physiological response. However, the magnitude of the insulin response was significantly altered in a glucose dependent fashion. There was not a complete abolition of the metabolic response to insulin at high glucose levels. This leads to one of several possible concepts.

1. There may be multiple signaling pathways. As described above there are several proteins that become phosphorylated when insulin receptor is activated. Each of these proteins may have an impact upon activation of the phosphorylase enzymes responsible for initiation of the glycogen synthesis pathway. Collectively they potentially have a greater impact on glycogen deposition than individually. If AGE formation impairs only one, or a few of these signaling pathways, then glycogen deposition would be reduced, but not abolished.

2. The impact on glycogen deposition may not be in the actual synthesis of glycogen, but rather in the availability of substrate. Glucose must be transported into the cell and then phosphorylated before glycogen synthesis can begin. AGE impairment of the insulin receptor may result in decreased availability of intercellular phosphorylated glucose as opposed to an impairment in the glycogen synthetic process. If AGE formation is occurring on the
insulin receptor and impairing it's function, then AGE formation could also occur on the glucose transport proteins. This could also reduce the availability of phosphorylated intercellular glucose.

**Hexokinase**

This study measured the activity of the glucose phosphorylating enzyme, hexokinase in response to insulin stimulation. There was no significant difference in the activity of hexokinase as a function of the level of glycation, or amount of glucose supplementation.

Hexokinase functions to phosphorylate intracellular glucose. Phosphorylated glucose is the required substrate for all of the metabolic processes involving glucose. In addition non-phosphorylated intracellular glucose is toxic to the cell principally because of increased hydrostatic pressure.

**Glycohemoglobin**

The in-vivo rate of glycation of non-variant hemoglobin in erythrocytes from non-diabetic individuals was measured by periodate oxidation as described earlier. Erythrocyte viability was measured by counting the number of viable cells in a hemocytometer. There was considerable cellular destruction associated with increasing glucose supplementation in the growth media. This study
demonstrated a definite increase in the degree of hemoglobin glycation, adjusted for erythrocyte number, as a function of both glucose dose and time. Technical problems with erythrocyte viability prevented analysis of the degree of glycation beyond 5 days.

Glycation is a continuous process that occurs throughout an erythrocytes life span. It probably does not proceed at a constant rate. This study indicates that there is the potential for a considerable amount of glycation occurring within the first few days of the erythrocytes life span. This is appears to be contrary to clinical studies that determined that a steady state level requires approximately four months to achieve, and approximately 50% of the glycation is determined by the glucose concentration in the one month immediately prior to sample collection. (Goldstein et al 1986) To date no one has demonstrated linearity in the glycation rate for any of the different hemoglobin subfractions. (Koskinen 1994) Results of this study indicate that at least the early stages of glycation are not linear but are dose dependent. Dose dependency in the rate of glycation supports the clinical utility of glycohemoglobin as a measure of long term glycemia. If there is a lack of linearity in the rate of glycation, as is indicated by this and other studies then application of HbA_{1c} to non-A hemoglobin variants may be inappropriate.
CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

This study tested the global hypothesis: Formation of advanced glycosylation end product (AGEs) adducts on insulin receptors inhibits insulin receptor binding by altering the physical and chemical properties of the insulin receptor.

In testing this hypothesis, this study demonstrated that embryonic 3T3-L1 fibroblasts can be differentiated to adipocytes; and that the proteins within the adipocytes can be glycated in a glucose dose dependent fashion. Further, this study provided evidence that glycation alters the physical and chemical properties of the insulin receptor. Specifically these properties included alterations in the isoelectric point and increased protein cross-linking.

Next the concept that AGE formation on the insulin receptor is a potential mechanism for increasing insulin resistance seen in type II diabetic individuals was tested. This phase of the study explored the effect that AGE-receptor adducts have upon the ability of the insulin receptor to generate a signal through it's intrinsic tyrosine kinase activity as well as the ability to influence glucose metabolism within
the cell. Changes in the physical and chemical properties of the insulin receptor had a mixed impact upon its functional properties. Tyrosine kinase phosphorylation was significantly diminished, but not abolished with glycation. Hexokinase activity was unchanged. Glycogen deposition was also significantly diminished, but not abolished.

A study of in vitro hemoglobin glycation provided a bridge between experimental and clinical applications of AGE formation. Glycated hemoglobin (gHb) is a clinical measure of glycemic control.

Nonenzymatic glycation of the insulin receptor does alter the physical and chemical properties of the insulin receptor. There is an increase in the amount of crosslinking within the protein, as evidenced by an increase in the amount of holoreceptor, along with decreased amounts of receptor subunits present in denaturing gel electrophoresis. The isoelectric point shifted with increasing amounts of glycation. There was also an increased affinity in the ligand-receptor binding interactions.

Non-enzymatic glycation does reduce the ability of the insulin receptor to effect intercellular metabolic processes. At a minimum the fact that insulin was able to bind to the receptor and effect metabolic changes such as glycogen deposition and hexokinase activation, in the cell argues for the concept that signal generation still occurs
in the presence of AGE formation. However the decreased responsiveness to insulin, as seen in glycogen deposition, suggests an impairment in the signaling system as a result of AGE formation. Whether this impairment is a direct result of impaired insulin receptor function is not totally clear from this study. It can, however, be inferred from the physical and chemical changes, that AGE formation on the insulin receptor does have a negative impact on at least some of the aspects of the signalling system.

**Model**

This study proposes a novel model for insulin resistance. In this model increased serum glucose levels result in an increase in the amount of AGE formation on proteins, including receptor proteins. In insulin receptors, AGE formation on or near the insulin binding sites alters the binding site such that insulin is not bound appropriately. Inappropriate ligand binding is expressed as a decrease in metabolic activity of the cell. Clinically a decrease in metabolic activity at the cellular level is described as a decrease in insulin sensitivity, or insulin resistance.

**Future Directions**

The results of this study raise several questions that should be explored in future research. To identify the
mechanisms of glucose driven changes more clearly, the following studies are proposed:

1. Identify that AGEs actually exist on insulin receptors. Identification of AGE's is hampered by the fact that very few AGEs have been identified in vivo. This is due in large part to the fact that the AGE reaction proceeds through any one of several complex pathways, resulting in a large number of structures (Glomb and Monnier 1995, Ledl et al 1989). Those AGEs that have been identified, pentosidine and CML, have been localized to collagen and the lens crystallin protein of the eye. It is not clear whether these compounds are tissue/protein specific, or are ubiquitous. One thing is clear, the areas where Pentosidine and CML have been localized have either a relatively high concentration of reactive amino acid sites, or are readily accessible to highly sensitivity tests.

2. Identify where AGE products are actually attached to the insulin receptor. There are several locations on the insulin receptor where there is a high probability that AGE formation can occur. Many of these sites are located in or near sequences that have been identified as important to ligand-receptor binding. This study found a decrease in the magnitude of a metabolic function as opposed to an abolition in activity. This argues in favor of the concept of multiple components in the ligand-receptor binding interaction. These multiple components may activate or
modulate the signal to effect different metabolic and mitogenic responses. In light of this possibility, identification of the specific location of AGE formation, and subsequent determination of the effect of that formation, at a specific site, could shed considerable light on the insulin signaling system.
APPENDIX A

Analytical Methods Protocols

Cell Growth and Differentiation
A. Thawing Cells
   1. Remove cells from liquid nitrogen and rapidly thaw.
   2. Pipette thawed cells into T-150 flasks
   3. Pipette 10 mL high glucose growth media.
   4. Incubate cells at 37°C with 5% CO₂ in high glucose growth media until confluent.
   5. When cells are confluent (7-10 days) perform 1-10 split and incubate until confluent.

B. Cell Culture of NIH 3T3-L1 Adipocytes.
   1. Incubate cells at 37°C with 5% CO₂ in high glucose growth media until confluent.
   2. Change media every 4 days.

C. Differentiation of 3T3-L1 fibroblasts to adipocytes
   1. Begin differentiation at 2 days post confluence.
   2. Incubate with differentiation media for 48 hours.
   3. Incubate 3 to 6 days changing growth media every 48 hours using low glucose growth media.

D. Confirmation of Differentiation
   1. When T-150 flasks are approximately 50% confluent, separate 1 flask into a 12 well plate.
      a. Subject cells in 6 of 12 wells to differentiation when they achieve 50-75% confluence.
      b. Confirm differentiation of these cells by staining 3 of the 6 wells with Oil-Red-O.
      c. Stain cells in 3 of the undifferentiated wells as controls.

   2. Confirmation of differentiation to adipocytes by Oil-Red O stain.
      a. Rinse cell monolayers 2 times with PBS.
      b. Fix for 1 hour @ 22°C with PBS containing 3.7% formaldehyde.
      c. Stain cells for neutral lipid with Oil-Red O
d. Destain cells with a 1:3 methanol:glycerol solution.
e. Rinse cells with 60% isopropanol and DDI.
f. Counter stain with Giemsa stain.
g. Count 500 cells/well and score for presence or absence of Oil Red O-stainable lipid droplets.

Bradford Method for Protein Determination
1. Turn on spectrophotometer, set lamp to 595 and allow to warm up for at least 30 min.
2. Pipet 3 ml of bradford reagent into a glass tube.
3. Pipet 100 μL sample into bradford reagent.
   a. Sample can be neat.
   b. For small samples dilute 1:10 with DDI.
   c. Be sure to mix well
4. Prepare a BSA standard curve and pipet 100 μL of each standard into a glass tube containing 3 mL of bradford reagent.
   a. For 100 μg/100 μL standard curve from 10 g/ml stock BSA.
      10 μL BSA (10 g/mL) in 1 mL DDI = 100 mg/mL
      10 μL BSA (100 mg/mL) in 1 mL DDI = 1 mg/mL
      100 μL BSA (1 mg/mL) in 1 mL DDI = 100 μg/mL
   b. Pipet 10, 20, 40, 60, & 80 μL of 100 μg/mL BSA into microfuge tubes and QNS to 100 μL with DDI.
   c. Use DDI for the 0 standard and 100 μg/mL for the 100 μg standard.
5. Prepare samples and standards in triplicate.
6. Allow all samples and standards to incubate at room temperature for approximately 15 min.
7. Measure the A595 of the samples and standards
   a. Compare all samples to a blank containing only Bradford reagent.
   b. The A595 of a 100 μg ia approximately 0.4.

Determination of Glucose by the Glucose Oxidase Method
1. Prepare fresh fresh reagents:
   a. PGO enzyme solution is prepared by adding the contents of 1 PGO enzyme capsule to 100 mL of distilled water in an amber bottle. Invert bottle several times with gentle shaking to dissolve contents.
   b. Color reagent solution is prepared by reconstituting one vial of o-Dianisidine dihydrochloride with 20 mL distilled water.
   c. Combined enzyme-color reagent solution is prepared by combining 100 mL of enzyme solution with with 1.6 mL of color reagent solution.
2. Prepare fresh standards.
3. Aliquot 0.2 mL of each sample, blank (distilled water) or standard into a 13 x 100 mm test tube.
4. Add 4.0 mL of combined enzyme-color reagent to each sample, blank or standard.
5. Incubate at 37 °C for 30 min.
6. Read absorbance at 500 nm.

**Solubilization of Insulin Receptors from Adipocytes**
1. Incubate each flask of confluent differentiated adipocytes with 10⁻⁶ M insulin for 30 min at 37°C.
2. Remove media and replace with 5 mL cold PBS.
3. Scrape cells from flask and transfer to siliconized centrifuge tube.
4. Centrifuge cells for 10 min @ 2000 r. Remove and discard supernatant.
5. Immerse cells in 2 mL of iced Solubilization Solution for 5 minutes.
6. Sonicate sample 3 times for 5 sec each.
7. Incubate cells in iced Solubilization Solution for an additional 5 minutes.
8. Purify receptors by WGA chromatography.

**Receptor Isolation by Wheat Germ Agarose Chromatography**
1. Wash and equilibrate a 2 mL column of wheat germ-sepharose with 4 bed volumes of equilibration buffer at 24 °C.
2. Add sample from solubilization procedure to wheat germ column and recycle 4x at 24 °C.
3. Eulate contains all proteins except the insulin receptor protein.
4. Wash column with 50 mL wash buffer.
5. Wash column with 10 mL elution buffer.
6. Dialyze eulate for 1 hour against dialysis buffer.
7. Concentrate eulate to 4 mL on Amicon PM-10 membrane.

**Determination of Glycation by Periodate Oxidation**
1. Prepare 40 µL aliquots of sample in triplicate.
   a. Prepare fructose calibrators with samples.
   b. Adjust any short sample to 40 µL with DDI water.
2. Add in order:
   a. 20 µL, 0.1 M HCL
   b. 20 µL, 0.05 M NaIO₃
3. Incubate sample for 45 minutes at room temperature.
4. Terminate oxidation by cooling samples in ice water bath for 10 minutes.
5. Precipitate periodate by adding precooled reagents in order:
   a. 20 µL, 15% ZnSO₄
   b. 20 µL, 0.7 M NaOH
   c. Vortex samples throughout addition.
   d. A milky white precipitate should form.
6. Centrifuge for 10 min at 9000 xg to remove precipitated zinc periodate.
7. Transfer 100 μL aliquots of supernatant to microtiter plate wells.
8. Add and thoroughly mix 200 μL freshly prepared formaldehyde detection reagent.
9. Develop for 1 hr at 37°C.
10. Determine fluorescence by reading on microplate reader at 410 nm.

**Albumin Glycation**

**A. Sample Preparation**
1. Sterile filter bovine serum albumin (10 g/dL) in phosphate buffered saline (pH 7.4).
2. Sterile filter glucose stock solution
3. Prepare specimens in according to the following:

<table>
<thead>
<tr>
<th>Specimen #</th>
<th>mL PBS</th>
<th>mL BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9 (no glucose)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>2,3</td>
<td>9 (100 mg/dL glucose)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>4,5</td>
<td>9 (300 mg/dL glucose)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>6,7</td>
<td>9 (1000 mg/dL glucose)</td>
<td>1.0 mL</td>
</tr>
</tbody>
</table>

**B. Procedure**
1. Prepare Samples and controls as described above.
2. Incubate all specimens at 37° for indicated time.
3. Aliquot 1 mL from each specimen at each time period and store at -20 °C until assay for glycation.
   - Time periods: 0, 2, 4, 8, 12, 24, 36, 48, 60, 72, 84, 96
4. Dilute sample with 20 mL PBS and concentrate to 1 mL with a PM-10 filter (Amicon)
5. Determine glycation at each time point by periodate oxidation.

**Denaturing Gel Electrophoresis**

**A. Gel Preparation**
1. Prepare gel (5 mL) according to quantities listed in Appendix 6B.
   a. Add in order
      1) Distilled deionized water
      2) Tris
      3) Acrylamide
   b. Degas mixture
   c. Add SDS
   d. Add ammonium persulfate
   e. Add TEMED
   f. Immediately pour into gel apparatus to a level 1 cm below where the well forming comb will lie.
   g. Carefully overlayer the gel with DDI
h. Allow gel to hardened (approx 30 min)

2. Prepare stacking gel in same manner as separation gel.
3. Add stacking gel on top of electrophoresis gel
4. Insert comb into apparatus and allow gel to harden approximately 30 minutes.

B. Sample Preparation
1. Add 18 uL sample to 2 uL warm gel loading buffer.
2. Boil mixture at 98 °C for 2 min.

C. Electrophoresis
1. Remove comb from gel.
2. Insert gel into apparatus.
3. Fill apparatus with running buffer.
4. Load samples and markers into wells.
5. Attach anode and cathode.
6. Turn on power supply and electrophorese at 95 mV constant power until marker reaches the bottom of the gel.

D. Stain Gel for Protein
1. Stain gel if it is not to be used for a blot analysis.
2. Stain gel with stain solution for 20 minutes.
3. Destain for 20 minutes with 50% methanol destain solution.
4. Destain for 20 minutes with 10% methanol destain solution.

Isoelectric Focusing
A. Preparing gel.
1. Clean cooling surface of IEF with DDI water.
2. Add approximately 1 mL of DDI water to the cooling surface.
3. Evenly distribute distilled/deionized water on the cooling plate.
4. Carefully place gel on top of cooling surface.
   - Be careful not to trap air bubbles between gel and cooling plate.
5. Thoroughly blot any excess water from the gel periphery with a clean paper towel.
6. Center gel on the cooling plate.
7. Prepare electrode wick.
   a. Place electrode wick, rough side down, on a clean paper towel.
   b. Evenly saturate the wick with anolyte solution.
   c. Gently blot wick with a dry paper towel until the wick just begins to appear dry.
   d. Place saturated wick on the appropriate side of the gel.
e. Run a finger along the total length of the wick to assure even contact between the wick and gel. Ensure there are no bubbles under the wick.

f. Wash hands of residual electrolyte solution before preparing the other electrode wick.

e. Repeat for catholyte.

B. Sample Application
1. Soak 1 mm x 2 mm filter paper in sample solution
2. Place soaked filter paper approximately 2 mm from anolyte wick.

C. Electrophoresis
1. Center the electrodes over the electrode wicks. Ensure that there is an even, centered contact between the electrodes and the wicks.
2. Connect the electrode leads into the electrophoresis unit.
3. Place the safety cover over the unit and connect the leads to the power supply.
4. Set the cooling temperature to 15°C.
5. Set running mode to constant power (watts).
6. Turn power supply on. Limit watts to 10 W
7. Electrophorese for 6 hours, or until marker bands appear crisp and straight.
8. Fix proteins at their isoelectric points by immersing in fixing solution for ten minutes.
9. Elute the ampholytes and clear by transferring the gel to DDI water and soak for 1 hour.

Detection of Protein by Western Blot
A. Non-electrophoretic Blotting Protocol
1. Place "wicks" on top of 2 layers of brown paper towel, place on top of transfer support and place assembly into transfer dish.
2. Rinse gel briefly in transfer buffer and carefully place on top of paper towels.
3. Prepare nitrocellulose (NC) membrane.
   a. Float membrane on transfer buffer until evenly wet and then submerge it.
   b. Drain membrane, and lay on top of the gel. careful to exclude air bubbles.
4. Place 2 inch layer of dry brown paper towels on top of NC membrane.
5. Place plastic plate on top of towels and place weight on top of plate.
6. Add sufficient transfer buffer such that the wicks are barely touching the transfer buffer.
   a. Ensure that the wicks are sufficiently covered by transfer buffer to take up the buffer.
b. If the wicks are covered by too much buffer, then a reverse capillary action could occur and the protein could transfer to the buffer reservoir.

B. Electrophoretic Blotting
1. Soak filter paper in transfer buffer, drain them, and place on lower electrode (anode).
2. Rinse gel briefly in transfer buffer and carefully place on top of filter papers.
3. Prepare nitrocellulose (NC) membrane.
   a. Float membrane on transfer buffer until evenly wet and then submerge it.
   b. Drain membrane, and lay on top of the gel.
      - careful to exclude air bubbles.
4. Soak filter paper in transfer buffer and place on top of NC membrane.
5. Place upper electrode (cathode) firmly on top of the stack.
6. Connect the power pack and apply constant current at a density of 0.8-1.0 mA/cm² for 0.5-2.0 hr.
7. Disassemble stack, remove NC membrane, rinse it in TBS and store it in transfer buffer at 4°C.

C. Detection of Protein
1. Block non-specific binding by washing NC membrane with TBST plus 1% gelatin for 30 min at 27°C.
2. Wash with TBST (no gelatin) 2 times for 10 min each.
3. Incubate NC membrane with primary antibody for 60 min at 27°C.
   a. Dilute antibody 1:1000 with TBST (1% gelatin)
   b. Collect primary antibody when incubation is complete.
4. Wash with TBST (no gelatin) 2 times for 10 min each.
5. Incubate with secondary antibody enzyme conjugate for 60 min.
   a. Dilute antibody 1:3000 with TBST (1% gelatin)
   b. Collect antibody when incubation is complete.
6. Wash with TBST (no gelatin) 3 times for 10 min each.
7. Wash with TBS (no tween/no gelatin) for 5 min.
8. Develop with detection reagent
9. Allow to develop for 15 min in complete darkness or until adequate color development is seen.
10. Fix development by washing NC membrane with DDI water.

Determination of Phosphotyrosine Activity
1. Incubate cells in glucose media at 37°C to prevent heat shock.
2. Add insulin (0, 10⁻¹⁰, & 10⁻⁹ M).
4. Incubate for 0, 2, 5, 10 and 20 min.
5. Remove media and wash with iced PBS.
6. Scrape cells in iced PBS.
7. Sonicate cells.
8. Boil sonicated cells in SDS for 5 min.

**Determination of Hexokinase Activity**

1. Incubate cells in glucose media at 37°C to prevent heat shock.
2. Add insulin ($0$, $10^{-10}$, & $10^{-9}$ M).
3. Collect 0.1 mL of cellular extract.
4. Incubate for 0, 2, 5, 10 and 20 min.
5. Remove media and wash with iced PBS.
6. Scrape cells in iced PBS.
7. Sonicate cells on ice.
8. Add 950 uL reagent mixture to cuvette.
9. Add 50 uL cell lysate to reagent mixture and immediately measure the absorbance.
10. Measure absorbance at 340 nm at 0.5 sec intervals until reaction reaches equilibrium.
11. Assay aliquots (0.010 mL, 0.005 ml, and 0.003 mL) of purified hexokinase to validate the method.
12. Calculate the concentration of glucose phosphorylated per unit time as a function of the absorbance using Beer's Law and a molar extinction coefficient for NADPH of $6.22 \times 10^3$.
13. Calculate the rate of phosphorylation calculating the NADPH (nM) production per minute per unit volume (uL) reaction mixture added to the aliquot.
14. The initial rate of the reaction is used as an indication of hexokinase activity.

**Hemoglobin Glycation**

A. Sample Collection and Preparation
   1. Draw approximately 30 cc of venous blood in heparin.
      a. Remove aliquot for base line glycation determination.
      b. Determine relative glycemia with glucometer.
   2. Centrifuge cells @ 1000 xg for 10 min.
   3. Decant and discard plasma and buffy coat.
   4. Wash erythrocytes 3 times in 10 volumes of iced PBS.
      - recentrifuge cells at 1000 xg for 5 min after each wash.

B. Incubation of Erythrocytes.
   1. Resuspend washed erythrocytes in PBS and pipette to a concentration of 1% into T-150 flasks.
   2. Incubate erythrocytes at 37 °C and 5% CO₂ in Earls with 10% PBS plus the indicated glucose concentration.
   3. Change media by transferring media and cells to a sterile centrifuge tube and centrifuging at 1000 xg for 10 min.
a. Decant supernatant and resuspend cells in Earls/PBS media and return to culture flasks.
b. Media should be changed every 8-10 days.

C. Glycation of Hemoglobin
1. Incubate samples in varying concentrations of glucose.

<table>
<thead>
<tr>
<th>Glucose Level</th>
<th>Glucose Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Glucose (NG)</td>
<td>100 mg/dL</td>
</tr>
<tr>
<td>Normal High Glucose (NHG)</td>
<td>200 mg/dL</td>
</tr>
<tr>
<td>Moderate High Glucose (MHG)</td>
<td>300 mg/dL</td>
</tr>
<tr>
<td>High Glucose (HG)</td>
<td>400 mg/dL</td>
</tr>
</tbody>
</table>

2. Prepare samples in triplicate.
3. Draw daily aliquots of each sample.
4. Determine extent of glycation by periodate oxidation.

Ligand-Receptor Binding
A. Ligand Binding
1. Split confluent differentiated cells to a final concentration of 7.5 x 10^4 cells/flask. Prepare all samples in dupliplicate for each unbound insulin level.
2. Incubate cells for 96 hours in media containing 5.5 mM, 11.6 mM, 16.6 mM or 22 mM glucose.
3. Remove media and wash cells carefully with iced PBS two times.
4. Incubate cells with 10 mL low glucose media (5.5 mM) containing 0.9 µCi ^125I-Insulin (Binax) for 24 hours at 4 °C.
5. Replace media with low glucose media containing increasing concentrations of unlabeled insulin and incubate cells for four hours at 27 °C.
6. Transfer cells to plastic test tubes and centrifuge cells for 10 min @ 2000 r. Remove and discard supernatant.
7. Gently wash cells with PBS two times.
8. Remove PBS and lyse cells with 1 M NaOH.
9. Count on gamma counter for 1 minute.

B. Ligand Dissociation
1. Repeat steps 1 through 6 in Ligand Binding procedure above.
2. Wash cells in 10% polyethlyene glycol two times.
3. Remove polyethylene glycol and gently wash cells with PBS two times.
4. Remove PBS and lyse cells with 1 M NaOH.
5. Pipette lysate into glass tubes and count on gamma counter for 1 minute.

C. Determination of Total Binding
1. Label tubes as follows:

<table>
<thead>
<tr>
<th>Label</th>
<th>Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Count (TC)</td>
<td>1 &amp; 2</td>
</tr>
<tr>
<td>Non Specific Binding (NSB)</td>
<td>3 &amp; 4</td>
</tr>
<tr>
<td>Maximum Binding ($B_0$)</td>
<td>5 &amp; 6</td>
</tr>
<tr>
<td>Calibrators A1 - F</td>
<td>7 - 20</td>
</tr>
<tr>
<td>Controls</td>
<td>21 - 24</td>
</tr>
</tbody>
</table>

3. Pipet 200 µL Assay buffer into $B_0$ tubes 5 & 6.
4. Pipet 200 µL of calibrators and controls into respective tubes.
5. Pipet 100 µL Tracer into all tubes.
6. Pipet 100 µL Insulin Antiserum into all tubes except TC and NSB tubes.
7. Vortex all tubes gently, cover and incubate for 90 minutes at room temperature.
8. Shake bottle of Precipitating REagent well to ensure an even suspension, then add 1 mL of reagent to all tubes except TC.
9. Vortex tubes and incubate for 10 minutes at room temperature.
10. Centrifuge at 1500 x g for 10 minutes at 2 - 8 °C.
11. Decant the supernatant of each tube (except TC tubes) into a suitable radioactive waste container. Decanting must be done smoothly and carefully to avoid dislodging any of the pellet. Blot the rim of each tube on absorbent paper.
12. Count radioactivity in all tubes for one minute.

D. Data Analysis
1. Determine the total bound insulin from the standard curve.
2. Determine the total free (unbound) insulin by deducting the bound insulin from the total unlabeled insulin that was added.
3. Use Scatchard analysis to compare the bound vs bound/free.
APPENDIX B

SOLUTIONS AND BUFFERS

Cell Growth and Differentiation

A. High Glucose Growth Media
   1. Dulbecco’s Modified Eagles Media (DMEM)
   2. 10% Bovine Calf Serum (BCS)
   3. 16.7 mM (450 mg/dL) glucose
   4. No antibiotics

B. Low Glucose Growth Media
   1. DMEM
   2. 20% BCS
   3. 5.5 mM (100 mg/dL) glucose

C. Differentiation Media
   1. DMEM (55 mM glucose)
   2. 10% FBS
   3. Dexamethasone (1 μM)
   4. methylisobutylxanthine (0.5 mM)
   5. insulin (10.0 μg/mL)

D. Trypsinizing Solution (500 mL)
   1. NaCl (4 g)
   2. KCl (0.2 g)
   3. Dextrose (0.5 g)
   4. NaHCO₃ (0.29 g)
   5. EDTA (0.1 g)
   6. Trypsin (0.25 g)
   Add in order
   Stir until clear (40-60 min @ room temp)
   Bring to volume with distilled water
   Sterile filter with 0.2 μ filter
   Freeze 40 mL in 50 mL conical tubes

Bradford Reagent

1. Coomassie Brilliant Blue G-250 - 100 mg
   (final concentration = 0.01%)
2. Ethanol (95%) - 50 mL
   (final concentration = 4.7%)
3. Phosphoric acid (85% w/v) - 100 mL
   (final concentration = 8.5%)
4. Distilled Deionized Water - QNS to 1 L

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Receptor Solubilization
A. Solubilization solution
1. PBS (pH 7.6.
2. 150 mM NaCl (438.3 mg/50 mL)
3. 5% glycerol (2.5 mL/50 mL)
4. 1 mM PMSF (8.7 mg/50 mL)
5. 1 mM Aprotinin
B. Phosphate Buffered Saline (2X)
1. KH\textsubscript{2}PO\textsubscript{4} - 12.25 g
2. NaOH - 2.05 g
3. NaCl - 17.55 g
4. DDI - 1 L
5. Adjust pH to 7.6

Receptor Purification
A. Equilibration Buffer
1. PBS (pH 7.6.
2. 10 mM MgCl\textsubscript{2} (100 mg/50 mL)
3. 0.1% Triton (50 μL/50 mL)
4. 1 mM PMSF (8.7 mg/50 mL)
B. Wash Buffer
1. PBS (pH 7.6.
2. 150 mM NaCl (2.18 g/ 250 mL)
3. 0.1 % Triton (250 μL/ 250 mL)
4. 1 mM PMSF (43.5 mg/250 mL)
C. Elution Buffer
1. 0.3 M N-acetyl-D-glucosamine (3.3 g/ 50 mL)
2. 0.025% Triton (12.5 μL/ 50 mL)
3. 1 mM PMSF (8.7 mg/50 mL)
D. Dialysis Buffer
1. PBS (pH 7.6)
2. 0.025% triton
3. 1 mM PMSF (8.7 mg/50 mL)

Denaturing Gel Electrophoresis
A. Denaturing Gel
1. Water 2.3 2.8 2.1
2. 1.0 M Tris (pH 6.8) 1.3 1.3 0.39
3. 30% Acrylamide (8%) 1.35 0.83 0.50
4. 20% SDS 0.025 0.025 0.015
5. TEMED 0.003 0.003 0.003
6. 10% Ammonium Persulfate 0.05 0.05 0.03
B. Gel loading buffer (1 mL)
1. 50 mM Tris (pH 6.8) 0.05 mL of 1 M Tris
2. 1% SDS 0.10 mL of 20 % SDS
3. Bromophenol Blue 0.005 mL of 0.05%
4. DDI 0.85 mL
C. 5X Running Buffer (1000 mL)
1. 25 mM Tris 15.1 g
2. 250 mM Glycine 94 g
3. 0.1% SDS 25 mL of 20% SDS stock

D. Stain Solution (100 mL)
1. Coomassie Brilliant Blue R-250 0.6 mg
2. Water 40 mL
3. Methanol 50 mL
4. Glacial acetic acid 10 mL

E. 50% Methanol Destain Solution (100 mL)
1. Methanol 50 mL
2. Glacial acetic acid 10 mL
3. Water 40 mL

F. 10% Methanol Destain Solution (100 mL)
1. Methanol 10 mL
2. Glacial acetic acid 10 mL
3. Water 80 mL

Non-denaturing Gel Electrophoresis
A. Non-Denaturing Gel 8% Gel 5% Gel 3.5% Gel
1. Water 2.35 3.50 2.00
2. 1.0 M Tris (pH 6.8) 1.30 0.83 0.50
3. 30% Acrylamide (8%) 1.30 0.63 0.38
4. TEMED 0.003 0.005 0.003
5. 10% Ammonium Persulfate 0.05 0.05 0.03

B. 5X Running Buffer (1000 mL)
1. 25 mM Tris 15.1 g
2. 250 mM Glycine 94 g
3. Water 1000 mL

Isoelectric Focusing
A. Ampholytes pH range 4.0 - 6.5
1. Anolyte 0.1 M glutamic acid in 0.5 M H₃PO₄
2. Catholyte: 0.1 M β-alanine

B. Fixing solution (250 mL)
1. 29 g trichloroacetic acid (TCA)
2. 8.5 g sulfosalicylic acid
3. Dissolve in 250 mL DDI

C. Destain solution (2.0 L)
1. 500 mL ethanol.
2. 160 mL acetic acid.
3. Quesce to 2.0 L with DDI.
D. Protein stain
   1. 2.4 mg Coomassie Brilliant Blue R-250.
   2. 400 mL destain solution.
   3. Stir with magnetic stirrer
   4. Heat solution to 60 °C.
   5. Filter before use, use only once.

E. Preserving solution (250 mL)
   1. 25 mL glycerol.
   2. Quesce to 250 mL with destaining solution.

**Western Blot Analysis**

**A. TBST**
   1. Tris-HCL pH 8.0 (12.1 g/L)
   2. NaCl (8.7 g/L)
   3. Tween-20 (0.5 mL/L)

**B. TBS**
   1. Tris-HCL pH 8.0 (12.1 g/L)
   2. NaCl (8.7 g/L)

**C. Detection Reagent**
   1. 10 mL AP substrate
   2. 0.05 mL substrate A
   3) 0.05 mL substrate B

**Hexokinase Activity**

Reaction Mixture (10 mL)
   1. 0.1 M MgCl2 (203 mg)
   2. 0.03 M glucose (54 mg)
   3. 0.1 M ATP (551 mg)
   4. glucose-6-phosphodehydrogenase (G6PD)
   5. NADP (TPN) 20 mg/


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