Relationship of Non-Enzymatic Glycation to Altered Renal Structural and Functional Changes in the Diabetic Rat

by

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ABBREVIATIONS

ATPase	Adenosine triphosphatase
cpm	Counts per minute
°C	Degrees of Celcius
CV	Coefficient of variation
DNase	Deoxyribonuclease
dpm	Disintegrations per minute
g	Gram
x g	Gravitational force
GFR	Glomerular filtration rate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Kda	Kilodalton
Kg	Kilogram
leucine equiv.	Leucine equivalents
L	Litre
uCi	Microcurie
ug	Microgram
ul	Microlitre
um	Micrometer (Micron)
umol	Micromole
uM	Micromolar
mCi	Millicurie
mg	Milligram
ml	Millilitre

mmol	Millimole
mM	Millimolar
M	Molar
NADPH	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide
nm	Nanometer
nmol	Nanomole
N	Normal
PEG	Polyethylene glycol
pKa	Negative log of the association constant
RIA	Radioimmunoassay
RPM	Revolutions per minute
SDS	Sodium dodecylphosphate
SD	Standard deviation
SEM	Standard error of the mean
TCA	Trichloroacetic acid
v	Volume
w	Weight

ABSTRACT

Renal failure is one of the most common secondary complications of diabetes mellitus and ultimately develops in about 40% of all diabetics. Furthermore, after 15-20 years of diabetes, all diabetics exhibit some renal impairment. This renal failure occurs as a result of specific lesions which have developed over many years.

The factors responsible for the development of diabetic nephropathy, as with the other complications of diabetes, are unknown; however, hyperglycemia which is found in poorly controlled diabetics, is believed to be involved. One way in which hyperglycemia may result in the development of nephropathy is through non-enzymatic glycation.

Non-enzymatic glycation is a common post-translational modification which occurs in many proteins; it can be expected to occur for any protein with a sterically available amino group to which glucose can bind. Non-enzymatic glycation can alter both a protein structure and function. There is evidence to suggest that non-enzymatic glycation of glomerular basement membrane may be the etiologic factor resulting in diabetic nephropathy.

In our studies, renal functional parameters including creatinine clearance, urinary albumin excretion, basement membrane thickening, and levels of non-enzymatic glycation of glomerular basement membrane were studied

in rats rendered diabetic with streptozotocin. Diabetic animals had elevated glycated hemoglobin levels ($p<0.05$), increased creatinine clearance, and urinary albumin excretion rates ($p<0.05$) as compared to insulin treated diabetic (euglycemic), age-matched, and streptozotocin non-diabetic animals. The level of non-enzymatic glycation of glomerular basement membrane was significantly elevated ($p<0.05$) in the diabetic animals as well. The level of non-enzymatic glycation of glomerular basement membrane correlated ($r=0.86$) to the average blood glucose level of each animal. We also examined the levels of non-enzymatic glycation of muscle capillary basement membrane and found that the levels correlated ($r=0.66$) with those of glomerular basement membrane.

Despite changes in functional parameters, and increased levels of non-enzymatic glycation between the hyperglycemic and euglycemic diabetic animals, there was no difference in glomerular basement membrane thickness. However, there was a difference in thickness between the diabetic euglycemic and the age-matched control animals.

We conclude that non-enzymatic glycation of glomerular basement membrane does not cause an increased thickness of this structure; further more, a relationship between glomerular basement membrane thickness and renal function was not found, thus casting doubt upon the importance of the former in the development of diabetic nephropathy.

I. INTRODUCTION

A. GENERAL INTRODUCTION:

Diabetes is a complex syndrome or grouping of a number of anatomical and biochemical problems resulting from an absolute or relative deficiency of insulin. The disease, if untreated, leads to slow body wasting of body tissues due to metabolic starvation, and can ultimately result in specific microvascular and macrovascular complications, which involve specific organs.

1. HISTORICAL ASPECTS: (1,2,3)

Knowledge of diabetes dates back to 1500 B.C. when it was first described as a condition resulting in "melting down of flesh and limbs into urine" (1). During the sixth century, scholars in the Far East described a condition of polyuria, in which the urine was sweet and sticky. The presence of sugar in the urine was confirmed in the 1700's. However, it was not until the twentieth century that anything was really found about the cause of the disease. It was found that removal of the pancreas of a dog, produced a diabetic-like syndrome. In the early 1900's it was noted that the beta cells of the islets of Langerhans were damaged in subjects with the disease (4).

In 1921 Banting and Best prepared active extracts from the pancreas of dogs which, when administered to diabetic dogs, could decrease elevated blood glucose levels (5). In humans, the extract (which was subsequently

determined to contain insulin) resulted in improved conditions and was found to be beneficial in the treatment of diabetics (6). This same treatment is still in use today. In the 1940's however, it was found that the patients treated with insulin in the early 1920's were developing characteristic complications of diabetes including retinopathy, nephropathy, and nervous and cardiac system disorders (7,8). Thus, treatment by insulin improved the immediate condition of the patient, but patients later developed complications which frequently lead to death. It was found that retinopathy effected 80% of diabetics and 50% were dying of renal failure (2). Even with improved treatment and blood glucose monitoring, these complications are still very much manifested in the diabetic state in the 1980's.

Insulin, a hormone produced by the B-cells of the islets of Langerhans, is released in response to increased blood glucose and other factors, including amino acids. Its role is to promote storage of glucose, when available in excess, via lipid formation, protein synthesis and glycogen synthesis for future energy needs (1,9). A deficiency of insulin results in the breakdown of lipid, carbohydrate, and protein stores of the body for the formation of glucose by the liver, resulting in marked body wasting due to destruction of cellular storage reserves (9). The elevated blood glucose levels in diabetes results from the inability of blood glucose to be transferred into the cell, due to the absence of insulin (Type I diabetes), or to a relative decrease in the number and/or affinity of insulin receptors at target tissues causing a decrease in insulin action (Type II diabetes).

Treatment with exogenous insulin promotes utilization of blood glucose in type I diabetes. This is the most common treatment which is used today to normalize blood glucose in such diabetics. In type II diabetes, however dietary restrictions, which improve receptor affinity and numbers, may be sufficient to maintain normal blood glucose levels.

2. CLASSIFICATION AND FEATURES OF DIABETES

Diabetes mellitus occurs in all parts of the world and is the third leading cause of death in the United States, with 150,000 deaths annually (10). The disease affects ten million people in the U.S. alone. In Canada the mortality rate from diabetes is 9.5 per 100,000, with over 2500 people dying per year (11), which is slightly less than the rate in the United States. Diabetes mellitus can be classified into two major forms (12,13); the first which occurs primarily in children is called type I, juvenile, or insulin-dependent diabetes mellitus. This type of diabetes is associated with an absolute deficiency of insulin secretion. The second form is type II, maturity-onset, or non-insulin-dependent diabetes mellitus. This form is more common than type I diabetes. These patients can still secrete insulin, however there appears to be some insulin resistance at the tissue level which is responsible for the relative deficiency of insulin (14). Table 1 summarizes the differences in these two types of diabetes.

The clinical features of the two types of diabetes are quite different (15,16). The onset of type I diabetes is rapid and usually occurs in people

Table 1. Features of Type I and Type II Diabetes Mellitus.

FEATURE	TYPE I DIABETES	TYPE II DIABETES
Age at onset	Usually under 30 yrs	Usually over 40 yrs
Onset	Often rapid	Insidious
Weight	Non-obese	Often obese
Ketosis	Common	Rare
Complications	Frequent	Frequent
Sex	Slight male preference	Female preference
Seasonal variation	Present	?
HLA association	Present	Absent
Concordance in identical twins	<50%	>90%
Islet antibodies at onset	60 - 80%	<5%
Islet mass	Severely reduced	Moderately reduced

under 30 years of age, with the highest incidence occurring between 10 and 14 years of age. Males have a slightly increased susceptibility than do females. There is some seasonal variability in the onset, with the highest incidence usually occurring in early winter and in summer. The lowest incidence rates are found in Japan, the Caribbean, and southern Europe; and the highest in Scandinavia and the United States. The incidence rate in Canada is 9.0 per 100,000 of population per year (17,18), which is less than that of the United States (9.5-18 per 100,000 per year depending on the study) but higher than Japan (0.6 per 100,000 per year) (11). The negro population of the United States has half the likelihood of developing type I diabetes when compared to caucasian Americans (15).

The loss of insulin secretion in type I diabetes appears to result from an autoimmune process which is directed at insulin-producing beta cells of the pancreas, ultimately leading to their destruction and thus development of the disease (19). However, the exact cause of diabetes is still unknown. Islet cell antibodies are found in 60-80% of type I diabetics at the onset of the disease, with a severely reduced mass of islet cells.

Type II diabetes, in contrast, usually occurs in patients over 40 years of age, with a parallel increase in incidence with age and has a slight female preference. The rate of incidence of type II is 4 times as high as is type I diabetes. There is substantial variability in occurence of diabetes among caucasians, depending on their locality, suggesting that an environmental component may be important in the development of the disease (15). The

United States, Canada, and Saudi Arabia have some of the highest incidence rates, with the lowest found in Japan, China, and Singapore. Obesity is a common condition associated with type II diabetes. The relative risk of diabetes increases with increasing degree of obesity, with 80% of patients with type II diabetes being obese.

The precise nature of genetic influence in the onset of diabetes mellitus is unclear. Type I is associated with certain antigens of the HLA system (20). HLA DR3 and HLA DR4 are associated with a three to five fold increase in risk. In contrast, there is no association of the HLA antigens with type II diabetes (21). There is some genetic influence in this type however, since the concordance of diabetes in identical twins is over 90% in Type II diabetes, while the concordance is 50% in Type I diabetes.

B. COMPLICATIONS OF DIABETES MELLITUS

The complications associated with diabetes mellitus present very serious problems and can affect the eyes, kidneys, nervous system, and both large and small blood vessels. The complications affect both insulin dependent and non-insulin dependent diabetics, although the former are at a greater risk. The various complications can be classified as follows:

1. Retinopathy
2. Neuropathy
3. Vascular complications
4. Nephropathy

1. RETINOPATHY

Diabetic retinopathy and other ocular complications pose a serious threat to the vision of diabetics (22). In North America, diabetes is the leading cause of blindness. Blindness from diabetic retinopathy is responsible for 12% of blindness at all ages and for 20% of blindness between the ages of 45 and 74 (23). The prevalence of retinopathy is very low before five years of diabetes, however it eventually progresses to affect some 80% of diabetics to some degree after 30 years duration of the disease. Diabetics also show an increase in the prevalence of cataracts over the general public (24).

The earliest lesion in retinopathy is the formation of microaneurysms or outpouchings of the capillary walls in the retina (25). Eventually this progresses to proliferative retinopathy with the formation of new blood vessels in the retina (neovascularization). As these vessels form, they shrink and cause traction on the retina, and result in detachment of the retina and subsequent blindness. The blood-retinal barrier is also effected. The vessels may hemorrage due to the traction, resulting in regional ischemia and capillary occlusion, causing death to the cells of the retina.

2. NEUROPATHY

Diabetic neuropathy refers to a heterogeneous group of neurologic syndromes associated with diabetes mellitus, and effects both the peripheral and the autonomic nervous system. At least 50% of all diabetics have some clinical impairment of neural function (26). The peripheral sensory motor

nervous system changes include numbness in the extremities, unsteadiness in walking, excessive tenderness and pain, muscle weakness and cramps (25). The autonomic nervous system changes result in vasomotor disturbances affecting the gastro-intestinal and urogenital system causing irregular food absorption, impotence and other endocrine disorders (27). The neurologic changes may also play an important role in the increased incidence of cardiac arrests and strokes which are found in diabetics over the general population (25,28).

These changes, many of which are specific for diabetes, are due to altered nerve electrophysiological properties. They arise from morphological and biochemical changes in the axon and the myelin sheath (29). In general, there is a slowing of nerve conduction velocities which may be due to segmental demyelination and/or axonal degeneration (29).

3. MACROVASCULAR COMPLICATIONS

Macrovascular disease is one of the most common, as well as most serious chronic complications of diabetes. Diabetics are susceptible to disease of the large muscular arteries (atherosclerosis) particularly those supplying the myocardium, the brain, and the lower limbs (25). Atherosclerotic vascular disease generally occurs at a younger age and progresses more rapidly in diabetics than in non-diabetic patients, resulting in an increased incidence of heart attacks, strokes, and gangrene (30).

The vascular changes, which occur in diabetics, plus abnormalities in blood components, including increased viscosity, is thought to be a result of decreased red blood cell deformability and increased platelet aggregation. As well, alterations in the lipoprotein profiles have been found, resulting in decreased high density lipoproteins (HDL) which are throught to be a protective agent against atherosclerosis, and an increase in low density lipoproteins (LDL) (31,32). Hormonal alterations may also play some role in the development of these macrovascular changes.

4. NEPHROPATHY

Diabetic nephropathy which eventually results in renal failure is a common complication of diabetes (33). Renal failure ultimately develops in about 40% of patients with insulin dependent diabetes mellitus, and diabetes represents the single most common cause of renal failure in the adult population (34). In humans, who develop diabetes before the age of twenty, roughly 70% will develop some proteinuria after 20 years (25). Nephropathy occurs less frequently in type II diabetes but when present it progresses at an accelerated rate.

C. ETIOLOGY OF THE COMPLICATIONS

The etiology of the complications of diabetes is unknown. Some investigators believe that genetic and immunological factors may be involved. Others, believe that hyperglycemia, which is the hallmark of diabetes, may be involved.

In this section, the evidence and problems of each of these hypotheses will be examined.

1. GENETIC AND IMMUNOLOGICAL FACTORS

Genetically, type I diabetes is etiologically distinct from type II diabetes, as there appears to be no HLA association with type II diabetes, whereas, HLA and immunological factors such as islet cell antibodies are found in type I diabetics (35,36).

The human leukocyte antigens or HLA, are a large group of antigens (so far over 60 have been identified) found on the surface of leukocytes and are responsible for recognition of self, and are important in transplantation and the rejection process. The genes which control this specific immune response are located on chromosome six. These antigens, may play a role in influencing susceptibility to disease, particularly ones which are immunological in origin (35,36). Many studies have been performed to establish evidence for an association between one or more HLA antigens and the disease in question.

Studies have been performed to dissociate the role that the HLA antigens may have in the development of the diabetic complications. Larkins et al (37) characterized the degree of retinopathy in 16 patients and their HLA frequencies to a well matched group of diabetics without significant retinopathy. There was some association between retinopathy and the HLA B8

antigen. Similarly, Standle et al (38) found increased prevalence of HLA B8 and a decreased prevalence of HLA B18 in patients with severe retinopathy when compared to patients with mild or no retinopathy. The alleles B8 and B15 are inherited with DR3 and DR4, the latter which appear to be more relevant, with the previous two in dis-equilibrium with the latter two (39). Barbosa and Sanar (40) have recently summarized the results of 21 previous studies on the subject. Of 12 studies on retinopathy, five reported association between DR4 and retinopathy, however, seven did not. Only two studies reported an association between retinopathy and DR3. The evidence of HLA association with the diabetic complications remains inconclusive. There are problems in such comparative studies; they are difficult to design and to control.

Recently, attention has focused on C4, the fourth component of the complement system, which plays a role in the clearance of immune complexes (41). Insulin-antibody complexes may be involved in aggravating the microvascular pathology, increasing the susceptibility to the complications. Immune complexes were found to be elevated in patients with retinopathy by Cudworth and Bodansky (35); however, they did not correlate this to HLA or other phenotypes. These complexes may aggravate microvascular disease, or may just simply reflect non-specific tissue damage. The C4 protein is coded by two genes, both of which display a high degree of polymorphism. Twenty five percent of insulin dependent diabetics were found to have low plasma C4 concentrations in a study by Barnett et al (41). Furthermore, this study suggested that the deficiency may be inherited. A highly significant association

between C4B3 (a variant of C4)) and retinopathy has been observed by Mijovic et al (42).

The role that insulin antibodies and immune complexes may have in the development of diabetic complications is not known. Recently, insulin antibodies have been found to be associated with immunoglobulin heavy chain markers (Gm) (43). Nakao et al (43) have found that insulin antibodies were associated with Gmzagx haplotypes. Further more, Mijovic et al (44) have suggested that this Gmzagx haplotype is associated with retinopathy in type I diabetics.

Genetic factors are not likely to be the cause of the complications of diabetes. Since genetic factors influence Type I diabetes mainly, and the complications are common to all types of diabetes, it is unlikely that they are responsible for the development of the latter.

2. ROLE OF HYPERGLYCEMIA IN DEVELOPMENT OF DIABETIC COMPLICATIONS

Controversy exists regarding the role hyperglycemia plays in the development of the secondary complications of diabetes. There is evidence to support the hypothesis that the secondary complications of diabetes are directly associated with hyperglycemia, but there is also evidence which disputes this hypothesis.

There is inconsistency between the frequency of the diabetic complications and blood glucose levels. Some poorly controlled diabetics do not develop complications, where as patients with good control of blood glucose levels do. Furthermore, Bondy et al (45) have failed to show a relationship between the degree of hyperglycemia and the risk of complications, casting uncertainty on the importance of hyperglycemia. Siperstein et al (46) have observed diabetic-like lesions in normal patients with a strong family history of diabetes. There is also evidence that strict glycemic control has no effect on the rate of progression of established nephropathy (47).

In contrast, there is much more evidence which supports the view that hyperglycemia is cause of the long term complications of diabetes. Very often, typical complications have been observed in all types of human diabetes providing that hyperglycemia has been present for many years. As well, the major complications seen in human diabetes have been observed in experimental diabetes (48).

In streptozotocin-induced diabetic models, a relationship has been observed between the degree of hyperglycemia and the degree of microangiopathy (49,50). Similar findings in clinical studies on humans have been suggested by Vlassara et al (51). Poorly controlled diabetics have been shown to have an increased rate of incidence of nephropathy. Pirart et al (52) have shown a correlation between the degree of hyperglycemia and the rate of appearance and progression of human diabetic retinopathy and nephropathy.

Bennion et al (53) have shown that mitigation of hyperglycemia can often reverse the complications of diabetes. Similarly, in other studies where hyperglycemia was controlled, there have been cases demonstrating a marked reversal of retinopathy (54) and neurological complications.

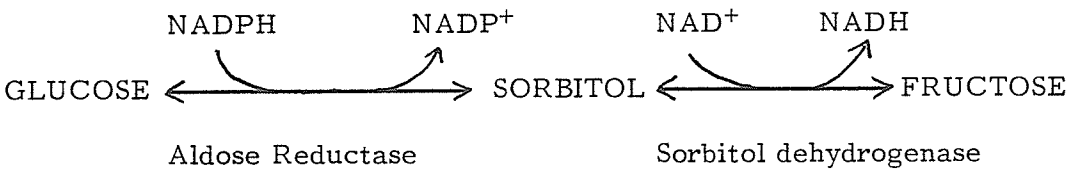
Other evidence to support the importance of hyperglycemia in diabetic complications comes from studies performed with renal transplant patients. Doud (55) and Mauer (56) have observed that kidneys from non-diabetic donors develop glomerulosclerotic lesions when transplanted into diabetic subjects. Steffes (57) has also shown reversal of these lesions in diabetic rats which have received beta-cell transplants, thereby restoring the insulin levels and hence the correction of elevated blood glucose levels. However, Gotzche et al (58) could not demonstrate the reversibility of glomerular basement membrane thickening, despite reversing the hyperglycemic state of diabetic rats by islet transplantation, possibly due to the long half-life of glomerular basement membrane. Steffes et al (59) studied glomerular basement membrane from identical twins discordant for diabetes, and found that the non-diabetic twins all had normal basement membrane thickness, whereas the diabetic twins demonstrated increased thickening. This suggests that a metabolic abnormality due to the diabetes, perhaps hyperglycemia, is responsible for the development of the glomerular lesions seen.

These studies seem to suggest that hyperglycemia may be the direct cause of the secondary complications of diabetes mellitus. A relationship has been observed between the degree of hyperglycemia and the risk of

retinopathy, neuropathy, and nephropathy; however, the evidence to relate hyperglycemia to macrovascular complications is less conclusive. This may be due inpart to the high frequency of arterial disease in the general population. Also, other factors may be involved including genetic and environmental ones. The mechanism through which hyperglycemia could cause the secondary complications is not known but two hypotheses have been proposed; increased expression of the polyol pathway and increased non-enzymatic glycation of proteins.

3. POLYOL PATHWAY:

One hypothesis for the role of hyperglycemia in the etiology of the diabetic complications involves the polyol pathway which is shown below:



The enzymes involved in the polyol pathway are aldose reductase and sorbitol dehydrogenase, which are responsible for the conversion of glucose to sorbitol and fructose (60). The enzymes are highly substrate dependent and are regulated by the cellular glucose levels. The Km for aldose reductase is 28 mmol which is much higher than that of hexose kinase (0.15 mmol); so that, under normal conditions, most of the glucose is metabolized to glucose-6-phosphate by hexokinase. When glucose levels rise, aldose reductase

metabolizes an increasing portion of glucose to sorbitol. Similarly, under hyperglycemic conditions, the enzyme sorbitol dehydrogenase is more active. It can be expected that in hyperglycemic states, such as in poorly controlled diabetes, both enzymes will be more active resulting in the increased formation of sorbitol. In fact, in humans (61) and in animals (62), concentrations of glucose, sorbitol, and fructose are elevated in peripheral nerves in poorly controlled diabetes.

Increased levels of sorbitol have been postulated to be involved in the formation of cataracts in diabetics (24,63). Since sorbitol diffuses across membranes slowly, it can accumulate intracellulary under these conditions, resulting in osmotic swelling (since sorbitol is highly osmotic). This can lead to the inhibition of transport of water into the eye. The millimolar levels of sorbitol concentrated in the eye result in the formation of sugar aggregates which lead to the opacity of the lens, impairing vision. Decreased action of the sodium-potassium ATPase is also observed (64), which may also play some role.

Inhibition of sorbitol formation using aldose reductase inhibitors, have been shown to prevent cataract formation (65). Likewise, it has been found that normalization of blood glucose levels, and strict glycemic control, can reverse the formation of early cataracts in diabetic subjects (63). This data suggests that the accumulation of sorbitol may be etiologically important in the development of diabetic cataracts.

The polyol pathway may also be involved in diabetic neuropathy. The increased activity of the polyol pathway has been shown to result in derangements of nerve myo-inositol metabolism by decreasing the uptake of tissue myo-inositol by as yet some unidentified mechanism (66). Myo-inositol is a cyclic polyalcohol membrane phospholipid which is involved in transmission of nerve impulses. Tissue myo-inositol levels are decreased in the nerves of both diabetic animals and patients (66,67). As its levels decrease in nerve tissues, there is a drop in motor nerve conduction velocity (67). Figure 3 depicts the postulated relationship between the polyol pathway and possible neurological changes as proposed by Greene et al (68).

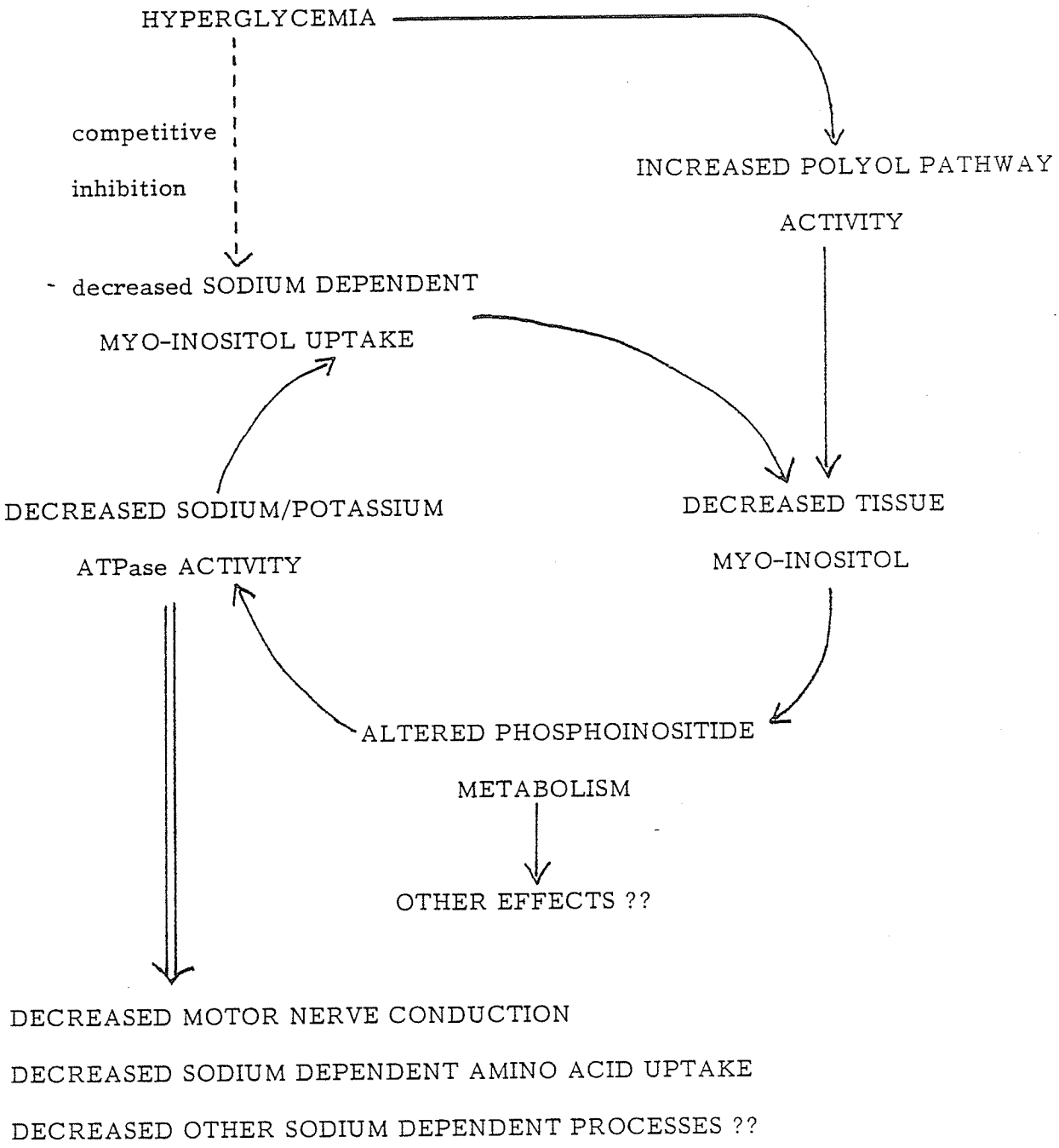
D. NON-ENZYMATIC GLYCATION

1. THE MAILLARD REACTION

The discovery of the non-enzymatic browning reaction by Maillard over 70 years ago has been studied in great detail by food scientists since its discovery because of the adverse effects it has on the appearance, nutritional quality, and safety of food (69). Only recently have biochemists begun to explore the relationship between this reaction and the pathological changes in diabetes.

In 1912, L.C. Maillard conducted pioneering work on sugar-amino acid condensations. He observed that yellow-brown pigments were formed when glucose was heated in the presence of amino acids or proteins (70). The

Figure 1. The Postulated Relationship between Hyperglycemia, Polyol Pathway, Myo-inositol, ATPase, and Nerve Conduction.



Modified from Greene et al (68).

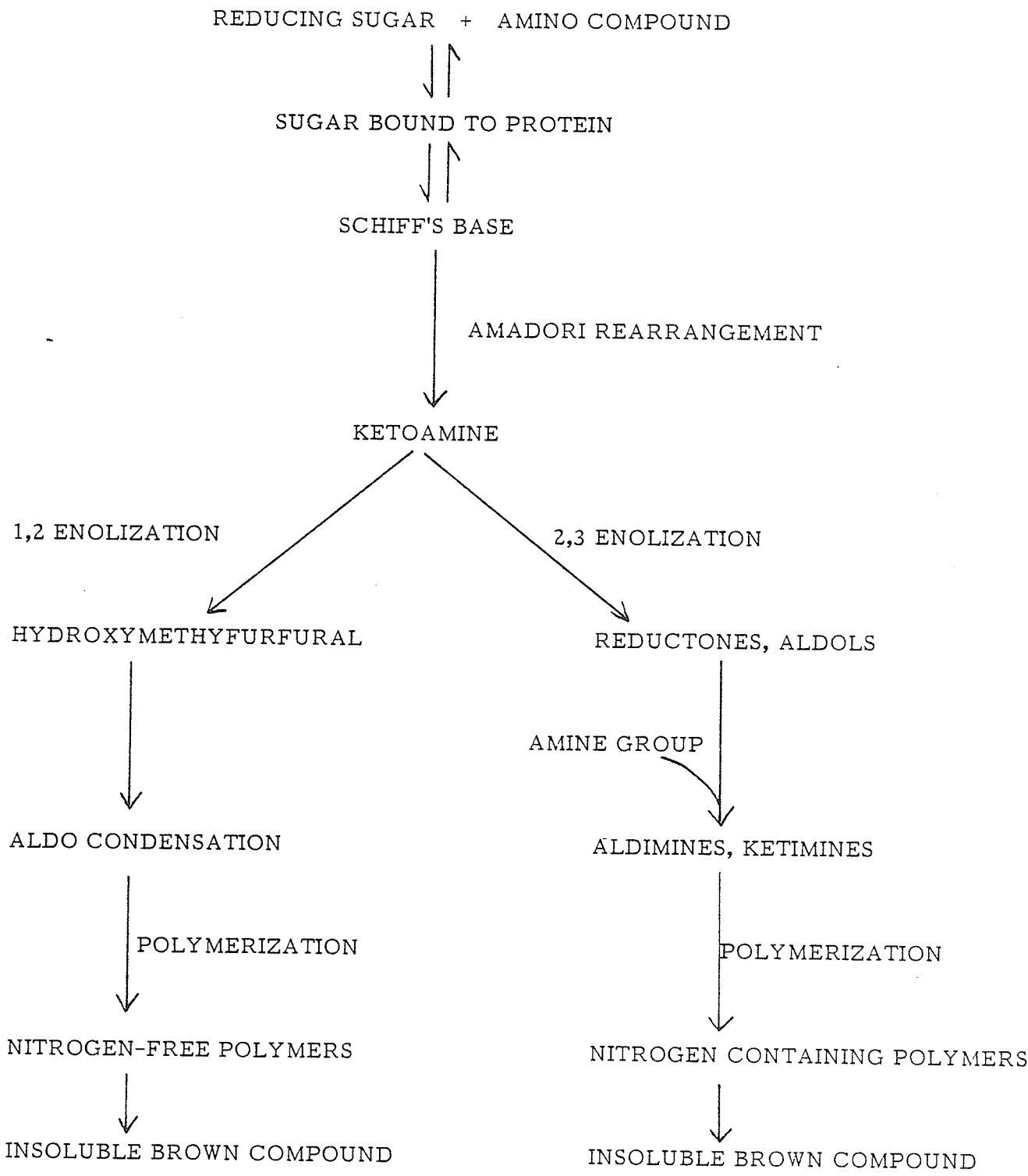
reaction occurs readily in food preparations and leads to irreversible modification of the dietary protein resulting in a decrease in its nutritional value by two mechanisms; first the reaction leads to modification of lysine, making it biologically unavailable; secondly, it leads to a decrease in the total nitrogen that can be digested.

The non-enzymatic browning reaction, or the Maillard reaction, evolves through several steps (71) as shown in Figure 2. The first step involves a condensation reaction between an amino group and the carbonyl group of a reducing sugar such as glucose, resulting in the formation of a Schiff's base. Subsequent cyclization and isomerization (Amadori rearrangement) results in formation of secondary products via enolization. This causes removal of amino groups from the carbohydrate complex with subsequent dehydration and cyclization, fragmentation, or amine condensations. The products formed include dicarbonyls, ketols, aldehydes, and reductones. These products formed are responsible for the flavor and aroma developed in processed food. The third step involves complex polymerization reactions of the secondary products, resulting in formation of both soluble and insoluble melanoidin pigments.

2. NON-ENZYMATIC GLYCATION REACTION:

Non-enzymatic glycation is analogous with the early stages of the Maillard reaction and is shown in detail in Figure 3. The reaction is dependent on the substrate concentration of the carbohydrate and the availability of an amino group. Pentose sugars are more reactive than hexoses which in

Figure 2. Simplified scheme of the Maillard reaction.



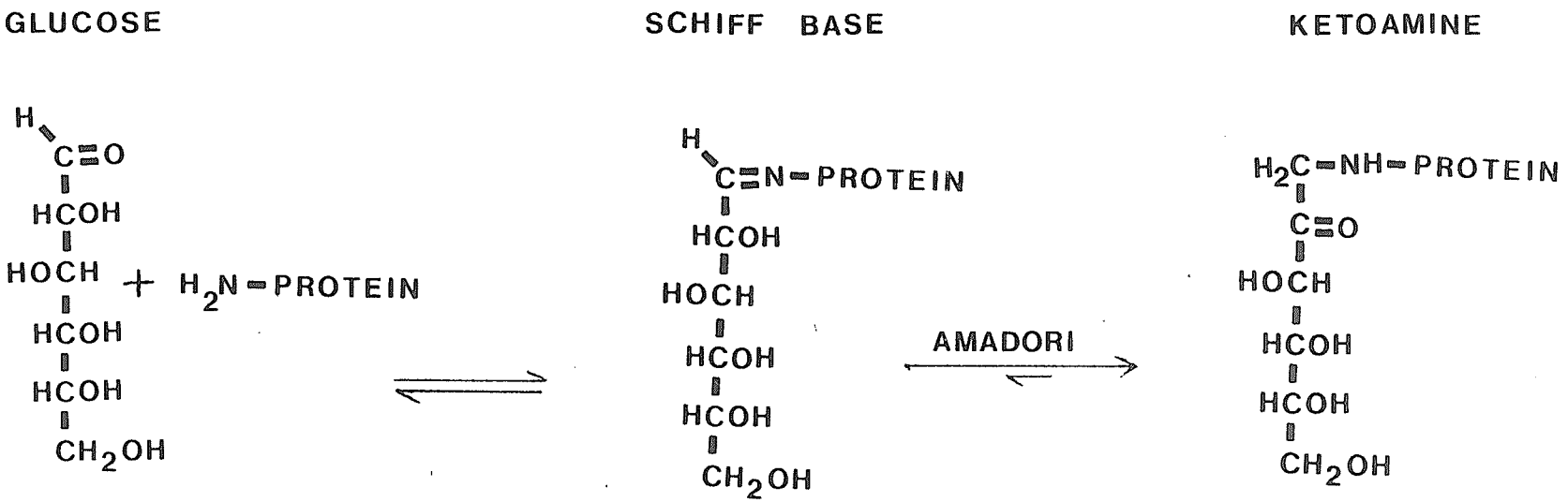


Figure 3. Reaction scheme for non-enzymatic glycation of the primary amino groups of a protein.

turn are more reactive than D-fructose. Only the aldehydic form of the carbohydrate is reactive, and the rate of the reaction depends on the ability of the carbohydrate to open its ring formation to form the aldehyde (72). Glucose is the least reactive of the hexoses, with only 0.001% in the aldehyde form; however, it is the carbohydrate of most importance, since it is found in significant concentrations in the blood.

The ability of the reducing carbohydrate to react with the amino group of proteins is dependent on the pKa of the amino group (73,74). Alpha-amino groups have a lower pKa than do epsilon-amino groups of lysine residues, and are more reactive. However, the lysine groups are present in a greater number over the alpha-amino groups and are more significantly involved in the reaction. Primary amines are sterically less hindered than are secondary amines, and are therefore more reactive. At physiological pH, the amino groups of all proteins are readily available for reaction with carbohydrates if they are sterically available.

The formation of the Schiff's base is acid catalyzed. The base itself is formed rapidly, is very unstable, and can readily dissociate (73,74). In water, it will rapidly dissociate into the original carbohydrate and the amino group. The base can readily undergo Amadori rearrangement to form a stable and irreversible product (75). The formation of the ketoamine from the aldimine is very slow however, in the order of days, and is the rate limiting step in the process.

In diabetes, where hyperglycemic conditions are encountered, it has been shown that the level of non-enzymatic glycation is increased (76). The level of non-enzymatic glycation is dependent only on the blood glucose levels in vivo, and the half-life of the protein in question. The protein should have a relatively long half-life to facilitate the reaction.

3. STRUCTURAL AND FUNCTIONAL ASPECTS OF THE PROTEINS INVOLVED:

Non-enzymatic glycation has been shown to occur for many proteins (see Table 2) (71,75-86). It can be expected that any protein, providing it has a relatively long circulatory half-life or slow turnover rate with a sterically available amino group, can be involved in non-enzymatic glycation. The rate of the reaction is also dependent on the prevailing glucose concentration. The reaction occurs under normal glycemic conditions but is elevated during hyperglycemic conditions (75) and occurs both in vivo and in vitro (71,75). The process can lead to alterations in a protein's structure and function, some of which are listed in Table 2.

The addition of a bulky hydrophillic group to a protein can have many effects on its structure. The addition of glucose to the epsilon-amino group of a protein will remove a positive charge of the protein at physiological pH. This will affect its overall net charge, and may interfere with the folding and secondary structure of the polypeptide chain. The removal of the positive charge will reduce the pKa of the protein, which in the case of hemoglobin

Table 2.

A) Proteins previously shown to undergo non-enzymatic glycation.

Hemoglobin	Low density lipoprotein
Collagen	Spectrin
basement membrane	
tendon	Peripheral nerve proteins
skin	
aorta	Erythrocyte membrane proteins
Ferritin	Fibrinogen
Insulin	Myelin
Albumin	Serum/plasma proteins

B) Some possible effects of non-enzymatic glycation on structure and function of a protein.

CHANGES IN STRUCTURE	CHANGES IN FUNCTION
Charge characteristics	enzymatic activity
Solubility	Immunogenicity
Stability	Hormonal activity
resistance to heat	
resistance to enzyme attack	Cellular uptake
Size, shape, and viscosity	Interaction with other molecules
Molecular cross-links	Biological half-life

allows separation of the glycated from non-glycated form by isoelectric focusing and ion-exchange chromatography (76,87).

Non-enzymatic glycation of proteins has been shown to alter protein turnover in diabetic patients. Increased glycation of fibrin reduces its susceptibility to plasmin degradation, resulting in increased accumulation of fibrin (88). Increased glycation of lipoproteins LDL and HDL has been shown to decrease and increase, respectively, their rate of clearance from the circulation (89). This may have some role in the development of macrovascular disease seen in diabetics. Non-enzymatic glycation of collagen also results in a decreased susceptibility to proteolytic digestion by proteases (90) leading to a decreased turnover. This may be responsible for the accumulation of basement membrane seen in diabetics. Non-enzymatic glycation of albumin results in a decrease in its solubility over that of the native form (78), suggesting a change in its structure.

Non-enzymatic glycation can also cause alterations resulting in resistance to enzymatic attack. Trypsin, a proteolytic enzyme which cleaves proteins between lysine and arginine residues, should be inhibited in its attack on non-enzymatically glycated protein, which has had its lysine residue glycated (91).

The non-enzymatic glycation of a protein can result in increased resistance to heat denaturation. Non-enzymatically glycated collagen is found to be more stable to heat and less susceptible to thermal rupture (92).

Non-enzymatic glycation of proteins has been shown to alter the immunogenicity of proteins. In vitro glycated rat skin collagen injected into the same strain of rats elicited an immune response where as unmodified collagen did not (93). The specificity of the generated antibodies was shown to be directed towards the non-enzymatically glycated residues.

Non-enzymatic glycation of a protein can affect its interaction with other molecules. Non-enzymatic glycation of a lysine residue at the active site of an enzyme may affect the enzymes activity, especially if the lysine residue was important for the binding or interaction of the active site with the ligand. Collagenase activity has been shown to be decreased in experimental diabetes in rats (94), perhaps due to non-enzymatic glycation of the enzyme's active site which involves a lysine residue. Non-enzymatic glycation of erythrocyte membrane proteins has been shown to decrease erythrocyte deformability, thus decreasing the ability of erythrocytes to traverse capillary beds (85,95).

Increased non-enzymatic glycation of lens protein in diabetics is thought to result in increased protein cross-linking, forming high molecular weight aggregates causing lens opacity (96) and affecting vision. Similarly, Eble et al (97) have developed a model system using RNase to study non-enzymatic glycation and glucose dependent cross-linking of proteins. Glycation was shown to lead to a decrease in the primary amino groups of the protein. There was a time-dependent formation of dimers and trimers of the RNase molecule. The primary cross-linking reaction occurred by condensation of a glycated amino acid on one RNase molecule with a free amino group on another.

Glycated protein could polymerize in the absence of glucose with a native molecule, suggesting that short term increases in glucose, can exert long term physiological impact by activating proteins for subsequent cross-linking reactions. These cross-links may be important in the pathophysiology of the diabetic complications.

E. - ROLE OF NON-ENZYMATIC GLYCATION IN ALTERED RENAL FUNCTION

1. NORMAL RENAL FUNCTION:

The primary function of the kidney is to help regulate the water, electrolyte, and acid-base content of the blood and other body fluids. Ultrafiltration of plasma across the glomerular wall is the primary event in urine formation. The glomerular capillary wall is a complex membrane which is highly permeable to water and small solutes and normally prevents passage of albumin and other plasma proteins.

The major determinants of glomerular permeability include; the number and/or radius of the glomerular pores, hemodynamic determinants of the glomerular filtration rate (GFR), and the electrostatic properties of the glomerular capillary wall (98).

a. Glomerular capillary wall components and organization (98,99).

In mammals, the glomerular filtration apparatus consists of three layers: a thin fenestrated enothelium with openings 50-100 nanometers in diameter; the glomerular basement membrane (GBM) with an electron dense central layer (lamina densa); and the epithelial cell layer, which is composed of podocytes with inter-digitating foot processes embedded in the inner layer of the GBM.

The surface of the endothelial and epithelial cells are coated with hydrophilic branched oligosaccharides attached to the membrane glycoproteins. These components are rich in sialic acid residues, giving rise to a polyanionic nature of the cells.

The glomerular basement membrane is a heterogenous substance containing collagen and non-collagenous components (100). The GBM is synthesized by the endothelial cells and is removed or degraded by the mesangial cells. The turnover of the material is slow, with a half-life of several months.

The collagenous component is composed of three identical alpha-chains arranged in a triple helix (type IV collagen), which is especially rich in the amino acids glycine, hydroxyproline, and hydroxlysine. It differs from the other types of collagen in that it contains three times as much hydroxlysine and large amounts of half-cystine (100). The carbohydrate components of this material are of two types. The first type is a complex asparagine-linked

heteropolysaccharide that contains a galactose, mannose, fucose, sialic acid, and glucosamine. These heteropolysaccharides are not unique to basement membrane, but are common to most glycoproteins. The second type is a disaccharide unit of glucose-galactose attached to hydroxylysine.

The non-collagenous component consists of various proteoglycans and glycoproteins. Proteoglycans including heparan sulfate, dermatan sulfate, and chondroitin sulfate are highly charged anionic molecules found in a lattice-like pattern along the glomerular basement membrane (160,161). Glycoproteins including laminin, fibronectin, and entactin are found bound to the surface of the basement membrane as well. Laminin and entactin are glycoproteins found exclusively in association with basement membranes (162). These glycoproteins are believed to play a role in linking cells to the basement membrane (163). They have also been shown to bind various proteoglycans, including heparan sulfate, ultimately forming an integrated component of the overall structure of basement membrane (164). These proteoglycans and glycoproteins are though to contribute to the change selectivity of the filtration barrier, ultimately determining the net charge and permeability of the basement membrane (163).

b. Factors determining glomerular permeability.

Tracer studies have been used to study the effect of molecular size or effective radius of the glomerular basement membrane with regards to

glomerular permeability (99,100,101). The GBM is the main barrier in the process of filtration. The endothelial fenestrae allow passage of molecules with a molecular weight up to 500 Kda (99). Similar tracer studies by Graham (102), showed that molecules as large as 160 Kda could pass through the glomerular basement membrane. Molecules smaller than this will not normally pass through the barrier. The molecular shape of a molecule is also important in the passage through the GBM. Linear, rod-shaped, flexible molecules migrate easier across the membrane than do globular molecules of the same size or radius (99).

The charge of the membrane itself, and the charge of the molecules in question are also important parameters in the passage of molecules across the glomerular capillary wall. The polyionic glycoproteins of the glomerular capillary wall (GCW) play an important role in the permeability of macromolecules. Negatively charged dextran sulphate has a lower fractional clearance as compared to a neutral dextran of similar molecular radius (98). Similarly, the excretion rate of horseradish peroxidase, a cationic molecule, is six times greater than a neutral protein of the same size, and fifty times as much as a negatively charged one. Glomerular transport of polyanionic molecules is restricted by the GCW compared to neutral or cationic molecules.

Glomerular transport of molecules is also influenced by the functional parameters that determine the filtration of water, namely, the effective filtration pressure, glomerular plasma flow, and the capillary ultrafiltration coefficient (103). The exact role that these components play is at present unknown.

In summary, the GCW is the main barrier in the filtration of negatively charged macromolecules. Negatively charged ones will be hindered from crossing the GCW by both steric factors as well as electrophysical forces of repulsion, due to the polyanionic nature of the GCW. These forces are, however, attractive for polycationic molecules. This results in their passage through the membrane at a much greater rate than that of similar sized polyanionic molecules. The dynamics of blood flow may also influence the entry of molecules in a yet to be determined mechanism.

2. THE STAGES OF DIABETIC RENAL FAILURE:

The natural history of deterioration of renal function in diabetes follows a very characteristic and unique pattern, ultimately resulting in end stage renal failure (100,104). Five characteristic stages in this progression can be recognized (105,106).

Stage 1 is characterized by early hyperfunction and hypertrophy (105). These changes are found at diagnosis of diabetes and can continue for many years in the presence of poor diabetic control (107). Renal size is increased with a concomitant increase in the GFR (and hence renal function). Urinary albumin excretion may be increased during exercise if the glycemic control is poor. Renal plasma flow can be normal or increased, but the blood pressure is usually normal (105,108). Good glycemic control can reverse the above effects, suggesting that the effects of hyperglycemia are responsible for the observed changes.

Stage 2 develops silently over many years eventually resulting in structural lesions of the kidney without visible presentation of clinical nephropathy. This stage can be detected within two years of diabetes in some cases, and progresses slowly over several years (105,107). Fifty to seventy percent of all diabetics stay at this stage throughout their lives with the remaining 30-40% progressing to clinical nephropathy. The GFR is increased 20-30% in poorly treated diabetics. Blood pressure and renal plasma flow are normal but may be increased slightly in some patients (109). Baseline urinary albumin levels are normal, but after a few years may become elevated during exercise. Despite normal renal function, structural and biochemical changes are evident. On renal biopsy, basement membrane is thickened over normal subjects and the mesangial region is expanded (109). It is unknown whether strict glycemic control can reverse these lesions; however, islet transplantation can prevent the lesions in streptozotocin induced diabetic rats (58).

Stage 3 or incipient diabetic nephropathy is characterized by visible functional changes, including microalbuminuria, despite good glycemic control and can occur after ten to fifteen years of the disease in 30-40% of diabetics (105). The GFR is still increased at this stage, however there is a slow decline in renal plasma flow. Blood pressure is increased, particularly during exercise, but can be normalized by anti-hypertensive treatment (109). Renal structural changes that occur are more pronounced than those described for stage 2, with a further increase in expansion of the mesangium and build up of basement membrane material (110). During the early stages of microalbuminuria, strict glycemic control can help to decrease it; however, as it progresses, glycemic control has less of an effect.

Microalbuminuria is a condition characterized by increased urinary excretion of albumin in the absence of visible nephropathy (111). Urinary albumin levels can be used to identify abnormalities in renal function in the early course of diabetic nephropathy before the presence of proteinuria (111,112). Since the levels are low, highly sensitive assays such as radioimmunoassays (113,114) or fluorescence immunoassays (115) are required for quantification. The clinical range of microalbuminuria is about 15 ug/min to 200 ug/min in humans. Recently, the urinary albumin level has been shown to be an excellent predictor of the progression to overt nephropathy from stage 3 in both insulin dependent and insulin non-dependent diabetes mellitus (111,112,116). It is unlikely that a diabetic with a resting level less than 30 ug/min will progress to overt clinical nephropathy within a decade. In contrast, diabetics with resting urinary albumin levels greater than 30 ug/min will show yearly increases of about 25 ug/min and will progress much faster to overt nephropathy.

Stage 4, or overt clinical nephropathy, which occurs in 30-40% of patients after 15 to 20 years since the onset of diabetes is characterized by persistant and pronounced non-specific proteinuria (105). The GFR decreases below normal levels and correlates well to the increase in diastolic blood pressure (117). Anti-hypertensive drugs can reduce the decline of the GFR (117), but good control of blood glucose levels has little effect on improvement of function at this stage (118). Proteinuria is of glomerular origin, until the stage when GFR falls, at which time it is of both glomerular and tubular in origin. As the GFR falls the degree of proteinuria increases (101,119), ultimately resulting in progression to end stage renal failure.

Stage 5, or end stage renal failure due to diabetic nephropathy occurs on average after 25 to 30 years of diabetes, eventually due to progressive glomerular capillary occlusion (101). The GFR is less than 10% of normal, and the blood pressure is high. 25% of all renal failure patients in North America are diabetic and at this stage, renal transplantation or dialysis is necessary.

3. Diabetic basement membrane chemistry and metabolism.

The composition of glomerular basement membrane isolated from diabetics both in animal and human subjects differ from that of normal subjects. The major differences are summarized in Table 3. The glomerular basement membrane isolated from diabetics shows an increase in hydroxlysine content with a proportional decrease in lysine content, such that the sum of the two is constant, when compared to normals. Diabetic basement membrane also exhibits elevations in glycine and hydroxproline with decreased levels of valine, tyrosine, half-cystine and sialic acid content (100). Increased levels of non-enzymatic glycation has also been observed both in vivo and in vitro (83,84,120).

Electron microscopy studies of renal biopsy tissue obtained from diabetic subjects with nephropathy show marked thickening of the glomerular basement membrane with the accumulation of basement membrane material (109). Overall, there is an excessive accumulation of basement membrane material, which is a result of increased synthesis and/or decreased degradation.

Table 3. Compositional Changes in Diabetic Glomerular Basement Membrane.

	INCREASED	DECREASED
Hydroxlysine	+	
Lysine		+
Hydroxyproline	+	
Glycine	+	
Half-cystine		+
Glucose-galactose disaacharide	+	
Sialic acid		+
Heparan sulfate		+
Level of non-enzymatic glycation	+	

F. NON-ENZYMATIC GLYCATION AND ALTERED RENAL FUNCTION:

Diabetic glomerulopathy is characterized by a slow development of basement membrane accumulation expressed as thickening of the basement membrane and as accumulation in the mesangial area. This build up of material ultimately results in glomerular occlusion, resulting in decreased renal function. The exact mechanisms that may influence these developments is unknown, but it is believed that metabolic abnormalities of diabetes is responsible (109). Non-enzymatic glycation of proteins, including basement membranes, may be an explanation for this phenomenon.

The accumulation of basement membrane may be due to increased synthesis and/or decreased degradation (121). Non-enzymatic glycation of collagen has been reported to result in a collagen which is more resistant to collagenase attack (90). Similarly, it has been reported that the activity of proteases and other enzymes which may be involved in the degradation of basement membrane are effected by non-enzymatic glycation, especially if a lysine residue is essential for normal function. Reduced collagenase activity has been reported in the kidneys of streptozotocin diabetic rats (94). Diabetes can also result in an increase in synthesis of basement membrane as has been demonstrated in streptozotocin diabetic rats (122). Both these ultimately result in increased amounts of basement membrane material.

It appears that non-enzymatic glycation may directly result in increased basement membrane accumulation by decreasing the susceptibility of the

membrane to degradation in two ways: decreased enzymatic activity of collagenase, and production of basement membrane which is resistant to enzymatic attack. Accumulation of basement membrane is undoubtably responsible for glomerular occlusion, but the role that basement membrane thickening plays is uncertain. The degree of thickening does not correlate to functional changes, including creatinine clearance and urinary albumin excretion (123), casting doubt on its importance.

Another aspect of diabetic nephropathy involves alterations in the glomerular basement membrane barrier resulting in altered filtration properties of the kidney ultimately resulting in non-selective proteinuria. The altered filtration is thought to be due to changes in the pore size of the membrane and/or changes in membrane charge.

The loss of negative charges on the basement membrane is responsible, at least in some part for the occurence of proteinuria (88). It has been found that diabetic basement membrane has a reduced amount of sialic acid (a negatively charged protein) resulting in a reduction of the negative charge of the membrane (124). This results in a more net positive charge of the membrane, making them more permeable to negatively charged proteins such as albumin. The mechanism leading to the decrease in the sialic acid content, however, is unknown. Non-enzymatic glycation of proteins, removes a positive charge which would further facilitate the passage of the protein. The basement membrane itself is non-enzymatically glycated, which would act to counteract these changes, by making the membrane more net negative by the removal of the positive charge on the lysine during the process.

Defects in the structure of the glomerular basement membrane, may result in increased pore sizes of the membrane (124). It has been suggested (98) that increased non-enzymatic glycation (due to hyperglycemic conditions) results in incorporation of glucose into the membrane on lysine and hydroxy-lysine residues, thereby removing a positive charge important in the formation of cross links. This would result in the formation of a membrane with larger pores than normal and would, as a result, lead to an increased passage of molecules across the glomerular barrier.

In rats with strict glycemic control the progression of nephropathy was halted or its progession inhibited (125). However, this was not shown in humans, suggesting that early renal damage is irreversible, and that a primary metabolic abnormality is responsible for the development of tissue damage. Recently, the accumulation of advanced glycosylated end products (AGE products) and their role in the long term complications of diabetes has been investigated. Proteins which turn over at a slow rate, such as collagen and elastin, undergo excessive glycation and subsequent modification (late stages of the Maillard reaction) giving rise to these irreversible AGE proteins. These products are believed to covalently trap serum proteins in the extra-cellular matrix and may contribute to capillary occlusion and the build up of basement membrane material (121). If this is the case, initial hyperglycemia in a newly diagnosed diabetic may give rise to these irreversible AGE proteins and the production of irreversible damage to renal function.

The role that non-enzymatic glycation plays in the development of diabetic nephropathy has not been fully elucidated. Is it responsible for the changes resulting in progression to renal failure or is it just a consequence of the disease state? If it is involved, then it would be strong evidence for the need of tight control of blood glucose levels to prevent the long term complications of diabetes, especially with the recent evidence which suggests that early damage, may be responsible for the long term changes seen at a later time (121).

II. RATIONALE AND OBJECTIVES:

There is evidence to suggest that non-enzymatic glycation of glomerular basement membrane may be the etiologic factor for diabetic nephropathy. Previous studies on the topic have failed to investigate the relationship between non-enzymatic glycation of glomerular basement membrane to renal morphological and functional changes simultaneously in the same group of animals. Without such a study, the role of non-enzymatic glycation in glomerular basement membrane thickening and diabetic nephropathy will remain speculative.

In this study we propose to:

1. Determine whether increased levels of blood glucose in diabetes results in an increase in the levels of non-enzymatic glycation of glomerular capillary basement membrane.

2. Ascertain whether increased levels of non-enzymatic glycation are related to basement membrane thickening.

3. Correlate the changes in functional impairment of the kidney; namely, urinary albumin excretion and creatinine clearance, to glomerular basement membrane thickening and to the level of non-enzymatic glycation of glomerular basement membrane.

4. Ascertain whether the levels of non-enzymatic glycation of muscle capillary basement membrane correlate to those of glomerular basement membrane.

III. METHODS:

A. PROCUREMENT OF TISSUES:

At the appropriate time, the animals were anesthetized with sodium pentobarbital and blood drawn via cardiac puncture for subsequent analysis of creatinine, glycated hemoglobin, and blood glucose. Kidneys were rapidly excised, a slice of the left kidney obtained for electron microscopy, and the remaining tissue placed in cold isotonic saline for subsequent isolation of glomeruli. A muscle sample was obtained from the left quadriceps muscle and placed in cold isotonic saline, and frozen for future analysis.

B. FUNCTIONAL AND METABOLIC METHODS:

1. MEASUREMENT OF BLOOD GLUCOSE:

Whole blood was obtained by tail bleed from the study rats. The glucose was quantitated using Dextrosticks by a glucose oxidase method and read on a glucometer (Ames Division, Miles Laboratory LTD.; Rexdale, Ontario).

2. MEASUREMENT OF URINARY PROTEIN:

Urinary protein was quantitated by the Department of Clinical Chemistry at the Health Sciences Centre. Briefly, it was measured turbidometrically following precipitation with 3% sulphosalicylic acid / 7% sodium sulphate (126) with the turbidity measured at 620 nm.

3. CREATININE CLEARANCE:

Creatinine in urine and serum was measured by autoanalyzer (Beckman Astra, Beckman Instruments INC., Brea, Ca.) by modification of the Jaffe rate method (alkaline picrate) (127). Analysis was performed by the Department of Clinical Chemistry at the Health Sciences Centre and calculated as follows:

ml/min. = urine creatinine/serum creatinine X volume/time

The values were corrected for weight and expressed as ml/min/100 gr.

4. MEASUREMENT OF GLYCATED HEMOGLOBIN

Glycated hemoglobin was quantitated by affinity chromatography using m-aminophenylboronic acid immobilized on 5% beaded agarose as previously described (87). M-aminophenylboronic acid has an affinity for coplanar cis-diol groups of glucose, such as that found in glycated hemoglobin. All reagents for glycated hemoglobin quantitation were provided in a commerically available kit (128), (Glycotest 100, Pierce Chemical Co., Rockford, Il.).

A hemolysate was prepared by mixing 50 ul of whole blood with 500 ul of the sample preparation reagent. Subsequently, 50 ul of the hemolysate was then added to a column which was previously equilibrated with 2 ml of equilibrium wash buffer (0.25 mol/L ammonium acetate, 0.05 mol/L magnessium chloride, and 0.02% sodium azide, pH=8.5). The sample was washed onto the column with 500 ul of the equilibrium wash buffer followed by an additional

5 ml of the buffer (the total volume added was 5.55 ml). This fraction consisted of the non-bound, non-glycated hemoglobin. Subsequently, 3 ml of the glyco-elution buffer (0.2 mol/L sorbitol, 0.1 mol/L TRIS, and 0.02% sodium azide) was added and the fraction collected in a separate tube. All tubes were vortexed well and the absorbance of each measured at 414 nm against deionized water and the % glycated hemoglobin calculated as follows:

$$\% \text{ GlyHB} = \frac{3.0 \text{ (Absorbance B)}}{5.55 \text{ (absorbance NB)} + 3.0 \text{ (absorbance B)}} \times 100$$

The coefficient of variation for this method was found to be 7% and 4.5% at a low and a high level (8% and 20%) respectively.

5. MEASUREMENT OF URINARY ALBUMIN:

Urinary albumin was measured by Radioimmunoassay by modification of previous methods (113,114,129) as described below.

A. Reagents.

1. Rabbit anti-rat albumin antisera was obtained from National Biological Laboratories (Dugald, Manitoba). Briefly, the antibody was raised to globulin free rat albumin obtained from Sigma Chemical Company (St. Louis, Mo.) by injection into New Zealand rabbits with adjuvent. 25 ml of serum was collected monthly, the titre was determined, and frozen in 2 ml aliquots. The titre for the antisera used in all assays was 1/16. A working solution was prepared by a dilution of 1/16 with saline.

2. Non-immune rabbit serum was obtained from rabbits not injected with rat albumin, and was diluted in a manner similar to the antisera (1/16) to prepare a working solution.

3. Iodinated Bovine serum albumin (specific activity of 2.0 mCi/ml) obtained from Dupont Canada LTD. (New England Nuclear Products, Lachine, Quebec) was used as a tracer and diluted with saline so that the working solution had about 50,000 dpm/100 ul.

4. 0.5 mg/ml rat albumin in saline was prepared as a stock standard solution and frozen in 1 ml aliquots. The working standard was prepared from this stock solution by diluting 1/100 with saline.

5. 1% (w/v) solution of gamma-globulins (Sigma Chemical Co.) in saline was prepared to enhance antibody precipitation.

6. 25% (w/v) Polyethylene glycol 6000 (Koch-Light LTD, Suffolk, England) in distilled water.

B. Procedure.

Total counts, non-specific binding (NSB), and standard tubes were prepared as described in Table 1. Two hundred ul of each rat urine previously diluted 1/50, 1/100 and 1/200 with saline was analyzed in duplicate. If the values obtained for the urine specimen was out of the range of the standard curve, the sample was reanalyzed using more appropriate dilutions. One

Table 4. Preparation of standards for radioimmunoassay for urinary albumin.

TUBES	IDENTIFICATION	AMOUNT OF 10 ug/ml RAT ALBUMIN	SALINE
1,2	Total counts	-	200 ul
3,4	NSB	-	200 ul
5,6	0 standard	-	200 ul
7,8	2 ug/ml std	40 ul	160 ul
9,10	3 ug/ml std	60 ul	140 ul
11,12	4 ug/ml std	80 ul	120 ul
13,14	5 ug/ml std	100 ul	100 ul
15,16	6 ug/ml std	120 ul	80 ul
17,18	7 ug/ml std	140 ul	60 ul
19,20	8 ug/ml std	160 ul	40 ul
21,22	9 ug/ml std	180 ul	20 ul

hundred ul of the working solution of label was added to each tube and subsequently, 100 ul of working antigen was added to all tubes except the total counts and the NSB tubes. To the NSB tubes, 100 ul of the diluted non-immune rabbit serum was added. All tubes were vortexed, and incubated at room temperature for 120 minutes. Subsequently, 25 ul of the 1% (w/v) gamma-globulin in saline was added to all tubes except the total count tubes, along with 400 ul of 25 polyethylene glycol (PEG). The tubes were vortexed well and centrifuged 15 minutes at 1000 x g. The supernatant was aspirated off, and the radioactivity in the pellet was counted by gamma counting (LKB 1272 gamma counter, LKB, Turku, Finland). The results were calculated using the logit (B/Bo) vs log concentration curve fitting method and expressed as ug/hr, taking into account the urine volume and the time of collection.

C. BIOCHEMICAL PROCEDURES:

1. PREPARATION AND PURIFICATION OF GLOMERULAR BASEMENT MEMBRANE.

The method employed to isolate glomerular basement membrane was modified from the method of Carlson et al (130), which involves the isolation of glomeruli from the kidney cortex by differential sieving through a series of nylon meshes. This results in a pure and uniform preparation of glomeruli. The glomeruli are then treated with sequential detergent extraction (131) to isolate the glomerular basement membrane. The procedure, unlike ones which involve sonification (132), does not destroy the structural integrity of the basement membrane.

A. Preparation of Earle's balanced salt solution.

Solutions A, B, and C were made separately in 300 ml of deionized water.

A.	116 mM NaCl	6.774 g/L
	16 mM KCl	1.193 g/L
	0.8 mM $MgSO_4 \cdot 7H_2O$	0.197 g/L
	5.6 mM glucose	1.009 g/L
B.	1 mM $NaH_2PO_4 \cdot H_2O$	0.138 g/L
C.	1.8 mM $CaCl_2$	0.265 g/L

A and B were mixed slowly, with constant stirring, and then solution C was added (rapid addition will cause precipitation to occur). The solution was buffered by the addition of 6.672 g/L HEPES (28 mM), the pH adjusted to 7.4 by the addition of concentrated NaOH, and the volume adjusted to 1 litre. The buffer is stable 2-3 days if stored refrigerated.

B. Isolation of glomeruli.

The following isolation was carried out on ice or in a cold room whenever possible. For each kidney, the renal capsule and perirenal fascia was removed. The kidney was sliced into a series of sections (2 mm thick) with a sharp razor blade. Using pointed forceps, the cortex was "pinched off" (outer 1 mm) to separate it from the medulla. The cortex from both kidneys was weighed

and then minced with a razor blade to a fine mash. The cortical mash was then placed in the center of the 254 um wire mesh screen (W.S. Tyler Inc., Winnipeg, Manitoba) and placed over a 150 ml beaker. The mash was pressed through the screen with the aid of a scoopula and occasional washing with a stream of buffer. The wash was then centrifuged at low speed (300 rpm) for 2-3 minutes. The supernatant was removed and discarded. The pellet was then washed through the 153 um nylon screen (held taut with the embroidery screen) in a similar manner as above. The procedure was repeated for both the 105 and the 88 um nylon screens (B and SH Thompson, Scarborough, Ontario). The material on the 88 and 105 um screens was collected (these fractions contained the glomeruli), pooled, and washed with buffer. The glomeruli were then frozen until the basement membrane could be isolated.

C. Isolation of glomerular basement membrane.

The glomerular basement membrane was isolated as follows. The frozen glomeruli were thawed, placed in a plastic centrifuge tube, and suspended in 10 ml of distilled water. The tubes were shaken on the shaker bath at a low speed for 1-2 hours at 4°C, followed by centrifugation at 6000 x g for 10 minutes, and the supernatant discarded. The pellet was resuspended in 10 ml of 3% (w/v) Triton X-100 and shaken again at 4°C for 2-4 hours. The samples were then centrifuged as above with the supernatant being discarded. The pellet was suspended in 10 ml of a DNase solution (2 mg Type I DNase (Sigma Chemical Co., product no. D4527) in 0.1 M sodium chloride, 0.05 M tris-HCl, and 3 mM magnessium chloride, pH=8.0) (133), and incubated at 37°C for 1 hour. The samples were then centrifuged as before. The pellet

was then resuspended in 10 ml of 4% (w/v) sodium desoxycholate, shaken 2-4 hours at room temperature, and centrifuged as before, with the resulting pellet being washed five times with deionized water to remove traces of the detergents as above, and lyophilized.

2. PREPARATION AND PURIFICATION OF MUSCLE CAPILLARY BASEMENT MEMBRANE.

The muscle capillary basement membrane was prepared in a manner similar to the glomerular basement membrane, with some modifications. The isolated muscle was thawed, the excess connective tissue removed, and minced with a sharp razor blade or scissors. The minced muscle was then homogenized in saline using a polytron homogenizer at medium speed for five minutes. The homogenate was then centrifuged at 7,500 rpm for 10 minutes and the supernatant discarded. The pellet was then washed twice with distilled water and similarly centrifuged. The washed muscle extract was then treated with the detergent extractions to isolate the basement membrane as described above for the glomeruli, except that the volumes of the detergents was increased from 10 ml to about 50 ml. Similarly, the washed muscle capillary basement membrane was lyophilized as described above.

3. MEASUREMENT OF NON-ENZYMATIC GLYCATION:

a. THIOBARBITURIC ACID ASSAY.

Ketoamine linked glucose (non-enzymatically linked glucose) upon mild acid hydrolysis yields 5-hydroxymethylfurfural (5-HMF) which can be quanti-

tated with thiobarbituric acid (135) (see figure 4 for a chemical representation of the reaction). Sodium borohydride reduction of the ketoamine derivative will result in formation of a stable hexitol derivative which lacks the keto-group, and for this reason will not form a 5-HMF derivative. This principle is utilized in the assay to develop a blank. 2-thiobarbituric acid (TBA) will bind to 1,2 cis-diol groups of carbohydrates forming a yellow colored derivative, which can be quantitated at 443 nm. The method employed in these studies was developed by modification of methods used by other investigators (135,136,137).

Five mg of lyophilized muscle capillary basement membrane and 500 ul of deionized water was added, in duplicate, to a screw topped test tube. To one tube (blank), a drop of 1-octanol was added to reduce subsequent foaming, followed by the addition of 200 ul of sodium borohydride (0.68 g sodium borohydride/20 ml distilled water) followed by incubation of samples at room temperature for 60 minutes. Two ml of 10% (w/v) trichloroacetic acid (TCA) was added to both tubes slowly and the tubes were vortexed briefly and then centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded. One ml of 2.5 M phosphoric acid was added to each screw capped tube, and the tubes sealed and placed in the heating blocks on a hot plate at 105° C for 90 minutes. Subsequently, the tubes were cooled on ice, 1 ml of 10% TCA added, and the tubes centrifuged for 10 minutes at 1000 x g. 750 ul of the supernatant was aliquoted into 1.0 ml microcentrifuge tubes. To all tubes and the standards (as prepared in Table 5, using 5-hydroxymethyl-furfural, Sigma Chemical Co.), 750 ul of 50 mM thiobarbituric acid was added.

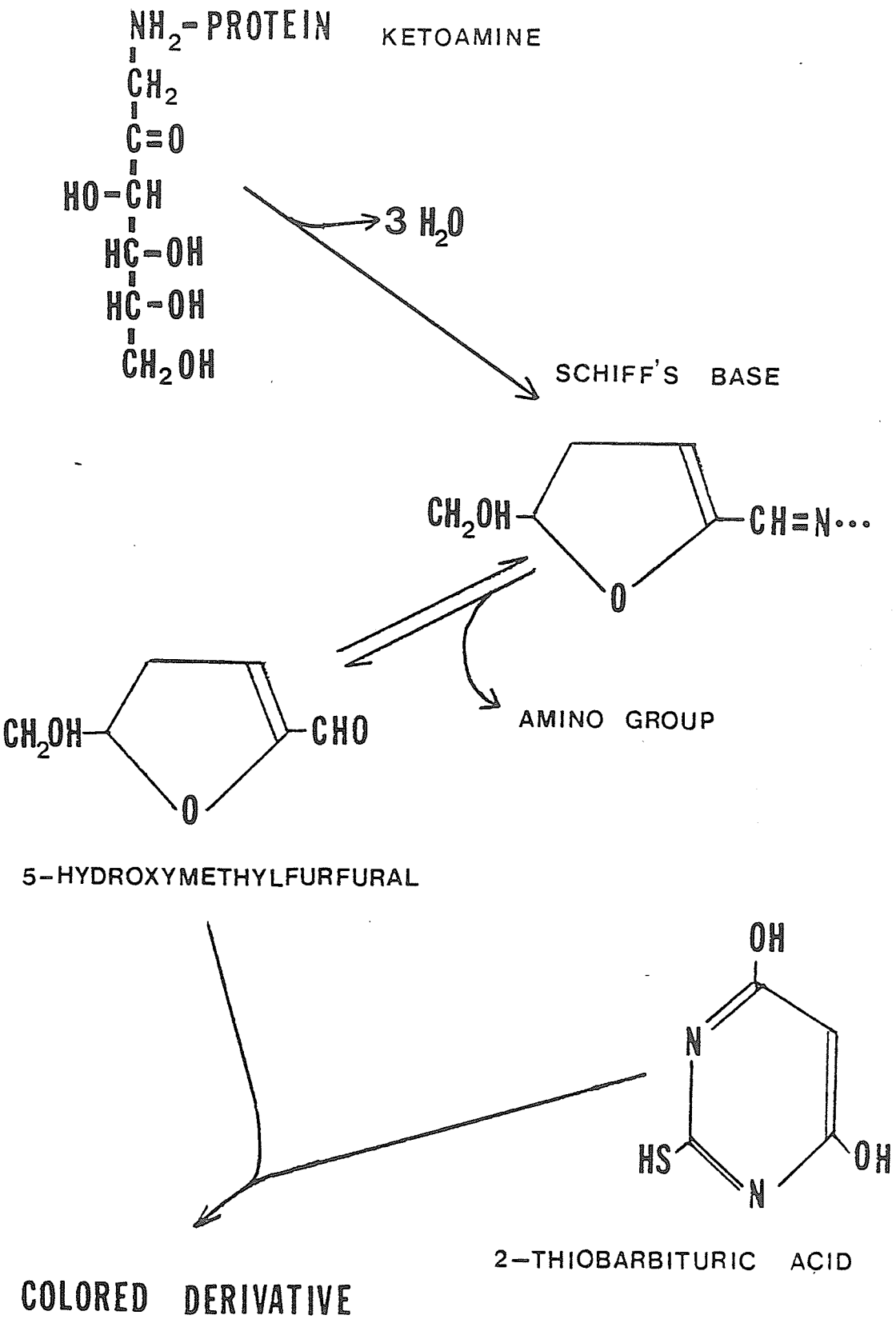


Figure 4. Schematic representation of the thiobarbituric acid assay.

Table 5. Preparation of the standard curve for the thiobarbituric acid assay using 5-hydroxymethylfurfural.

STANDARD CONCENTRATION	AMOUNT OF 100 nmol/ml 5-HMF	DISTILLED WATER
BLANK	-	750 ul
5.3 nmol/ml	40 ul	720 ul
11 nmol/ml	80 ul	670 ul
20 nmol/ml	150 ul	600 ul
30 nmol/ml	220 ul	530 ul
	AMOUNT OF 1000 nmol/ml 5-HMF	
40 nmol/ml	30 ul	720 ul
53 nmol/ml	40 ul	710 ul
67 nmol/ml	50 ul	700 ul
93 nmol/ml	70 ul	680 ul

The tubes were incubated at 40°C for 60 minutes, allowed to cool and centrifuged at 1,000 x g for 5 minutes in a microcentrifuge. The absorbance at 443 nm of all standards, samples, and blanks was measured spectrophotometrically. Subsequent to the subtraction of blank absorbances from each specimen, the concentration of HMF was determined from the standard curve (a typical standard curve is demonstrated in Figure 5), and the result expressed as mmol 5-HMF/mg protein.

b. TRITIATED SODIUM BOROHYDRIDE PROCEDURE

Reduction of the ketoamine linkage of non-enzymatically glycated proteins with tritiated sodium borohydride will result in the incorporation of tritium into the double bonds of the sugar resulting in the formation of a stable hexitol derivative (see Figure 6). However, this reduction is non-specific and will also reduce and incorporate tritium into the other double bonds found in the protein. Strong acid hydrolysis of the protein will result in breakage of all of the peptide bonds, resulting in the release of free amino acids. These amino acids are passed on an m-aminophenyl boronic acid affinity column, which will specifically bind carbohydrates containing a 1,2 cis-diol conformation (such as glucose) or compounds bound to glucose. The radioactivity of the bound fraction can be measured by standard liquid scintillation procedures and will be a measure of the extent of non-enzymatic glycation of the protein.

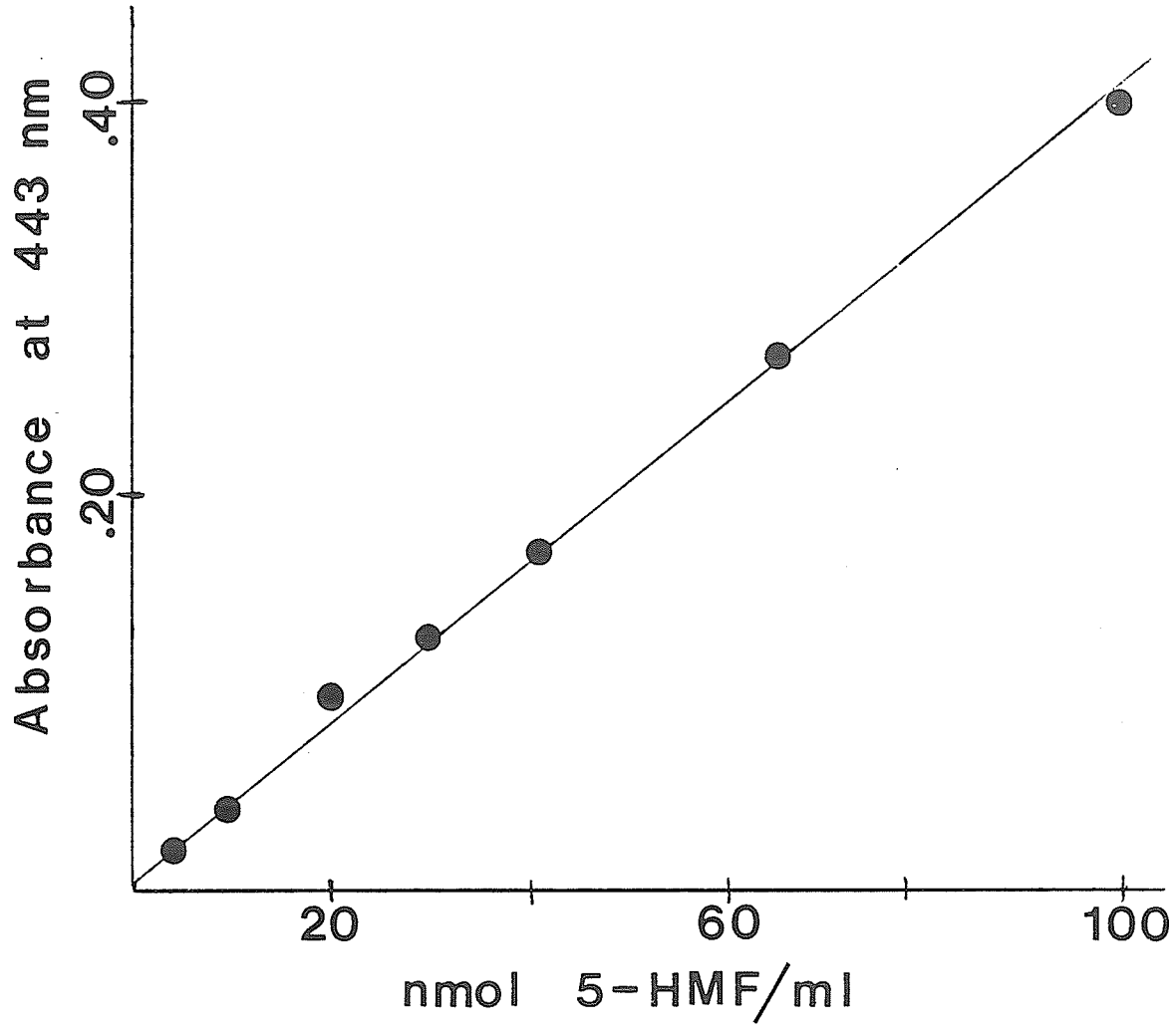


Figure 5. Typical standard curve of the thiobarbituric acid assay. Various concentration of 5-HMF were incubated with 50 mmol/L thiobarbituric acid. The absorbance was measured at 443 nm.

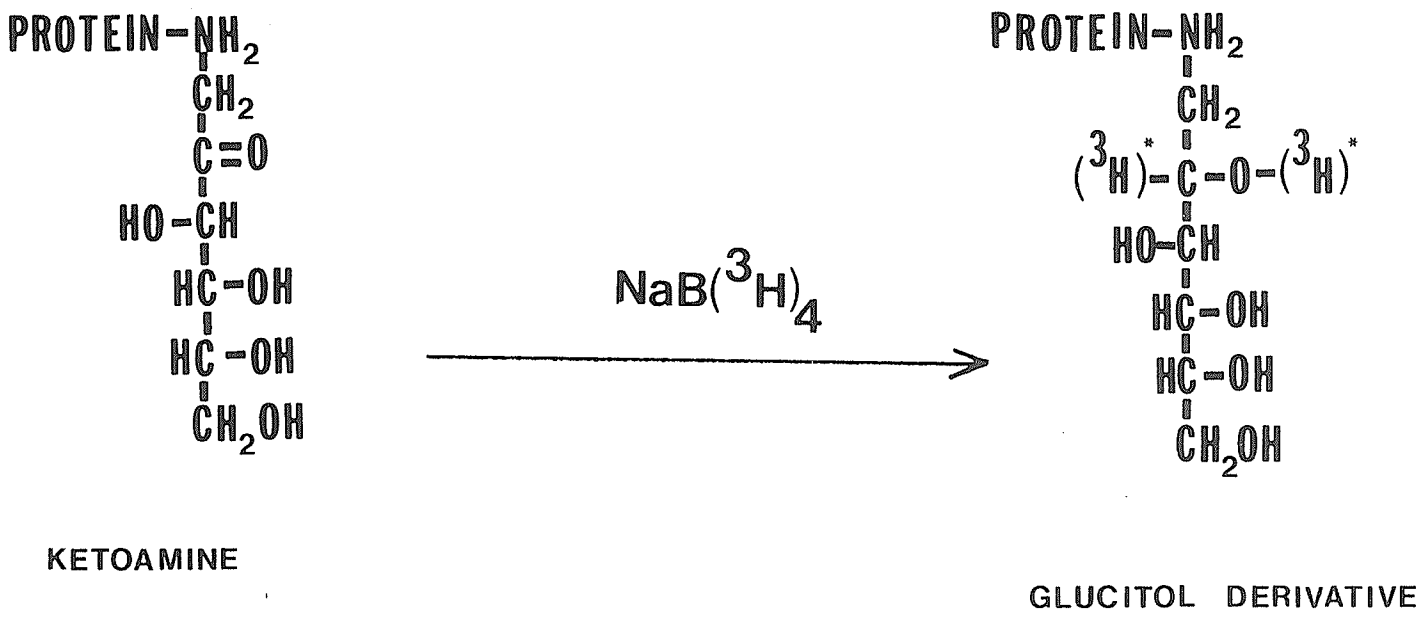


Figure 6. Chemical representation of tritiated sodium borohydride reduction of the ketoamine linkage.

i. Procedure. (138,139,140)

Two mg of lyophilized basement membrane was suspended in 200 ul of water, followed by the addition of 200 ul of tritiated sodium borohydride (Amersham Canada LTD., Oakville, Ontario. Product no. TRA.45, 500 mCi/mmol) and a drop of 1-octanol. Reduction was carried out for 90 minutes on ice in a fumehood, and was terminated by the addition of 4 ml of 10% TCA. The reduced basement membrane was washed 8 - 10 times with 10% TCA, followed by centrifugation and aspiration of the supernatant. The washed basement membrane was hydrolyzed with 1.5 ml of 6 M HCl for 16 hours at 110°C, in sealed screw top tubes, then the pH was adjusted to 8.5 by the addition of concentrated NaOH. The amino acid concentration of the hydrolysate was determined by the ninhydrin procedure (141), using a standard curve produced with known concentrations of leucine and expressed in leucine equivalents. Four hundred ul of the hydrolysate was loaded onto a m-aminophenylboronic acid affinity column (Glyc-affin GSP columns, Isolab Inc., Akron, OH) which was previously equilibrated with 3 ml of 50 mM Na_2HPO_4. The column was then washed with 15 ml of the 50 mM NA_2HP0_4. The bound fraction was eluted from the column by the addition of 2 ml of 0.1 M HCl and was collected. One ml of the eluate was added to 10 ml of PCS scintillation cocktail (Amersham Inc., Arlington Heights, Il.) and counted by standard scintillation procedures using a quench corrected counting program (LKB Rackbeta liquid scintillation counter, LKB, Turku, Finland).

ii. Measurement of amino acid concentration by the ninhydrin procedure (141).

One ml of the standards (0.05 to 0.25 mmol/L) were prepared in screw top tubes using a stock solution of L-leucine (500 umol/L in 0.1 M citrate buffer, pH 5.0), by various dilutions using 0.1 M citrate buffer, pH 5.0. The samples were diluted in the citrate buffer, usually 1/20 to 1/100 with 1 ml being added to a screw topped tube. One ml of ninhydrin reagent (Sigma Chemical Co., product no. 1632) was added, the tubes sealed, and placed on heating blocks for 15 minutes at 105°C. Subsequently, 2 ml of 50% (v/v) ethanol was added, and the tubes allowed to cool. The absorbances of the tubes were read against the blank at 570 nm and the results calculated from the standard curve and expressed as umol leucine equivalents.

iii. Calculations

The results for the tritiated sodium borohydride were expressed as cpm/ umol leucine equivalents.

4. LOWRY METHOD OF PROTEIN MEASUREMENT (142)

A standard curve, using a 1 mg/ml solution of bovine serum albumin in distilled water, was prepared so that the amount of protein in the standards ranged from 20 to 100 ug. To 200 ul of each standard and sample (diluted so that the protein concentration would be in the ug/ml range). One ml of the following reagent (1:1:2; 1% (w/v) copper sulfate, 2% (w/v) sodium

potassium tartrate, and 2% (w/v) sodium carbonate; respectively) was added to every sample, blank, and standard. The tubes were mixed and allowed to stand 10 minutes at room temperature. Subsequently, 100 ul of 1 N Folin-phenol reagent (Fisher Scientific) was added, the tubes mixed, and then allowed to stand 30 minutes at room temperature. Finally, the absorbances were read at 710 nm against the blank, the standard curve plotted, and the concentrations of the samples determined from the standard curve.

D. MORPHOLOGICAL INVESTIGATIONS

The morphological investigations were carried out by Dr. J.A. Thliveris from the Department of Anatomy, University of Manitoba. Briefly, small pieces of kidney cortex were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for two hours at 4°C. Tissues were rinsed for 24 hours at 4°C, in 0.1 M phosphate buffer (pH 7.4) containing 0.2 M sucrose. The tissues were then postfixed for two hours at 4°C, in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), dehydrated in ascending concentrations of ethanol and embedded in Epon 812. Thick sections were stained with toluidine blue and examined for routine orientation. Thin sections were stained with uranyl acetate and lead citrate, viewed and photographed in a Philips EM 201 electron microscope. Quantification of kidney glomerular basement membrane thickness was performed utilizing the orthogonal intercept method of Jensen et al (143). The actual measurements were carried out on micrographs (magnification 20,000 X) with an electronic planimeter (Hewlett Packard digitizer, model 9874A) equipped with an electrosensitive cursor connected to a Hewlett Packard 9875A calculator/computer.

The thickness of glomerular basement membrane for each rat was performed on 25 randomly selected micrographs per animal.

E. STATISTICS

The experimental data was analyzed by the unpaired T-test to compare the means of the various groups. The level of significance was $p=0.05$. The statistical analysis was performed on a commercial package (Crunch Interactive Statistics Package (CRISP), Crunch Software, San Francisco, Ca). The statistical analysis for the morphological studies was by analysis of variance (ANOVA) and by Tukey's procedure (144).

IV. METHOD DEVELOPMENT

A. THE ISOLATION OF GLOMERULI AND BASEMENT MEMBRANE.

The glomerular isolation procedure was investigated to determine the actual amount of glomerular protein that was isolated. The protein yields for the procedure were determined and the results shown in Table 6. Protein was measured by the method of Lowry et al (142) as described previously. It was found that approximately 10 to 15 percent of the cortical protein was found in the glomerular fraction (40 mg/g wet kidney weight).

The isolated muscle basement membrane was examined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis following digestion with pepsin (Figure 7) to ensure the presence of Type IV collagen. Fifty mg of muscle basement membrane was digested with 5 mg pepsin (Sigma Chemical Co.) in 0.5 M acetic acid, pH 2.5 at 4°C for 48 hours as previously described (120). The reaction mixture was then centrifuged at 1,000 x g for ten minutes and the supernatant collected. Five ug of protein from the supernatant (as measured by the procedure of Lowry et al (142)) was loaded onto a 7.5% polyacrylamide gel with a 5% stacking gel prepared according to Laemmli (134) and electrophoresed at 100 mA for 4 hours following pre-electrophoresis at 50 mA for 30 minutes. The gels were stained with 0.25% coomassie blue (R250) in 10% acetic acid, 50% ethanol, and destained with 10% acetic acid and 25% ethanol. Electrophoresis following pepsin digestion was not performed on glomerular basement membrane since it was not available in the quantities required for such analysis.

Table 6. Protein yields during the isolation of glomeruli.

STEP	AMOUNT OF PROTEIN[1]	YIELD
Starting material (cortex)	786 ± 42 mg	100%
Through 154 micron screen	315 ± 23 mg	40%
Through 108 micron screen	301 ± 42 mg	38%
Material on 108 micron screen	73 ± 4 mg	9%
Material on 88 micron screen	43 ± 4 mg	5%

[1] Analyses carried out in duplicate. Results expressed as mean ± range.

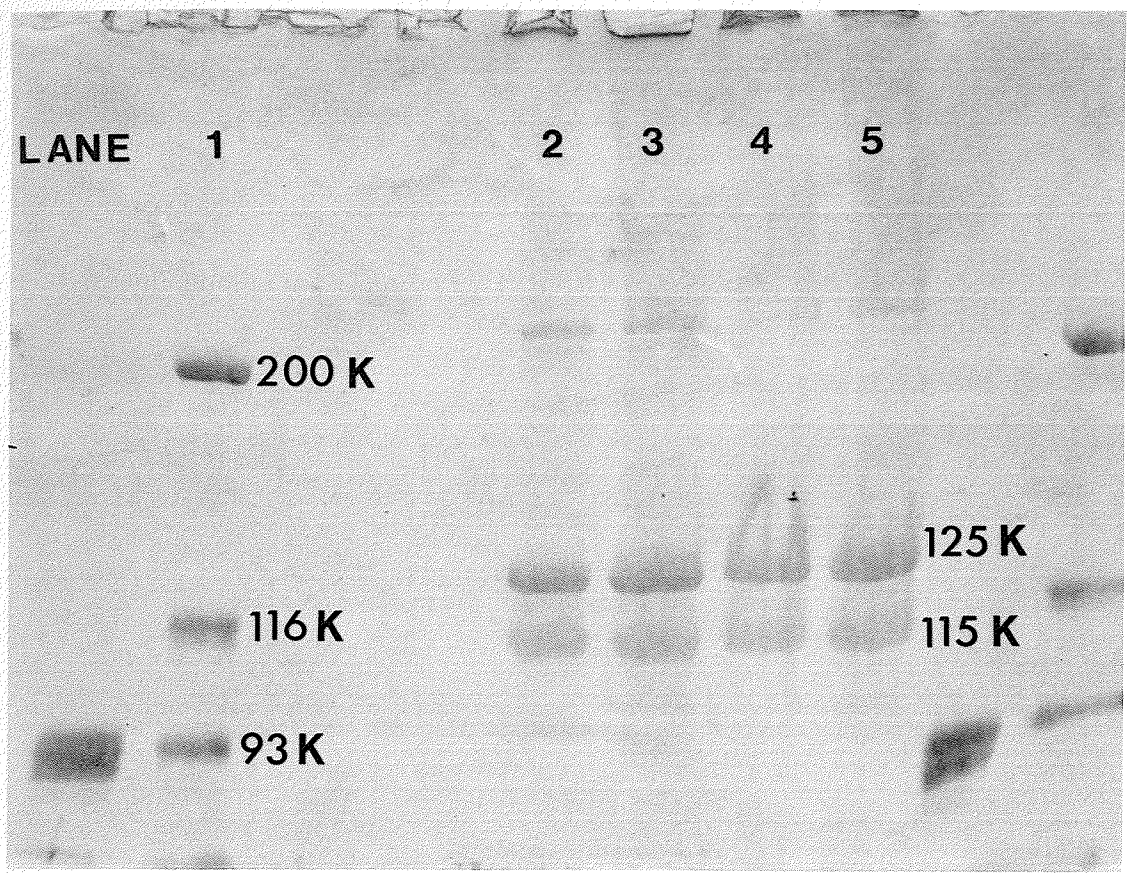


Figure 7. Sodium dodecylsulphate (SDS) polyacrylamide electrophoresis of pepsin digested basement membrane. 50 mg of muscle capillary basement membrane was previously digested with 5 mg of pepsin in 0.5 M acetic acid, pH=2.5 at 4°C for 48 hours. 5 mg of the digest was analyzed on a 7.5% polyacrylamide gel with a 5% polyacrylamide stacking gel essentially by the method of Laemmli (134). Lane 1 represents molecular weight markers; 92,00 (phosphorylase b), 116,250 (β-galactosidase), and 200,000 (myosin). Lanes 2 through 5 represent the pepsin digested basement membrane.

The presence of fragments of 125 and 115 kDa is consistent with the electrophoretic pattern of type IV collagen (basement membrane) after pepsin digestion as seen by others (120). The prepared basement membrane appeared to be composed of mainly type IV collagen, since it demonstrated an electrophoretic pattern characteristic of this protein. The lack of other protein bands on the electrophoretogram further suggest this. Although electrophoretic analysis was only performed on basement membrane isolated from muscle, it is anticipated that similar results would be obtained with glomerular basement membrane. It should be noted that muscle basement membrane was used for much of the preliminary work and method development in the place of glomerular basement membrane since it was easily obtainable in larger quantities than was glomerular basement membrane.

B. RADIOIMMUNOASSAY FOR URINARY ALBUMIN

Initially, a commerical radioimmunoassay (RIA) for the measurement of urinary albumin (Diagnostic Products Corporation, Los Angeles, Ca.) was investigated as a method to quantitate urinary albumin excretion. However, it was found that the antibody in the kit was specific for human albumin, and showed no cross reactivity with rat albumin. For this reason, we developed a RIA to measure albumin in rat urine.

Rabbit anti-rat albumin antisera was obtained from National Biological Laboratories as described under the methods section. The titre of each sera was determined to establish the optimal amount of antibody which would bind 50% of the tracer present (iodinated bovine serum albumin).

Concentrations of antibody that result in a higher binding will result in a reduced sensitivity in the lower range of the standard curve. In contrast, an antibody concentration resulting in less than 50% binding of tracer will result in a decrease in the dynamic range on the assay (145, 146).

The rabbit antisera was serially diluted (1/2 to 1/128) with saline. To 100 ul of each dilution, 100 ul of iodinated bovine serum albumin (500,000 dpm/ml), and 200 ul saline was added and incubated two hours at room temperature. The bound fraction (antibody/antigen complex) was obtained by centrifugation (1,000 x g for 15 minutes at 4°C) subsequent to the addition of 25 ul 1% (wt/vol) gamma globulin in saline and 400 ul of 25% polyethylene glycol (PEG). The radioactivity of the bound fraction for each sample was measured and the counts per sample over the total counts (corrected for non-specific binding) was plotted against the antibody dilution as in Figure 8. It was found that a 1/16 dilution of the antisera gave 50% binding of the radiolabelled bovine serum albumin. This was the dilution of antibody which was used for subsequent analysis.

The final step in a RIA is the separation of antibody bound and free fractions. In our assay, a separation using polyethylene glycol (PEG) was employed. PEG is a water soluble polymer which precipitates proteins. The effectiveness of PEG depends on the percent used, the pH and salt content of the solution, and the amount of protein present in the assay tube (145). To optimize the separation, the percentage of PEG which will give the least amount of non-specific binding with the maximal amount of antibody precipitation was determined. Four hundred ul of various concentrations of PEG

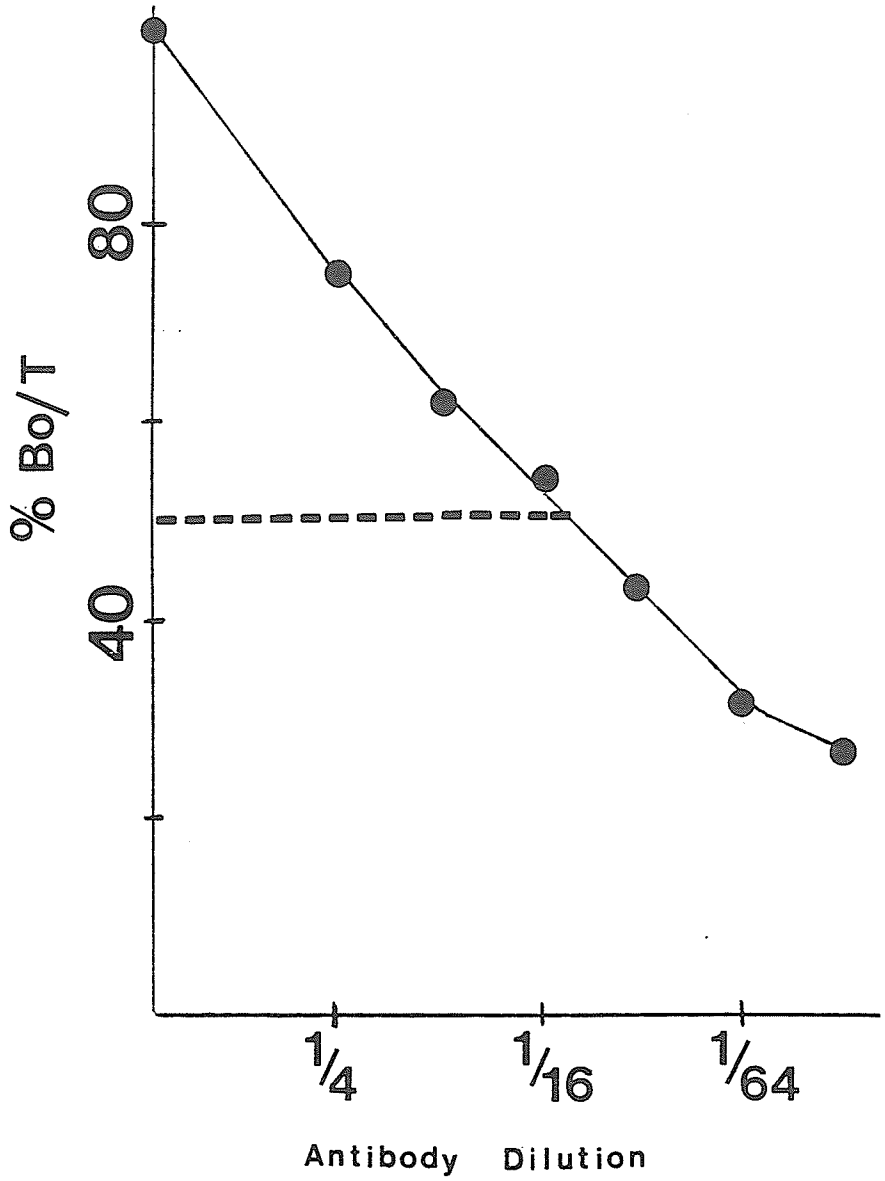


Figure 8. The determination of optimal titre of rabbit anti-rat albumin. The dilution of antisera, which would give 50% binding of the tracer in the absence of antigen was determined as follows. To 100 ul of antisera which was diluted serially with saline, 100 ul of iodinated bovine serum albumin was added and the tubes incubated. The bound fraction was obtained by precipitation with 25 ul of 1% (w/v) gamma-globulin and 400 ul of 25% (w/v) polyethylene glycol. The radioactivity was determined in this fraction and the percentage bound to total counts was calculated and plotted against the antisera dilution.

were added to assay tubes containing 100 ul of label (500,000 dpm/ml), 200 ul saline, and 100 ul of antisera, which were previously incubated at room temperature for two hours. The radioactivity in the pellet (bound fraction) was determined after centrifugation, and the percentage bound over total counts (% Bo/T) plotted against the percentage PEG used, as in Figure 9. Similarly, various concentrations of PEG were used to obtain the bound fraction from assay tubes containing 100 ul of label and 100 ul of non-immune rabbit anti-sera (titre of 1/16) and plotted as above and shown in Figure 9. This represented non-specific binding. It was found that with 25% PEG the optimal precipitation of the bound fraction, with the least amount of non-specific binding (NSB) was obtained. This was the percentage of PEG used in all further analyses. As well, 25 ul of 1% (wt/vol) gamma-globulin was added prior to addition of the PEG to further enhance to separation of the bound from free fraction.

Once the conditions of the RIA were optimized, the performance of the RIA was evaluated by various methods. Precision studies were undertaken using urine from two hyperglycemic diabetic rats. The results of the precision studies undertaken are shown in Table 7. The assay had a within run co-efficient of variation (CV) of three percent, and a between run CV of six to seven percent. Typically, the non-specific binding of this assay system was about 20 percent.

The accuracy of the assay was evaluated by determining the analytical recovery of albumin added to urine as shown in Table 8. Rat urines were

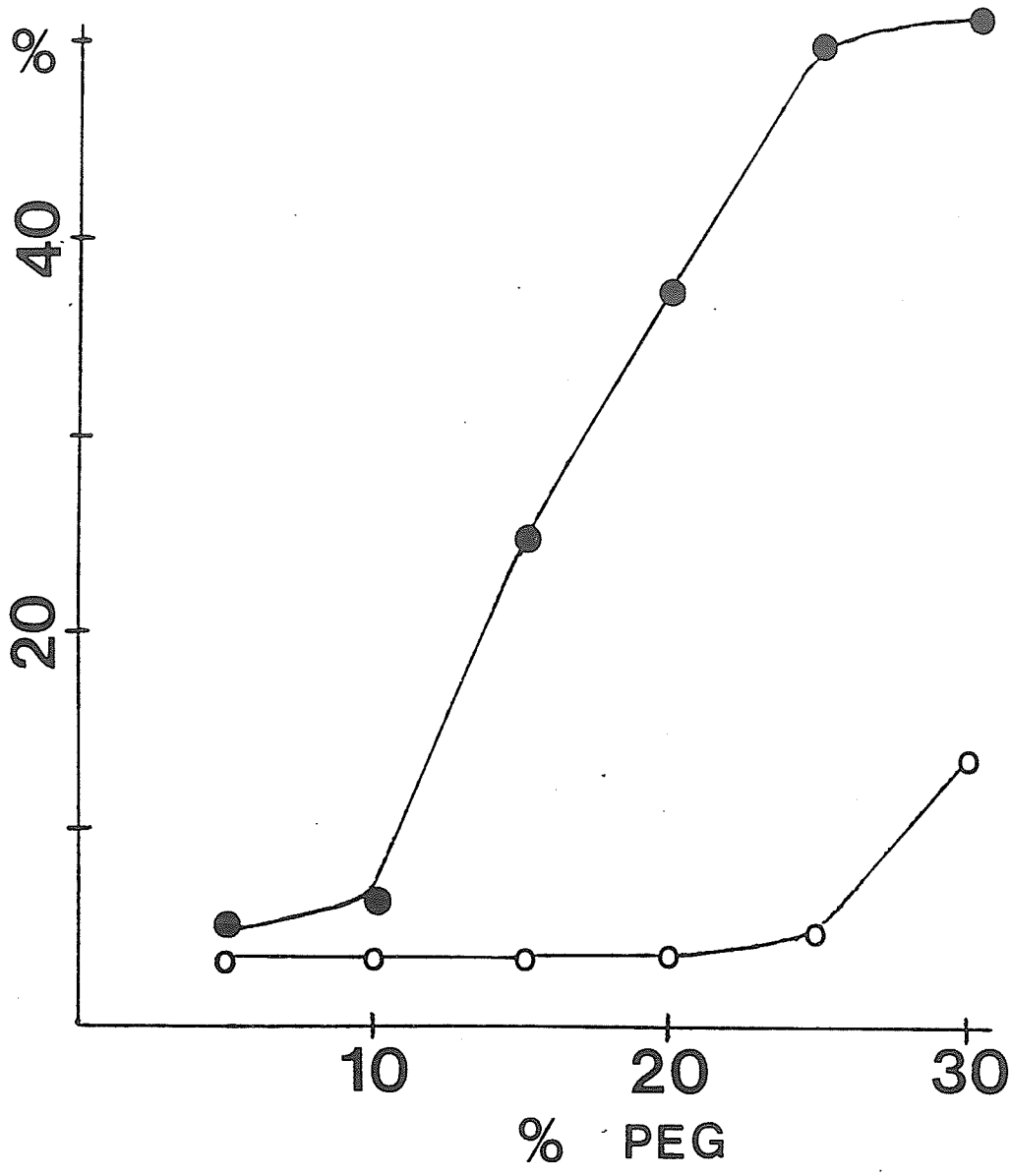


Figure 9. Determination of the maximal amount of polyethylene glycol (PEG) required for the separation of the bound fraction from the free fraction for the RIA for urinary albumin. Four-hundred ul of various percentages of PEG were added to assay tubes containing 100 ul antisera, 100 ul of iodinated bovine serum albumin (500,000 dpm/ml), and 200 ul of saline, previously incubated two hours at room temperature. The assay tubes were centrifuged and the radioactivity determined in the pellet (bound fraction), expressed as a percentage of total counts and graphed against the percent PEG used (●-●). The above was repeated with non-immune rabbit serum to determine the percent non-specific binding (○-○) for each percentage of PEG used.

Table 7. Precision studies for RIA for rat urinary albumin.

1. Within run precision.

	Sample 1	Sample 2
n	10	10
mean	2.24 ug/ml	4.16 ug/ml
standard deviation	0.08	0.06
coefficient of variation	3.6%	1.4%

2. Between run precision.

	Sample 1	Sample 2
n	10	10
mean	321 ug/hr	51.9 ug/hr
standard deviation	25	2.3
coefficient of variation	7.8%	4.4%

Table 8. Recovery studies for measurement of urinary albumin by Radioimmunoassay.

Conc. Measured	Conc. Added	Conc. Recovered	% Recovery
3.23 ug/ml (Baseline)	-	-	-
5.43 ug/ml	2.03 ug/ml	2.20 ug/ml	109%
7.45 ug/ml	3.98 ug/ml	4.23 ug/ml	106
2.50 ug/ml (Baseline)	-	-	-
4.35 ug/ml	2.03 ug/ml	1.85 ug/ml	91
6.80 ug/ml	3.98 ug/ml	4.3 ug/ml	108
1.50 ug/ml (Baseline)	-	-	-
3.75 ug/ml	2.03 ug/ml	2.25 ug/ml	111
5.18 ug/ml	3.98 ug/ml	3.68 ug/ml	93
4.20 ug/ml (Baseline)	-	-	-
7.35 ug/ml	3.20 ug/ml	3.15 ug/ml	98
8.75 ug/ml	4.20 ug/ml	4.55 ug/ml	108
3.14 ug/ml (Baseline)	-	-	-
7.35 ug/ml	4.20 ug/ml	4.22 ug/ml	100

Mean Recovery $\bar{x}$ = 103%

SD = 7%

spiked with a weighed out amount of purified rat albumin, and the albumin concentrations measured RIA. The recovery was calculated as follows:

$$\% \text{ Recovery} = \frac{\text{Spiked - baseline albumin value}}{\text{concentration of albumin added}}$$

Each sample was evaluated at two different albumin levels (2 and 4 ug/ml). The assay showed a mean recovery of 103% with a co-efficient of variation of seven percent.

To assess the linearity of the assay, a rat urine with a high albumin concentration was diluted with saline to give concentrations falling over the entire range of the standard curve. The concentration of each dilution was measured by the RIA and the observed concentration was plotted vs the expected (calculated) concentration for each dilution. All results obtained fell on or near the line of identity (see Figure 10). This indicates that specimens having albumin values above the upper limit of the standard curve can be appropriately diluted to bring them into the range of the latter.

C. DEVELOPMENT OF THE THIOBARBITURIC ACID ASSAY.

The thiobarbituric acid assay used was a modification of the method used by previous investigators (135,136). Initially, the characteristics of the colored complex formed between 5-hydroxymethylfurfural (5-HMF) and 2-thiobarbituric acid were examined. Thiobarbituric acid was incubated 60 minutes with 5-HMF and the absorbance spectra measured with a scanning spectrophotometer. The complex had a peak absorbance at 443 nm (the complete spectral scan is shown in Figure 11). Similarly, the time of maximal

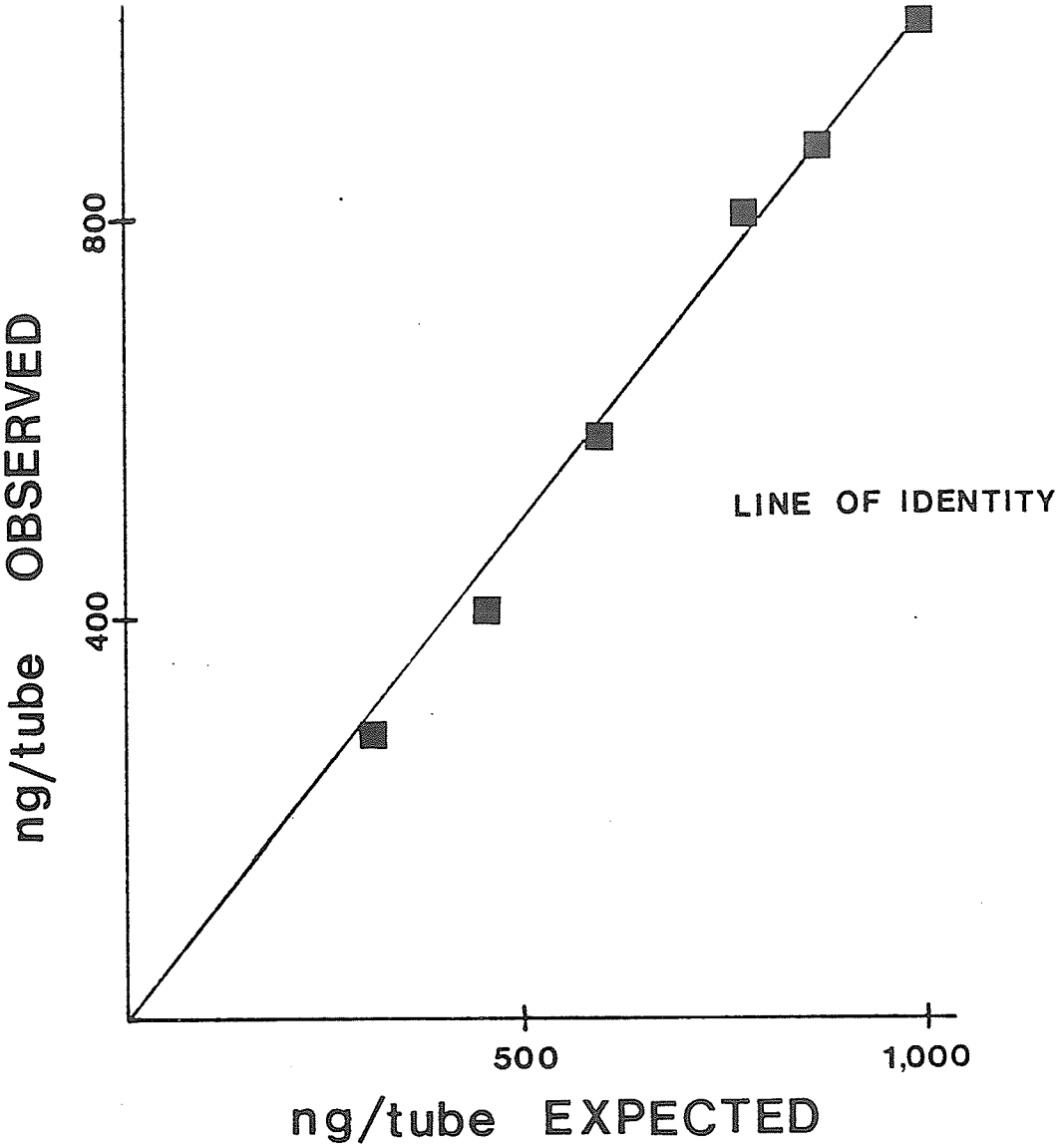


Figure 10. Demonstration of linearity for the RIA for urinary albumin. A rat urine (6 ug/ml albumin) was diluted in saline and the concentrations of these dilutions determined by RIA (see methods section). The observed concentrations were graphed against the expected (calculated) concentrations.

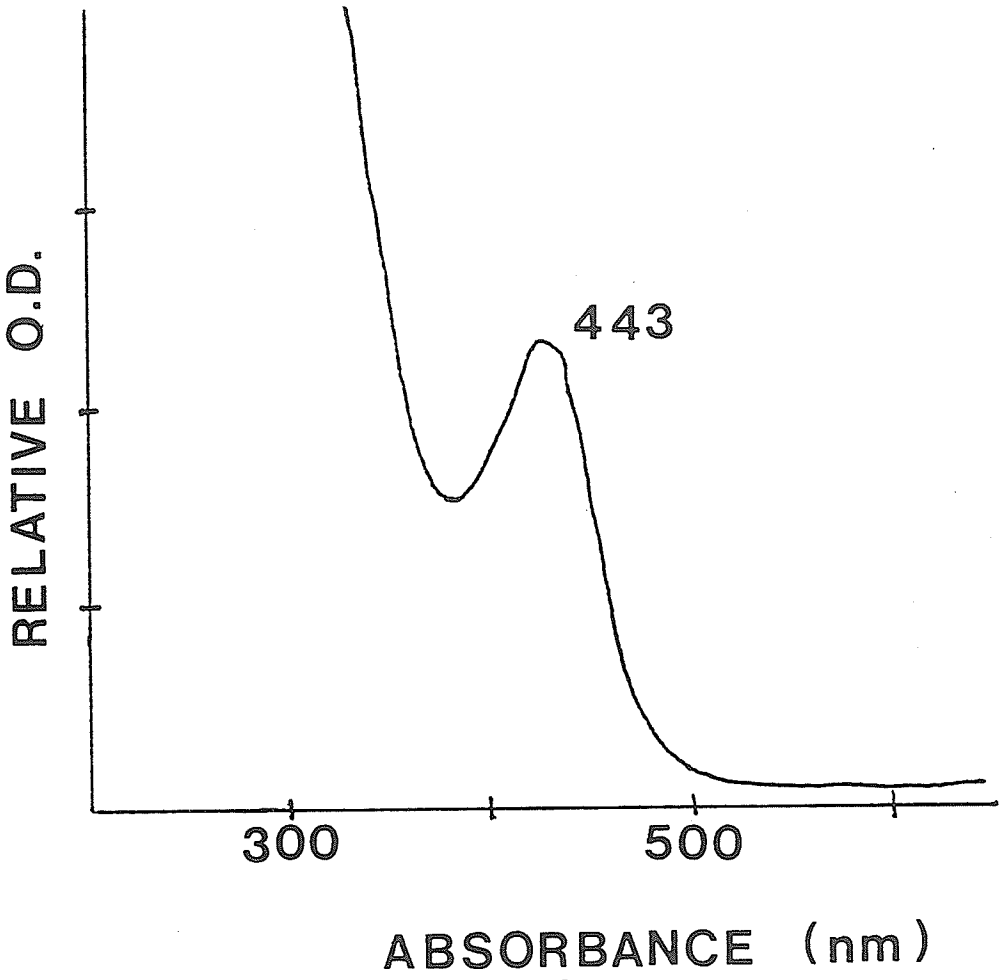


Figure 11. Absorption spectra of 2-thiobarbituric acid. One hundred mmol 5-HMF was incubated with 50 umol 2-thiobarbituric acid for one hour at 40°C. The solution was cooled to room temperature and the absorbance of the solution measured by a scanning spectrophotometer from 200 to 850 nm. The peak absorbance was found at 443 nm.

color development was determined (Figure 12) for the complex at 443 nm, with the maximal level occurring after 60 minutes.

Weak acid hydrolysis of the ketoamine derivative with phosphoric acid, results in formation of the 5-HMF derivative. The optimal time of acid hydrolysis at 105°C was determined to be 90 minutes (Figure 13) as evidenced by the maximal production of the 5-HMF derivative. In a similar manner, the optimal acid concentration was determined to be 2.5 M phosphoric acid (Figure 14).

The thiobarbituric acid assay has been previously shown to be somewhat dependent on protein concentration (136). This feature was investigated further. It was found that there was a protein dependency (Figure 15), however, between 10 and 30 mg of protein, there was little effect on the results obtained. At low protein levels, there is a high degree of error, most likely due to the low absorbance of the resulting 5-HMF product.

A sample blank which consisted of 10 to 30 mg of the sample was reduced with sodium borohydride, and ran in conjunction with each sample. This treatment reduced the ketoamine linkage and prevented the formation of a 5-HMF derivative. Any color formed by the blank would be due to various interferences. It has been found that free glucose and fructose can form 5-HMF derivatives and will react with the thiobarbituric acid to form a colored derivative (136). The blank value was subtracted from the sample result to determine the actual amount of 5-HMF produced, and hence the extent of non-enzymatic glycation.

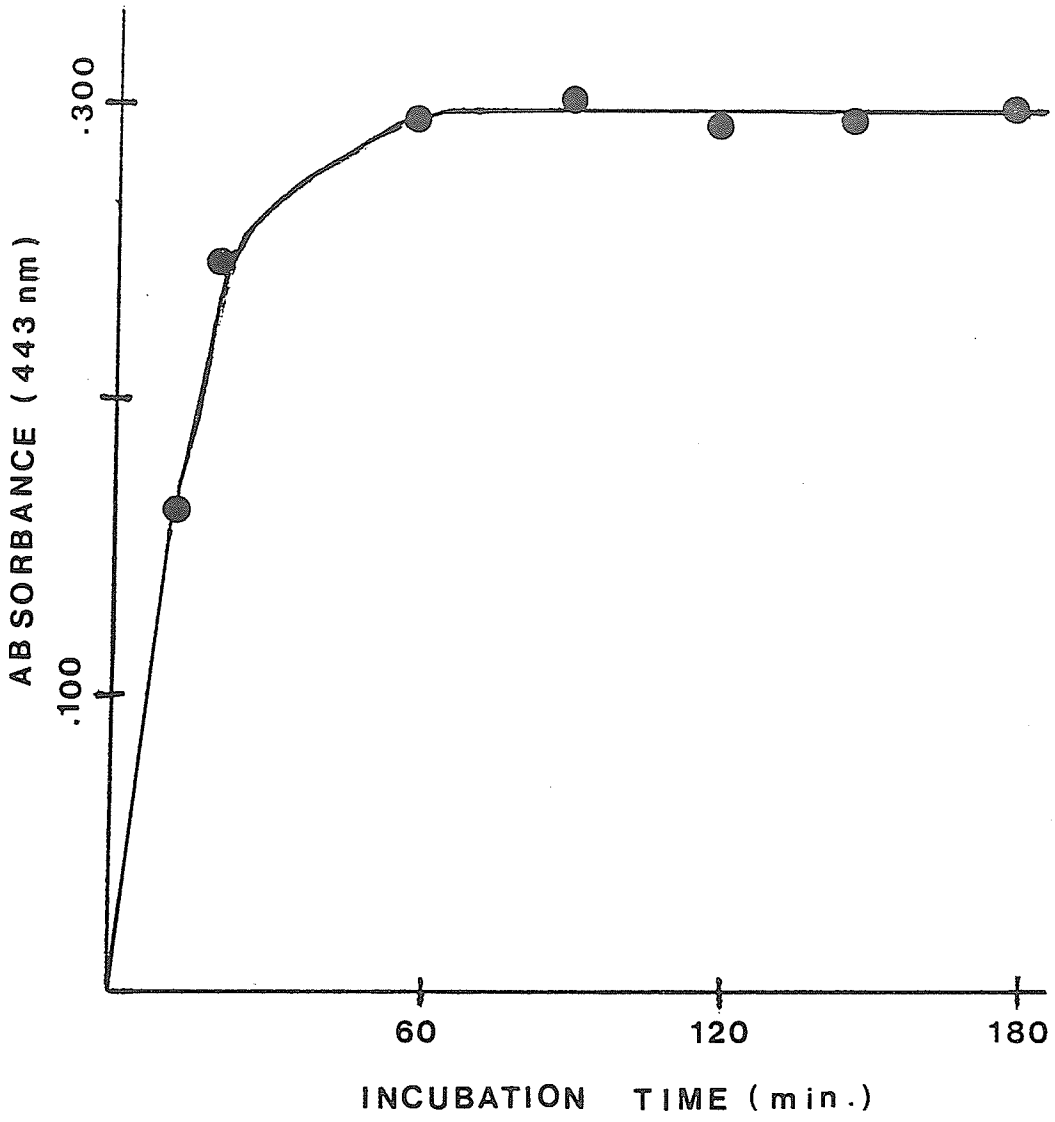


Figure 12. Determination of maximal color development for the thiobarbituric acid assay. 50 nmol 5-HMF was incubated with 50 umol 2-thiobarbituric acid for various times (15 minutes to 3 hours) at 40°C. Subsequently after cooling to room temperature, the absorbance was measured at 443 nm.

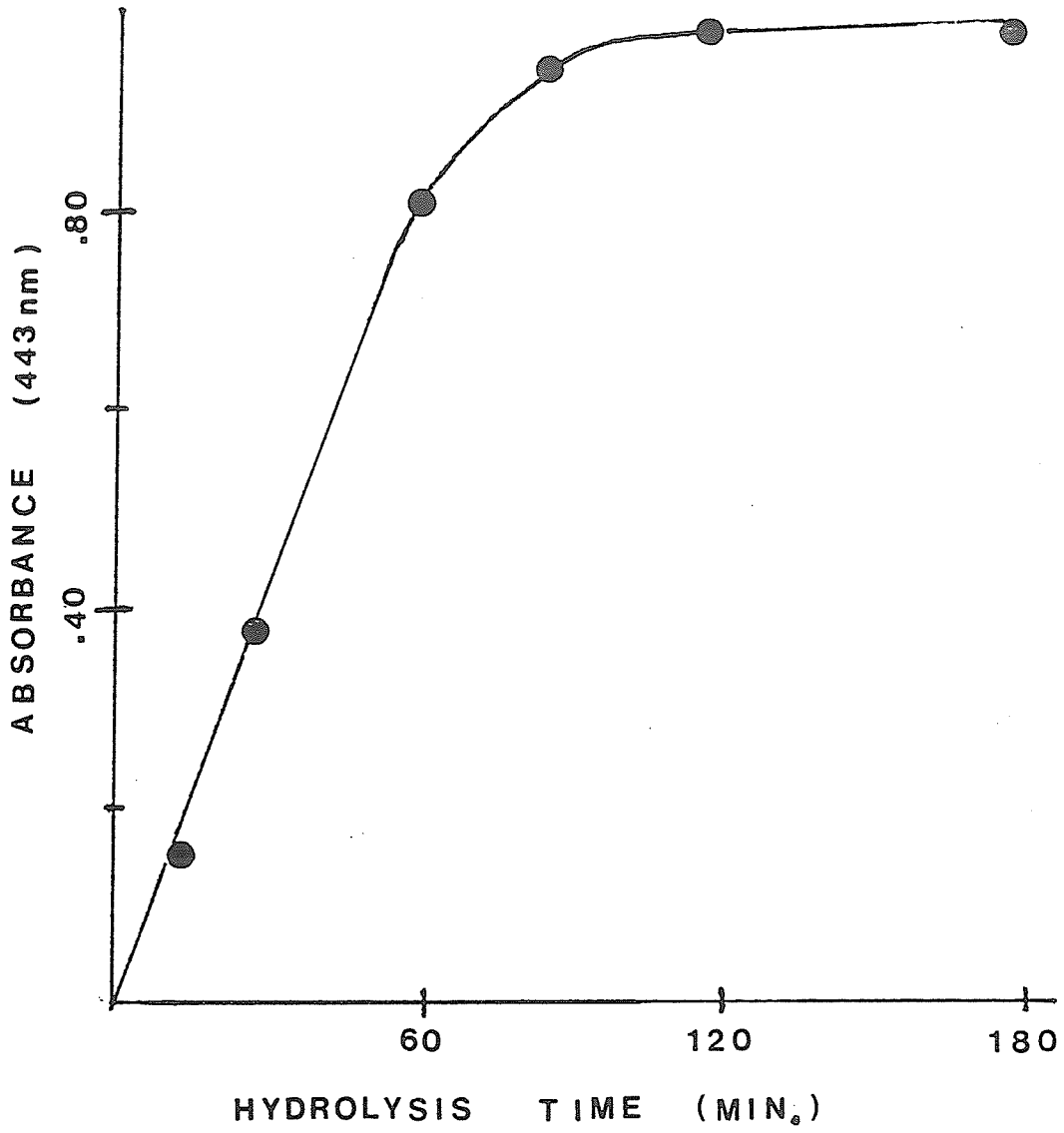


Figure 13. Determination of maximal time of phosphoric acid hydrolysis for conversion of a ketoamine derivative to the 5-HMF derivative in the thiobarbituric acid assay. A pooled diabetic serum sample (56 mg/ml protein) was hydrolyzed with 2.5 M phosphoric acid at 105°C for various times (15 minutes to 3 hours). Subsequently, the release of 5-HMF was quantitated using thiobarbituric acid.

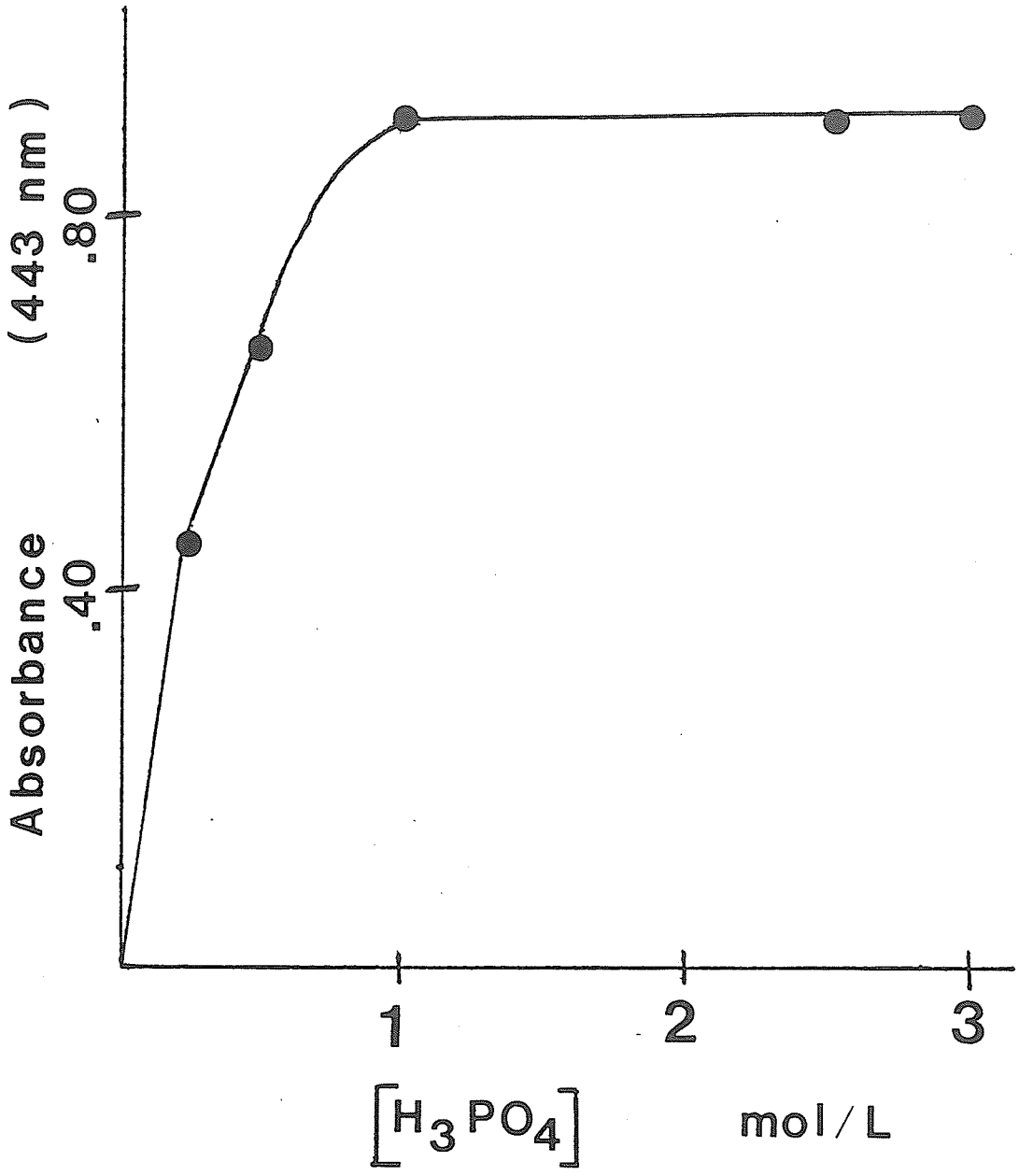


Figure 14. The effect of phosphoric acid concentration on the production of 5-HMF from a ketoamine derivative in the thiobarbituric acid assay. A pooled diabetic serum sample (28 mg/ml protein) was hydrolyzed at 105°C for 90 minutes with varying concentrations of phosphoric acid (0.25 M to 3.0 M). Subsequently, the release of 5-HMF was quantitated using thiobarbituric acid.

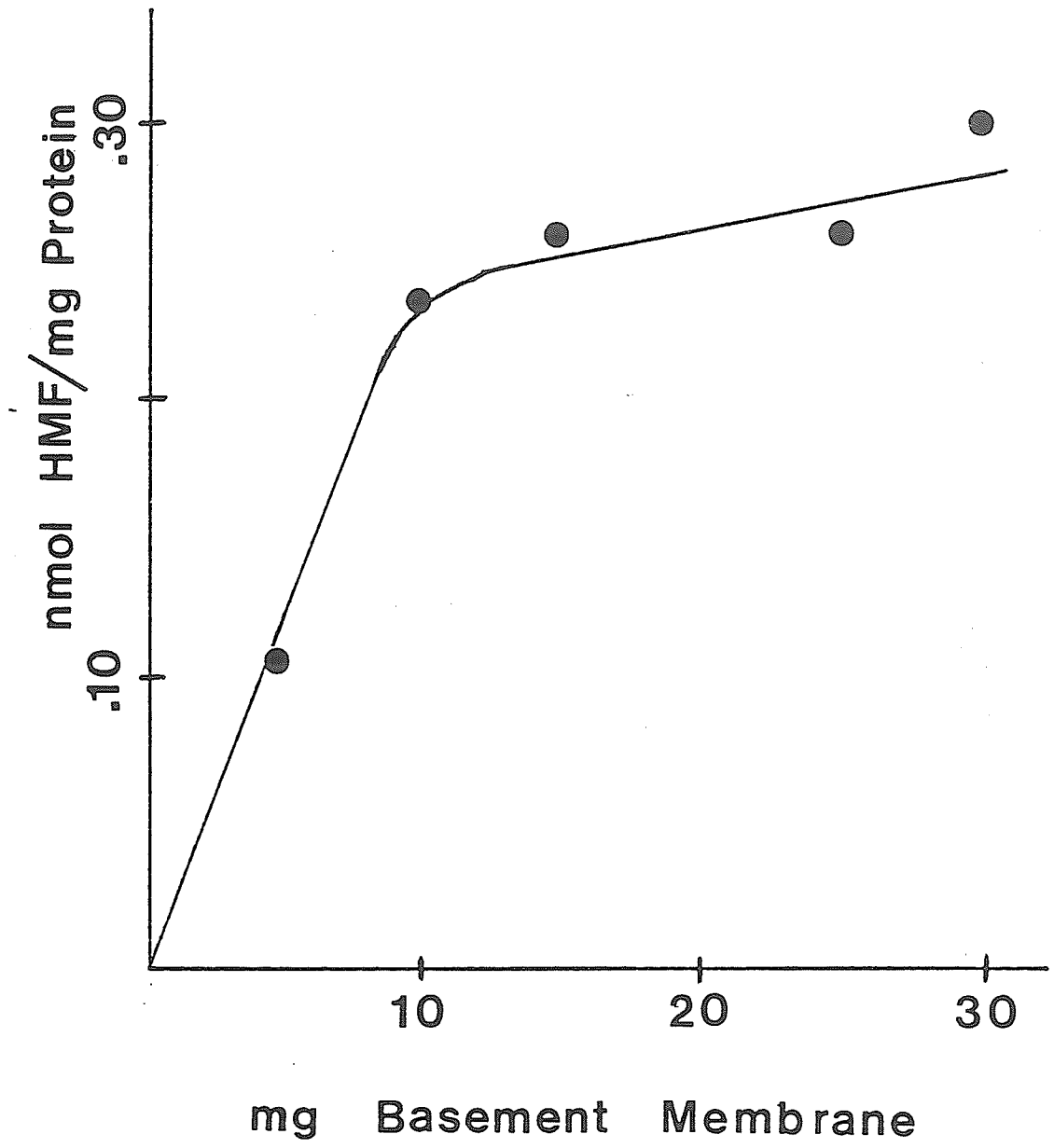


Figure 15. Effect of protein concentration on the results of the thiobarbituric acid assay. Various amounts of muscle capillary basement membrane (5 to 30 mg) were analyzed by the thiobarbituric acid assay (see methods section). The results are expressed as nmol HMF/mg protein vs protein concentration.

Muscle capillary basement membrane, which was previously glycated to varying degrees by in vitro incubation with increasing concentrations of glucose, was analyzed by the thiobarbituric acid assay (Figure 16). It was found that the extent of non-enzymatic glycation was proportional to the glucose concentration in which the basement membrane was incubated. Incubation in 100 mmol/L glucose resulting in a four fold increase in levels of non-enzymatic glycation over that of control levels.

The with-in run precision of the assay as determined using glycated muscle capillary basement membrane was found to have a CV of 19% (mean of 94.9 ± 18.3 nmol HMF/10 mg, n=10) which is consistent with what has been reported by others (136).

The thiobarbituric acid assay is a useful assay for the measurement of non-enzymatic glycation. However, the assay requires large amounts of basement membrane (10-30 mg range). The samples in some cases have high blank values, with optical densities nearly as high as the sample itself, resulting in low net absorbances and low results. The assay is useful for the measurement of non-enzymatic glycation of muscle capillary basement membrane, where large sample sizes are available. However, when only a few milligrams of material is available, for example from a rat kidney, the assay does not exhibit the sensitivity required to determine the extent of non-enzymatic glycation from these samples. Further more, the high degree of imprecision, may prevent discernment of two samples with similar extents of non-enzymatic glycation.

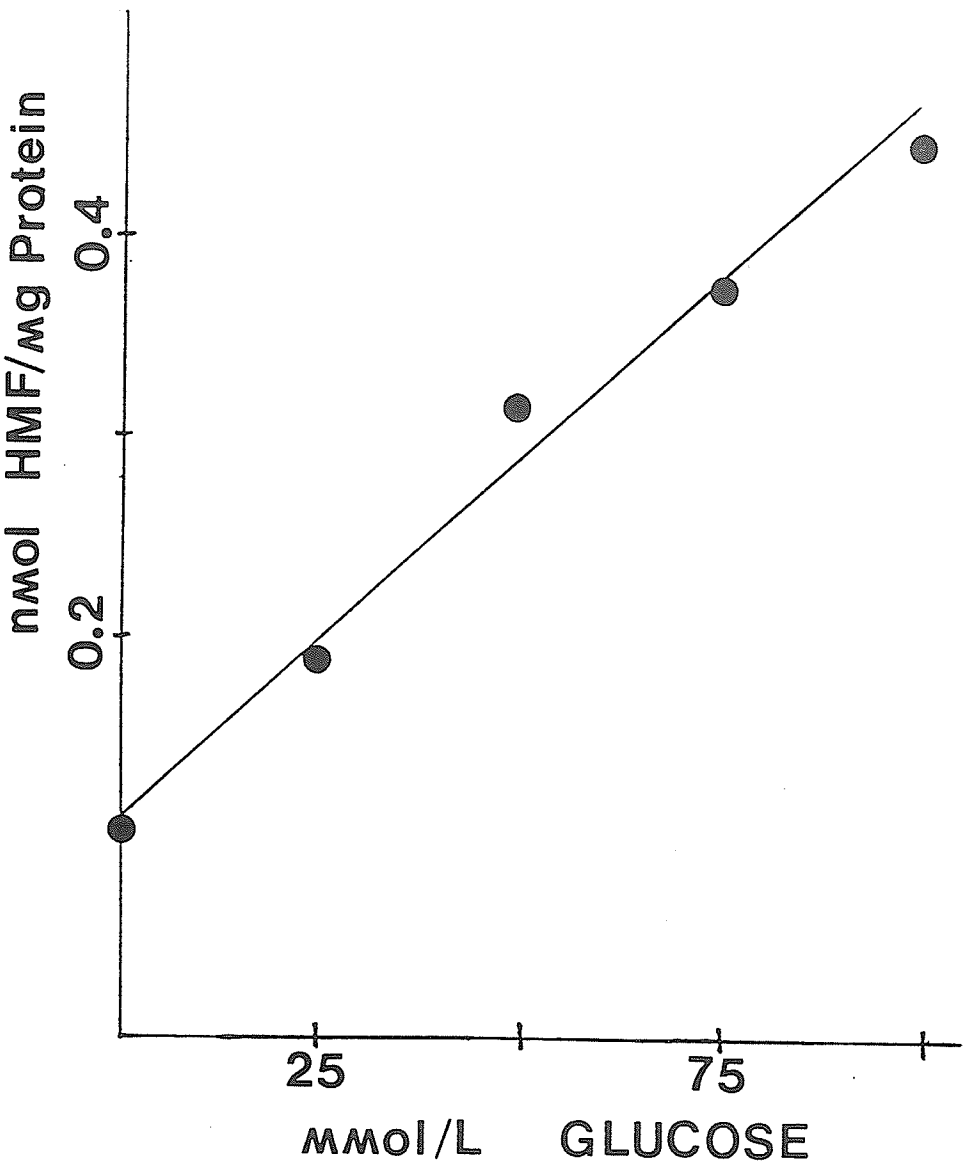


Figure 16. Quantitation of the level of non-enzymatic glycation of in vitro glycated muscle capillary basement membrane determined by the thiobarbituric acid assay. The basement membrane was glycated to various degrees by incubation in increasing concentrations (25 to 100 mmol/L) glucose in 0.2 M phosphate buffer, pH=7.4 for seven days at 34°C.

D. TRITIATED SODIUM BOROHYDRIDE ASSAY.

The tritiated sodium borohydride assay was developed by modification of the methods used by others (138,139) as a means of measuring non-enzymatic glycation. The assay, as described under the methods section, uses tritiated sodium borohydride to reduce, and in the process, introduce a radioactive label into the ketoamine linkage.

After reduction of the ketoamine linkage by the tritiated sodium borohydride, the excess radioactivity was removed from the reaction mixture by precipitation of the protein with 10% (wt/vol) trichloroacetic acid (TCA), followed by centrifugation and the removal of the radioactive supernatant. The material was washed in this manner a number of times with the radioactivity measured in the supernatant for each wash, and plotted as shown in Figure 17. It was found that eight to ten such washes with five ml of 10% TCA were required to remove the excess radioactivity from the protein. Dialysis could have been used as an alternative method, however, it would be more time consuming and results in a dilutional effect. On the other hand, TCA precipitation results in a concentration of the protein.

The separation of the labeled (glycated) amino acids in the hydrolysate, following acid hydrolysis, was necessary. This was accomplished by affinity chromatography using Glyc-affin GSP affinity columns containing m-aminophenylboronic acid, which has an affinity for 1,2 cis-diol groups. The amino acids containing the labelled glycitol residues (the amino acids which were non-enzymatically glycated) bind the support, since the glucitol derivative

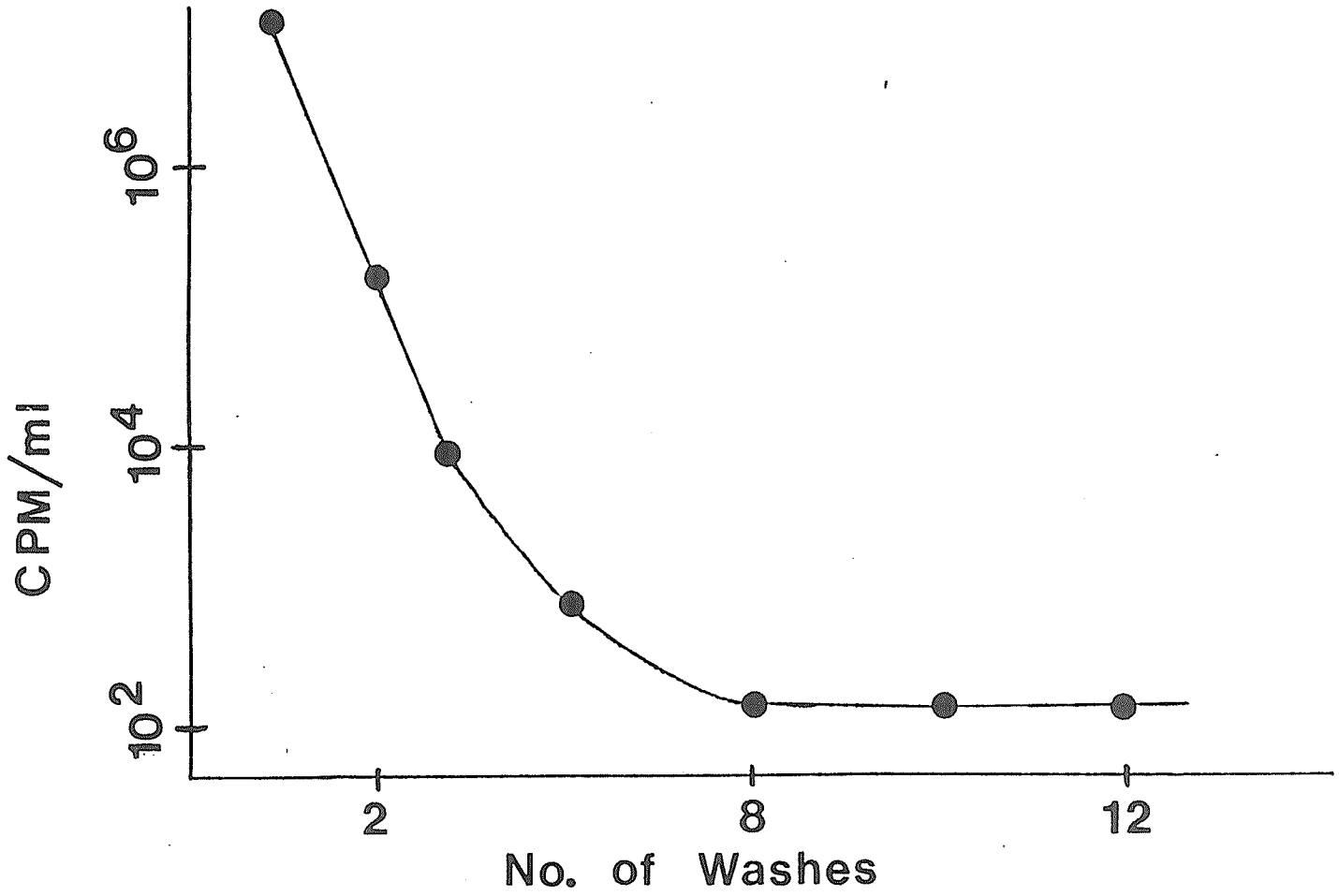


Figure 17. Effect of multiple washes of basement membrane with 10% (w/v) trichloroacetic acid (TCA) following reduction with tritiated sodium borohydride. Four mg of basement membrane was reduced with tritiated sodium borohydride (as described under methods section). The excess tritium was removed by a series of five ml washes with 10% (w/v) TCA, followed by centrifugation and removal of the supernatant. The radioactivity was determined in one ml of the supernatant by standard liquid scintillation procedures.

has a cis-diol group. The radioactivity in this fraction was measured, subsequent to elution from the column with 0.1 N HCl. A typical elution profile is shown in Figure 18. The radioactivity initially eluted, was due to non-specific reduction and subsequent labeling of other double bonds, including carboxyl groups, which may be present in the protein. These amino acids eluted more rapidly since they do not contain a cis-diol group, and will therefore not bind to the column. However, glycated amino acids containing a cis-diol group will bind the column and are eluted with 0.1 M HCl.

With the procedure, there was little dependence of protein concentration on the results obtained when two to eight mg of protein was used. However, excessive amounts of protein resulted in lower results, since there was a decrease in the ratio of label to protein. When less than two mg of protein was used, especially glomerular basement membrane from non-diabetic kidneys, the results were imprecise due to the low amount of radioactivity incorporated. This resulted in low counts per minute, sometimes fairly close to background levels. When the results for these samples were expressed per umol leucine equivalents, the error compounded due to the small amount of material. In general, two to four milligrams of basement membrane was used and gave reproducible results.

The assay, when using 10 mg of glycated muscle capillary basement membrane, had a with-in run precision CV of 16% (487 ± 79 cpm/umol leucine equivalent (mean ± standard deviation)). similarly, when two mg of in vitro glycated basement membrane and basement membrane isolated from normal rats were analyzed, the assay demonstrated a CV of 13% and 18% respectively.

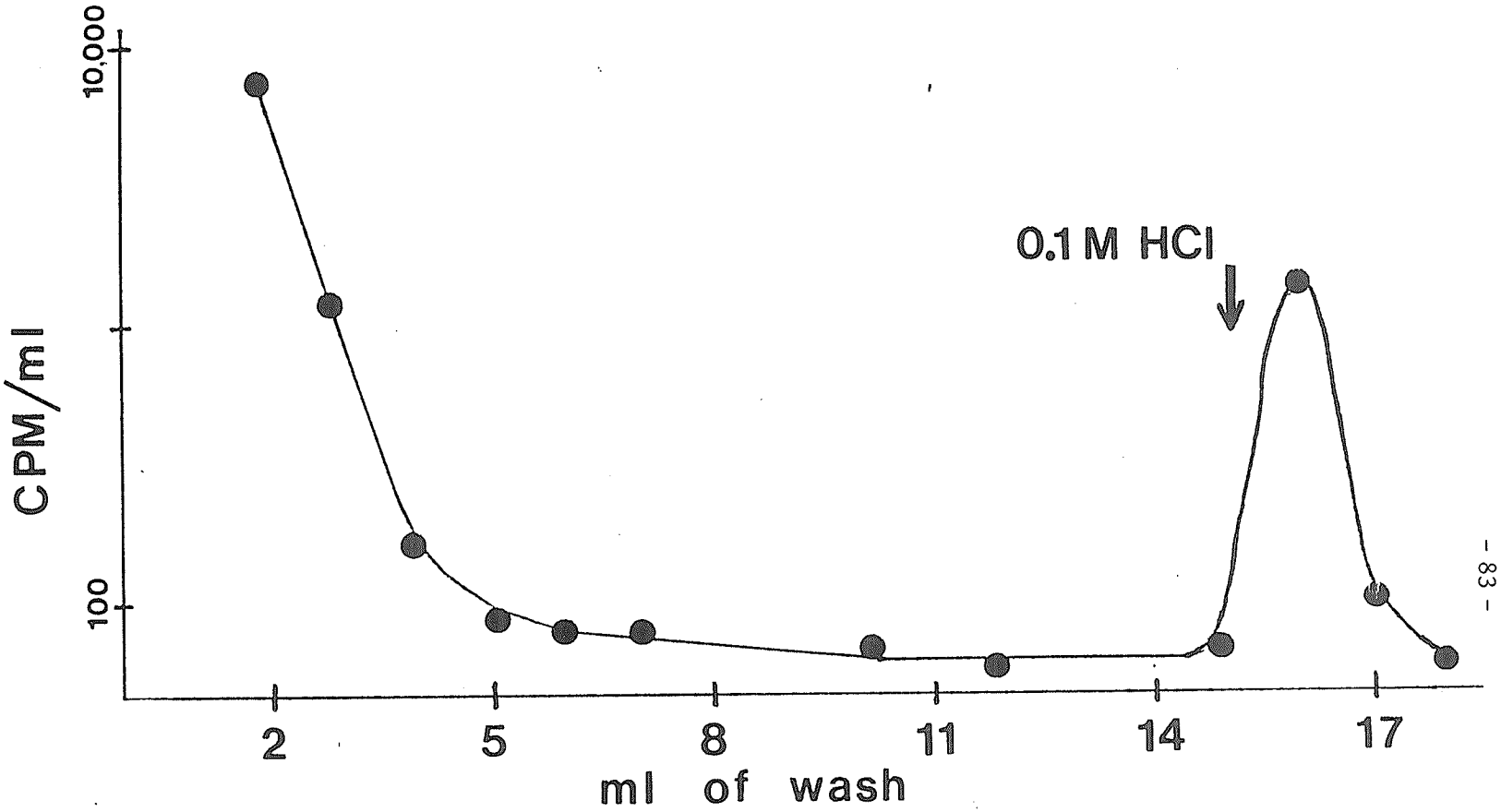


Figure 18. Elution profile of a Glyc-affin GSP affinity chromatographic column to isolate glycated amino acids in the tritiated sodium borohydride assay. Four hundred ul of previously hydrolyzed basement membrane (as described under the methods section) was applied to a Glyc-affin GSP disposable affinity column and washed with 15 ml of 100 mmol/L sodium phosphate, dibasic, pH=9.0; one ml fractions of eluate were collected. Subsequently, five ml of 0.1 M HC1 was added, and five ml fractions further collected. The radioactivity in each fraction was determined by standard liquid scintillation procedures.

The results of the precision studies are shown in Table 9. Basement membrane from normal rats exhibited some degree of background levels of non-enzymatic glycation, since the reaction is a normal occurrence; however, the extent of the reaction is elevated in hyperglycemic conditions.

In vitro glycated basement membrane was also analyzed (Figure 19). It was found that the level of non-enzymatic glycation was a function of both the time of incubation (11 as opposed to seven days) and the concentration of glucose.

The level of non-enzymatic glycation of muscle capillary basement membrane from a group of the study animals was measured by both the thiobarbituric acid assay and the tritiated sodium borohydride assay (Figure 20). A proportional relationship (or correlation) between the level of non-enzymatic glycation of muscle basement membrane, as measured by the above two procedures, was found. Similar results were also found, using in vitro glycated basement membrane (results not shown).

The tritiated sodium borohydride assay developed, exhibits a much higher sensitivity and hence, much less protein is required (two mg as opposed to 20 mg) as compared to the thiobarbituric acid assay. This fact is particularly important when working with small amounts of protein, including glomerular basement membrane which is only available in small quantities.

Table 9. Between run precision of the tritiated sodium borohydride procedure for the measurement of non-enzymatic glycation. Both two mg and 10 mg of basement membrane was used.

1. 10 mg sample size.

n = 8

mean = 487 cpm/umol leucine equiv.

SD = 79

CV = 16%

2. 2 mg sample size.

a) Low Control

n = 6

mean = 165 cpm/umol leucine equiv.

SD = 30

CV = 18%

b) High Control

n = 6

mean = 3180 cpm/umol leucine equiv.

SD = 413

CV = 13%

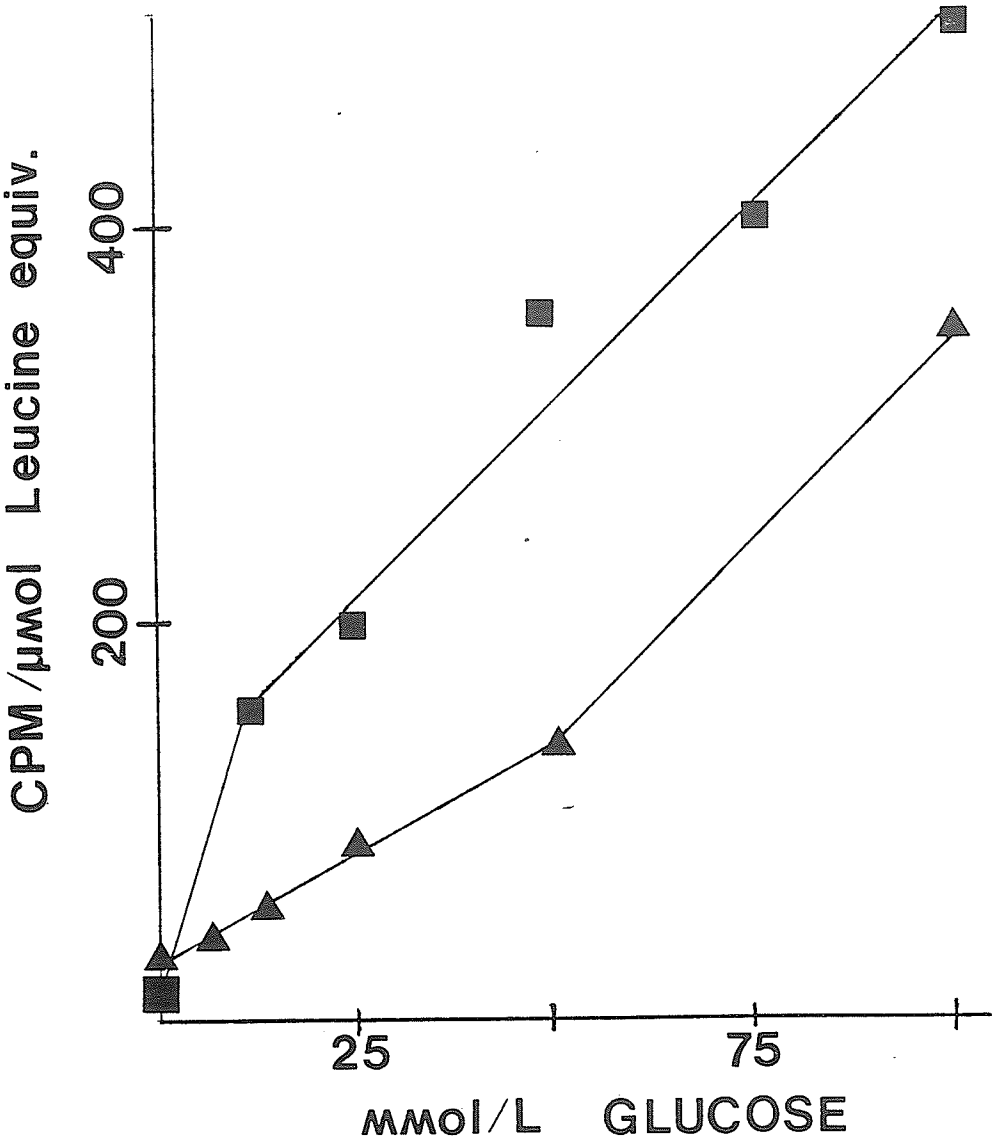


Figure 19. Non-enzymatic glycation of <u>in vitro</u> glycated muscle capillary basement membrane. Muscle capillary basement membrane was glycated <u>in vitro</u> by incubation in various concentrations (25 to 100 mmol/L) glucose in 0.2 M phosphate buffer, pH=7.4 for seven days (▲—▲) and 11 days (■—■) at 37°C. The level of non-enzymatic glycation was subsequently measured by the tritiated sodium borohydride procedure (as previously described).

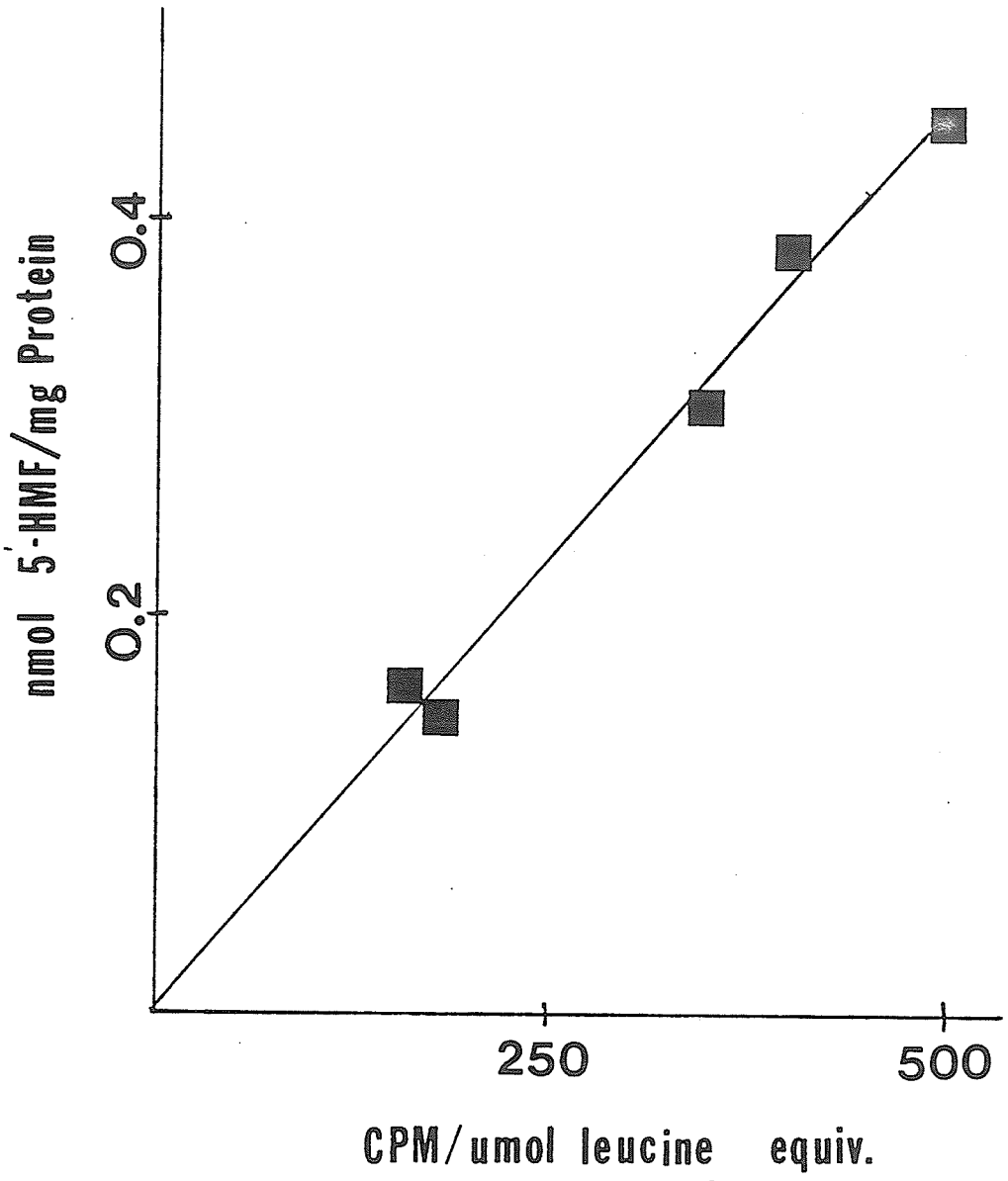


Figure 20. Correlation between the tritiated sodium borohydride assay and the thiobarbituric acid assay for the measurement of non-enzymatic glycation. Muscle capillary basement membrane was isolated from diabetic and non-diabetic rats (as previously described) and analyzed for the level of non-enzymatic glycation by both the tritiated sodium borohydride (results expressed as CPM/μmol leucine equiv.) and the thiobarbituric acid assay (results expressed as nmol 5-HMF/mg protein).

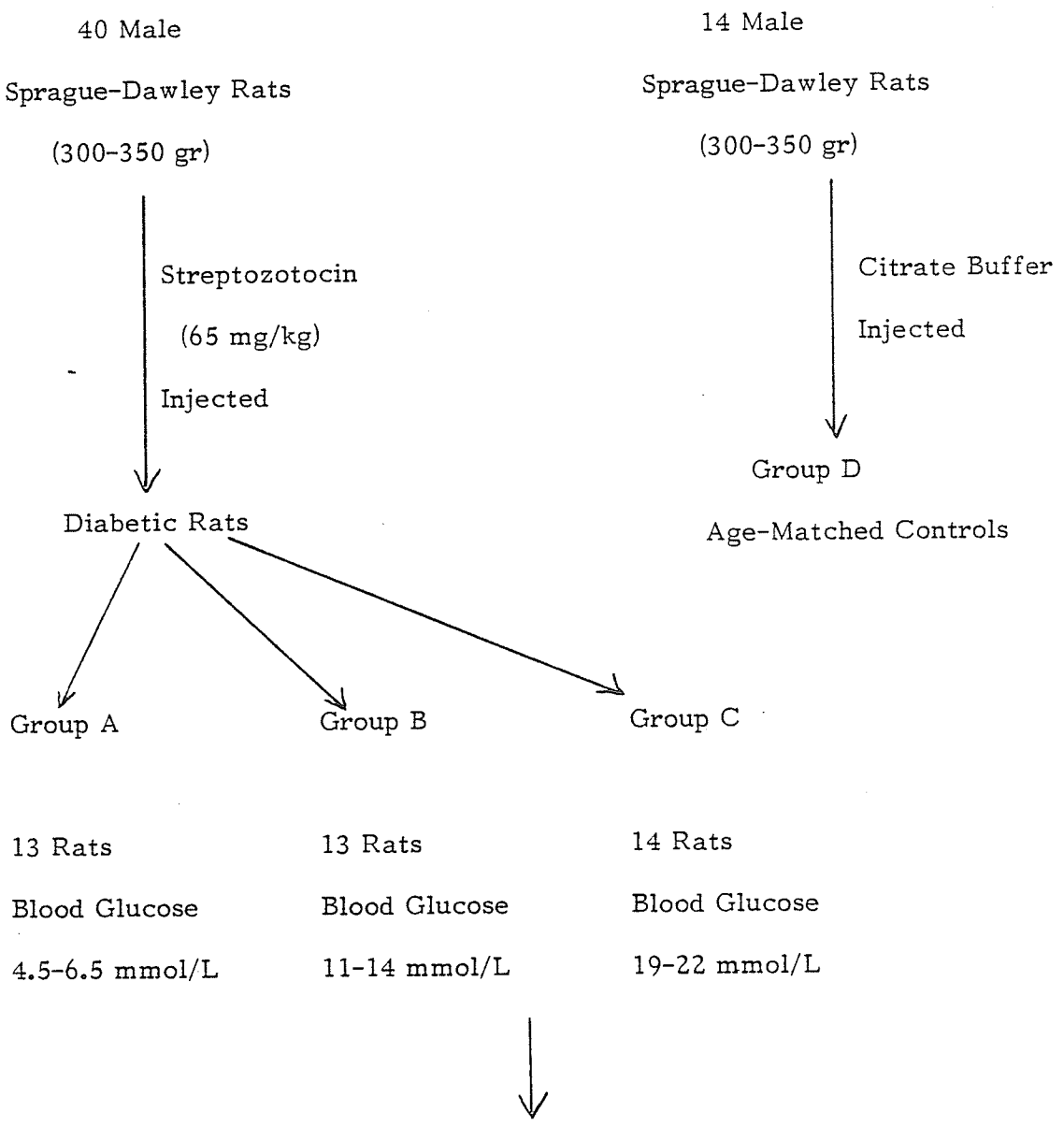
V. EXPERIMENTAL PROTOCOL

A. STUDY 1.

Adult male (300-350 g) Sprague-Dawley rats (n=40) were injected intraperitoneally with a single injection of streptozotocin (65 mg/kg) dissolved in cold citrate buffer (pH 4.5). Hyperglycemia (blood glucose greater than 20 mmol/L) and glycosuria were present three days after injection in all rats. A control group of citrate-injected rats (n=14) was age-matched to the diabetic groups. The animals were fed Wayne F6 rodent blox and kept for 6 months with food and water ad libitum. The diabetic animals were divided into 3 groups (see Figure 21). Thirteen animals were maintained at a blood glucose level of 4.5-6.5 mmol/L by daily injection of protoamine-zinc insulin (Connaught Laboratories, Willowdale, Ontario). The dosage ranged from 2-12 units/day depending on the animal, and was adjusted according to bi-weekly blood glucose monitoring. Another 13 animals were treated similarly with the exception that the blood glucose levels were maintained at 11-14 mmol/L. The final 14 animals were maintained similarly as well, with the exception that the blood glucose was maintained at 19-22 mmol/L.

At times 0, 2, 4 and 6 months of duration, 3 animals from each group were sacrificed (12 animals in total). Prior to death, 24 hour urine collections and blood samples were obtained for analysis of creatinine clearance, urinary protein, and glycated hemoglobin. Body weights of all animals were recorded weekly for the duration of the study and glycated hemoglobin was quantitated monthly on all animals.

Figure 21. Study 1 Experimental Protocol



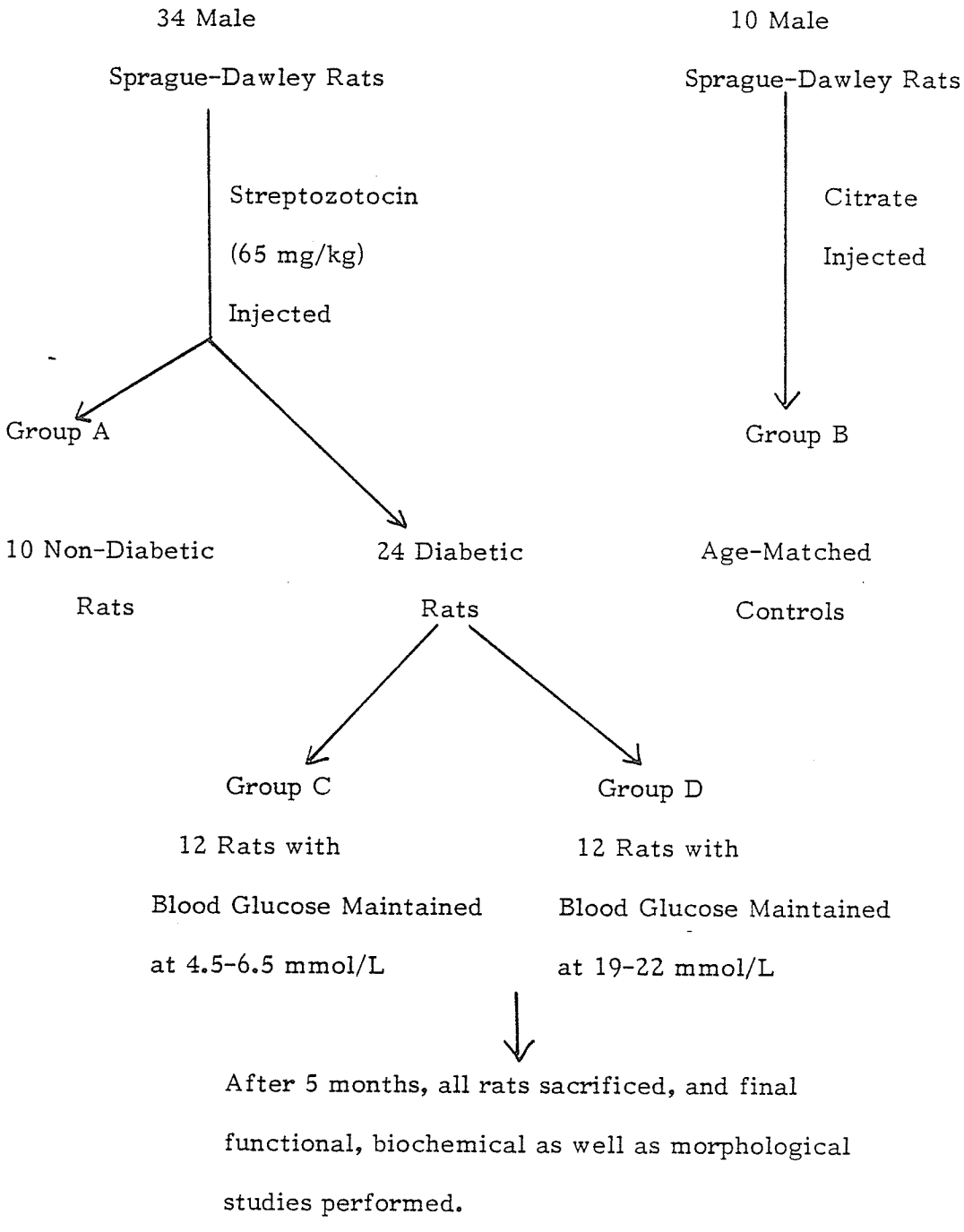
At times 0, 2, 4 and 6 months three animals from each group sacrificed, and functional biochemical and morphological studies performed.

B. STUDY 2.

Adult male (300-350 g) Sprague-Dawley rats were injected intraperitoneally with a single injection of streptozotocin (65 mg/kg) as in the above study. Hyperglycemia and glycosuria was present three days after injection in 24 animals while another 10 were maintained in a euglycemic state. A control group of citrate-injected rats (n=10) was age-matched to the diabetic group. The groups were fed Wayne F6 rodent blox and kept for five months with food and water ad libitum. Half the diabetic animals (n=12) (see Figure 22) were maintained at a blood glucose level of 4.5-6.5 mmol/L by daily injection of protoamine-zinc insulin as above. The dosage ranged from 2-14 units/day depending on the animal and was adjusted according to bi-weekly blood glucose monitoring. The remaining group of animals (n=12) was treated similarly, with the exception that the blood glucose levels were maintained at 19-22 mmol/L.

Twenty-four urine collections and blood samples were obtained for analysis of creatinine clearance, glycated hemoglobin, and urinary albumin at time 0, 2, and 5 months of the study. Body weights of all animals were recorded weekly for the duration of the study. The animals were all sacrificed after 5 months.

Figure 22. Study 2 Experimental Protocol



VI. STUDY 1

A. RESULTS OF STUDY 1:

1. METABOLIC AND FUNCTIONAL PARAMETERS.

The mean blood glucose levels for the four groups of rats sacrificed after 6 months of the study are shown in Table 10. The metabolic and functional results at six months parallelled those of the rats sacrificed at previous months (two and four months). The diabetic hyperglycemic group had a mean blood glucose level of 16.4 mmol/L which was significantly elevated ($p<0.05$) over the other three groups; namely, the age-matched control and the diabetic euglycemic and moderate hyperglycemic groups. Similarly, the average blood glucose of the diabetic animals which were maintained at a moderate level of hyperglycemia (a blood glucose of 11-14 mmol/L) was elevated ($p<0.05$) over the remaining two groups. However, the euglycemic diabetic animals had an elevated blood glucose when compared to the age-matched control group, possibly due to difficulty in maintaining euglycemia in these animals. A correlation existed between the blood glucose levels and the levels of glycated hemoglobin ($r=0.81$, see Figure 23), with the hyperglycemic diabetic animals having significantly elevated glycated hemoglobin values over those of the three remaining groups. The percent glycated hemoglobin values for the final four months of the six month study are shown in Table 10. The euglycemic diabetic animals had glycated hemoglobin values which were similar to those of the age-matched controls, with the moderate hyperglycemic diabetic animals having values which were elevated over these two groups.

Table 10. Percent Glycated Hemoglobin for Animals of Study 1.

GROUP	Ave. Blood Glucose (mmol/L)	6 Months (n=3)	5 Months (n=3)	4 Months (n=3)	3 Months (n=3)
Control	5.5[1]	6.9%	7.5%	6.8%	7.6%
	5.2-5.8[2]	5.9-8.0	6.2-8.0	6.5-7.1	7.4-7.9
Euglycemic	7.0	6.6	5.8	6.5	6.9
	6.8-7.1	4.4-8.1	4.9-7.0	5.5-7.1	5.9-8.8
Moderate	12.5	8.7	9.2	9.0	8.8
	12.0-13.1	7.0-11.0	8.6-9.8	7.6-10.9	8.5-9.4
Hyperglycemic	16.4	11.1	11.2	15.5	16.4
	10.8-20.4	9.6-13.8	8.7-13.8	12.7-18.7	13.3-18.4

[1] Values represent mean for the three rats in each group.

[2] Range of the individual values.

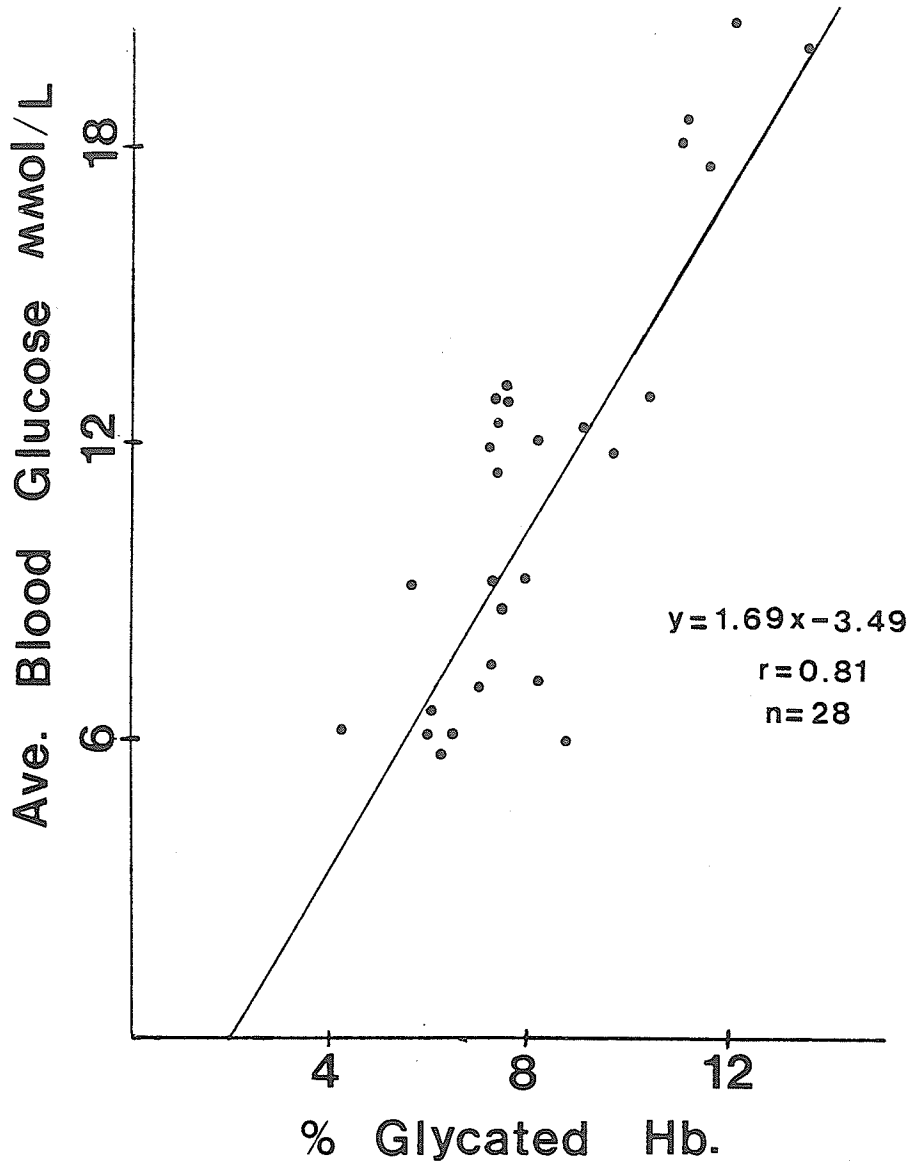


Figure 23. Correlation between the average blood glucose level and the glycated hemoglobin for the animals in Study 1.

The body weights of all animals at the beginning of the study were approximately the same. However, the mean body weight of the diabetic hyperglycemic animals was significantly decreased ($p<0.05$) when compared to the other three groups at two, four, and six months of the study as shown in Table 11. There was no significant weight differences between the moderate and euglycemic diabetic animals and the age-matched control animals at all times investigated.

Renal function was monitored via two methods; namely, urinary protein and creatinine clearance. The data for urinary protein excretion for the rats at the time of sacrifice is shown in Table 12. The values obtained were quite variable, with no significant conclusions being able to be made. The mean glomerular filtration rate (GFR) for the animals, as measured by creatinine clearance, is summarized in Table 13. As with the urinary protein results, the values were highly variable; and once again, few conclusions could be drawn (see discussion for a detailed explanation).

2. MORPHOLOGICAL STUDIES.

The morphological data representing glomerular basement membrane thickness is shown in Table 14. The age-matched control animals demonstrated an age-dependent thickening, as evidenced by the differences in thickness at times 0 and 6 months. At 6 months, the diabetic animals (both euglycemic and hyperglycemic) showed an increase in thickness ($p<0.05$) over that seen for the age-matched controls; however, there was no statistical difference

Table 11. Body weights of rats from Study 1.[1]

GROUP	0 MONTHS (n=8)	2 MONTHS (n=9)	4 MONTHS (n=6)	6 MONTHS (n=3)
Control	293 ± 27 g[2]	523 ± 28 g	608 ± 32 g	644 ± 41 g
Euglycemic		566 ± 48	663 ± 55	716 ± 24
Moderate		528 ± 45	592 ± 30	660 ± 54
Hyperglycemic		488 ± 37	537 ± 26	546 ± 27

[1]Values represent mean plus or minus the standard deviation of the body weight of each rat at death.

[2]Body weights of animals prior to division into individual groups.

Table 12. Urinary protein excretion rates for each individual rat of Study 1 at time of sacrifice.

(Results expressed as mg protein/24 hrs.)

GROUP	0 MONTHS	2 MONTHS	4 MONTHS	6 MONTHS
Control		8,3,4	2,13	2,54,11
Euglycemic		3	9,63,10	16,64,11
	35 ± 16[1]			
	n = 16			
Moderate		6,2	17,49,2	17,20,22
Hyperglycemic		12	36,30,17	5,10

[1]Value represents mean plus or minus the standard deviation prior to division of the animals into the various groups.

Table 13. Creatinine clearance for each individual rat of Study 1 at time of sacrifice.

(Results expressed as ml/min.[1])

GROUP	0 MONTHS	2 MONTHS	4 MONTHS	6 MONTHS
Control		1.3; 0.6; 0.4	0.1; 0.8; 0.2	0.4; 2.2; 2.1
Euglycemic		0.4	0.3; 0.8; 1.0	1.9; 0.9; 0.9
	1.6 ± 0.6[2] (n = 10)			
Moderate		0.5; 0.8	1.0; 1.5; 0.3	2.1; 1.0; 1.3
Hyperglycemic		2.4; 0.9; 1.0	2.0; 1.7; 1.0	0.5; 0.9

[1]Results not corrected for body weight.

[2]Value represents mean plus or minus the standard deviation prior to the division of the animals into the various groups.

Table 14. Glomerular Basement Membrane thickness for Study 1 animals.

GROUP	n[1]	MEAN THICKNESS (microns)	STANDARD ERROR
Control T=0 months	3	0.075	.002
Control T=6 months	3	0.205	.006
Hyperglycemic T=6 months	3	0.231	.008
Euglycemic T=6 months	3	0.247	.006

[1] n=3 as follows: The thickness of the glomerular basement membrane was determined on 25 random micrographs for each of the three rats per group, with all values being pooled for that particular group.

between the two diabetic groups previously mentioned. The glomerular basement membrane thickness of the diabetic rats maintained at a moderate level of hyperglycemia was not determined, since at first we only wanted to determine whether changes had occurred between the hyperglycemic diabetics and control animals. The glomerular basement membrane thickness was not measured from any rats at the intermediate times of the study, nor was muscle capillary basement membrane thickness measured on any of the study animals.

3. BIOCHEMICAL STUDIES

The level of non-enzymatic glycation of basement membrane was quantitated via the thiobarbituric acid and the tritiated sodium borohydride assays. The thiobarbituric acid assay was used to measure the extent of non-enzymatic glycation of muscle capillary basement membrane isolated from the animals sacrificed at six months, and the results shown in Table 15. It was found that the hyperglycemic diabetic animals had an average level of non-enzymatic glycation of 18.5 nmol HMF/10 mg protein, which was significantly elevated ($p<0.05$) over the levels for the remaining groups. The euglycemic diabetic animals had values similar to those of the age-matched control group, 9.4 and 8.3 nmol HMF/10 mg protein respectively. Due to the large sample requirement, the assay was not suitable for use on glomerular basement membrane which was available in only small quantities.

The tritiated sodium borohydride assay was also used to measure the extent of non-enzymatic glycation of muscle capillary basement membrane as shown in Table 16. As with the thiobarbituric acid assay, the values for

Table 15. Thiobarbituric Acid assay to measure the extent of non-enzymatic glycation.

The level of non-enzymatic glycation was determined on muscle capillary basement membrane isolated from animals sacrificed at six months.

GROUP	Level of non-enzymatic glycation (nmol/10 mg protein)	AVERAGE
Hyperglycemic (n=3)	18.6	18.5
	15.2	
	21.6	
Moderate (n=3)	15.2	12.0
	10.8	
	10.0	
Euglycemic (n=3)	9.6	9.4
	7.8	
	10.8	
Control (n=3)	7.8	8.3
	9.6	
	7.6	

Table 16. The extent of non-enzymatic glycation of muscle capillary basement membrane isolated from the animals sacrificed in Study 1.

Non-enzymatic glycation was determined by the tritiated sodium borohydride procedure and the results expressed as cpm/umol leucine equivalents.

GROUP	6 MONTHS	4 MONTHS	2 MONTHS
Control	364[1]	395	328
	274-484[2]	189-541	243-413
	n=5	n=4	n=6
Euglycemic	309	335	370
		279-430	320-445
	n=1	n=5	n=3
Moderate	575	646	400
	366-782	416-768	336-506
	n=2	n=4	n=3
Hyperglycemic	1067	759	587
	782-1319	640-897	502-669
	n=5	n=6	n=3

[1]Result expressed as a mean for each group.

[2]Range of values for each group.

the diabetic hyperglycemic groups were elevated over those of the age-matched control and diabetic euglycemic animals at six months. The diabetic animals which were maintained at a moderate degree of hyperglycemia had values somewhere in between those of the hyperglycemic animals and those of the euglycemic diabetic and age-matched control animals. At two months of the study the levels of non-enzymatic glycation for all groups was similar. However, there appeared to be an increase over time in the levels of non-enzymatic glycation in the diabetic hyperglycemic group, with the levels doubling between the two and six months, 587 ± 68 and 1069 ± 214 cpm/umol leucine equivalent, respectively. This was not seen in the age-matched control animals which had a constant level of non-enzymatic glycation for all measured times of the study.

The extent of non-enzymatic glycation of glomerular basement membrane was also quantitated using the tritiated sodium borohydride assay, the results expressed in Table 17. The levels of non-enzymatic glycation of glomerular basement membrane was similar at all times for basement membrane isolated from the age-matched control animals and the values were similar to those obtained for the diabetic euglycemic animals. The values for the diabetic hyperglycemic animals were elevated over the age-matched controls, although not to a great extent. As with muscle capillary basement membrane, the diabetic animals maintained at a moderate level of hyperglycemia had levels of non-enzymatic glycation of glomerular basement membrane somewhere between the values of the other groups.

Table 17. The extent of non-enzymatic glycation of glomerular basement membrane isolated from the animals sacrificed in Study 1.

Non-enzymatic glycation was determined by the tritiated sodium borohydride procedure and the results expressed as cpm/umol leucine equivalents.

GROUP	6 MONTHS	4 MONTHS	2 MONTHS
Control	318[1]	316	264
	254-408[2]	296-335	211-304
	n=4	n=2	n=3
Euglycemic	286	254	331
	271-296	198-314	n=1
	n=3	n=3	
Moderate	407	316	384
	320-452	248-425	371-401
	n=3	n=3	n=3
Hyperglycemic	510	472	499
	n=1	295-627	411-611
		n=3	n=3

[1]Result expressed as a mean for each group.

[2]Range of values for each group.

C. DISCUSSION

The overall glycemic state of the euglycemic diabetic animals paralleled that of the age-matched control animals as evidenced by the similar glycated hemoglobin values for both groups. However, whole blood glucose levels were elevated in the former group, possibly due to problems in maintaining a normal glycemic state in these animals. Insulin doses were adjusted according to bi-weekly blood glucose measurements. If the blood glucose level was elevated, the dose was increased to return the animal to a euglycemic state. The animals, in some cases, went from being hyperglycemic to hypoglycemic when the dose was changed or visa versa. This led to an overall increase in the average blood glucose levels when all values for each rat were averaged; where as, the glycated hemoglobin for these animals, a long term indicator of glycemic control (148), remained comparable to the values for the age-matched control animals.

Another aspect of diabetes, especially in hyperglycemic conditions, is marked wasting of body tissues. This was evidenced by the lower body weight of the hyperglycemic animals. A deficiency in insulin levels, as evidenced by increased blood glucose levels, results in increased catabolism of protein and lipolysis, leading to body wasting and breakdown of cellular reserves of lipids and proteins (9). A decrease in glucose transport, due to the lower insulin levels, leads to increased production of glucose due to increased utilization of the cellular reserves in order to maintain normal glucose transport and utilization. In the process, the excess glucose is excreted

into the urine (glycosuria) as the maximum level of renal tubular reabsorption of glucose is exceeded. Further more, the urine volume is increased (polyuria) due to osmotic effects of the glucose.

These biochemical abnormalities lead to substantial caloric loss, and results in weight loss despite normal or even increased caloric intake. Reversal of hyperglycemia will correct the abnormalities (106,125). Since the euglycemic diabetic animals had near normal blood glucose levels, their weight corresponded to that of the age-matched control animals and did not exhibit any body wasting. Further more, the urine output for this group of rats was similar to the age-matched control rats. The diabetic animals maintained at a moderate level of hyperglycemia, maintained a normal weight profile, suggesting that the degree of hyperglycemia in this group was not severe enough to cause these metabolic abnormalities, at least up to six months of diabetes.

The methods to monitor renal function, namely urinary protein and creatinine clearance, produced results which could not be readily interpreted due to the following factors: 1) small numbers of animals per group, 2) small urine volumes, and 3) problems in the methodology used to measure urinary protein. Another problem with the functional tests was the collection of the urine. In many cases, especially with normal and euglycemic diabetic animals, the urine volume was small (in some cases less than 1 ml). The possible effects due to evaporation or the addition of water from the animal's water bottle was not investigated. This may have added uncertainty and imprecision to the results.

A further problem with the measurement of urinary protein was the method employed. The sulphosalicylic acid method is not that sensitive, with a lower limit of detection of 50 mg/L (149), with the best precision being obtained between 250 and 1400 mg/L. The values obtained for the rat urines were all near the detection limit (50 to 100 mg/L in most cases), producing results with a high degree of imprecision. The measurement of urinary protein has been shown not to be a good indicator of renal function, since it does not detect subtle changes in renal function. Urinary protein levels are not increased until diabetic nephropathy has progressed to a fairly late stage (98,105), and therefore the use of this method is limited in the detection of early renal changes. Overall, no conclusions as to renal functional status could be drawn from these studies.

The measurement of non-enzymatic glycation was performed by two procedures, the thiobarbituric acid assay and the tritiated sodium borohydride assay. Both methods did detect increases in the level of non-enzymatic glycation of muscle capillary basement membrane isolated from the hyperglycemic animals when compared to the diabetic euglycemic and age-matched control animals; however, both procedures did have certain drawbacks. The major drawback of the thiobarbituric acid assay was the large sample requirement, which was addressed earlier. For this reason, the assay could not be used to quantitate the level of non-enzymatic glycation of glomerular basement membrane, which was only obtainable in small quantities (a few milligrams). The tritiated sodium borohydride assay, in contrast, had a much higher degree of sensitivity, and as a result less basement membrane was required. For this reason, this method was chosen to measure the level of non-enzymatic glycation of glomerular basement membrane isolated from the rats included

in this study. The assay, was however, more time consuming, and did require the use of a radioactive label (which required special precautions in the handling and disposal of it).

The levels of non-enzymatic glycation of muscle capillary basement membrane isolated from the diabetic hyperglycemic animals was elevated over the levels found for muscle basement membrane isolated from the euglycemic and moderate hyperglycemic diabetic and age-matched control animals, as measured by both assays. The levels of non-enzymatic glycation of glomerular basement membrane was measured by the tritiated sodium borohydride assay. As with muscle capillary basement membrane, there was no difference in the levels of non-enzymatic glycation of glomerular basement membrane isolated from the diabetic euglycemic and age-matched control animals. The levels of non-enzymatic glycation of the glomerular basement membrane did correlate somewhat to the average blood glucose levels, suggesting that increased non-enzymatic glycation is a result of hyperglycemia, which has been suggested by others (84).

There was no difference in glomerular basement membrane thickening between the euglycemic and hyperglycemic diabetic animals after six months of diabetes, despite the fact that these groups exhibited differences in the extent of non-enzymatic glycation of both muscle capillary and glomerular basement membrane. However, both of these groups of diabetic animals showed an increase in glomerular basement membrane thickness over that of the age-matched control animals. This suggests that non-enzymatic glycation may not be an etiologic factor in basement membrane thickening.

Since the results from the functional studies were inconclusive, the relationship of basement membrane thickness, non-enzymatic glycation, and functional changes could not be appropriately assessed with this study.

VII. STUDY 2.

A second study, in which many of the deficiencies of the original study were corrected, was undertaken to further elucidate the relationship between non-enzymatic glycation, basement membrane thickness, and renal functional parameters. To correct the problem of the small number of animals per group, the following changes were made. The moderate hyperglycemic diabetic group (blood glucose in the range of 11-14 mmol/L) was deleted since this group did not differ substantially from the diabetic euglycemic group. Secondly, the sacrifice of rats at two and four months was also deleted to increase the number of animals available for sacrifice at 5 months. The rats were to be initially sacrificed at 6 months, but due to an increased incidence of mortality than anticipated and in order to maintain as many animals per group as possible, they were sacrified at five months. These modifications allowed us to increase the number of animals per group without causing a drastic increase in the total number of animals in the study.

In the second study, to insure that the streptozotocin used to induce experimental diabetes did not have any effects on the results, a group of animals which were given the drug, but failed to produce experimental diabetes was added to the study protocol. If the drug has no effect on renal function, the results obtained with this group should parallel the age-matched control group with respect to functional, biochemical, and morphological findings.

The urinary protein results were inconclusive and a more specific and sensitive method was required to measure subtle changes in renal function which may occur. Also, more care was taken with the collection of urine.

Smaller collection bottles were used to improve the collection of small volumes of urine. As well, the containers were held tight up against the collection funnel, to help prevent evaporation.

In the second study renal protein excretion was monitored using a more sensitive and specific assay. A radioimmunoassay was used to quantitate urinary albumin. Since the method is much more sensitive than the measurement of urinary protein by sulphosalicylic acid precipitation, subtle changes in early renal function impairment can be detected. Urinary albumin has been shown to be an important predictor of clinical nephropathy (111). Changes in urinary albumin excretion are detected during the early stages of diabetic nephropathy before the presence of clinical nephropathy, and are therefore a good indicator of early renal functional changes.

The measurement of non-enzymatic glycation of both isolated glomerular and muscle capillary basement membrane was measured by the tritiated sodium borohydride assay. Non-enzymatic glycation was not measured by the thiobarbituric acid assay since it lacked the sensitivity required for analysis of glomerular basement membrane.

A. RESULTS

1. METABOLIC AND FUNCTIONAL PARAMETERS.

The mean monthly blood glucose levels of the four groups of animals for the duration of the study are shown in Figure 24. The diabetic

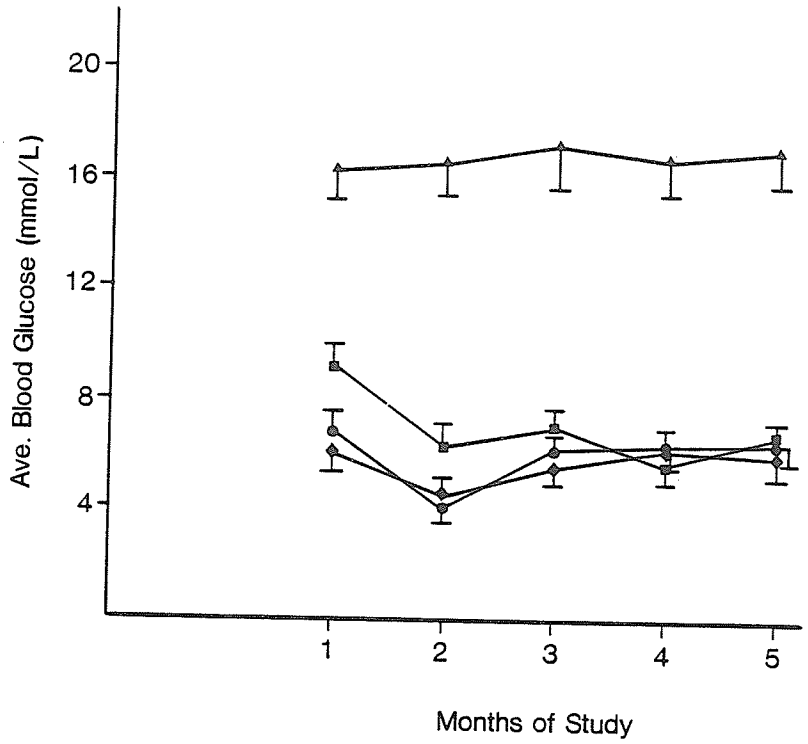


Figure 24. Monthly blood glucose profiles for the four groups of animals in Study 2. Values are represented as mean ± SEM for each group. The symbols represent: ▲, hyperglycemic diabetic rats, n=8; ●, euglycemic diabetic rats, n=5; ■, age-matched control rats, n=7; and ◆, streptozotocin injected non-diabetic rats, n=6. The blood glucose levels for the hyperglycemic diabetic rats was significantly elevated ($p<0.05$) over the other three groups.

hyperglycemic group had a mean blood glucose level of 16.4 ± 2.6 mmol/L which was significantly elevated ($p<0.05$) over the other three groups; namely the age-matched control, streptozotocin injected non-diabetic, and diabetic euglycemic groups of animals. There was no signficant difference between the mean monthly blood glucose levels of these latter three groups. Similarly, the diabetic hyperglycemic animals had glycated hemoglobin values which were significantly elevated ($p<0.05$) over the other three groups of animals (see Figure 25). The monthly weight profiles for all groups of animals is shown in Figure 26. At the beginning of the study, all animals had similar body weights; however, it was found that the hyperglycemic maintained diabetic animals had significantly decreased ($p<0.05$) body weights compared to the other three groups at 2 and 5 months of the study. These three groups, all demonstrated similar weight profiles through out the study.

The mean body weight of the diabetic hyperglycemic animals was 523 ± 49 g, which was significantly decreased ($p<0.05$) when compared to the other three groups. The kidney weights of the study animals are summarized in Table 18. The kidney weights of the hyperglycemic animals was significantly increased ($p<0.05$) over that seen in the diabetic euglycemic, age-matched control, and streptozotocin non-diabetic group of animals.

The mean glomerular filtration rate (GFR), measured by creatinine clearance, for the 4 groups of animals is summarized in Figure 27. The diabetic hyperglycemic animals had an elevated creatinine clearance ($p<0.05$) at both 2 and 5 months of the study over the three other groups. At 2 and 5 months,

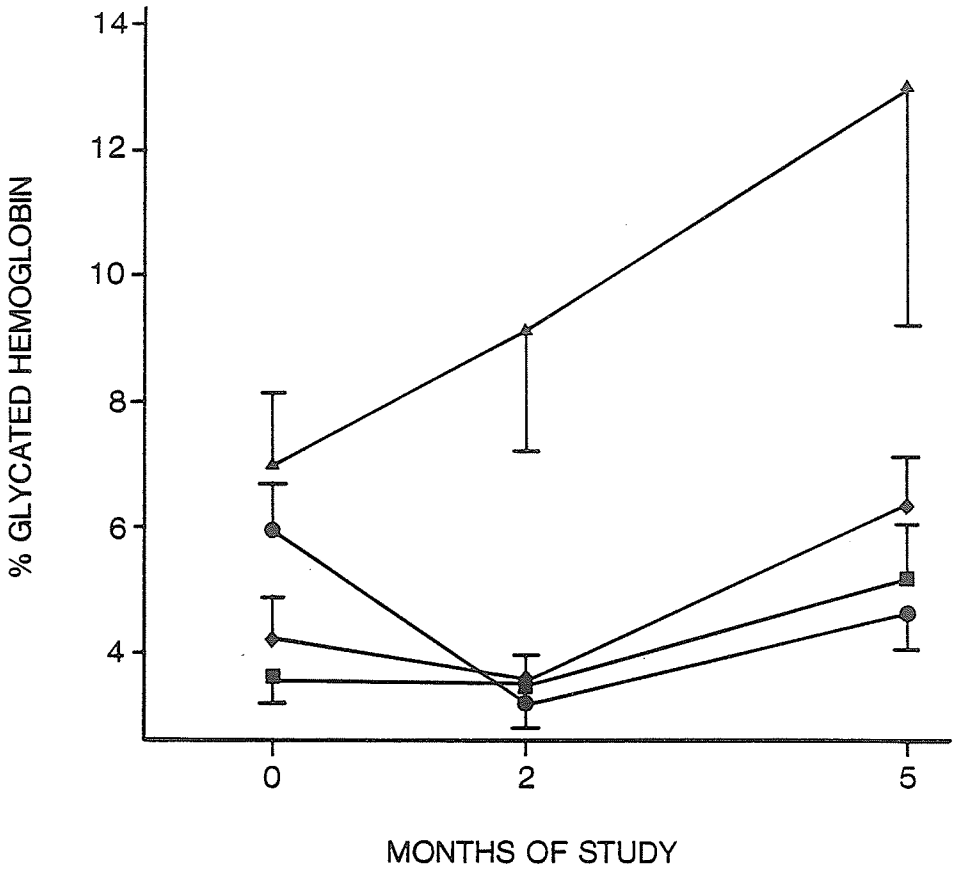


Figure 25. Percent glycated hemoglobin profiles for the four groups of animals in Study 2. Values represent mean ± SD. The symbols represent: ▲, hyperglycemic diabetic rats, n=8; ●, euglycemic diabetic rats, n=5; ■, age-matched control rats, n=7; and ◆, streptozotocin injected non-diabetic rats, n=6. The percent glycated hemoglobin for the hyperglycemic diabetic rats was significantly elevated ($p<0.05$) over the three other groups at both 2 and 5 months of the study.

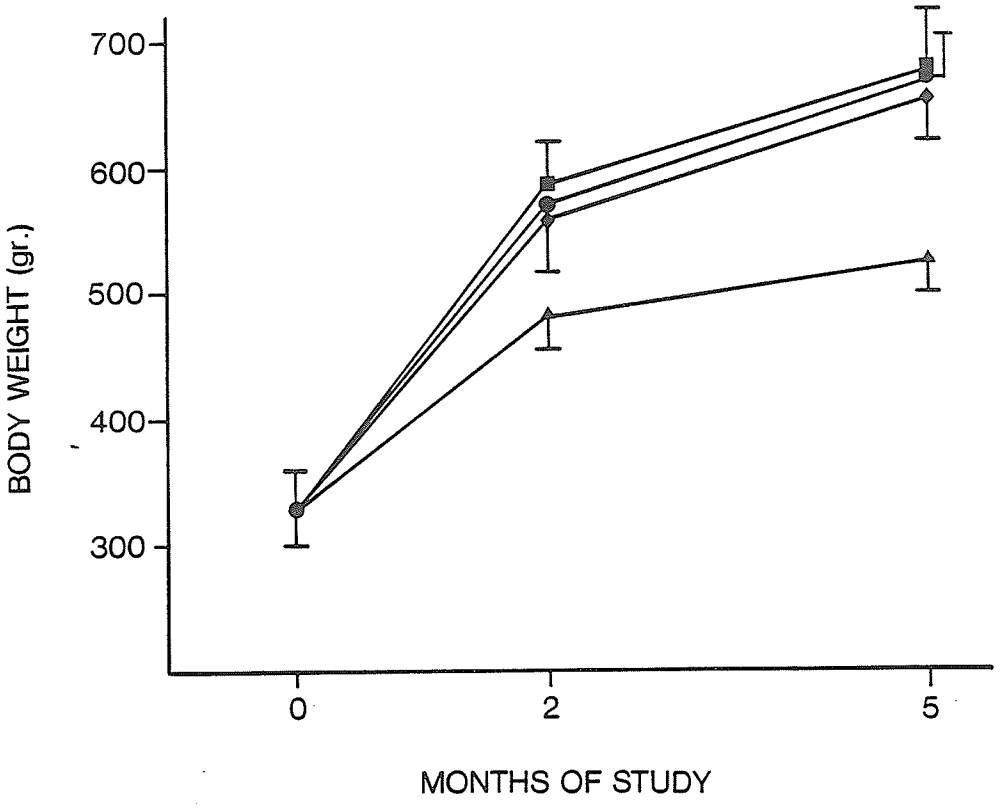


Figure 26. Body weight profiles for the four groups of animals in Study 2. The values represent mean ± SD. The symbols represent: ▲, hyperglycemic diabetic rats, n=8; ●, euglycemic diabetic rats, n=5; ■, age-matched control rats, n=7; and ◆, streptozotocin injected non-diabetic rats, n=6. The hyperglycemic diabetic rats had weights significantly decreased ($p<0.05$) over the three other groups at both 2 and 5 months of the study.

Table 18. Weights of the isolated kidneys from the animals of study 2 at the time of death.

GROUP	KIDNEY WEIGHT[1] (g/100 g body weight)
Control	0.29 ± 0.02
Drug Control	0.28 ± 0.02
Diabetic Euglycemic	0.30 ± 0.03
Diabetic Hyperglycemic	0.42 ± 0.07[2]

[1]Values expressed as mean ± SD.

[2]Significantly different at $p<0.05$ from the other groups.

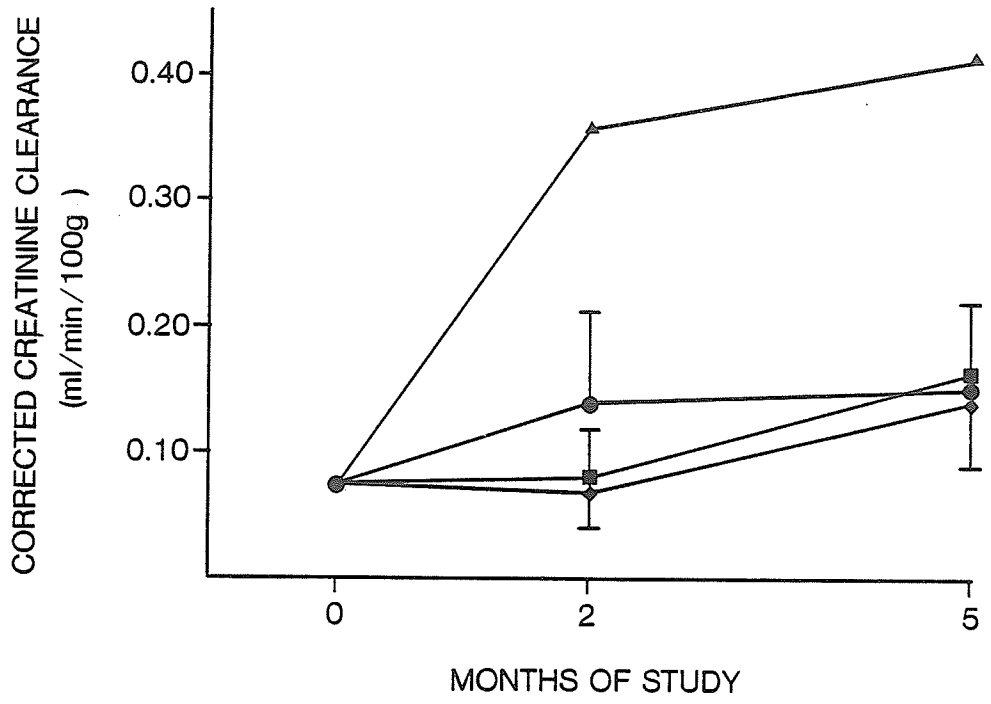


Figure 27. Corrected creatinine clearance profiles for the four groups of animals of Study 2. The values represent mean ± SD except for the diabetic hyperglycemic group in which only the means are listed due to the large SD's encountered. The symbols represent: ▲, hyperglycemic diabetic rats, n=8; ●,euglycemic diabetic rats, n=5; ■, age-matched control rats, n=7; and ♦, streptozotocin injected non-diabetic rats, n=6. The creatinine clearance for the hyperglycemic rats was significantly ($p<0.05$) elevated at both 2 and 5 months of the study.

the diabetic euglycemic, age-matched controls, and streptozotocin non-diabetic groups had similar values.

Urinary albumin excretion profiles for the various groups are shown in Figure 28. The excretion appears somewhat age-dependent. The hyperglycemic diabetic animals had values significantly elevated ($p<0.05$) over the other three groups of animals. Within each group, there was quite a variable range of values, in albumin excretion by the rats. A correlation between the functional results and the blood glucose levels was also found in our study; blood glucose levels and GFR correlated directly ($r=0.68$, $p<0.01$) as did urinary albumin levels ($r=0.63$, $p<0.01$) with blood glucose levels. Similarly, the % glycated hemoglobin correlated with the GFR ($r=0.71$, $p<0.05$).

The functional and metabolic data at the completion of the study is summarized in Table 19. The diabetic hyperglycemic group differed with respect both to functional and metabolical parameters when compared to the three other groups. In contrast, the diabetic euglycemic group paralleled the control and drug treated non-diabetic animals with respect to these parameters.

2. MORPHOLOGY

The morphological data representing glomerular basement membrane thickness, is shown in Table 20. All animals showed an age-dependent thickening over the course of the study. After 5 months, the diabetic animals (both euglycemic and hyperglycemic) showed an increase ($p<0.05$) in thickness

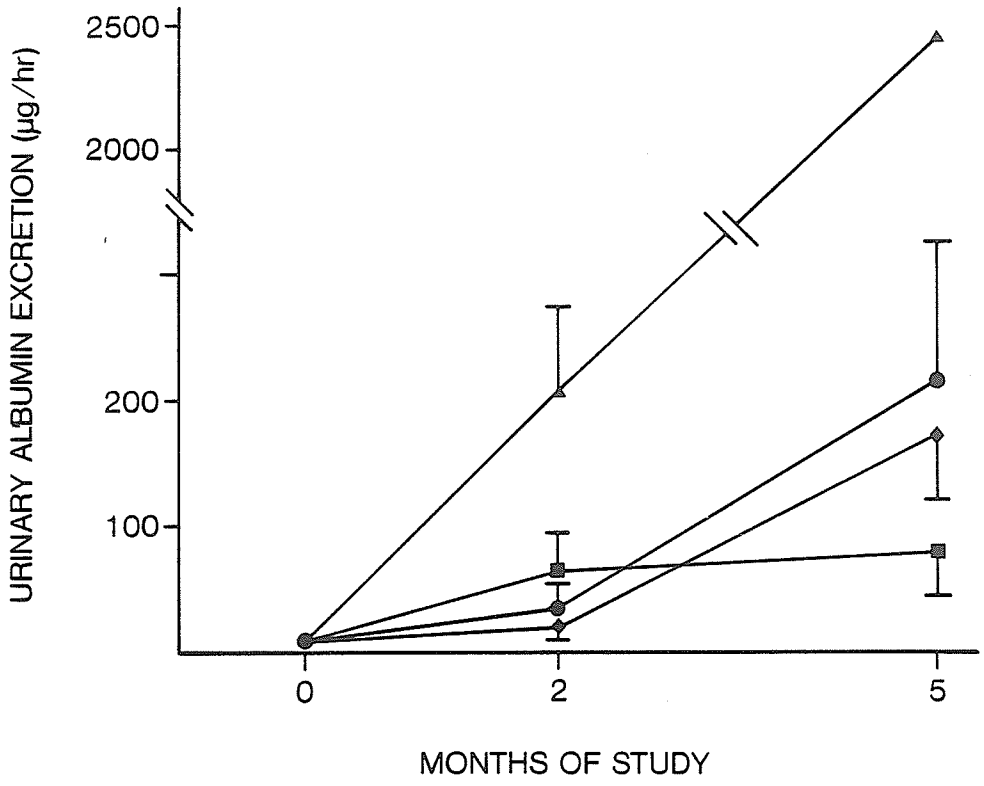


Figure 28. Urinary albumin excretion profiles for the animals of Study 2. The values represent mean ± SD, except for the diabetic hyperglycemic group, in which only the means are listed due to the large SD's encountered. The symbols represent: ▲, hyperglycemic diabetic rats, n=8; ●, diabetic euglycemic rats, n=5; ■, age-matched control rats, n=7; and ◆ streptozotocin injected non-diabetic rats, n=6. The urinary albumin excretion for the hyperglycemic diabetic rats was significantly elevated ($p<0.05$) at both 2 and 5 months of the study.

Table 19. Metabolic and functional results for diabetic and control animals at 5 months of study 2.[1]

Group	Weight (g)	Average Blood Glucose (mmol/L)	Corrected[2] Creatinine Clearance (ml/min/100g)	% Glycated Hemoglobin
- Control n=7	676 ± 38	6.4 ± 0.4	0.165 ± 0.056	5.21 ± 1.13
Drug Control n=6	657 ± 66	5.9 ± 0.6	0.154 ± 0.065	6.42 ± 0.79
Diabetic Euglycemic n=5	668 ± 58	7.0 ± 0.5	0.152 ± 0.074	4.65 ± 0.61
Diabetic Hyperglycemic n=8	523 ± 49[3]	16.4 ± 2.6[3]	0.409 ± 0.230[3]	13.08 ± 3.96[3]

[1]Results are expressed as mean ± SD.

[2]Values corrected for body weight.

[3]Significantly different at $p<0.05$ from the other groups.

Table 20. Morphometric analysis of glomerular basement membrane thickness in control and diabetic rats at 0 and 5 months of study 2.

GROUP	BASEMENT MEMBRANE THICKNESS (microns)[1]
Rats at time 0 (n=6)	0.085 ± .002
Control at 5 months (n=6)	0.197 ± .004
Drug Control at 5 months (n=6)	0.191 ± .003
Diabetic Euglycemic at 5 months (n=6)	0.217 ± .004[2]
Diabetic Hyperglycemic at 5 months (n=6)	0.220 ± .004[2]

[1]Values represent mean plus or minus the standard error of the mean.

[2]Significantly different at $p<0.05$ from other groups but not from each other.

over that seen in the two control groups of animals; however, there was no significant difference in glomerular basement membrane thickening between the hyperglycemic or the euglycemic animals. The thickness of muscle capillary basement membrane was not investigated.

3. BIOCHEMISTRY

The levels of non-enzymatic glycation of muscle capillary basement membrane measured by the tritiated sodium borohydride assay at the duration of the study are shown in Table 21. The diabetic euglycemic animals showed levels of non-enzymatic glycation which paralleled those of the age-matched control animals. In contrast, the level of non-enzymatic glycation of muscle capillary basement membrane from the diabetic hyperglycemic animals were significantly elevated ($p<0.05$) over those of the age-matched control and diabetic euglycemic animals.

The level of non-enzymatic glycation of glomerular basement membrane isolated from the study animals at 5 months is shown in Figure 29 and Table 22. The hyperglycemic diabetic animals had significantly elevated levels ($p<0.05$) in comparison to the other three groups. There was no statistical difference in non-enzymatic glycation of both the control groups and the diabetic euglycemic group. The level of non-enzymatic glycation of glomerular basement membrane and average blood glucose values correlated directly ($r=0.86$, $p<0.01$) as shown in Figure 30.

Table 21. Non-enzymatic glycation of muscle capillary basement membrane isolated from the rats of Study 2.

The level of non-enzymatic glycation was determined on muscle capillary basement membrane isolated from the rats of Study 2 and quantitated using the tritiated sodium borohydride assay.

GROUP	Levels of non-enzymatic glycation[1] (CPM/μmol leucine equiv.)
Control (n=4)	855 ± 57
Drug Control (n=3)	952 ± 127
Diabetic Euglycemic (n=5)	906 ± 103
Diabetic Hyperglycemic (n=8)	1792 ± 151[2]

[1]Results represent mean plus or minus the standard deviation.

[2]Significantly different at $p<0.05$ from other groups.

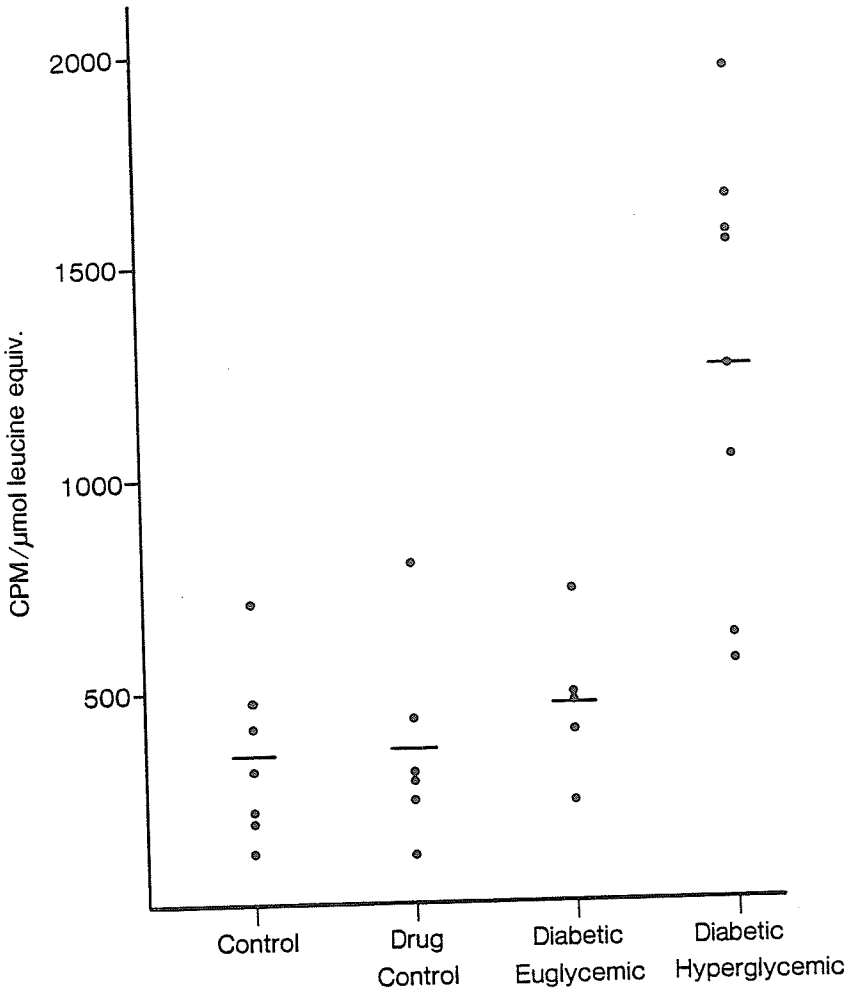


Figure 29. The levels of non-enzymatic glycation of glomerular basement membrane for the rats of Study 2. The mean of each group is represented by the horizontal bar.

Table 22. Non-enzymatic glycation of glomerular capillary basement membrane isolated from the rats of Study 2.

The level of non-enzymatic glycation was determined on glomerular basement membrane isolated from the rats of Study 2 and quantitated using the tritiated sodium borohydride assay.

GROUP	LEVEL OF NON-ENZYMATIC GLYCATION[1] OF GLOMERULAR BASEMENT MEMBRANE (CPM/umol leucine equiv.)
CONTROL n=7	351 ± 203
DRUG CONTROL n=6	366 ± 237
DIABETIC EUGLYCEMIC n=5	468 ± 182
DIABETIC HYPERGLYCEMIC n=8	1260 ± 521[2]

[1]Values expressed as mean ± SD.

[2]Significantly different at $p<0.05$ from the other groups.

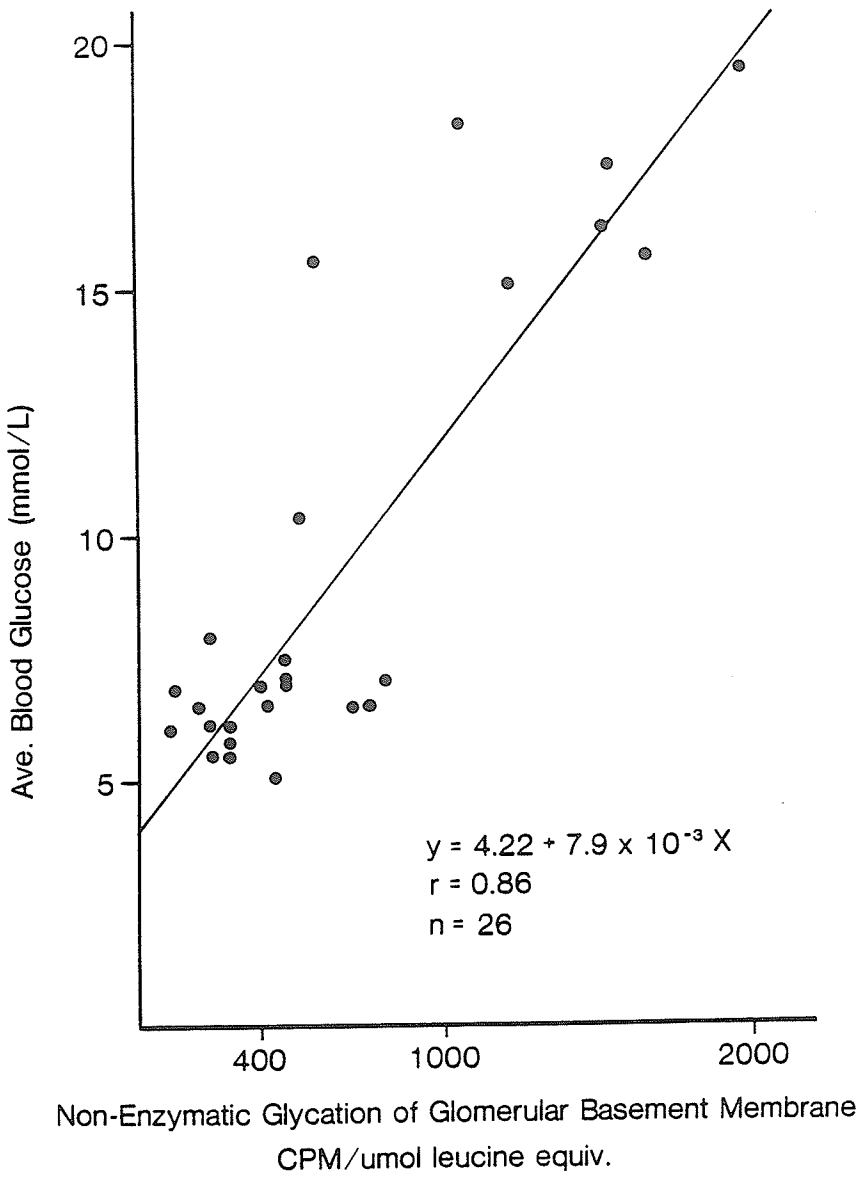


Figure 30. The relationship between average blood glucose and the level of non-enzymatic glycation of glomerular basement membrane.

The levels of non-enzymatic glycation of isolated glomerular basement membrane and muscle capillary basement membrane from the study animals were compared (Figure 31). It was found that the levels of non-enzymatic glycation of both tissues for each animal correlated significantly ($r=0.66$, $p<0.01$).

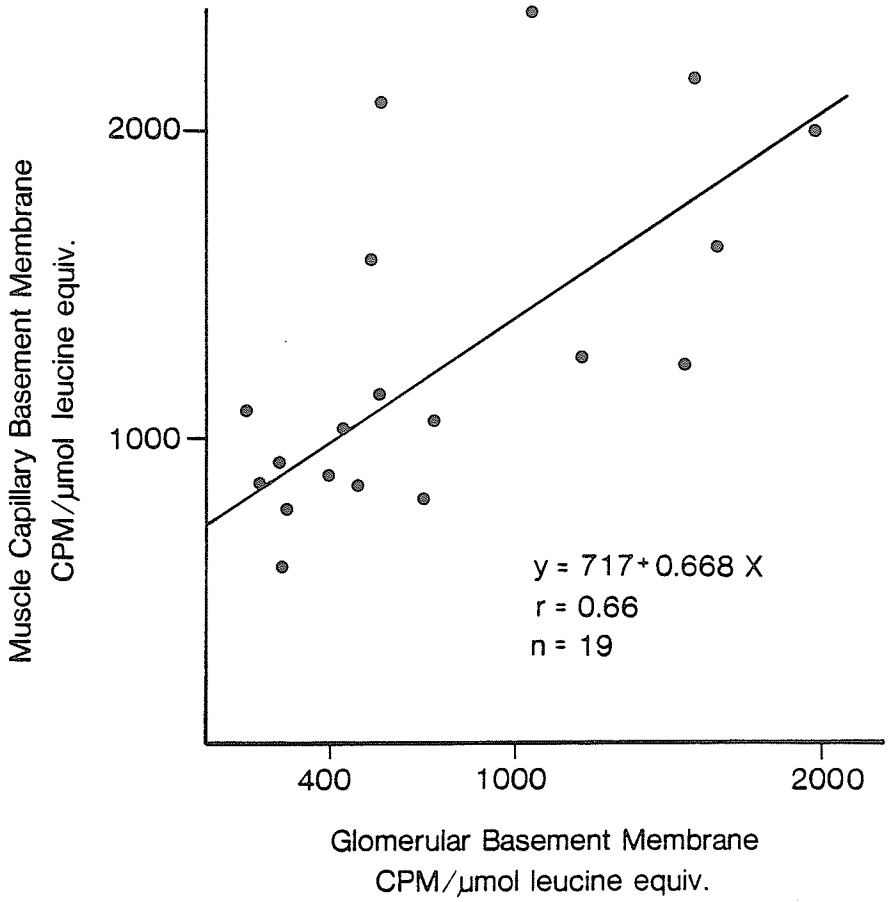


Figure 31. The relationship between the extent of non-enzymatic glycation of isolated muscle capillary and glomerular basement membrane from the animals of Study 2.

B. DISCUSSION

The etiology of diabetic nephropathy is unknown, but hyperglycemia experienced in poorly controlled diabetics may be involved. The diabetic hyperglycemic animals of this present study exhibited elevated blood glucose levels and percent glycated hemoglobin values over the other three groups; namely the diabetic euglycemic, streptozotocin injected non-diabetic, and age-matched control animals. In contrast, the overall glycemic state of the diabetic euglycemic animals paralleled that of the age-matched control and streptozotocin non-diabetic animals as evidenced by the similar blood glucose and percent glycated hemoglobin profiles. The above results reflected those of the first study.

In the hyperglycemic diabetic state, marked body wasting can occur due to biochemical abnormalities involved in insulin and blood glucose balance. This marked body wasting was evidenced in the study by the significant ($p<0.05$) decrease in body weights of the hyperglycemic diabetic animals over the euglycemic diabetic and control animals. The hyperglycemia ultimately leads to increased catabolism of protein and lipolysis leading to the breakdown of cellular reserves (as discussed in study 1). As stated earlier, the abolishment of hyperglycemia will correct these abnormalities (106,125).

Similarly, another aspect of hyperglycemia in diabetes is a marked increase in renal weight. Induction of experimental diabetes in the rat results in an almost immediate increase in the kidney weight if hyperglycemia is maintained (150). In humans with type I diabetes, similar findings have been shown (151). It has been found that there is enhanced RNA and DNA synthesis which is responsible for increased protein production and content in the kidney (150). The reason for this increased protein synthesis is not known; however, blood glucose levels may be involved. In rats, after six weeks of streptozotocin induced diabetes, the average renal cell is 25% larger and the cell numbers have increased by nearly 50% (150). The increase in weight is not due to the accumulation of water, but is due to increased protein content. There is a close correlation between blood glucose levels and the rate of kidney growth (152). Since the diabetic euglycemic animals had blood glucose levels similar to those of the control (non-diabetic) animals, it would be expected that the kidney weights be similar for both groups, which was in fact found. In contrast, the hyperglycemic diabetic animals would be expected to, and did have, significantly elevated ($p<0.05$) kidney weights over these euglycemic animals.

The hyperglycemic diabetic animals demonstrated an elevation in the glomerular filtration rate (as measured by creatinine clearance) as well as urinary albumin excretion over the three other groups. These changes suggest that the hyperglycemic diabetic animals were at an early stage of renal impairment or diabetic nephropathy (105). The increase in the GFR of the diabetic hyperglycemic group could be due to several possible mechanisms.

The GFR has been shown to increase with an increase in kidney size (153) subsequently leading to glomerular enlargement and an increase in the glomerular filtration area. Glomerular hyperfiltration has been shown to result in an increase in GFR (106); however, the exact cause is not known. Hyperglycemia is also thought to have some role in the increase in GFR observed. The correlation between glycated hemoglobin and GFR in our study animals ($r=0.71$) would support this statement.

The mechanisms by which hyperglycemia may lead to an increased GFR are uncertain; however, metabolic and hormonal mechanisms are likely involved. High blood glucose level may induce vasodilatation of the glomerular arterioles (154) which has been seen to occur in the retinal circulation (155). This vasodilation could lead to hyperfiltration in the kidney resulting in an increase in the GFR. It has been found that strict glycemic control, as evidenced by our diabetic euglycemic animals, can prevent such an increase, especially during the early stages of diabetes. Other factors, including growth hormone, angiotensin, and catecholamines, have been shown to elevate the GFR (156,157). The role that these may play in diabetic nephropathy was not investigated in this study.

Another aspect of the early changes of renal function in diabetes is an elevation in urinary albumin levels. Due to the low concentrations of this analyte in early diabetic nephropathy, sensitive assays, such as radio-immunoassays are required for its measurement. The increase in urinary albumin excretion is due to alterations of the glomerular filtration barrier

resulting in a selective increase in its permeability. Factors responsible for the increase in glomerular filtration include the pore size of the basement membrane, which separates molecules due to their molecular size, GFR, and the net molecular charge of the membrane (158).

Non-enzymatic glycation may be involved in the urinary albumin excretion described above. Increased blood glucose levels, as seen in the hyperglycemic diabetic animals, has been shown to result in increased levels of non-enzymatic glycation of many proteins, including basement membranes (83,90) and serum proteins (73,78,86) over animals maintained at euglycemic levels. This increase in non-enzymatic glycation can be expected to lead to structural and functional alterations of the glomerular basement membrane which may lead to the development of diabetic nephropathy.

Non-enzymatic glycation of glomerular basement membrane can be expected to alter its net charge. The addition of a glucose molecule to a lysine or hydroxylysine residue will remove a positive charge from the basement membrane or physiological pH. This will result in an increase in the overall net negative charge of the membrane. Schober et al (165) have shown that the immunoelectrophonetic pattern of <u>in vitro</u> glycated basement membrane was different than that of native basement membrane, thus confirming that non-enzymatic glycation can alter the net charge.

The charge relationship between the glomerular basement membrane and a protein in question is important in the passage of molecules through

the filtration barrier (100). The negative charge of the glomerular basement membrane will normally repel the passage of negatively charged proteins, such as albumin; and facilitate the passage of neutral and especially cationic ones (98). The non-enzymatic glycation of albumin can be expected to increase its anionic nature, further repelling its passage. However, due to its relatively short half-life (<30 days) in comparison to that of glomerular basement membrane, it can be expected that it will not be glycated to the same extent as the latter. This could change its charge relationship with glomerular basement membrane such that albumin will have a more net positive charge. This could result in passage of albumin through the filtration barrier, resulting in microalbuminuria.

Non-enzymatic glycation of glomerular basement membrane, may also be responsible for increasing the pore size of the glomerular filtration barrier (another factor responsible for renal filtration). The removal of the positive charge on hydroxylysine by non-enzymatic glycation may prevent interchain crosslinking (98), and could interfere with the packing of the collagen fibrils, resulting in increased pore size and increased membrane permeability.

Other factors, independent of non-enzymatic glycation may also affect the passge of molecules across the filtration barrier. Decreased sialic acid (124) and heparan sulfate (166) incorporation have been observed in glomerular basement membrane isolated from diabetics. This is thought to result in the reduction in its negative charge, decreasing the repulsion of anionic

molecules such as albumin. The influence that this may have on our results was not examined.

Diabetic glomerulopathy is characterized by a very slow development of basement membrane accumulation. This basement membrane thickening is thought to start at the onset of diabetes, for it may be detected within a few years subsequent to the diagnosis of the disease (147). The pathogenesis is unclear, however biochemical studies have been interpreted to suggest that hyperglycemia causes the increased thickness of glomerular basement membrane (83,84,120). Previous investigators have suggested that non-enzymatic glycation may be responsible for the increase in glomerular basement membrane thickness. Trueb et al (120) have suggested that increased non-enzymatic glycation of basement membrane may inhibit its turnover, ultimately resulting in increased basement membrane thickness. Cohen et al (83,84) have also shown similar results and have also suggested the importance of non-enzymatic glycation in the increase in glomerular basement membrane seen in diabetics.

The diabetic animals (both hyperglycemic and euglycemic) exhibited increased levels of glomerular basement membrane thickness over that of non-diabetic control animals. However, only the hyperglycemic diabetic animals exhibited an elevated level of non-enzymatic glycation. This suggests that the two processes, namely, non-enzymatic glycation and glomerular basement membrane thickening, are not related, which we have recently reported (see Appendix). Furthermore, the results of this study suggest that

non-enzymatic glycation does not cause basement membrane thickening, which is in contrast to what has been suggested by others (83,120).

Several studies, to date, have examined the non-enzymatic glycation of glomerular basement membrane (84,120,165,167), however all have failed to examine the role of the latter in glomerular basement membrane thickening and renal functional changes. All studies have demonstrated an increase in the non-enzymatic glycation of basement membrane in hyperglycemic conditions over that seen in euglycemic conditions, both in humans and in various animal models of diabetes. Generally, the extent of non-enzymatic glycation found is similar in all studies, regardless of the method employed to quantitate it. We have observed that the hyperglycemic animals had values about twice that seen in the euglycemic animals which is similar to what has been reported by others (84,120). The majority of the above mentioned studies conclude with speculative statements to the effect "whether non-enzymatic glycation causes morpho- logical and functional changes requires further investigation" (165) or else they hypothesize that "it can be expected that non-enzymatic glycation may lead to glomerular basement membrane thickening" (167).

Our study, unlike previous ones, addresses the relationship that non-enzymatic glycation may play in basement membrane thickness and renal functional changes simultaneously in the same set of animals. Mauer and Steffes (123) have examined glomerular basement membrane thickness and renal function including microalbuminuria and creatinine clearance in

streptozotocin-induced diabetic rats. Their conclusion that glomerular basement membrane thickness is not responsible for renal functional changes is similar to what we propose. However, they did not examine the role that non-enzymatic glycation may play in renal functional changes, which is important in addressing diabetic nephropathy.

Brenner et al (169) believe that hyperperfusion and hyperglycemia must be dissociated to better address the factors responsible for diabetic nephropathy. Wen et al (170) have addressed this problem, by studying diabetic nephropathy in streptozotocin induced diabetic rats on a low protein diet. Since a low protein diet can reduce the GFR (171), it can be used to prevent glomerular hyperfiltration (171). Thus, the role hyperglycemia plays in diabetic nephropathy can be investigated independent of hyperfusion. They found that a low protein diet ameliorates but does not prevent nephropathy, and suggest that both hemodynamic factors as well as hyperglycemic related factors may be important. As with Maurer & Steffes (123) they did not examine the levels of non-enzymatic glycation of glomerular basement membrane in their experiments.

Recently, it has been suggested that the renal lesions, including glomerular basement membrane thickening, are thought to be due to the diabetic state of the animal and not genetic factors (159). In rat models of diabetes, it has been found that a kidney from a normal rat when transplanted into a diabetic rat, will develop the early lesions characteristic of diabetic nephropathy (159). Similarly in human diabetes, it has been shown

that characteristic diabetic lesions, including glomerular basement membrane thickening, have been seen in kidneys from normal donors which were transplanted into a diabetic subject (56). This evidence suggests that the glomerular basement membrane thickening may arise from the diabetic environment, with genetic factors not being directly involved.

In long term diabetic animals, in which normalization of blood glucose levels was obtained by islet cell transplantation, the glomerular basement membrane thickening did not revert to normal values after one year, despite the improved glycemic environment (57). In contrast, functional changes, including urinary albumin excretion returned to normal. This data further suggests that glomerular basement membrane thickening is not directly responsible for urinary albumin excretion. This is similar to what we have observed, and suggests that, at least early on in diabetic nephropathy, glomerular basement membrane thickening is not likely the direct cause of renal function impairment. Mauer et al (123) have suggested that mesangial expansion rather than glomerular basement membrane thickening may lead to glomerular functional deterioration in diabetes by restricting the glomerular capillary vasculature. Further investigation in this area is required.

In man, kidney biopsies to obtain glomerular basement membrane from diabetics is not possible due to ethical reasons. In contrast, muscle capillary basement membrane can easily be obtained via muscle biopsy. If muscle capillary basement membrane is shown to reflect the changes seen in

glomerular basement membrane from diabetic subjects, then muscle biopsies could be used to monitor changes in glomerular basement membrane. In our study, the levels of non-enzymatic glycation of muscle capillary basement membrane correlated ($r=0.66$) to those of glomerular basement membrane, and also to the average blood glucose ($r=0.84$). However, we did not examine muscle capillary basement membrane thickness. From our results, it appears that the levels of non-enzymatic glycation of muscle capillary basement membrane are related to glycemic control.

The importance of measurement of muscle BMT as an indicator of renal basement membrane change may be limited since the latter has been shown in our study not to be related to renal functional changes. Few studies to date have examined the significance of non-enzymatic glycation of muscle capillary basement membrane to diabetic nephropathy. One such study by Monniers group (168), found no correlation between the above two parameters. Similarly, no relationship has been found between muscle capillary basement membrane thickness and diabetic nephropathy (59). However, Steffes et al (59) found that glomerular basement membrane width correlated to muscle capillary basement membrane width. This muscle capillary basement membrane thickening did not correlate to changes in the mesangial region, which is thought to be important in the pathogenesis of diabetic nephropathy (123).

It therefore appears that the levels of non-enzymatic glycation of muscle capillary basement membrane reflects glycemic control only. It is not known what role, if any, it may play in diagnosing renal structural and functional changes associated with diabetic nephropathy at this time.

VII CONCLUSIONS:

1. Non-enzymatic glycation is not involved in the development of glomerular basement membrane thickening. The diabetic hyperglycemic animals had increased levels of non-enzymatic glycation over the euglycemic diabetic animals, despite the fact that no differences in glomerular basement membrane thickness was observed.

2. Glomerular basement membrane thickening appears to be a consequence of the diabetic state as evidenced by the fact that all diabetic animals exhibited increased glomerular basement membrane thickness over that of non-diabetic animals. Further more, glomerular basement membrane thickness does not appear to be the primary cause of the development of early renal functional changes, such as increased urinary albumin excretion. The euglycemic diabetic animals exhibited increased levels of glomerular basement membrane thickness over those found in non-diabetic animals, but did not differ with respect to urinary albumin excretion rates. Similarly, the hyperglycemic diabetic animals exhibited changes in urinary albumin excretion, when compared to the euglycemic diabetic animals, while having a similar degree of glomerular basement membrane thickness.

3. Hyperglycemia, and particularly non-enzymatic glycation may be responsible for the early renal functional changes seen in diabetic nephropathy, including increased urinary albumin excretion. However at this time, the role that non-enzymatic glycation plays is speculative. It does, however, offer a mechanism by which hyperglycemia may result in the development of diabetic nephropathy.

REFERENCES

1. Cahill GF: Current concepts in diabetes. In Joslin's Diabetes Mellitus, Twelfth edition. Lea and Fibiger, New York, pg. 1-11, 1985.

2. Free HM, Free AH: Analytical chemistry in the conquest of diabetes. Anal Chem 56:664A-684A, 1984.

3. Levine R: Historical view of the classifications of diabetes. Clin Chem 32:B5-B6, 1986.

4. Hedon E: Sur la secretion interne du pancreas. CR Soc Biol Paris 71:124-127, 1911.

5. Bliss M: The discovery of insulin. McClelland and Stewart Ltd., Toronto, 1982.

6. Best CH: The first clinical use of insulin. Diabetes 5:65-67, 1956.

7. Bell ET: Renal vascular disease in diabetes mellitus. Diabetes 2:376-389, 1953.

8. Kimmelstiel P, Wilson C: Intercapillary lesions in glomeruli of kidney. Am J Pathol 12:83-97, 1936.

9. Lehninger AL: Biochemistry, second edition, Worth Publishers, Inc. New York, pg. 820-822, 1975.

10. National Institute of Health. Report of the national conference on diabetes. NIH publication 80-273. Bethesdas, MD: NIH, 1980.

11. Entmacher PS, Krall LP, Kranczer JN: Diabetes mortality from vital statistics. In Joslin's Diabetes Mellitus, Twelfth edition. Lea and Fibiger, New York, pg. 278-297, 1985.

12. National Diabetes Data Group. Classification and diagnosis of diabetes mellitus and other catagories of glucose intolerance. Diabetes 28: 1039-1057, 1979.

13. WHO Expert Committee on Diabetes Mellitus. Second report. Geneva, World Health Organization, 1980, Technical report series 646.

14. Weir GC: Non-insulin-dependent diabetes mellitus: Interplay between B-cell inadequacy and insulin resistance, Am J Med 73:461-464, 1982.

15. Kahn CR: Pathophysiology of diabetes mellitus: An overview. In Joslin's Diabetes Mellitus, Twelfth edition. Lea and Fibiger, New York, pg. 43-50, 1985.

16. Vranic M, Hollenberg CH, Steiner G, eds.: Comparison of type I and type II diabetes. Advances in Experimental Medicine and Biology, 1985.

17. West R, Bellmonte MM, Colle E, et al: Epidemiologic survey of juvenile-onset diabetes in Montreal. Diabetes 28:690-693, 1979.

18. Ehrlich RM, Walsh LJ, Falk JA, et al: The incidence of type I (insulin-dependent) diabetes in Toronto. Diabetologia 22:289-291, 1982.

19. Kalandy A: Autoantibodies to islet cells in diabetes mellitus. Diabetes 28:102-105, 1979.

20. Sachs JA, Cudworth AG, Jaraquemada D, Gorsuch AN, Festenstein H: Type I diabetes and the HLA-D locus. Diabetologia 18:41-43, 1980.

21. Cudworth AG, Woodrow JC: Genetic susceptibility in diabetes mellitus: Analysis of HLA association. British Med J 2:846-848, 1976.

22. Rand LI: Recent advances in diabetic retinopathy. Am J Med 70:595-602, 1981.

23. Klein R, Klein BEK: Vision disorders in diabetes. In Harris MJ, Hamman RF eds., Diabetes in America. NIH Publication 85-1468, Bethesda, MD: NIH, Chapter 13, 1985.

24. Prchal J, Skalka H, Clements RS, et al: Diabetes and risk of cataract development. Metabolic, Pediatric and Suplemic Ophthalmology 4:185-189, 1980.

25. Santiago JV: Overview of the complications of diabetes. Clin Chem 32:B48-B53, 1986.

26. Clements RS, Bell DSH: Complications of diabetes: Prevalence, detection, current treatment, and prognosis. Am J Med 79 (suppl. 5A):2-6, 1985.

27. Clark BF, Ewing DJ, Campbell IW: Diabetic autonomic neuropathy. Diabetologia 17:195-212, 1978.

28. Boulton AJM, Ward JD: Diabetic neuropathies and pain. Clinics Endo Metab 15:917-931, 1986.

29. Greene DA: Metabolic abnormalities in diabetic peripheral nerve. Relation to impaired function. Metabolism 32 (suppl. 1):118-123, 1983.

30. Kannel WB, McGee DL: Diabetics and cardiovascular risk factors: the Framingham study. Circulation 59:8-13, 1979.

31. Gordon T, Castelli WP, Hjortland ML et al: High density lipoprotein as a protective factor against coronary heart disease. Am J Med 62:702--714, 1977.

32. Santen RJ, Willis PW, Fajans SS: Artherosclorosis in diabetes mellitus: Correlations with serum lipid levels, adoposity, and serum insulin levels. Arch Intern Med 130:833-843, 1972.

33. Hostetter TH: Diabetic nephropathy (editorial). New Engl J Med 312:642-643, 1985.

34. Grenfell A, Watkins PJ: Clinical diabetic nephropathy: Natural history and complications. Clinics Endo Metab 15:783-805, 1986.

35. Cudworth AG, Bodansky HJ: Genetic and metabolic factors in relation to the prevalence and severity of diabetic complications. In Complications of Diabetes. Keen H, Jarrett J, eds., Edward Arnold (Publishers) Ltd., pg. 1-12, 1982.

36. Bodansky H, Wolf E, Cudworth A, et al: Genetic and immunological factors in microvascular disease in type I (insulin dependent) diabetes. Diabetes 31:70-74, 1982.

37. Larkins RG, Martin FIR, Tait BD: HLA patterns and diabetic retinopathy. British Med J 1:1111, 1978.

38. Standle E, Dexel T, Alberti ED, Scolz AS, Greite JH, Mehnert H: Diabetic retinopathy: Association with the human leukocyte antiserum -(HLA-) System (Abstract). Diabetes 28:396, 1979.

39. Barnett AH, Pyke DA: The genetics of diabetic complications. Clinics Endo Metab 15:715-726, 1986.

40. Barbosa J, and Saner B: Do genetic factors play a role in the pathogenesis of diabetic microangiopathy? Diabetologia 27:487-492, 1984.

41. Barnett AH, Mijovic C, Fletcher J, et al. Low plasma C4 concentrations: associations with microangiopathy in insulin dependent diabetes. British Med J 289:943-945, 1984.

42. Mijovic C, Fletcher J, Bradwell AR, Harvey T, Barnett AH: Relationship of gene expression (allotypes) of the fourth component of complement to insulin dependent diabetes and its microangiopathic complications. British Med J 291:9-10, 1985.

43. Nayao Y, Matsumoto H, Miyazaki T, et al: IgG heavy chain (Gm) allotypes and immune response to insulin in insulin requiring diabetes mellitus. New Engl J Med 304:407-409, 1981.

44. Mijovic C, Fletcher J, Bradwell AR, Barnett AH: Phenotypes of the heavy chains of immunoglobulins in patients with diabetic microangiopathy: Evidence for an immunogenetic predisposition. British Med J 292:433-435, 1986.

45. Bondy PK, Felig P: Relation of diabetic control to development of vascular complications. Medical clinics of North America 55:889-898, 1971.

46. Siperstein MD, Finegold KR, Bennett PH: Hyperglycemia and diabetic microangiopathy. Diabetologia 15:365-367, 1978.

47. Witzlum J, Mahoney EM, Branks MJ, Fisher M, Elam R, Steinberg D: Nonenzymatic glycosylation of low-density lipoprotein alters its biologic activity. Diabetes 31:283-291, 1982.

48. West KM: Hyperglycemia as a cause of long-term complications. In Complications of Diabetes, second edn., edited by Keen H, Jarrett J. Edward Arnold (Publishers) Ltd., pg. 13-18, 1982.

49. Rash R: Prevention of diabetic glomerulopathy in streptozotocin diabetic rats by insulin treatment. Glomerular basement membrane thickness. Diabetologia 16:319-324, 1979.

50. Fox CJ, Darby SC, Ireland JT, Sonksen PH: Blood glucose control and glomerular capillary basement membrane thickening in experimental diabetes. British Med J 2:605-607, 1977.

51. Vlassara H, Brownlee M, Cerami A: Excessive nonenzymatic glycosylation of peripheral and central nervous system myelin components in diabetic rats. Diabetes 32:670-674, 1983.

52. Pirart J: Diabetes mellitus and its degenerative complications: A prospective study of 4400 patients observed between 1947 and 1973. Diabetes Care 1:168-188, 1978.

53. Bennion KJ, Grundy SM: Effects of diabetes mellitus on cholesterol metabolism in man. New Engl J Med 296:1365-1371, 1977.

54. Cunha-vaz JG, Fonseca JR, Abreu JF, Ruas MA: Detection of early retinal changes in diabetes by vitreous fluorophotometry. Diabetes 28:16-19, 1979.

55. Doud R, Lee DBN, Waisman J, Bergstein JM: Development of a lesion resembling diabetic nephropathy in renal homograft. Arch Intern Med 137:945-947, 1977.

56. Mauer SM, Steffes MW, Connett J, Najarian JS, Sutherland DER, Barbosa J: The development of lesions in the glomerular basement membrane and mesangium after transplantation of normal kidneys to diabetic patients. Diabetes 32:948-952, 1983.

57. Steffes MW, Brown DM, Basgen JM, Mauer SM: Amelioration of mesangial volume and surface alterations following islet transplantation in diabetic rats. Diabetes 34:509-515, 1985.

58. Gotzsche O, Gundersen HJG, Osterby R: Irreversibility of glomerular basement membrane accumulation despite reversibility of renal hypertrophy with islet ransplantations in early experimental diabetes. Diabetes 30:481-485, 1981.

59. Steffes MW, Sutherland DER, Goetz FC, Rich SS, Mauer SM: Studies of kidney and muscle biopsy specimens from identical twins discordant for type I diabetes mellitus. New Engl J Med 312:1282-1287, 1985.

60. Gabbay KH: The sorbitol pathway and the complications of diabetes. N Engl J Med 288:831-836, 1973.

61. Finegold D, Lattimer S, Nolle S, Bernstein M, Greene DA: Polyol pathway activity and myo-inositol metabolism. Diabetes 32:988-992, 1983.

62. Gillon KRW, Hawthorne JN: Sorbitol, inositol, and nerve conduction in diabetes. Life Sci 32:1943-1947, 1983.

63. Bron AJ, Cheng H: Cataract and retinopathy: Screening for treatable retinopathy. Clinics Endo Metab 15:971-999, 1986.

64. Cohen MP: Reduced glomerular sodium-potassium adenosine triphosphatase activity in acute streptozotocin diabetes and its prevention by oral sorbinil. Diabetes 34:1071-1074, 1985.

65. Datiles M, Fukui H, Huwabara T, Kinoshita JH: Galactose cataract prevention with sorbinil, an aldose reductase inhibitor: a light microscopic study. Investigative Ophthalmology 22:174-179, 1982.

66. Greene DA, Lattimer SA: Biochemical alterations and complications in diabetes. Clin Chem 32:B42-B47, 1986.

67. Greene DA, Lattimer S, Sima AA: Nerve Na^+-K^+-ATPase, conduction, and myo-inositol in the insulin-deficient BB rat. Am J Physiol 247:E534-E539, 1984.

68. Greene DA, Lattimer S, Vibrecht J, Carroll P: Glucose-induced alterations in nerve metabolism: Current perspective on the pathogenesis of diabetic neuropathy, and future directions for research and therapy. Diabetes Care 8:290-299, 1985.

69. Lee T-C, Pintauro SJ, Chichester CO: Nutritional and toxicologic effects of nonenzymatic Maillard browning. Diabetes 31 (suppl. 3):37-46, 1982.

70. Maillard LC: Action des acides amines sur les sucres; formation des melanoidens par voie methodique. C R Acad Sci 154:66-68, 1912.

71. Kirschenbaum DM: Glycosylation of proteins: Its implications in diabetic control and complications. Pediatric Clinics of North America 31:611-621, 1984.

72. Means GE, Chang MK: Nonenzymatic glycosylation of proteins. Structure and function changes. Diabetes 31 (suppl. 3):1-4, 1982.

73. Fantl WJ, Stevens VJ, Peterson CM: Reactions of biologic aldehydes with proteins. Diabetes 31 (suppl. 3):15-21, 1982.

74. Hodge JE: The amadori rearrangement. Adv Carbohydrate Chem 10:169-210, 1955.

75. McFarland KF, Cataland EW, Day JF, Thorpe SR, Baynes JW: Non-enzymatic glycosylation of serum proteins in diabetes mellitus. Diabetes 28:1011-1014, 1979.

76. Garlick RL, Bunn HF: Characterization of glycosylated hemoglobins. J Clin Invest 71:1062-1072, 1983.

77. Guthrow CE, Morris MA, Day JF, Thorpe SR, Baynes JW: Enhanced nonenzymatic glycosylation of human serum albumin in diabetes mellitus. Proc Natl Acad Sci USA 76:4258-4261, 1979.

78. Day JF, Thorpe SR, Baynes JW: Nonenzymatically glycosylated albumin. J Biol Chem 254:595-597, 1979.

79. Witztum JL, Mahoney EM, Branks MJ: Non-enzymatic glycosylation of low density lipoprotein alters its biologic activity. Diabetes 31:283-291, 1982.

80. Gonen B, Farrar P: Non-enzymatic glycosylation of low density lipoproteins in vitro. Diabetes 30:875-878, 1981.

81. Vlassara H, Brownlee M, Cerami A: Nonenzymatic glycosylation of peripheral nerve protein in diabetes mellitus. Proc Natl Acad Sci USA 78:5190-5192, 1981.

82. Zaman Z, Verwilighen RL: Non-enzymatic glycosylation of horse spleen and rat liver ferritins. Biochim Biophys Acta 699:120-124, 1981.

83. Cohen MP, Urdaniva E, Surma M: Nonenzymatic glycosylation of basement membranes: in vitro studies. Diabetes 30:367-371, 1981.

84. Cohen MP, Urdaniva E, Surma M: Increased glycosylation of glomerular basement membrane collagen in diabetes. Biochem Biophys Res Commun 95:765-769, 1980.

85. McMillan DE, Brooks SM: Erythrocyte spectrin glycosylation in diabetes. Diabetes 31 (suppl. 3):64-69, 1982.

86. Miller JA, Gravallese S, Bunn HF: Nonenzymatic glycosylation of erythrocyte membrane proteins. J Clin Invest 65:896-901, 1980.

87. Yatscoff RW, Tevaarwerk GJ, Clarson CL, Warnock LM: Evaluation of an affinity chromatographic procedure for the determination of glycosylated hemoglobin (HbA_1). Clin Biochem 16:291-295, 1983.

88. Brownlee M, Vlassara H, Cerami A: Nonenzymatic glycosylation reduces the susceptibility of fibrin to degradation by plasmin. Diabetes 32:680--684, 1983.

89. Kennedy L, Baynes JW: Non-enzymatic glycosylation and the chronic complications of diabetes: An overview. Diabetologia 26:93-98, 1984.

90. Lubec G, Pollack A: Reduced susceptibility of non-enzymatically glycosylated glomerular basement membrane to proteases. Renal Physiol 3:4-8, 1980.

91. Coradello H, Lubec G, Pollack A, Steinberg M: Enzyme activities of native non-enzymatically glycosylated trypsin, chymotrypsin, and papain. Paediatr Paedol 17:457-464, 1982.

92. Yue DK, McLennan S, Delbridge L, Handelsman DJ, Reeve T, Turtle JR: The thermal stability of collagen in diabetic rats: Correlation with severity of diabetes and non-enzymatic glycosylation. Diabetologia 24:282-285, 1983.

93. Bassiouny AR, Rosenberg H, McDonald TL: Glycosylated collagen is antigenic. Diabetes 32:1182-1186 , 1983.

94. Lubec G, Leban J, Peyroux J, Steinberg M, Pollak A, Latzka U, Coradello H: Reduced collagenolytic activity of rat kidneys with streptozotocin diabetes. Nephron 30:357-360, 1982.

95. McMillan DE, Viterback NG, La Puma J: Reduced erythrocyte deformability in diabetes. Diabetes 27:895-901, 1978.

96. Pande A, Ganner WH, Spector A: Glycosylation of human lens protein and cataractogenesis. Biochem Biophys Res Commun 89:1260-1266, 1979.

97. Eble AS, Thorpe SR, Baynes JW: Non-enzymatic glycosylation and glucose dependent crosslinking of protein. J Biol Chem 258:9506-9512, 1983.

98. Ireland JT, Viberti GC, Watkins PJ: The kidney and renal tract. In Complications of Diabetes, second edition. Edited by Keen H, Jarrett J, Edward Arnold (Publishers) Ltd., pg. 137-178, 1982.

99. Rennke HG, Venkatachalam MA: Structural determinants of glomerular permselectivity. Federation Proc 36:2619-2626, 1977.

100. Alberti KGMM, Press CM: The biochemistry of the complications of diabetes mellitis. In Complications of Diabetes, second edition, edited by Keen H, Jarrett J, Edward Arnold (Publishers) Ltd., pg. 231-270, 1982.

101. Friedman S, Jones HW, Golbetz HV, Lee JA, Little HL, Myers BD: Mechanism of proteinuria in diabetic nephropathy II: A study of the size-selective glomerular filtration barrier. Diabetes 32 (suppl. 2):40-46, 1982.

102. Graham RC, Karnovsky M: Glomerular permeability. Ultrastructural cytochemical studies using peroxidases as protein tracers. J Exp Med 124:1123-1134, 1966.

103. Deen WM, Dohrer MP, Robertson CR, Brenner BM: Determinants of the transglomerular passage of macromolecules. Federation Proc 36:2614-2618, 1977.

104. Mauer SM, Steffes MW, Goetz FC, Sutherland DER, Brown DM: Diabetic nephropathy. A perspective. Diabetes 32 (suppl. 2):52-55, 1983.

105. Mogensen CE, Christensen CK, Vittingus E: The stages of diabetic renal disease with emphasis on the stage of incipient diabetic nephropathy. Diabetes 32 (suppl. 2):64-78, 1983.

106. Viberti GC, Wiseman MJ: The kidney in diabetes. Significance of the early abnormalities. Clinics Endo Metab 15:753-782, 1986.

107. Christainsen JS, Gammelgaard J, Tronier B, Svendsen P, Parving H-H: Kidney function and size in diabetics before and during initial insulin treatment. Kid Int 21:683-688, 1982.

108. Mogensen CE, Steffes MW, Deckert T, Christiansen JS: Functional and morphological renal manifestations in diabetes mellitus. Diabetologia 21:89-93, 1981.

109. Osterby R: Diabetic glomerulopathy. Structural characteristics of the early and advanced stages of diabetes mellitus. Diabetes 32 (suppl. 2):79-82, 1983.

110. Wiseman M, Viberti G, Jarrett RJ, Keen H: Glycemia, arterial pressure, and microalbuminuria in type I (insulin-dependent) diabetes mellitus. Diabetologia 26:410-415, 1984.

111. Parving H-H, Andersen AR: Early detection of patients at risk of developing diabetic nephropathy. A longitudinal study of urinary albumin excretion. Acta Endo 100:550-555, 1982.

112. Mogensen CE: Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. New Engl J Med 310:356-360, 1984.

113. Woo J, Floyd M, Cannon DC, Kahan B: Radioimmunoassay for urinary albumin, Clin Chem 24:1464-1467, 1978.

114. Miles DW, Mogensen CE, Gundersen HJG: Radioimmunoassay for urinary albumin using a single antibody. Scand J Clin Lab Invest 26:5-11, 1970.

115. Fielding BA, Price DA, Houlton CA: Enzyme immunoassay for urinary albumin. Clin Chem 29:355-357, 1983.

116. Viberti GC, Jarrett RJ, Mahmud U, Hill RD, Argyropoulos A, Keen H: Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. Lancet i:1430-1432, 1982.

117. Mogensen CE: Long-term antihypertensive treatment inhibiting the progression of diabetic nephropathy. Br Med J 285:685-688, 1982.

118. Viberti GC, Bilous RW, Mackintosh D, Keen H: Monitoring glomerular function in diabetic nephropathy. A perspective study. Am J Med 74:256-264, 1983.

119. Viberti GC, Mackintosh D, Keen H: Determinants of the penetration of proteins through the glomerular barrier in insulin-dependent diabetes mellitus. Diabetes 32 (suppl. 2):92-95, 1983.

120. Trueb B, Fluckiger R, Winterhalter KH: Nonenzymatic glycosylation of basement membrane collagen in diabetes mellitus. Collagen Rel Res 4:239-251, 1984.

121. Vlassara H, Brownlee M, Cerami A: Non-enzymatic glycosylation: Role in the pathogenesis of diabetic complications. Clin Chem 32: B37-B41, 1986.

122. Grant ME, Harwood R, William IF: Increased synthesis of glomerular basement membrane in streptozotocin diabetes. J Physiol 257:56-57, 1976.

123. Mauer SM, Steffes MW, Ellis EN, Sutherland DER, Brown DW, Goetz FC: Structural-functional relationships in diabetic nephropathy. J Clin Invest 74:1143-1155, 1984.

124. Myers BD, Wietz JA, Chui F, Michaels AS: Mechanisms of proteinuria in diabetic nephropathy: A study of glomerular barrier function. Kidney Int 21:633-641, 1982.

125. Mauer MW, Steffes MW, Brown DM: The kidney in diabetes. Am J Med 70:603-612, 1981.

126. Pennock CA, Passant LP, Bolton FG: Estimation of cerebrospinal fluid protein. J Clin Path 21:518-520, 1968.

127. Fabing DL, Ertingshausen G: Automated reaction-rate method for determination of serum creatinine with the Centrifichem. Clin Chem 17:696-699, 1971.

128. Glycotest 100 package insert. Pierce Chemical Company. Rockford, Il., 1985.

129. Radioimmunoassay for urinary albumin package insert. Diagnostic Products Limited, 1985.

130. Carlson EC, Brendel K, Hjelle JT, Mezan E: Ultrastructural and biochemical analyses of isolated basement membrance from kidney glomeruli and tubules and brain and retinal microvessels. J Ultrastructural Res 62:26-53, 1978.

131. Cohen MP, Carlson EC: Preparation and analysis of glomerular basement membrane. In, Methods of Diabetes Research. Edited by J Larner and SL Pohl, John Wiley and Sons, Inc., pg. 357-375, 1984.

132. Westberg NG, Michael AF: Human glomerular basement membrane. Preparation and composition. Biochemistry 9:3837-3838, 1970.

133. Shack J, Bynum BS: Interdependence of variables in the activation of deoxynuclease I. J Biol Chem 239:3842-3848, 1964.

134. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685, 1970.

135. Pecoraro RE: Comparison of a colorimetric assay for glycosylated hemoglobin with ion-exchange chromatography. Diabetes 28:1120-1125, 1979.

136. Dolhofer R, Wieland OH: Improvement of the thiobarbituric acid assay for serum glycosyl-protein determination. Clin Chem Acta 112:197-204, 1981.

137. Paisey R: Correlation between skin glycosylation and glycemic control in human diabetics. Clin Endo 20:521-525, 1984.

138. Garlick RL, Mazer JJ: The principle site of nonenzymatic glycosylation of human serum albumin in vivo. J Biol Chem 258:6142-6146, 1983.

139. Shaklai N, Garlick RL, Bunn HF: Nonenzymatic glycosylation of human serum albumin alters its conformation and function. J Biol Chem 259:3812-3817, 1984.

140. Yue DK, McLennan S, Handlesman DJ, Delbridge L, Reeve T, Turtle JR: The effect of salicylates on nonenzymatic glycosylation and thermal stability of collagen in diabetic rats. Diabetes 33:745-751, 1984.

141. Moore SJ: Amino acid analysis: aqueous dimethyl sulfoxide solvent for the ninhydrin reaction. J Biol Chem 243:6281-6293, 1968.

142. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275, 1951.

143. Jensen EB, Gundersen HJG, Osterby R: Determination of membrane thickness distribution from orthogenal intercepts. J Microscopy 115:19-33, 1978.

144. Sokal RR, Rohlof FF: Biometry. The principle and practice of statistics in biological research. W.H. Freeman and Company, San Francisco, 1984.

145. Praither JD: Principles of radioimmunoassay testing: A simple approach. J Nuclear Medicine Technology 13:24-43, 1985.

146. Pimputkar MR, Feldkamp CS: Radioimmunoassay kit evaluation and selection. J Nuclear Medicine Technology 13:88-94, 1985.

147. Osterby R: Structural changes in the diabetic kidney. Clinics Endocrinology and metabolism 15:733-751, 1986.

148. Goldstein DE, Little RR, Wiedmeyer H-M, England JD, McKenzie EM: Glycated hemoglobin. Methodologies and clinical applications. Clin Chem 32:B64-B70, 1986.

149. Tietz NW (editor): Textbook of Clinical Chemistry. WB Saunders Co., Philadelphia, 1986.

150. Syer-Hansen K: Renal hypertrophy in experiment diabetes. Kidney Int 23:643-646, 1983.

151. Ellis EN, Steffes MW, Goetz GC, Sutherland DER, Mauer MW: Relationship of renal size to nephropathy in type I (insulin-dependent) diabetes. Diabetologia 28:12-15, 1985.

152. Syer-Hansen K: Renal hypertrophy in experimental diabetes: relation to severity of diabetes. Diabetologia 13:141-143, 1977.

153. Mogensen CE: Renal functional changes in diabetics. Diabetes 25 (suppl. 2):872-879, 1979.

154. Kasiske BL, O'Donnell MP, Keane WF: Glucose-induced increase in renal hemodynamic function. Possible modulation by renal prostaglandins. Diabetes 34:360-364, 1985.

155. Atherton A, Hill DW, Keen H, Young S, Edwards EJ: The effect of acute hyperglycemia on the retinal circulation of the normal cat. Diabetologia 18:233-237, 1981.

156. Dworkin LD, Ichikawa I, Brenner BM: Hormonal modulation of glomerular function. Am J Physiol 244:F95-F104, 1983.

157. Bohrer MP, Deen WM, Robertson CR, Brenner BM: Mechanism of angiotensin II-induced proteinuria in the rat. Am J Physiol 233:F13-F21, 1977.

158. Carrier BJ, Myers BD: Proteinuria and functional characteristics of the glomerular barrier in diabetic nephropathy. Kidney Int 17:667-676, 1980.

159. Lee CS, Mauer SM, Brown DM, Sutherland DER, Micheal AF, Najarian JS: Renal transplantation in diabetes mellitus in rats. J Exp Med 39:793-800, 1974.

160. Klein DJ, Brown DM, Oegema TR: Glomerular proteoglycans in diabetes. Diabetes 35:1130-1142, 1986.

161. Kanwar YS, Farquhar MG: Presence of heparan sulfate in the glomerular basement membrane. Proc Natl Acad Sci USA 76:1303-1307, 1979.

162. Kanwar YS, Linker A, Farquhar MG: Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. J Cell Biol 86:688-693, 1980.

163. Woodley DT, Rao CN, Hassell JR, et al: Interactions of basement membrane components. Biochim Biophys Acta 761:278-283, 1983.

164. Kleinman HK, Klebe RJ, Martin GR: Role of collagenous matrices in the adhesion and growth of cells. J Cell Biol 88:473-485, 1981.

165. Schober E, Pollak A, Coradello H, Lubec G: Glycosylation of glomerular basement membrane in type I (insulin-dependent) diabetic children. Diabetologia 23:485-487, 1982.

166. Cohen MP, Surma ML: ^{35}S sulfate incorporation intoglomerular basement membrane glycosaminoglycans is decreased in experimental diabetes. J Lab Clin Med 98:715-722, 1981.

167. Perejda AJ, Vitto J: Non-enzymatic glycosylation of collagen and other proteins: Relationship to development of diabetic complications. Collagen Rel Res 2:81-88, 1982.

168. Vishwanath V, Frank KE, Elmets CA, Monnier VM: Glycation of skin collagen in type I diabetes mellitus. Diabetes 35:916-921, 1986.

169. Zatz R, Meyer TW, Rennke HG, Brenner BM: Predominance of hemodynamic rather than metabolic factors in the pathogenesis of diabetic glomerulopathy. Proc Natl Acad Sci USA 82:5963-5967, 1985.

170. Wen S-F, Huang T-P, Moorthy AV: Effects of low-protein diet on experimental diabetic nephropathy in the rat. J Lab Clin Med 106:589-597, 1985.

171. Meyer TW, Hostetter TH, Rennke HG, Noddin JL, Brenner BM: Preservation of renal structure and function by long term protein restriction in rats with reduced nephron mass. Kidney Int 23:218-222, 1983.

APPENDIX

Manuscript submitted to Kidney International.

RELATIONSHIP OF NON-ENZYMATIC GLYCATION TO ALTERED RENAL STRUCTURE AND FUNCTION IN THE DIABETIC RAT

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ABSTRACT

Renal functional parameters including creatinine clearance, urinary albumin excretion, basement membrane thickening, and levels of non-enzymatic glycation of glomerular basement membrane were studied in rats rendered diabetic with streptozotocin. Diabetic animals had elevated glycated hemoglobin levels ($p<0.05$), increased creatinine clearance, and urinary albumin excretion rates ($p<0.05$) as compared to insulin treated diabetic (euglycemic), age-matched, and streptozotocin non-diabetic animals. The level of non-enzymatic glycation of glomerular basement membrane was significantly elevated ($p<0.05$) in the diabetic animals as well, with the level of non-enzymatic glycation of all animals, correlating ($p<0.05$) to the average blood glucose level of each animal. Despite changes in functional parameters, and increased levels of non-enzymatic glycation between the diabetic and euglycemic animals, there was no difference in glomerular basement membrane thickness between the two groups. However, there was a difference between all diabetic euglycemics and the age-matched control animals. We hypothesize that increased glycation of glomerular basement membrane may alter renal function, possibly by affecting the net charge of the glomerular filtration barrier. However, glomerular basement membrane thickening does not affect the functional changes which have been observed, thus casting doubt upon its role in the development of diabetic nephropathy.

KEYWORDS: Non-enzymatic glycation of glomerular basement membrane, Diabetic Nephropathy, Basement Membrane Thickening, Experimental Diabetes.

INTRODUCTION

Diabetic nephropathy is one of the most common secondary complications of diabetes. It has been shown that 40% of patients with diabetes eventually develop nephropathy with increased proteinuria and decreased glomerular filtration rate (GFR) (1). The etiology of diabetic nephropathy is not known, but one hypothesis suggests that the increased blood glucose levels encountered in diabetes in some way may lead to renal changes (2).

An increase in blood glucose has been shown to result in increased levels of non-enzymatic glycation (3). Non-enzymatic glycation is a common post-translational modification which has been shown to occur for many proteins (4,5). It is a result of direct chemical reaction between reducing sugars and primary amino groups of proteins, ultimately resulting, via Amadori rearrangement, in the formation of a stable ketoamine derivative (6). The reaction is glucose dependent and is increased in hyperglycemic situations. Non-enzymatic glycation has been shown to alter the structure and function of many proteins, both in vitro and in vivo (5,7,8).

The hyperglycemia found in diabetes has been shown to result in increased non-enzymatic glycation. This may precipitate changes in the composition and synthesis of glomerular basement membrane (9,10), ultimately leading to functional and morphological changes in the kidney. The functional changes

that occur early on in diabetic nephropathy include an increase in the GFR (11) and development of microalbuminuria (12), notably in poorly controlled diabetic subjects. The specific role that non-enzymatic glycation plays in the changes in renal function remains unclear; it may alter membrane permeability, causing a gradual loss of selective filtration resulting in non-selective proteinuria (13).

Morphological studies have previously shown an increase in glomerular basement membrane thickening in human diabetics over that of normal individuals (14). Many investigators, however, have reported poor correlation between functional changes, such as GFR and microalbuminuria, and basement membrane thickness in long term diabetic subjects. In experimentally induced diabetes, Cohen et al (18) have demonstrated an increase in non-enzymatic glycation of glomerular basement membrane but did not investigate its relationship to morphological and functional changes in the kidney.

In view of the conflicting data sited in the aforementioned clinical studies and paucity in the studies on experimental animals, the present investigation was designed to examine the relationship between hyperglycemia, non-enzymatic glycation, and renal structure and function concurrently in experimentally induced diabetic rats.

METHODS

1. ANIMALS:

Adult male (300-350 gr) Sprague-Dawley rats were injected intraperitoneally with a single injection of streptozotocin (65 mg/Kg) dissolved in cold citrate buffer (pH=4.5). Hyperglycemia (blood glucose greater than 20 mmol/L) and glycosuria were present three days after injection in 24 animals while another 10 maintained euglycemic. A control group of citrate-injected rats (n=10) was age-matched to the diabetic group. These three groups were fed Wayne F6 rodent blox and kept for five months with food and water ad libitum. Half the diabetic animals (n=12) were maintained at a blood glucose level of 4.5-6.5 mmol/L by daily injection of protoamine-zinc insulin (Connaught Laboratories, Willowdale, Ontario). The dosage ranged from 2-14 units/day depending on the animal and was adjusted according to bi-weekly blood glucose monitoring. The remaining group of animals (n=12) was treated similarly, with the exception that the blood glucose levels were maintained at 19-22 mmol/L.

Twenty-four hour urine collections and blood samples were obtained for analysis of creatinine clearance, glycated hemoglobin, and urinary albumin at time 0, 2, and 5 months of the study. Body weights of all the animals were recorded weekly for the duration of the study.

2. FUNCTIONAL STUDIES

Blood glucose was monitored bi-weekly in whole blood obtained by tail bleed and quantitated using Dextrosticks and read on a glucometer (Ames Division, Miles Laboratories LTD., Rexdale, ON).

Glycated hemoglobin was measured by affinity chromatography using a commercially available kit (Glyco-Test, Pierce Chemical Co., Rockford, IL), as previously described (19). The between run coefficient of variation for this method was less than 10%.

Creatinine clearance was expressed as ml/min/100 gm, and corrected for body weight but not body surface area. Serum and urine creatinine was measured by an autoanalyser (Beckman Astra, Beckman Inc., Brea, CA) using an alkaline picrate method (20).

Urinary albumin was measured by radioimmunoassay by modification of (21,22). Rabbit anti-rat albumin was obtained from (National Biological Laboratories, Winnipeg, Manitoba). Briefly, the antibody was raised to globulin free rat albumin (Sigma Chemical Co., St. Louis, Mo; A4538). Iodine 125 bovine serum albumin (Dupont Canada, Inc. (NEN products), Lachine, Quebec) was used as the tracer. Briefly 200 ul of sample, standard, or control was pipetted

into 12x75 mm glass tubes. 100 ul of antibody, and 100 ul of tracer (0.2 uCi/ml) was added. The mixture was incubated for 2 hours at room temperature; and the bound fraction collected after precipitation with 25% (w/v) polyethylene glycol (PEG) and centrifugation. The RIA showed good recovery, parallelism, and sensitivity (2 ug/ml). The intra- and inter-assay coefficient of variations were 3.2% and 6.2% respectively. The results were expressed in ug/hr.

3. PROCUREMENT OF TISSUES

After five months of study, the animals were anesthetized with sodium pentobarbital and blood drawn via cardiac puncture for subsequent analysis of creatinine, glycated hemoglobin, and blood glucose. Kidneys were rapidly excised, a slice of the left kidney obtained for electron microscopy, and the remaining tissue placed in cold isotonic saline for subsequent isolation of the glomeruli.

4. ISOLATION OF GLOMERULAR BASEMENT MEMBRANE

Glomeruli were isolated from renal cortex by differential sieving through a series of nylon meshes according to Cohen et al (23), with the material on the 88 um and 105 um screens collected as glomeruli. The basement membrane was isolated by osmotic lysis and sequential detergent extraction using 3% triton-X 100, deoxynuclease digestion, and sodium desoxycholate; as described by Carlson et al (24).

5. MEASUREMENT OF NON-ENZYMATIC GLYCATION

The level of non-enzymatic glycation was assayed for by borohydride reduction and boronic acid affinity chromatography as previously described (25,26,27). Two mg of lyophylized basement membrane was suspended in 200 ul of water, followed by the addition of 200 ul of 0.10 N NaOH containing 625 uCi of tritiated sodium borohydride (Amersham Canada LTD., Oakville, ON; specific activity 500 mCi/mmol) and 12.5 mg of unlabelled sodium borohydride. Reduction was carried out for 90 minutes on ice in a fumehood, and was stopped by the addition of 4 ml 10% (w/v) trichloroacetic acid (TCA). The reduced basement membrane was washed a number of times with 10 TCA, followed by centrifugation and aspiration of the supernatant. The washed pellet was hydrolyzed with 1.5 ml of 6 M HCl for 16 hours at 110°C, in sealed screw top tubes. The pH was adjusted to 8.5 by the addition of concentrated NaOH. The amino acid concentration of the hydrolysate was determined by the ninhydrin procedure (28) using a standard curve produced with known concentrations of leucine. Results were expressed in leucine equivalents. Four hundred ul of the hydrolysate was also loaded onto a m-aminophenylboronic acid column (Glycogel GSP columns, Isolab Inc., Akron, OH) and washed with 15 ml of 50 mM $(Na)_2HPO_4$. The bound amino acids were removed by the addition of 2 ml 0.10 M HCl. one ml of the eluate was counted on a LKB 12-19 scintillation counter (LKB. Turko, Finland) using a quench corrected counting program.

The results were expressed as CPM/umol leucine equivalents.

6. MORPHOLOGICAL STUDIES

Small pieces of kidney cortex were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH=7.4) for 2 hours at 40°C. Tissues were rinsed for 24 hours at 4°C in 0.1 M phosphate buffer (pH=7.4) containing 0.2 M sucrose. The tissues were then postfixed for two hours at 4°C in 1% osmium tetroxide in 0.1 M phosphate buffer (pH=7.4), dehydrated in ascending concentrations of ethanol and embedded in Epon 812. Thick sections were stained with toluidine blue and examined for routine orientation. Thin sections were stained with uranyl acetate and lead citrate, viewed and photographed in a Philips EM 201 electron microscope. Quantification of kidney glomerular basement membrane was performed utilizing the orthogonal intercept method of Jensen et al (29). The actual measurements were carried out on micrographs (magnification 20,000 X) with an electronic plaimeter (Hewlett Packard digitizer, model 9874A) equipped with an electrosensitive cursor connected to a Hewlett Packard 9875A calculator/computer.

The total number of micrographs assessed was 750. Glomerular basement membrane thickness for each rat was measured on 25 randomly selected micrographs.

7. STATISTICS

The experimental data was analyzed by the unpaired T-test to compare the means of the various groups. The level of significance was $p=0.05$. The statistical analysis was performed on a commercial package (Crunch Interactic Statistics Package (CRISP), Crunch Software, San Francisco, CA). The statistical analysis for the morphological studies was by ANOVA and Tukey's procedure (30).

RESULTS

METABOLIC AND FUNCTIONAL PARAMETERS

The mean monthly blood glucose levels for the four groups of animals are shown in Figure 1. The hyperglycemic group had a mean blood glucose level of 16.4 $\pm$ 2.6 mmol/L which was significantly elevated ($p<0.05$) over the other three groups; namely age-matched controls, streptozotocin non-diabetics, and diabetic euglycemics. There was no significant difference between the mean blood glucose levels of these later three groups. Similarly, the hyperglycemic group had glycated hemoglobin values which were significantly elevated ($p<0.05$) over the other three groups of animals (see Figure 2).

The mean body weights of the diabetic hyperglycemic animals were 523 $\pm$ 49 gm, which was significantly decreased ($p<0.05$) when compared to the three other groups. The kidney weights of the hyperglycemic animals expressed as mean plus or minus standard deviation was 0.42 $\pm$ 0.07 gm/100 gm body weight, which was significantly increased ($p<0.05$) over that seen in the diabetic euglycemic, age-matched controls, and streptozotocin non-diabetic groups; (0.30 $\pm$ 0.03, 0.29 $\pm$ 0.02, and 0.28 $\pm$ 0.02 gm/100 gm body weight, respectively).

The mean glomerular filtration rate (GFR), measured by creatinine clearance, for the 4 groups of animals is summarized in Figure 3. The diabetic hyperglycemic animals had an elevated creatinine clearance ($p<0.05$) at both 2 and 5 months of the study over the three other groups. At 2 and 5 months, the diabetic euglycemic, age-matched controls, and streptozotocin non-diabetic groups had similar values.

Urinary albumin excretion profiles for the various groups are shown in Figure 4. The excretion appears somewhat age-dependent. The hyperglycemic diabetic animals had values significantly elevated ($p<0.05$) over the other three groups of animals. Within each group, there was quite a variable range of values, in albumin excretion by the rats. A correlation between the functional results and the blood glucose levels was also found in our study; blood glucose levels and GFR correlated directly ($r=0.68$, $p<0.0001$) as did urinary albumin levels ($r=0.63$, $p<0.0006$).

The functional and metabolic data at the completion of the study is summarized in Table 1. The diabetic hyperglycemic group differed with respect both to functional and metabolical parameters when compared to the three other groups. In contrast, the diabetic euglycemic group paralleled the control and drug treated non-diabetic animals with respect to these parameters.

BIOCHEMISTRY

The level of non-enzymatic glycation of glomerular basement membrane isolated from the study animals at 5 months is shown in Figure 5 and Table 2. The hyperglycemic diabetic animals had significantly elevated values ($p<0.05$) in comparison to the other three groups. There was no statistical difference in non-enzymatic glycation of both the control groups and the diabetic euglycemic group. The level of non-enzymatic glycation of glomerular basement membrane and average blood glucose values correlated directly ($r=0.86$, $p<0.0001$) as shown in Figure 6.

MORPHOLOGY

The morphological data representing glomerular basement membrane thickness, is shown in Table 3. All animals showed an age dependent thickening over the course of the study. After 5 months, the diabetic animals (both euglycemic and hyperglycemic) showed an increase ($p<0.05$) in thickness over that seen in the two control groups of animals; however, there was no statistical difference in glomerular basement membrane thickening between the hyperglycemic or the euglycemic diabetic animals.

DISCUSSION

The cause of diabetic nephropathy is unknown, but one possible mechanism may result from the hyperglycemia experienced in poorly controlled diabetics. The changes in creatinine clearance and urinary albumin levels, suggest that the hyperglycemic diabetic animals in our study are at an early state of renal impairment. These changes, which did not occur in the control or euglycemic diabetic animals, could be due to the hyperglycemic state, as evidenced by the increased glycated hemoglobin levels in the diabetic hyperglycemic group.

The streptozotocin-induced diabetic rat is a useful experimental model to study diabetic nephropathy. In our study, approximately 25% of the animals given the drug failed to become diabetic which is consistent with other studies (31). There was no apparent difference in metabolic or functional parameters, including GFR and urinary albumin levels, between the age-matched control animals and those of the streptozotocin injected non-diabetic animals. Similarly, no changes in biochemical or morphological parameters between these two groups was observed. Thus, one may conclude that streptozotocin, per say, had no effects on the various parameters measured.

The glycemic state of the euglycemic diabetic animals paralleled that of the age-matched control animals. This was reflected in the lack of differences in either blood glucose or glycated hemoglobin levels for these animals. The hyperglycemic diabetic animals in contrast, exhibited significant elevations in blood glucose and glycated hemoglobin levels. These animals were probably in the early stages of diabetic nephropathy, as evidenced by increased GFR and urinary albumin levels over the values seen in the euglycemic diabetic and control animals. The increase in the GFR could be due to several possible mechanisms. The GFR has been shown to increase with an increase in kidney size (11), subsequently leading to glomerular enlargement and increasing the glomerular filtration surface area. The mechanism for the increase in GFR is unknown, but hyperglycemia may be involved since strict glycemic control as evidenced in the euglycemic diabetic animals can prevent such an increase, especially during the early stages of diabetes. Other factors including growth hormone, angiotensin and catacholamines may also be involved (32,33).

Factors responsible for glomerular filtration include the pore size of the basement membrane, which separates molecules due to their molecular size, GFR and the net molecular charge of the membrane. In our study, it is unlikely that pore size played a role, since only at late stages of diabetes do such changes occur (34). Therefore, increased GFR and changes in glomerular basement membrane charge may be involved in the increased urinary albumin excretion rates seen in the present study.

Non-enzymatic glycation may be involved in the changes of renal function observed. Increased blood glucose levels, as seen in the hyperglycemic diabetic animals can result in increased levels of non-enzymatic glycation of many proteins, including basement membranes (4). This was noted in our study, as evidenced by the good correlation between blood glucose levels and non-enzymatic glycation of glomerular basement membrane (r=0.86) and the level of glycated hemoglobin (r=0.86).The increased level of non-enzymatic glycation of glomerular basement membrane in diabetics may lead to structural and functional changes including alterations in its net change.

There was no difference in glomerular basement membrane thickness between the euglycemic and the hyperglycemic diabetic animals, despite the fact that these groups exhibited differences in renal function and in the extent of non-enzymatic glycation. However, all diabetic animals in the study showed increased glomerular basement membrane thickness over the control animals. Our data therefore suggests that basement membrane thickening may not be important in the development of diabetic nephropathy. This is consistent with the work of Mauer et al (15) which suggests that mesangial expansion rather than glomerular basement membrane leads to glomerular functional deterioration in diabetes by restricting the glomerular capillary vasculature and its filtration surface.

We have shown changes in renal function, increased GFR and increased urinary albumin excretion, without changes in glomerular basement membrane thickness which suggests and supports evidence that the latter may not be important in the renal changes associated with diabetic nephropathy. Hyperglycemia appears to be involved in some way in the development of renal functional changes, however its exact role is unknown. The increased level of non-enzymatic glycation that results from hyperglycemia may be responsible for the alteration of glomerular basement membrane, ultimately resulting renal functional impairment and the future development of chronic diabetic nephropathy. It is therefore important that diabetics are maintained under good glycemic control to prevent the development of diabetic nephropathy.

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REFERENCES

1. Knowles HC, Guest GM, Lamp J: The course of juvenile diabetics treated with measured diet. Diabetes 14:239-273, 1975

2. Sandahl Christiansen M, Frandsen M, Parving H-H: Effect of intervenous glucose infusion on renal function in normal man and in insulin-dependent diabetes. Diabetologia 21:368-374, 1981

3. Day JF, Thorpe SR, Baynes JW: Non-enzymatic glycosylation of rat serum proteins in vitro and in vivo. Fed Proc 38:418, 1979

4. Kennedy L, Baynes JW: Non-enzymatic glycosylation and the chronic complications of diabetes: an overview. Diabetologia 26:93-93, 1984

5. Kirschenbaum DM: Glycosylation of proteins: Its complications in diabetic control and complications. Pediatric Clinics of North America 31:611-621, 1984

6. Bunn HF, Haney DN, Kamin S, Gabbay, Gallop PM: The biosynthesis of human hemoglobin A1c. Slow glycosylation of hemoglobin in vivo. J Clin Invest 57:1652-1659, 1976

7. Shaklai N, Garlick RL, Bunn HF: Nonenzymatic glycosylation of human serum albumin alters its conformation and function. J Biol Chem 259:3812-3817, 1984

8. Means YE, Chang MK: Nonenzymatic glycosylation of proteins: structure and function changes. Diabetes 31:1-4, 1982

9. Trueb B, Fluckiger R, Winterhalter KH: Nonenzymatic glycosylation of basement membrane collagen in diabetes mellitus. Collagen Rel Res 4:239-251, 1984

10. Cohen MP, Urdanivia E, Surma M, Ciborowski CJ: Nonenzymatic glycosylation of basement membranes, in vitro studies. Diabetes 31:367-371, 1981

11. Mogensen CE: Renal functional changes in diabetics. Diabetes 25 (suppl.2):872-879, 1976

12. Viberti GC, Pickup JC, Jarrett RJ, Keen H: Effect of control of blood glucose on urinary albumin excretion of albumin and B2-microglobulin in insulin-dependent diabetes. N Engl J Med:639-641, 1979

13. Rennke HG, Venkatachalam MA: Structural determinants of glomerular permselectivity. Fed Proc 39:2619-2626, 1977

14. Brown DM, Andres GA, Hostetter TH, Mauer SM, Price R, Venkatachalam MA: Kidney Complications. Diabetes 31 (suppl. 1):71-81, 1982

15. Mauer SM, Steffes MW, Ellis EN, Sutherland DER, Brown DM, Goetz FC: Structural-functional relationships in diabetic nephropathy. J Clin Invest 74:1143-1155, 1984

16. Gellman DD, Pirani CL, Soothill JF, Muehrcke RC, Maduros W, Kark RM: Structure and function in diabetic nephropathy. The importance of diffuse glomerulosclerosis. Diabetes 8:251-256, 1959

17. Rasch R: Prevention of diabetic glomerulopathy in streptozotocin diabetic rats by insulin treatment. Glomerular basement membrane thickening. Diabetologia 16:319-324, 1979

18. Cohen MP, Urdanivia E, Surma M, Wu V-Y: Increased glycosylation of glomerular basement membrane collagen in diabetes. Biochem Biophys Res Commun 95:765-769, 1980

19. Yatscoff RW, Tevaarwerk, GJ, Clarson CL, Warnock, LM: Evolution of an affinity chromatographic procedure for the determination of glycosylated hemoglobin (HbA_1). Clin Biochem 16:291-295, 1983

20. Fabing DL, Ertingshausen G: Automated reaction-rate method for the determination of serum creatinine with the Centrifichem. Clin Chem 17:696-699, 1971

21. Miles DW, Mogensen CE, Gundersen HJG: Radioimmunoassay for urinary albumin using a single antibody. Scand J Clin Lab Invest 26:5-11, 1970

22. Woo J, Floyd D, Cannon DC, Kahan B: Radioimmunoassay for urinary albumin. Clin Chem 24:1464-1467, 1978

23. Cohen MP, Carlson EC: Preparation and analysis of glomerular basement membrane. In, Methods in Diabetes Research 1(c), edited by Larner J, Pohl SL, New York, John Wiley and Sons, Inc., 1984, 357-375

24. Carlson EC, Brendel K, Hjelle JT, Mezan E: Ultrastructural and biochemical analyses of isolated basement membrane from kidney glomeruli and tubules and brain and retinal microvessels. J Ultrastructural Research 62:26-53, 1978

25. Shaklai N, Garlick RL, Bunn HF: Nonenzymatic glycosylation of human serum albumin alters its conformation and function. J Biol Chem 259:3812-3817, 1984

26. Garlick RL, Mazer JJ: The principle site of nonenzymatic glycosylation of human serum albumin in vivo. J Biol Chem 258:6142-6146, 1983

27. Yue DK, McLennan S, Handlesman DJ, Delbridge L, Reeve T, Turtle JR: The effect of salicylates on nonenzymatic glycosylation and thermal stability of collagen in diabetic rats. Diabetes 33:745-751, 1984

28. Moore SJ: Amino acid analysis: aqueous dimethyl sulfoxide solvent for the ninhydrin reaction. J Biol Chem 243:6281-6283, 1968

29. Jensen EB, Gundersen HJG, Osterby R: Determination of membrane thickness distribution from orthogenal intercepts. J Microscopy 115:19-33, 1978

30. Sokal RR, Rohlof FF: Biometry. The principles and practice of statistics in biological research. W.H. Freeman and Company. San Francisco 1984

31. Mordes JP, Rossini AA: Animal models of diabetes, in Joslin's Diabetes Mellitus, edited by Marble A, Twelfth Edition, Philadelphia, Lea and Febiger, 1985, p. 110

32. Bohrer MP, Deen WM, Robertson CR, Brenner BM: Mechanism of angiotensin II-induced proteinuria in the rat. Am J Physiol 233:F13-F21, 1977

33. King SE, Baldwin DS: Production of renal ischemia and proteinuria in man by adrenal medullary hormones. Am J Med 20:217-224, 1956

34. Carrie BJ, Myers BD: Proteinuria and functional characteristics of the glomerular barrier in diabetic nephropathy. Kidney Int 17:667-676, 1980

TABLE 1

Metabolic and Functional Results for Diabetic and Control Animals at 5 Months of Study.[1]

Group	Weight (gm)	Average Blood Glucose (mmol/L)	Corrected[2] Creatinine Clearance (ml/min/100gm)	% Glycated Hemoglobin
Control n=7	676 ± 38	6.4 ± 0.4	0.165 ± 0.056	5.21 ± 1.13
Drug Control n=6	657 ± 66	5.9 ± 0.6	0.154 ± 0.065	6.42 ± 0.79
Diabetic Euglycemic n=5	668 ± 58	7.0 ± 0.5	0.152 ± 0.074	4.65 ± 0.61
Diabetic Hyperglycemic n=8	523 ± 49[3]	16.4 ± 2.6[3]	0.409 ± 0.230[3]	13.08 ± 3.96[3]

[1] Results are expressed as mean + SD.

[2] Values corrected for body weight.

[3] Significantly different at $p<0.05$ from the other groups

TABLE 2

Non-enzymatic Glycation of Glomerular Capillary Basement Membrane

GROUP	LEVEL OF NON-ENZYMATIC GLYCATION[1] OF GLOMERULAR BASEMENT MEMBRANE (CPM/umol leucine equivalents)
CONTROL n=7	351 ± 203
DRUG CONTROL n=6	366 ± 237
DIABETIC EUGLYCEMIC n= 5	468 ± 182
DIABETIC HYPERGLYCEMIC n=8	1260 ± 521[2]

[1] Values expressed as mean + SD

[2] Significantly different at $p<0.05$ from the other groups

TABLE 3

Morphometric Analysis of Glomerular Basement Membrane Thickness in Control and Diabetic Rats At 0 and 5 Months of the Study.

GROUP	BASEMENT MEMBRANE THICKNESS (microns)[1]
Rats at time 0	0.085 ± .002
Control at 5 months	0.197 ± .004
Drug Control at 5 months	0.191 ± .003
Diabetic Euglycemic at 5 months	0.217 ± .004[2]
Diabetic Hyperglycemic at 5 months	0.220 ± .004[2]

[1]Values represent mean plus or minus the standard error of the mean.

[2]Significantly different at $p<0.05$ from other groups but not from each other.

LEGENDS TO FIGURES

Fig. 1: Monthly blood glucose profiles for the four animal groups. Values are represented as mean ± SEM for each group. The symbols represent: ▲, hyperglycemic diabetic rats, n=8; ●, euglycemic diabetic rats, n=5; ■, age-matched control rats, n=7; and ◆, streptozotocin injected non-diabetic rats, n=6. The blood glucose levels for the hyperglycemic diabetic rats was significantly elevated ($p<0.05$) over the other three groups.

Fig. 2: % glycated hemoglobin profiles for the four animal groups in the study. Values represent mean ± SD. The symbols represent: ▲, hyperglycemic diabetic rats, n=8; ●, euglycemic diabetic rats, n=5; ■, age-matched control rats, n=7; and ◆, streptozotocin injected non-diabetic rats, n=6. The % glycated hemoglobin for the hyperglycemic diabetic rats was significantly elevated ($p<0.05$) over the three other groups at both 2 and 5 months of the study.

Fig. 3: Corrected creatinine clearance profiles for the four animal groups of the study. The values represent mean ± SD except for the diabetic hyperglycemic group in which only the means are listed due to the large SD's encountered. The symbols represent: ▲, hyperglycemic diabetic rats, n=8; ●, euglycemic diabetic rats, n=5; ■, age-matched control rats, n=7; and ◆, streptozotocin injected non-diabetic rats, n=6. The creatinine clearance for the hyperglycemic rats was significantly ($p<0.05$) elevated at at both 2 and 5 months of study.

Fig. 4: Urinary albumin excretion profiles for the four animal groups of the study. The values represent mean ± SD, except for the diabetic hyperglycemic group, in which only the means are listed due to the large SD's encountered. The symbols represent: ▲, hyperglycemic diabetic rats, n=8; ●, diabetic euglycemic rats, n=5; ■, age-matched control rats, n=7; and ◆ streptozotocin injected non-diabetic rats, n=6. The urinary albumin excretion for the hyperglycemic diabetic rats was significantly elevated ($p<0.05$) at both 2 and 5 months of study.

Fig. 5: The levels of non-enzymatic glycation of glomerular basement membrane for the study rats in each group. The mean of each group is represented by the horizontal bar.

Fig. 6: The relationship between average blood glucose and the level of non-enzymatic glycation of glomerular basement membrane.

Figure 1.

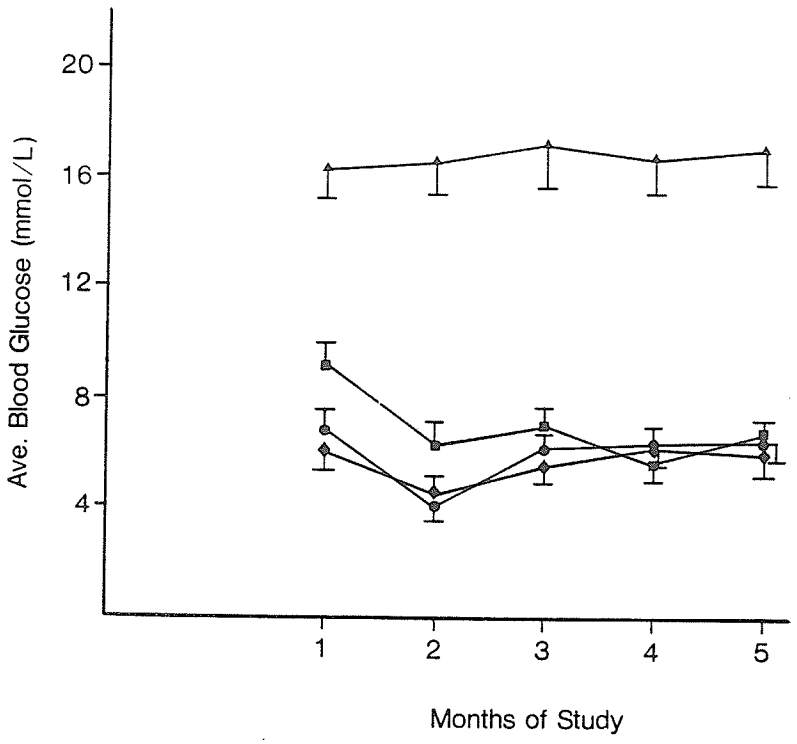


Figure 2.

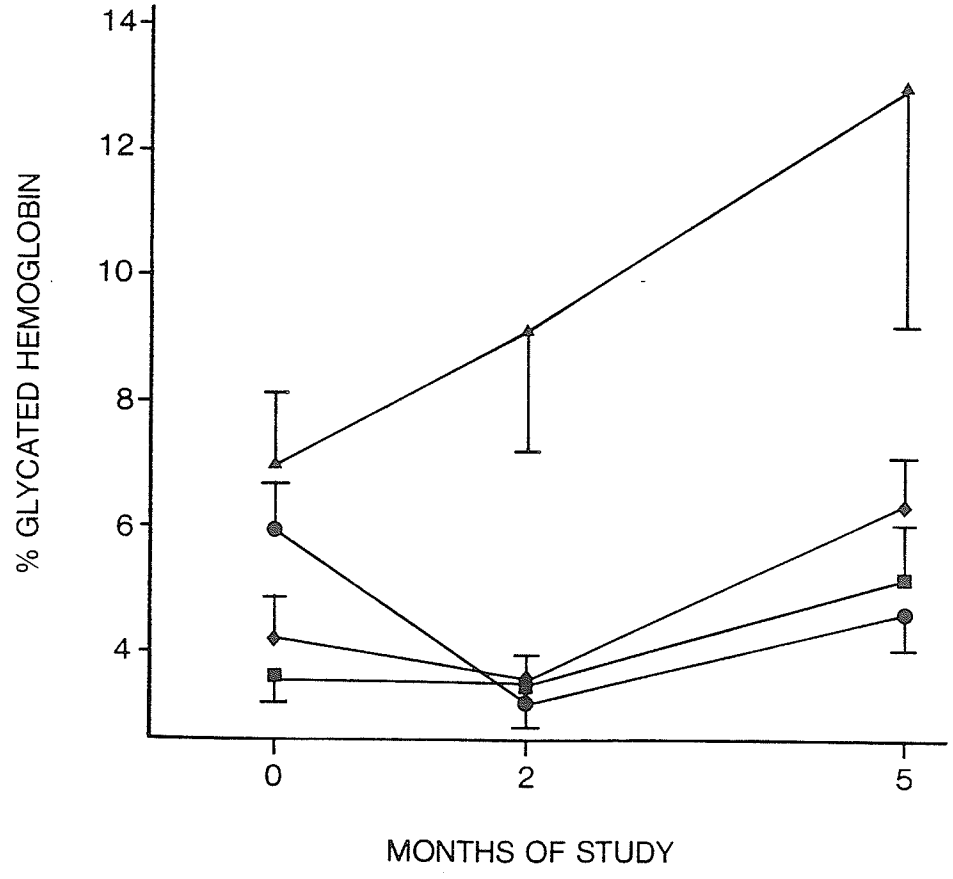
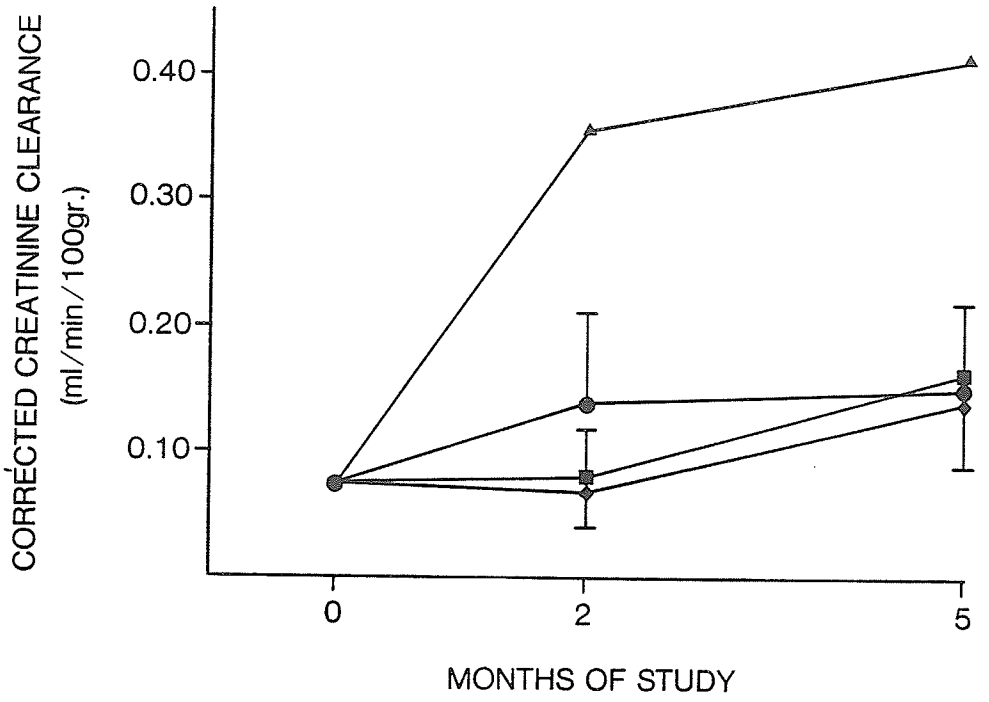


Figure 3.



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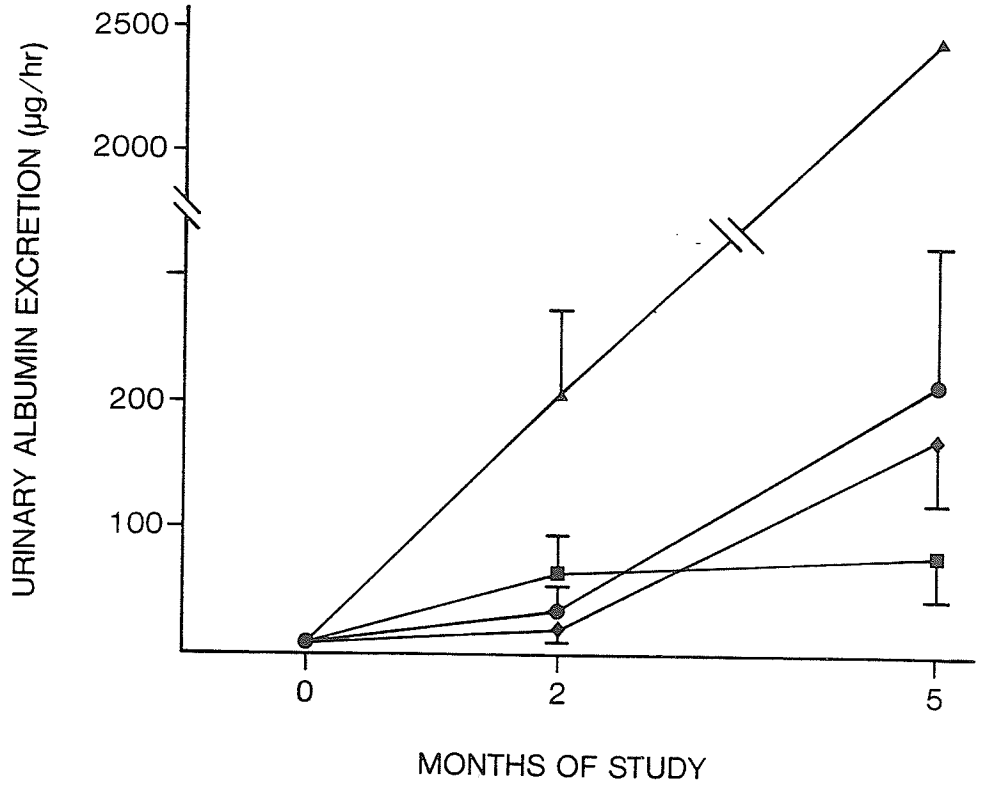

Figure 5.

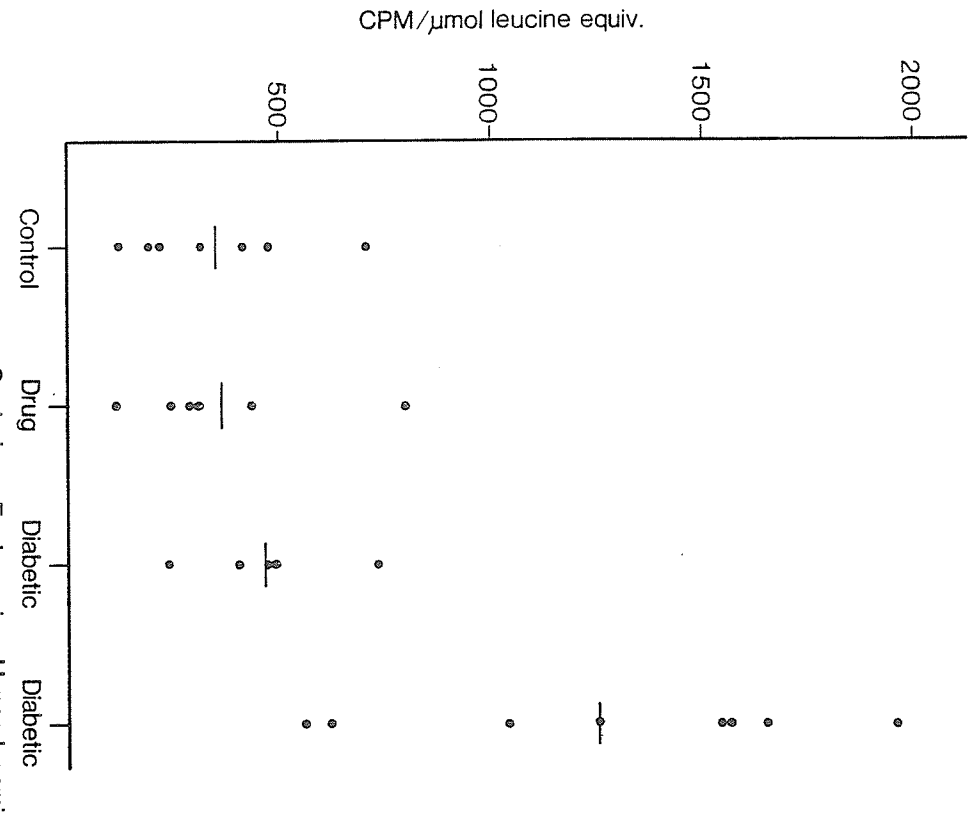

Figure 6.