MICROALBUMINURIA AS A PREDICTIVE TOOL IN DIABETIC NEPHROPATHY: SAMPLING PROTOCOLS, CROSS-SECTIONAL ANALYSIS AND ASSOCIATION WITH OTHER NEUROVASCULAR COMPLICATIONS

by

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This work is dedicated to
my brother, David
(1964 - 1978)

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ABSTRACT

The clinical hallmark of diabetic nephropathy is proteinuria. Subclinical microalbuminuria may denote early glomerular dysfunction and may thus serve a predictive role for diabetic renal disease.

We examined four urine collection protocols (overnight recumbent, morning ambulatory, 24-hour, exercise provocation) for albumin excretion rate (AER) reproducibility in five healthy subjects, and for sensitivity to early microalbuminuria in five short-term Type I diabetic subjects. AER was measured by a double antibody radioimmunoassay. β2-microglobulin excretion rate (β2-uER), measured by a commercially available kit (Phadebas, Sweden) indicated tubular function. Ambulatory samples had the least intra- and inter-individual AER variability and optimal AER sensitivity and specificity. β2-uER's were normal in the diabetic subjects.

For a cross-sectional analysis of albuminuria, recumbent (R) and upright (U) urine samples were collected by 65 Type I diabetic (duration 0 - 32 yr) and 20 nondiabetic persons. Glycemia was assessed by autoanalyzer assay of glucose in blood spots home collected on filter paper (ac and hs, 2 days) and by % glycosylated hemoglobin. Upper limit AER in both postures in health was 11 ug/min. In diabetes, hyperalbuminuria did not appear before 6 (R) and 2.5 (U) years duration. Thereafter, a duration-related increase in albuminuria was present in 26 (R) and 33 (U) subjects. 19 clinically non-proteinuric individuals had abnormal immunoassayable

AER (U). Skewness was such that normoalbuminuria was present in 5 of 12 subjects after more than 20 years duration. 13 of 15 hypertensives were hyperalbuminuric. No correlation of AER with prevailing glycemia or diastolic blood pressure was found. 6 patients had elevated β2-uER; three of these were clinically proteinuric.

Fingertip touch perception threshold (TPT) may denote early peripheral nerve dysfunction and may thus have predictive value for diabetic neuropathy. TPT was measured by a modified Von Frey technic. Impaired TPT occurred in 21 of 54 subjects after 2 years of diabetes and correlated with disease duration, but was normal in 2 of 9 subjects after 20 years. No correlation with prevailing glycemia was found. Neither TPT nor clinical neuropathy correlated with AER independent of diabetes duration.

Capillary dilatation (CD), assessed ophthalmoscopically, may serve a prognostic role for diabetic retinopathy. CD correlated with presence of clinical retinopathy. The presence of clinical retinopathy also correlated with the presence of microalbuminuria.

Conclusions: (1) Upright urine collection provides optimal AER reproducibility and sensitivity/discrimination; (2) Subclinical microalbuminuria is primarily a glomerular disorder and initial abnormalities are consistent in timing and frequency with glomerular basement membrane thickening (Osterby, R. Acta med Scand, Suppl 574:1, 1975); (3) Early functional glycemia-dependent albuminuria did not occur; (4) The progression of microalbuminuria to clinically overt levels is probably ramplike and gradual, such that early rises in AER may serve a prognostic role for nephropathy; (5) TPT and CD may have

analogous predictive value for neuropathy and retinopathy, respectively; (6) Neurovascular complications are not the inevitable fate of every patient; and (7) The vascular complications of diabetes (nephropathy and retinopathy) are probably linked to a common pathogenetic factor while the neuropathic complications may differ somewhat etiologically.

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LIST OF ABBREVIATIONS

General

AER	albumin excretion rate
AHAS-R	antihuman-albumin serum, rabbit
ARGS-G	antirabbit-gammaglobulin serum, goat
β2-uER	β2-microglobulin excretion rate
BG	blood glucose
BP	blood pressure
CD	capillary dilatation
GBM	glomerular basement membrane
GFR	glomerular filtration rate
ac	latin, after meals
hs	latin, before bed
NANA	N-acetyl neuraminic acid
% HbA_1	percent glycosylated hemoglobin
R	recumbent
R-AER	recumbent albumin excretion rate
TPT	touch perception threshold
U	upright
U-AER	upright albumin excretion rate

Units of Measure

cpm	counts per minute
cc	cubic centimetre

dl	decilitre (10^{-1} litre)
g	gram
hr	hour
kpm	kilopond-metre
l	litre
uCi	microcurie
ug	microgram (10^{-6} gram)
mg	milligram (10^{-3} gram)
mg%	milligram per decilitre
ml	millilitre (10^{-3} litre)
mm	millimetre (10^{-3} metre)
mmHg	millimetres of mercury
min	minute
M	molar
MW	molecular weight (daltons)
nm	nanometre (10^{-9} metre)
N	normal
rpm	revolutions per minute
yr	year

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LITERATURE REVIEW

Diabetes mellitus is associated with many alterations in renal structure and function. In order that the reader fully comprehends the significance of renal changes in diabetes, an introductory overview of kidney structure and function in health will be presented. Although general principles of renal physiology will be discussed, emphasis will be on the fate of macromolecules, particularly proteins, in the kidney, since early diabetic renal dysfunction is heralded by abnormal renal protein handling.

(A) KIDNEY FUNCTION IN HEALTH

I. Introduction

The early writings of Malpighi in 1666 were some of the first to promote the kidney as being a physiological sieve, separating clear urine from opaque blood (1). Bowman (in 1842) localized the sieve to the glomerulus, believing that its function was to separate only salts and water from the blood (2). The filtrate was thought to be essentially protein-free (3). Subsequent investigators refined concepts of glomerular filtration and in 1917, Cushny proposed that hydrostatic ultrafiltration of plasma occurs across the glomerular wall, with subsequent resorption of solutes and water across the tubular wall (1). The process involves separation of as much as 1/3 of the total amount of plasma entering the glomeruli into a nearly ideal ultrafiltrate. Though the permeability properties are such that water flows with little resistance offered to it,

the glomerulus impedes flow of all but the smallest plasma proteins (4) such that the bulk of these remain in the circulation (5). The small amount of protein that normally crosses the glomerular wall is, for the most part, resorbed by the proximal tubule (5).

Thus, proteins pass from serum into urine as a result of a combination of selective filtration and resorption. The structural basis of these two mechanisms will now be discussed.

II. The Structural Basis of Kidney Function

(1) Introduction

The nephron is the functional unit of the kidney, responsible for the formation of urine. There are approximately 2 million in each kidney. Along the length of the nephron are several morphologically distinct regions. Located proximally is thin-walled Bowman's capsule, which is indented by a tuft of capillaries, the glomerulus. These two structures comprise the renal corpuscle (Fig. 1). Embryonically, the renal corpuscle arises from the indentation of a blind terminal expansion of the epithelial uriniferous tubule by the glomerulus. In this way, there is a visceral layer of epithelium applied to the capillaries, and a parietal layer, comprising the capsular epithelium. Between these two is the slit-like cavity of Bowman's space. There is a vascular pole of the renal corpuscle, where afferent and efferent blood vessels enter and leave the glomerular tuft and a urinary pole, where Bowman's space is continuous with the lumen of the next segment of the nephron, the proximal tubule. The proximal tubule consists of a convoluted and straight portion and is followed by the thin segment of the loop of Henle.

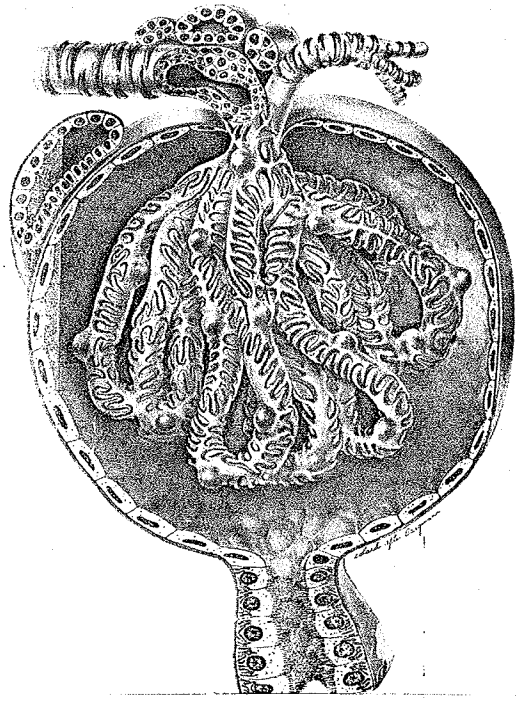

Fig. 1. Schematic representation of the renal corpuscle. Afferent and efferent arterioles enter and leave the vascular pole of the glomerular tuft at top left and right. Parietal layer of Bowman's capsule and visceral layer of podocytes overlying the capillaries are greatly oversimplified. Bowman's space is continuous with the proximal tubule at the urinary pole (bottom) (after 6).

This is in turn followed by the straight and convoluted portions of the distal tubule. The latter is continuous with the collecting ducts, which deliver urine to the papillary ducts and eventually out of the kidney (6).

The renal corpuscles are efficient ultrafiltration devices which clear blood of wastes. About 1/5 of the plasma volume that flows through the kidneys is filtered off at the glomerular tuft. This glomerular filtrate (about 125 ml/min) is modified as it passes through each of the nephron's segments, until the final product, urine (about 1 ml/min), drains through the collecting ducts to the renal pelvis and ureter for elimination (6).

(2) Glomerular filtration

The filtration pathway consists of three anatomically distinct layers: the endothelium, the glomerular basement membrane and the glomerular epithelium (Fig. 2). The innermost endothelium comprises flattened cells whose nuclei are located axially. The cytoplasm of the endothelial cell encloses the capillary lumen in a 30 nm-thick layer and is traversed by multiple fenestrae 50-100 nm in diameter (7, 8). A glycoprotein cell surface coat covers the endothelial plasma membrane (8) thereby partly narrowing the endothelial fenestrae (9). This coat, up to 12 nm in thickness, is a surface extension of the plasma membrane and consists of branching chains of sugar moieties. The cell coats are negatively charged (8), due to the presence of anionic radicals in the form of carboxyl groups of sialic acid (10), and other anionic sites.

The middle layer, the glomerular basement membrane, is bound on its inner and outer aspects by endothelium and epithelium, respectively

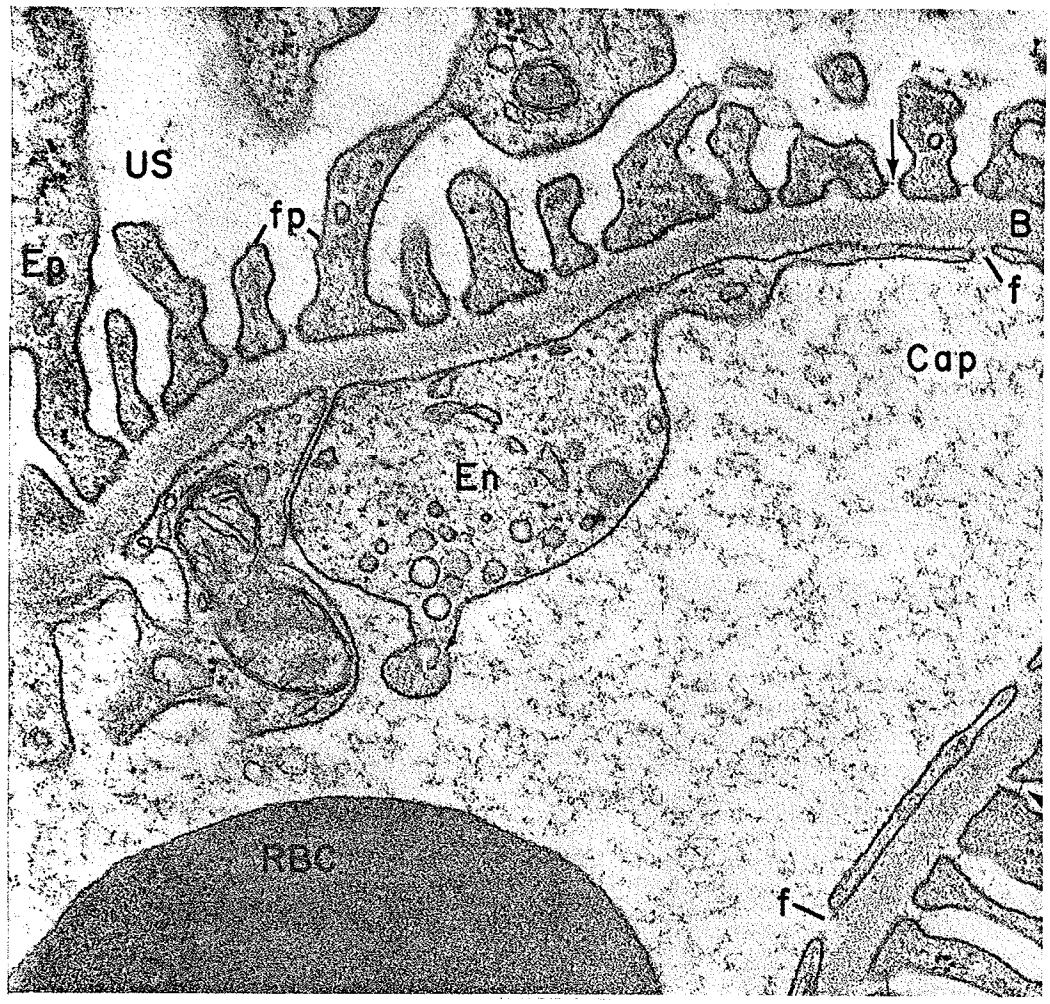

Fig. 2. Electron micrograph of an area of rat glomerulus, illustrating the three layers in the filtration barrier: the endothelium (En) with its periodic fenestrations (f), the continuous layer of basement membrane (B), and the epithelium (Ep) with its foot processes (fp). Arrows point to the thin slit membranes bridging adjacent foot processes. Cap (capillary lumen), US (urinary space), RBC (red blood cell) (x 40,000) (from 3).

(11). It is comprised of three layers, a subendothelial lamina rara interna, a central lamina densa, and a subepithelial lamina rara externa (9). The membrane is a network of 3-5 nm-wide fibrils (12) which appear to be most densely packed in the central lamina densa and much less so in the interna and externa. Fibrils in the latter two layers are arranged perpendicularly to the lamina densa and appear to attach it to the endothelial and epithelial cell layers (1, 9).

Glomerular basement membrane belongs to the collagen family of proteins and is characterized by a content rich in glycine, hydroxylysine, hydroxyproline, cystine and carbohydrate (13); the basement membrane contains no mucopolysaccharides (14) and its lipid content is less than one per cent (13). Glycine accounts for more than one fifth of its amino acid residues. Carbohydrate, in the form of glucose, galactose, glucosamine, galactosamine, mannose, fucose, and sialic acid, accounts for 10% dry weight of the glomerular basement membrane (14). Synthesis of the basement membrane appears to be the responsibility of the epithelial cells (7, 15) while endothelial cells have also been reported to participate (16). Turnover (synthesis and degradation) of the glomerular basement membrane is exceedingly slow (17).

The outermost layer of the glomerular capillary wall is composed of specialized epithelial cells, called podocytes (1). These complex cells have cytoplasmic projections from which secondary branches, the "foot processes" extend, to attach to the basement membrane and interdigitate with foot processes from adjacent podocytes (1) (Fig. 3). Each foot process is embedded in the lamina rara externa of the basement membrane to a depth of 40-50 nm (18). Between adjacent foot processes are the

Fig. 3. Scanning electron micrograph of an area of the glomerulus, illustrating the complexity of interdigitating podocyte foot processes surrounding the cylindrical capillaries (after 6).

filtration slits, which are completely filled by an epithelial cell coat derived from contiguous epithelial cell plasma membranes (8). The epithelial cell coat is similar in its anionic nature to the endothelial cell coat. At their narrowest, the filtration slits are 24 nm in diameter, and are bridged by a thin membrane (7nm thick), the filtration slit diaphragm (1). Viewed en face, toward the capillary lumen, the slit diaphragms may be visualized as flat narrow ribbons bridging the space between adjacent foot processes (9).

In addition to the endothelial and epithelial cells of the glomerulus, there is a third specialized cell type, the mesangial cell. Location of the mesangial cell is described as being centrolobular and deep, a region distinct from that occupied by endothelial and epithelial cells. The mesangium is bound largely by endothelial cells, and partly by the central layer of the basement membrane (Fig. 4). The intercellular mesangial matrix has a morphologic appearance similar to that of glomerular basement membrane, and is often referred to as "basement membrane-like material" (19). Immunohistochemical studies have revealed the presence of type IV procollagen, type I collagen, fibronectin, and laminin in the mesangial matrix (20). Continuity between the endothelial cell and the mesangium may provide a pathway for the movement of material from the peripheral capillary loop to the mesangium; this process is unique in that entry of a substance does not require its passage through the capillary basement membrane (19). A number of macromolecules have been shown to be sequestered by the mesangium (see 19 for review), but movement of large macromolecules is impeded compared to that of smaller molecules (19).

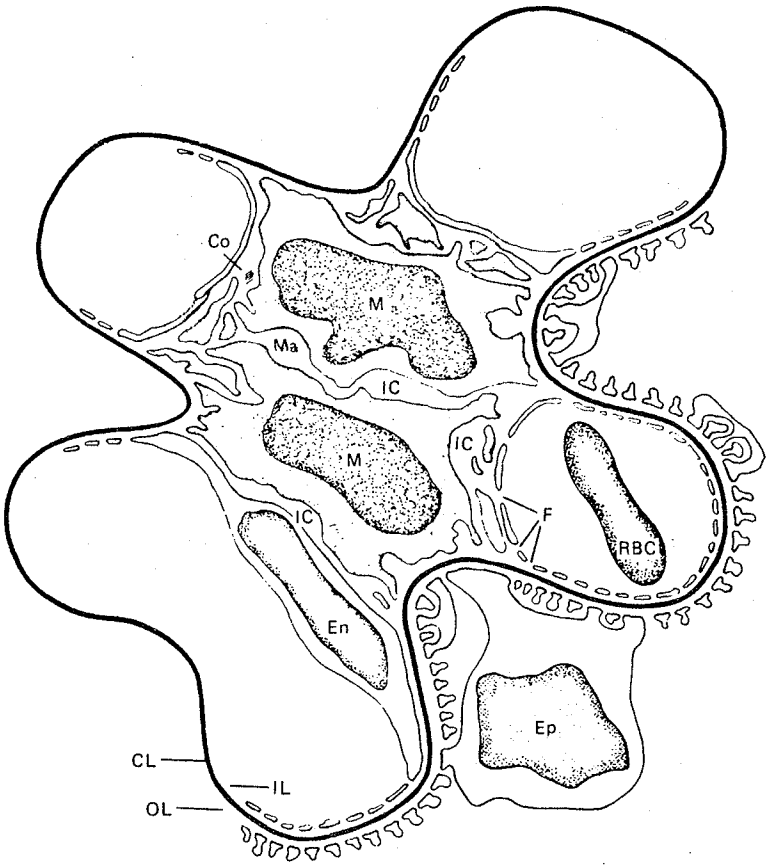

Fig. 4. Schematic representation of an area of rat mesangium, bound by endothelial cells (En) and by the central layer of the basement membrane (CL). Mesangial cells (M) are partially surrounded by mesangial matrix (Ma) in which bundles of collagen (Co) can be found. Fenestrations (F) in the endothelial cells allow movement of particles and plasma from inside the capillary to intercapillary and intercellular (IC) channels. Ep (epithelial cells), OL (lamina rara interna), IL (lamina rara externa), RBC (red blood cell) (from 12).

As exemplified in the above discussion, glomerular capillaries have a specialized structure/function relationship, which may be considered atypical of capillary systems in general. They differ from other capillary beds in that: (1) the endothelial fenestrae are larger than those found in intestinal, endocrine, or other fenestrated capillary systems; (2) the endothelial fenestrae lack diaphragms; (3) the basement membranes are much thicker because they arise as the result of fusion of endothelial and epithelial basement membranes; (4) there is no aspect of the basement membrane which abuts on connective tissue; (5) they possess interdigitating foot processes in the epithelial layer; and (6) they have, on the luminal side of the basement membrane, specialized mesangial cells (analogous to the pericyte) (3, 21).

To reiterate, the extracellular route that macromolecules follow in traversing the glomerular filter consists of (1) endothelial fenestrations; (2) the glomerular basement membrane; (3) the filtration slit diaphragms; and (4) the filtration slits. Although we know much of the physiological and ultrastructural properties of the glomerular filtration mechanism, the exact location of the site of ultrafiltration remains an enigma. The mesangial layer, being restricted to axial regions and therefore incomplete, was never considered in this context (3). Theoretically, the barrier should be located proximally with respect to filtration, to prevent the accumulation of unstirred layers of larger molecules within the filter which could progressively impair filtration efficiency (9), i.e., excluded molecules should be rejected at the most upstream surface, the endothelial fenestrae (9). However, it was soon realized that these large fenestrae (50-100 nm) could restrict passage of only the cellular elements of the blood. That

left two other potential candidates: the basement membrane and the epithelial slits (17).

Most morphologists believe the basement membrane to be the main filter, because it is the only continuous layer in the capillary wall. The basement membrane has charge discrimination because of the fixed negative charges attributable to the sialic acid groups of its glycoprotein matrix, i.e., molecules with a net negative charge would be repelled more so than those with a fixed positive charge. The basement membrane also appears to have size discrimination. Recently, the concept of biological thixotropy has been proposed to explain how molecules traverse the basement membrane (21). Thixotropy refers to the dependence of flow properties on time (22) and the function of basement membranes is suggested as resulting from their thixotropic nature. Thixotropic membranes, composed of an internal structure of fibre-like units arranged into a fully hydrated lattice (21) allow small molecules and solutes to pass freely through, while forcing larger molecules to cause localized deformation of the gel in order to traverse the membrane. Energy required to deform the lattice could come from increased intercapillary pressure (21).

As early as 1957, Hall (23) proposed the epithelial slits as the major filtration barrier for proteins. The filtration slits have comparable charge discrimination due to the anionic nature of the epithelial cell coat. The diameter of the filtration slit determines its capacity for size discrimination.

Work with tracer molecules has given mixed results as to the definitive "barrier" (3, 4, 9, 11). Overall it appears that different molecules are handled by different filtering elements according to their

steric and electrical properties (discussed in a later section). In other words, there is no single barrier to glomerular filtration, but instead a series of three filtering elements, each doing its part to keep the bulk of the plasma proteins in the circulation.

(3) Reabsorption

Most of the plasma proteins present in normal glomerular filtrate are subsequently reabsorbed by the cells of the proximal tubule (2). Concentrations of proteins in fluid from Bowman's space and proximal convoluted tubules (obtained by micropuncture) far exceed concentrations seen in normal urine, i.e., the filtered load is greater than the final urine concentration (24). Thus, reabsorption must take place along the nephron. It is apparent from loading and clearance studies (25, 26, 27) that the reabsorption of proteins is a transport maximum-limited process, i.e., it is saturable. In these experiments tubular cells are found to take up protein until a certain saturation dose is reached; beyond this point, increased excretion and decreased resorption occurs. However, it is generally thought that the reabsorptive capacity is not fully used (28). Theoretically, endocytosed proteins may be catabolized within the tubular cells or returned intact to the circulation by contraluminal transport (29). In general, absorbed proteins are thought to quickly associate with hydrolytic enzymes in lysosomes, with resultant intracellular digestion (2, 29). However, the relative contribution of intracellular digestion and contraluminal exocytosis to protein disposal is still disputed (2).

III. Factors Which Influence Urine Protein Concentrations

1. Introduction

Approximately 60% by weight of the proteins in normal urine originate from plasma, the remaining 40% being of renal and urogenital origin. Proteins originating from plasma include albumin (40%) and small-sized immunoglobulins (and their fragments), enzymes and peptide hormones (20%). Proteins of uroepithelial origin include tissue proteins, antigens and glycoproteins (40%) (30). This composition will, of course, be affected by many physiological and nonphysiological parameters (2).

Determinants of protein filtration and resorption include: (1) structural properties of the filtering and reabsorbing devices, as discussed; (2) properties of the molecules themselves; and (3) hemodynamics and blood rheology. These latter two will now be discussed. Since the kidney plays an important role in albumin homeostasis (29) and since albumin is the major protein component of normal serum and urine, discussion will focus on this protein molecule, although the principles raised will be generally applicable.

2. Factors Influencing Filtration of Proteins

(a) Properties of the Molecule

Two of the most important factors determining the fate of a molecule in the glomerulus are the size and charge of the molecule, since the glomerular filter discriminates largely on the basis of these two parameters. Fractional clearance studies (31) estimate the average "pore" radius of the glomerular capillary wall to be from 3.6nm based on the clearance of proteins, to 4-6 nm based on the clearance of uncharged molecular species (5). Studies using dextrans have shown that the filtration of

substances decreases progressively as the molecular size of serum albumin (3.6nm) is approached (1, 32, 33). This physical phenomenon is termed steric hindrance, the degree of which depends on the ratio of effective molecular radius of the molecule to the effective pore radius (2).

If molecular size were the only factor determining filtration of macromolecules, a fair amount of albumin would be lost in the urine, i.e., the filtration of albumin is restricted to a greater degree than one would expect on the basis of molecular weight alone (4). Furthermore, fractional clearance of neutral dextrans, having the same molecular radius as albumin, is much greater than that of albumin or of anionic dextrans of equal size (33). On the basis of these results, many investigators (9, 33, 34) propose that the restricted filterability of poly-anionic species, such as albumin, is due to the presence of fixed negative charges on the glomerular capillary wall. This phenomenon, termed electrostatic hindrance, depends on the electrical interaction between cationic or anionic groups on the solute molecule and areas of negative charge density on the glomerular cell coats and basement membrane (2). In addition to decreasing the filtration of negatively charged species, the highly anionic glomerular barrier enhances the filtration of positively charged species (4). Venkatachalam and Rennke (9) envisage the combined roles of molecular size and charge in the following manner: large molecular species are excluded from the filtration matrix, and hence the filtrate, on steric grounds alone. Entry into the matrix of molecules smaller than the pores is regulated by electrostatic interactions in addition to the disparity between molecular and pore radii. Cationic or neutral intermediate-sized molecular species are able

to penetrate the filtration matrix, whereas a functional electrostatically-induced narrowing of the pores excludes polyanionic species of intermediate size (for example, albumin). Small molecules enter the polymer matrix regardless of charge, but repulsive or attractive forces may determine the rate of molecule movement, and therefore, the quantity filtered (9) (Fig. 5). Robinson (2) sees charge interaction as playing a lesser role in determining transglomerular passage of the smallest macromolecules, increasing in importance as the effective radius of the macromolecule approaches that of the glomerular "pore." The relative contributions of molecular size and electric charge to transglomerular passage have not been elucidated for any significant number of plasma proteins (2).

Deformability of a macromolecule may also affect its permeability in the filtration matrix. Dextran molecules, for example, form loosely coiled hydrated spheres in free solution and are, therefore, elastically compliant under the stresses of ultrafiltration (9). These large polymeric molecules may unfold and elongate to slide through the matrix of the filtration barrier, a process called reptation (35). Globular proteins, on the other hand, are folded into compact units that approach spheroidal shapes, e.g. albumin, hemoglobin, fibrinogen, etc. (36). With their rigid crosslinking, they are much less likely to unfold under the same conditions (1).

(b) Hemodynamics and Blood Rheology

While charge and molecular size are important variables in determining transglomerular passage of macromolecules, convective and diffusive forces operating across the filtration apparatus must be considered important governing factors as well. Since the fractional clearance of

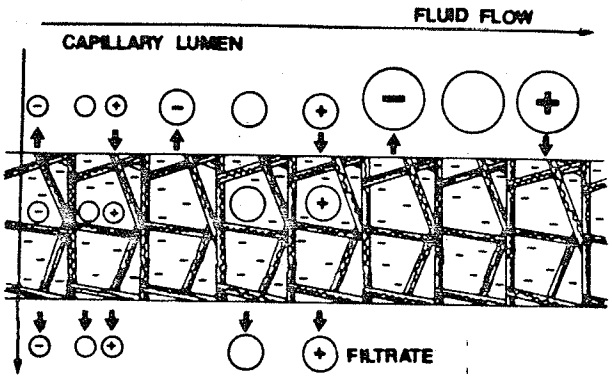

Fig. 5. Schematic representation of the filtration of macromolecules of different size and charge through a hypothetical anionic membrane. Circles with negative, positive, and no signs represent macromolecules with negative, positive, and zero net charge. Solvent flow (horizontal arrow) drives molecules axially along the membrane, while net driving pressure (vertical arrow) favors filtration across the membrane. Large molecules (at right) are excluded from the membrane on steric grounds alone. Intermediate-sized molecules (center) with net positive or zero charge may enter and pass through the membrane while those with net negative charge are restricted due to electrostatic repulsion. Small molecules (at left) can enter and pass through the matrix, but repulsive forces may reduce the rate of movement of negatively charged species (from 9).

a molecule is affected by the glomerular filtration rate (GFR) of water, hemodynamic determinants of GFR will influence solute movement (33). The major determinants of glomerular ultrafiltration are: (1) the glomerular capillary plasma flow rate; (2) the degree of imbalance between hydrostatic and oncotic or colloid osmotic pressures across the capillary wall as proposed by Starling (37); (3) the glomerular capillary plasma protein concentration; and (4) the ultrafiltration coefficient (water permeability of the filter times the surface area of the filter) (1, 33).

A renal blood flow or renal plasma flow rate of about 1200 ml/min restricts the passage of macromolecules through the glomerular filter (2). Albumin does not significantly penetrate beyond the endothelial fenestrae during normal blood flow; if blood flow is stopped, albumin passes through the basement membrane and enters the urinary space (38). These results conform with the proposals of Pappenheimer, involving the concept of molecular sieving across capillary walls; with an increase in rate of solvent flux, molecules are subject to increasing restriction from the theoretical pores (39). In other words, a decrease in capillary plasma flow rate results in a disproportionately greater increase in the average solute flux than volume flux (33), i.e., macromolecules approach diffusion equilibrium with the ultrafiltrate (1).

According to Poiseuille's formula, blood flow is inversely related to the viscosity of the blood and directly related to the driving pressure. Viscosity is the measure of internal friction resulting when a layer of fluid is made to move in relation to another layer (22). The progressive loss of glomerular filtrate from afferent to efferent arteriole results in a coincident increase in viscosity, such that the blood traversing the

efferent vessel is of a higher viscosity than that at the afferent. If viscosity increases such that the increase in pressure required to prevent blood stasis is able to deform the filtration membrane, protein will leak through (40).

The driving pressure for ultrafiltration depends on the balance between hydrostatic and oncotic forces across the glomerular capillary wall (37). Blood enters the glomerular tuft with a hydrostatic pressure of approximately 70 mmHg; this tends to force fluid out of the capillary into Bowman's space, and thus favors filtration. A plasma oncotic pressure of about 32 mmHg and a capsular interstitial pressure of about 20 mmHg oppose filtration. Net filtration pressure is, therefore, about 18 mmHg (6). An increase in the driving pressure for ultrafiltration (as a result of chronic ad libitum protein intake (41) or increased arterial blood pressure (42), for example) will result in increased protein flux and albuminuria (41).

Thus, it appears that excess protein leakage through the glomerular sieve may occur if alterations occur in the balance between convective and diffusive forces operating at the glomerular barrier. The glomerular sieve has a functional dependence in addition to its structural dependence, as proteinuria may result in the absence of morphological damage (1). The following two examples illustrate this point:

Posture and Protein Excretion. The earliest descriptions on the effects of posture on protein excretion are attributed to Ulzmann (in 43). According to Mogensen (44), urine albumin excretion rate during recumbency is on average 52% of that during upright ambulation, i.e., most of the albumin in normal urine is excreted during maintenance of

the upright posture (45). It does not appear to be the result of an intrinsic diurnal cycle since the increase in albumin excretion occurs regardless of the time of day the upright sample is collected (45). The mechanism of the increased protein excretion during upright ambulation is as yet unknown, but hemodynamic factors may play an important role. Upright tilting is known to decrease renal blood flow and glomerular filtration rate (46). This normal upright reduction of renal blood flow may facilitate protein transport across the glomerular wall (45).

The characteristic response of healthy people to assumption of the upright posture is identical to, albeit less dramatic than, that seen in postural or orthostatic proteinuria. According to Rytand and Spreiter (47) and Glassock (48), orthostatic proteinuria is an apparently benign disorder affecting 0.6 -5.8% of young people (48). In the majority of cases, no renal lesion can be demonstrated (49). The hypothesized mechanism is that the normal hemodynamic and humoral adjustments to the assumption of the upright posture (48) act together with a defective glomerular barrier to increase protein transfer (50) beyond the capacity of the tubular reabsorptive mechanism (2). Hemodynamic factors may then be considered to play a permissive, rather than primary, role (50).

Exercise and Protein Excretion. Exercise proteinuria was first noticed by von Leube in 1878 (in 43). There are many reports of increased protein excretion under physical exertion in healthy adults (43, 51-54). Huttunen et al. (55) report that albuminuria occurs in children and adolescents as well, affecting both sexes equally, regardless of subject age. Exercise-induced proteinuria appears to be the result of increased filtration of plasma solutes, as 82% of the proteins of

exercise urine are of plasma origin (compared to 60% at rest); albumin, in particular, constitutes 59% of the post-exercise urine protein (compared to 40% at rest) (52). The exact mechanism of exercise-induced proteinuria is unknown. Exercise causes a number of hemodynamic changes, including an increase in heart rate, blood pressure and cardiac output (55). The changes in renal blood flow are of particular interest. The body shunts blood from the kidneys to the working muscles by constriction of afferent and efferent renal arterioles, resulting in a fall in renal blood flow (54). The transport of water and macromolecules across the filtration barrier is related to the transcapillary pressure (determined in part by the blood pressure) and the capillary blood flow. When blood pressure rises in response to vigorous exercise, transport of both water and macromolecules is favored. The declining renal blood flow favors the clearance of macromolecules in relation to water flux (55). Glomerular filtration rate also declines during effort, but the decrease is small compared to the reduction in renal blood flow, so that filtration fraction is increased (56).

3. Factors Influencing Reabsorption of Proteins

According to Strober and Waldmann (29) molecular weight does play a role in protein reabsorption, but it is the permeability of the macromolecule with respect to filtration that largely determines the degree of reabsorption. Small proteins (< 50,000 MW) readily pass through the filtering mechanism and are largely taken up and catabolized by the tubules. The kidney is, therefore, a significant catabolic gate

for this class of proteins (29) . Intermediate and large proteins (> 60,000 MW) normally do not pass through the filtering mechanism in great quantities so that the tubules would not be the primary site of catabolism (29). The quantitative differences in tubular catabolism between small and large MW proteins suggests that a key factor determining tubular catabolism of protein is its tendency to traverse the glomerular filter (29). Albumin (MW 69,000) leaks through the glomerular filter in minute amounts, and appears in tubular fluid. Here, more than 90% of that filtered is reabsorbed and consequently digested, representing no more than 10% of total albumin catabolism (57).

The plasma protein concentration in the peritubular capillaries influences sodium and water resorption along the renal tubules through its affects on Starlings forces (58). Protein reabsorption capacity may be influenced similarly.

IV. Mechanisms of Pathologic Proteinuria

In examining the complex structure/function of renal filtration and reabsorption it becomes obvious that even slight defects in these two processes will lead to abnormal urine protein concentrations, i.e., proteinuria. Robinson (2) suggests four major mechanisms whereby proteinuria may occur: (1) "overflow" proteinuria due to elevated plasma concentrations of normal or abnormal proteins, e.g. Bence-Jones proteinuria (59); (2) elevated tubular protein secretion, e.g. Tamm-Horsfall proteinuria (60); (3) diminished tubular reabsorption of normal filtered amounts of protein, e.g. Wilson's disease (61); and (4) increased

glomerular permeability secondary to a structural or functional alteration of the filtration surface, e.g. diabetic proteinuria.

(B) KIDNEY FUNCTION IN TYPE I DIABETES MELLITUS

I. Introduction

Diabetes mellitus comprises a genetically and clinically heterogeneous group of disorders that share glucose intolerance as a basis. The subclass of interest is Type I, insulin-dependent diabetes mellitus (also inappropriately referred to as juvenile diabetes). The syndrome is characterized by insulinopenia, abrupt onset of symptoms, proneness to ketosis, and dependence on exogenous insulin to sustain life (62).

Vascular lesions are a prominent feature of long-term diabetes mellitus, the most characteristic of these being microangiopathy (literally "small vessel disease") involving the arterioles, venules and capillaries (63). Diabetic microangiopathy affects all the body organs, but is clinically significant in the kidneys and eyes (64); nephropathy and retinopathy are terms used to describe the pathology, clinical and structural, of these two organs in advanced diabetes. While the discovery of insulin sixty years ago greatly extended the expected life span of the diabetic individual, it has done little to resolve the problem of small vessel disease, which now poses the greatest threat to survival (17). There is a close association between nephropathy, retinopathy and neuropathy (nerve disease) in diabetes mellitus, according to the prospective study of Pirart (65).

According to Deckert et al. (66), mortality rates in a diabetic population are 2-6 times that in a comparable non-diabetic population,

even though 50% of subjects survive their disease for more than 35 years. Furthermore, his investigation revealed that the age of diabetes onset (0-30 years) did not influence the prognosis. The metabolic disturbance of diabetes mellitus, which prior to the discovery of insulin lead to 75% of the mortality, now accounts for only a small percentage of deaths (67). Deckert et al. (68) also examined the prevalence of diabetic nephropathy and concluded that it is the most serious diabetic complication, leading to early death. The risk of developing nephropathy has been reported to be about 30%, the remaining 70% never developing this serious complication despite long diabetes duration (68). Apparently, many people with Type I diabetes are "protected" against the deleterious effects of the disease on the renal apparatus (69). Sex, family history, age (65), body build, insulin sensitivity, stability of diabetes and renal tubular function are not features which distinguish these two groups; the nature of the protective mechanism is as yet unresolved (69). After renal function begins its decline, the progression is often relentless and accelerating (70). Maintenance dialysis is required about 2.8 years after the appearance of renal disease (71). Since the present methods of treating end-stage renal failure, dialysis and transplantation, are not optimally suited to the diabetic patient (72, 73), it is not surprising that 50% and 75% of patients who develop nephropathy die before the twenty-fifth and twenty-eighth year of their diabetes, respectively (74). Causes of death in those with diabetic nephropathy include uremia (80%), myocardial infarction (10%), and other, 10%; in those without nephropathy, deaths are due to myocardial infarction (40%), infectious disease (15%), and other,

43% (68). Diabetic nephropathy offers a grave prognosis, but it is substantially better than has been believed in the past (75).

II. Progression of Renal Dysfunction With Time

The clinical hallmark of diabetic nephropathy is glomerular proteinuria (76). The relationship between proteinuria and survival was dealt with by Deckert et al. (66): patients who did not develop proteinuria during 40 - 50 years of diabetes had an excess mortality of 200% over the background population, whereas those who did develop proteinuria had an excess mortality of 800%. In addition, only 10% of those patients living after 40 years of diabetes showed proteinuria, whereas 61% of the deceased patients had shown persistent proteinuria (66).

At first appearance, proteinuria is usually slight and intermittent, becoming pronounced and persistent with time (77). Persistent proteinuria is usually defined, in terms of available clinical tests, as being excretion of total protein in excess of 0.5g/24 hr. at several clinic visits (68).

The course of diabetic nephropathy has been documented by several investigators. In 1951, Wilson et al. (78) found that proteinuria appeared by about 13.9 years duration, followed by edema and death two and five years later. Analysis of 112 patients at the Joslin Clinic between 1962 and 1972 showed that mean duration of diabetes at onset of persistent proteinuria was 17 years, early renal failure occurred approximately two years later, and late renal failure by 21.6 years duration. The mean duration of life after onset of proteinuria was 4.8 years with no one surviving longer than 13 years (74). In 1971,

Knowles reported that five years after the onset of proteinuria there would be 50% mortality, rising to 77% by 10 years (79). More recently, Andersen et al. (80) demonstrated that average duration before onset of proteinuria was 19 years (range 4 - 41 years) and that, on average, death ensued six years later (range 2 - 32 years). The progression appears to show marked variation; the factors responsible for this highly variable course are unknown.

The pattern of proteins excreted in the progression towards renal failure is as follows: there is an initial rise in the glomerular filtration of proteins of intermediate MW (e.g. albumin: MW 69,000; transferrin: MW 60,000) and a delayed rise in excretion rate of larger proteins as well (e.g. immunoglobulin G: MW 160,000) (81). Tubular resorption of proteins is usually considered normal until end-stage renal failure, when the possibility exists of tubular damage or blockade (82). Tubular proteinuria is characterized by a tubular inability to take up and catabolize small proteins (e.g. β_2 - microglobulin MW 11,800) which pass freely through the filtration mechanism (29).

As albumin makes up about 75% of the total protein excreted in diabetic proteinuria, it is the most widely used protein for screening and diagnostic purposes (83). Albuminuria is defined by use of available clinical methods (e.g., Albustix®), as being albumin excretion in excess of 40 mg/24 hr. (83). The term microalbuminuria is used to describe the blind area between normal albumin excretion rates (0 - 10 ug/min.) and clinical albuminuria.

There is disagreement in the literature as to the existence and progressive nature of microalbuminuria. The advent of sensitive

radioimmunoassays has made possible the measurement of minute amounts of urine albumin, i.e., down into the normal range. The conflict surrounds the nature of the passage from normal to clinically overt proteinuria. Some investigators claim that the progression occurs rapidly with all clinically non-proteinuric individuals having immuno-assayable albumin within the normal range. Others claim the progression to be gradual, with a ramp-like rise in albumin excretion to clinically measurable levels. Evidence for both sides is provided by cross-sectional studies, but definitive proof requires longitudinal investigation.

Mogensen and colleagues (44, 84-86) found normal urinary albumin excretion rates in clinically non-proteinuric Type I diabetic subjects irrespective of diabetes duration (1 - 40 years) (44). These data tend to support the hypothesis of an abrupt progression from normal to pathologic excretion rates.

In apparent contradistinction to Mogensen's findings, in 1967, Panzram et al. (87) reported an increase in total protein excretion in 130 diabetic subjects which correlated with duration of diabetes. Using disc-electrophoresis, Hemmingsen et al. (88) found urinary protein excretion to be increased right from the onset of diabetes. Major increases in excretion were evident after more than ten years duration, and there was a correlation between duration and protein excretion (88) . Keen and Chlouverakis (89) found a clear tendency for clinically normal diabetic subjects to be better represented in the higher albumin

excretion rate categories, when measured by radioimmunoassay with the reverse being true for the healthy subjects. More recently, Viberti and colleagues (90, 91) found that under ordinary conditions of life, a considerable number of Type I diabetic subjects can have elevated, although subclinical, albumin excretion rates. However, most patients who do not have clinical proteinuria after long diabetes duration generally maintain normal excretion of albumin (91).

Although cross-sectional studies such as these do give us valuable information about microalbuminuria in the diabetic population as a whole, longitudinal studies allow us to elucidate the progressive nature of microalbuminuria in individual subjects. One such investigation was carried out by Viberti et al. (92). Eighty-seven Type I diabetic patients, all clinically non-proteinuric, were screened for albumin excretion rate in 1966-67 and again in 1980-81. Development of clinical proteinuria by follow-up was related to the 1966-67 albumin excretion rate values. Clinically detectable albuminuria developed in only 2 of 55 patients (4%) with AER below 30 ug/min., but in 7 of 8 (88%) with AER between 30 and 140 ug/min.; the risk of developing nephropathy was 24 times higher for the latter group. Viberti et al. (92) concluded that although two assessments 14 years apart do not elucidate the exact nature of the passage from microalbuminuria to clinical albuminuria, the change is not likely to occur abruptly. A longitudinal study out of Denmark (93) followed urinary albumin excretion rate (by radial immunodiffusion) in 23 clinically non-proteinuric diabetic subjects over a six-year period; measurements were carried out yearly. Eight of the patients had elevated albumin excretion rate at the time of screening.

The five with the highest excretion rate showed a progressive increase in albuminuria over time and eventually developed persistent clinical proteinuria. One developed intermittent proteinuria while two remained stable. Only 2 of 15 with normal urinary albumin excretion at screening developed intermittent or persistent proteinuria during the six-year follow-up. The investigators concluded that detection of patients at risk of developing diabetic nephropathy is possible by monitoring the degree of microalbuminuria over time (93).

III. Factors Influencing Renal Function in Diabetes

(1) Role of Glycemia

(a) "Control"--An Overview

The factor(s) responsible for development of the microvascular complications of diabetes mellitus are unknown. They may be a consequence of the deranged metabolic state of diabetes or the result of an inborn error, i.e., genetically determined (77). Clarification of the role that carbohydrate intolerance, and other metabolic disturbances attributed to insulinopenia, play in determining the development of microangiopathy is of utmost importance in establishing clinical and research goals (94) . There is a wide range of opinion regarding the benefits of "tight" control. Proponents of the "metabolic" hypothesis believe that normoglycemia delays or prevents the occurrence of microvascular complications (95); evidence has often been in the form of retrospective personal impressions. Opponents of the hypothesis believe that vascular disease will occur regardless of the level of glycemia (96); they point out the occurrence of complications in

apparently well-controlled patients, the emotional demands of rigorous diabetes care, and the dangers associated with risk of hypoglycemia (95).

For ethical and practical reasons, it is virtually impossible to compare perfectly controlled and uncontrolled diabetic patients; it is only feasible to compare varying degrees of imperfection. Many retrospective studies appear to correlate degree of control with microangiopathic complications. For example, Keiding et al. (97) and Harden et al. (98), assessing glycemia more or less on the presence or absence of glycosuria, concluded that degree of control does influence complication rate. Johnsson (99) published a report in 1960 on the incidence of vascular complications in 159 patients. Diabetic subjects treated with multiple insulin injections and strict dietary control had a much lower incidence of nephropathy (32%) compared to diabetic subjects treated with single injections and less regimented dietary control (56%), even though mean diabetes duration was 10 years longer in the former group (99). Pirart (65) examined 4,400 patients in a prospective study and found that poor control assessed over 26 years of diabetes was significantly related to the incidence of microangiopathy; the incidence was also clearly related to the degree of glycemic control achieved during the year immediately preceding the annual examination (65).

However, there are examples of studies which were unable to demonstrate a positive effect of quality control. Knowles (96) investigated more than 300 reports up to 1964 and concluded that sufficient proof was not at hand. Bondy and Felig (100) surmised that belief in the treatment effect was based on faith rather than

scientific proof. Retrospective analyses by Knowles et al. (101) and Dolger (102) reported that vascular complications occurred at an equal rate regardless of the level of control. Even so, most retrospective studies strongly suggest that poor diabetes control is associated with more severe forms of microangiopathy, even if they don't prove that good control is beneficial (103). In addition, very strict control is thought to slow down the progression of established lesions (104, 105).

(b) Glycemia and Microproteinuria

Although it is generally thought that the proteinuria characteristic of advanced nephropathy is not reversible (82, 106), the following few examples suggest that early microalbuminuria is amenable to improvement of the metabolic state. Mogensen (44) found that initiation of insulin treatment in newly diagnosed diabetic subjects induced a significant fall in albumin excretion rates. Lauritzen et al. (107) achieved near-normal levels of glycemia using continuous subcutaneous insulin infusion, and were able to demonstrate a 12% reduction in albumin excretion rate over six months; excretion rate in comparable subjects rose by 56% when left on conventional therapy. Viberti (91) found that strict metabolic control, even in the short term (1 - 3 days) caused a significant reduction in albumin excretion rate in seven diabetic subjects. Ciavarella et al. demonstrated the strict metabolic control could ameliorate the albumin excretion rate in microalbuminuric patients and also in patients with clinical evidence of nephropathy (108). In addition, studies have shown that the reverse is true too, i.e., moderate increases in urinary albumin excretion occur during periods of poor metabolic control (109,

110). Parving et al. (63) measured the transcapillary escape rate for albumin (a measure of microvascular permeability in extra-renal tissue) and found it to be elevated under conditions of poor metabolic regulation. This demonstrates how generalized the "leakiness" of the microvasculature is during poor control.

Animal studies provide further evidence for the role of glycemia in functional derangements. For example, streptozotocin-diabetic rats exhibit elevated urinary protein excretion with onset of glycosuria and polyuria; institution of insulin therapy results in a prompt and significant decline in proteinuria (111, 112). Transplantation of islet tissue into diabetic rats results in a rise in insulin levels, and a fall in glucose and albumin excretion rate (113-116). Exposure to cold water leads to hyperglycemia and an increased diffusion capacity to albumin in the rete capillaries of the eel (117).

One can only speculate on the significance of the reversible fluctuations in microalbuminuria. Whether this early glycemia-dependent microproteinuria is the functional counterpart of subsequent structural-dependent clinical proteinuria is unknown. If it is causal, early detection and strict treatment might prevent the development of clinical nephropathy (82). Only interventions exercised before clinical nephropathy develops can influence the outcome of diabetic nephropathy (118). In addition, it is not known to what extent the abnormal glomerular permeability contributes to the progression of structural abnormalities (108). Mogenson (119) suggests that hyperglycemia is likely to be active in initiation and maintenance of the abnormalities.

The link between early renal changes and overt nephropathy may be provided by one or more of the following mechanisms:

(1) increased extravasation of plasma proteins as a function of metabolic control (discussed above) may be important in the genesis of nephropathy;

(2) hemodynamic changes which operate in the early reversible fluctuations in renal function may contribute to the development of irreversible lesions; and

(3) gradual alterations in the filtration surface that influence the functional capacity of the glomerulus may eventually lead to overt proteinuria (119). These latter two aspects will now be discussed.

(2) Hemodynamics, Renal Size and Blood Composition

The phenomenon of renal hyperfunction has puzzled researchers because of the apparent paradox that early diabetes is associated with a supranormal state of kidney function while renal insufficiency and uremia are often long-term consequences (120). Changes in renal hemodynamics probably play a role in the pathogenesis of diabetic nephropathy.

There is general agreement in the literature that glomerular filtration rate (GFR) is elevated very early in Type I diabetes mellitus. As early as 1959, Stalder et al. (121) reported increased GFR in newly diagnosed diabetic children. Since then, Mogensen (85, 122-124), Ditzell and Junker (125), Christiansen et al. (126), Ditzell and Schwartz (127), and Ditzell (128) have found elevated GFR in early diabetes. Renal plasma flow is usually found to be decreased (125) or unchanged (124),

although a few have reported it to be increased (123, 126). As a result, filtration fraction is almost invariably elevated in diabetes (72), reflected in an increased extravasation of plasma proteins.

Since this functional disturbance may be normalized by strict insulin therapy (129), blood glucose is hypothesized to have a direct causative role. Brochner-Mortensen (130) found significantly elevated GFR in diabetic subjects during moderate hyperglycemia, but others (131) have been unable to produce increases in GFR by glucose infusion.

Humoral factors have been implicated in elevated GFR production, including growth hormone (122) and glucagon (132, 133), both of which are secreted in excess in young diabetics (132, 134). Other hormones such as catecholamines, renin angiotensin, prostaglandins, etc., may influence the GFR as well (135).

In addition to biochemical or hormonal perturbations, there are altered morphologies associated with early diabetes which may be responsible for an increased GFR. There is a generalized increase in kidney size in both experimental (120, 136) and human diabetes (137), and a correlation exists between kidney size and function (137). In experimentally-induced diabetes, the kidney grows by an admixture of cellular hypertrophy and hyperplasia (120). There is non-uniformity of growth such that initially, glomerular growth is more pronounced than whole kidney growth; glomerular volume increases by 30% after four days of streptozotocin diabetes (136). The changes in glomerular dimensions may account in part for the greater filtration rates (138). Experimental diabetic renal hypertrophy is related to the severity of diabetes and growth relates linearly to blood glucose (120). Rigorous insulin

therapy can reverse the renal hypertrophy (139). Mogensen and Anderson (137) were able to associate increased GFR in short-term insulin-dependent diabetic subjects with increased kidney size, which was measured by an invasive procedure. In 1981, Christiansen et al. (126) measured kidney size with noninvasive ultrasound and found similar size-function correlations. As in experimental diabetes, the increase in kidney size is accompanied by glomerular enlargement (140) and may involve expansion of the glomerular filtration surface. This morphological change could well represent the mechanism behind the increased GFR (141). Kidney size changes are amenable to insulin treatment in human diabetes, as in experimental diabetes (129).

Mogensen (86) suggested that elevated GFR may be the result of a combined effect of increased filtering area and filtration pressure. Hostetter et al. (138) found significant increases in glomerular transcapillary hydraulic pressure gradients in experimental diabetes mellitus which could partially account for glomerular hyperfiltration. Christiansen et al. (126) reported comparable increases in hydrostatic pressure gradients in 13 human diabetic subjects with elevated GFR. Evidence from several organs suggests increased filtration pressure in the microcirculation during periods of poor metabolic control (190). Increased filtration pressure can result from impedance of blood flow through the efferent arteriole (51). In periods of poor control, diabetes is associated with increased blood viscosity, impaired erythrocyte deformability and increased erythrocyte aggregation (142-144). The rise in intravascular pressure required to overcome the increased peripheral resistance of viscous blood in the efferent

arteriole (40) leads to intra-glomerular back pressure. It is hypothesized that proteinuria indicates a higher than normal intra-glomerular pressure at the time of filtration (51). Viberti et al (145) suggest that in microproteinuric individuals (AER <60 ug/min) increased intraglomerular pressure is primarily responsible for the higher proportional filtration of both albumin and IgG. In addition, the renal arterioles in diabetes may have an impaired ability to change calibre in response to pressure changes, due to increased rigidity; this may lead to increased wall stress and enhance permeability (146).

Hypertension and Diabetes

Parving et al. (42) have shown that albumin excretion rate is elevated in subjects with insufficiently treated benign essential hypertension, although these patients are clinically non-proteinuric; urinary albumin excretion rate correlated with systolic, diastolic and mean blood pressure. The rapid reversibility of albuminuria with treatment suggests glomerular capillary pressure as the major factor involved (76). These findings are important in view of the fact that a rise in arterial blood pressure to hypertensive levels is frequently present in young Type I diabetic patients with nephropathy (147, 148). A clear rise in serum creatinine and blood pressure is found in patients developing persistent proteinuria (93). A small increase in systolic pressure may be seen from early on in the disease, while diastolic hypertension is closely related to renal involvement (149). The close temporal association between onset of proteinuria and onset of hypertension suggests that the hypertension is renal in origin (150). It seems that not until the development of clinical nephropathy does blood

pressure begin to rise and serve as an accelerating factor in progression of renal disease (146), i.e., it is not responsible for nephropathy, but instead is an aggravating risk factor (151). For example, Berkman and Rifkin (152) reported a case of unilateral renal artery stenosis and diabetes. The kidney exposed to hypertension developed marked nodular glomerulosclerosis, whereas the stenotic kidney revealed little change attributable to diabetes. In addition, diabetic rats with unilateral nephrectomy show accelerated glomerulosclerotic changes, probably due to elevated glomerular filtration pressure (153).

Early detection of those at risk and placement on aggressive antihypertensive treatment may postpone end-stage renal failure (148, 154). Parving et al. (76) reported decreases in albuminuria with antihypertensive treatment, which they attributed to reduced intraglomerular filtration pressure. Similar results were obtained by Mogensen (155). Mogensen (156) was able to slow the usually inexorable rate of decline in glomerular filtration rate by lowering the blood pressure in six long-term diabetic subjects. The preservation of kidney function with aggressive antihypertensive treatment is so far the only method available in nephropathic individuals, short of dialysis and transplantation (156).

Exposure of diabetic glomeruli to increased flow and pressure accelerates the development of glomerulopathy (157) once the lesions are established (113). We know, however, that enhanced flow rates and pressures are not the only factors responsible for eventual decline

in kidney function towards renal failure. Kidneys protected from these increases often demonstrate diabetic renal disease, emphasizing once again the role that metabolic dysfunction plays in the sequalae of diabetes. Once clinical proteinuria is established, GFR begins its progressive decline. Although the progression varies considerably from patient to patient, the rate of fall of GFR in any one individual is rather constant (148).

The exact causative link between hemodynamic hyperfunction and renal hypertrophy and/or progression to renal failure has not been elucidated. The functional changes may participate in development of the long-term consequences of diabetes and may, in fact, be precursors of the degenerative vascular changes (158).

(3) Structural and Biochemical Alterations in the Filtration Surface

To this point, discussion has centered mainly on functional changes associated with diabetes, but there are also many structural changes seen in the diabetic kidney. In addition to the glomerular hypertrophy discussed previously, changes in the actual filtration surface range from the structural and biochemical alterations in the basement membrane seen early in the course of the disease to the pathologic lesions associated with long-term diabetes.

Basement membranes serve a supportive role as well as participating in selective filtration. They can be found in a variety of tissues, including the renal glomeruli and tubules, blood capillaries, alveoli, the retina and lens capsule, muscle, Schwann cells, sweat glands, mammary ducts and thyroid follicles (14). Histologic

changes in the amount of basement membrane material in diabetes have been noted by many authors. Initially, investigators noted increases in hydroxyproline content of whole glomeruli (159) or its basement membrane (in 14); this was interpreted as indicating an absolute increase in basement membrane material in diabetes. Beisswenger and Spiro (160) also found increased deposition of basement membrane material in human diabetic kidneys. In 1978, Yagihashi et al. (161) reported an increase in thickness of the glomerular basement membrane in spontaneously diabetic Wistar rats. A progressive age-related thickening was noted as well, but the mean values of basement membrane width were consistently higher in the diabetic versus healthy rats and the difference became larger with increasing age (161). Comparable changes have been reported in experimentally-induced diabetes as well. Osterby and Gundersen (162) found significantly increased masses of peripheral and total glomerular basement membrane in rats that had been diabetic for only four days. They interpreted it as indicating an acceleration of synthesis, rather than inhibition of breakdown, as even total degradative arrest could not account for such rapid changes (163). Assembly of the glomerular basement membrane involves several postribosomal enzyme-mediated events which can serve as points of regulation of the rate of basement membrane synthesis (17). Glycosyl-transferase enzyme activity appears to mirror the rate of synthesis of the basement membrane (164) and the levels of this enzyme are significantly elevated in the kidneys of experimentally-induced diabetic rats (165). Therefore, the basement membrane synthetic machinery may be overactive in diabetes.

The question of whether basement membrane thickness is normal at the onset of diabetes is still debated owing to variations in methodology and control group selection (77). In a detailed morphometric study, Osterby (77) found no difference in glomerular basement membrane thickness between healthy and recently diagnosed diabetic subjects. Osterby also found no difference in basement membrane thickness in newly diagnosed subjects before and after normalization of the metabolic state with insulin (166). Kilo et al. (94) reported that intercepts computed for muscle capillary basement membrane width at birth were the same for healthy and diabetic subjects, i.e., basement membrane thickening associated with diabetes is a postnatal phenomenon (94).

Many authors have found a correlation between duration of diabetes and basement membrane thickening. For example, Osterby (77) demonstrated thickening as early as $1\frac{1}{2}$ years after diabetes onset and found a more pronounced thickening by $3\frac{1}{2}$ - 5 years duration. Pardo et al. (167) found comparable duration-related increases in muscle capillary basement membrane width; in addition, the mean basement membrane thickness of patients with diabetes duration greater than five years was significantly higher than that of patients with duration less than five years.

Although there is no direct evidence to suggest that increased basement membrane thickness is associated necessarily with increased permeability (125), it is generally accepted that increased width of this structure is an expression of a more permeable state (72). However, the permeability properties of the glomerular filter may be altered prior to thickening of the basement membrane (at $1\frac{1}{2}$ years) (77) such

that basement membrane thickness and permeability to plasma proteins may be somewhat independent (72)). Viberti (72) suggests that increase in basement membrane width may be a consequence, rather than a forerunner, of increased glomerular permeability. Increased extravasation of plasma proteins and their deposition in the glomerular wall and mesangium would be a potent and continuous stimulus to mesangial matrix production, which may lead to the glomerulosclerotic changes (168) seen in advanced diabetes. Analagous to observed alterations in basement membrane structure, the content of mesangial basement membrane-like material is known to be elevated by $1\frac{1}{2}$ years diabetes duration (77). In addition, mesangium from diabetic rats exhibits an inability to clear effectively macromolecules localized therein (169) such that mesangial matrix production may be enhanced further.

As mentioned previously, two different views are held as to the causative factor operative in the pathogenesis of microangiopathy. Those who believe that capillary abnormalities are inherited independently of insulin deficiency and carbohydrate intolerance cite the work of Siperstein et al. (170). These investigators contend that basement membrane abnormalities are present at the onset of diabetes, show no relation to diabetes duration, and occur in a large proportion of so-called "pre-diabetic" subjects (offspring of two conjugal diabetic persons) (171, 172). In addition, Siperstein and colleagues propose that the basement membrane defect causes insulin deficiency by interfering with its diffusive properties. In a 1978 review, Tchobroutsky reviews and effectively rejects Siperstein's arguments (103).

There are numerous lines of evidence which provide support for the role of metabolic dysfunction in the structural pathogenesis of diabetic microangiopathy, i.e., the "metabolic hypothesis." First of all, basement membrane thickening is normal at the onset of diabetes (77, 166, 173) and there is a correlation between duration of diabetes and basement membrane width (77, 94). Jackson et al. (174) was able to correlate basement membrane thickening to degree of metabolic control. Characteristic lesions occur in patients with insulinopenia secondary to chronic pancreatitis, for example (175) and in animals with experimentally-induced (176) or spontaneous (177) diabetes. According to Gunderson et al. (64): "all reliable evidence indicates that the pathogenesis and the natural history of diabetic microangiopathy is the same in the kidney and in all other organs, i.e., it is secondary to the metabolic disturbance and therefore in principle preventable by optimal treatment."

In addition to morphologic changes in the glomerular basement membrane, there are certain biochemical changes that appear to be specific for diabetic renal disease (178). Reported differences include an elevation in hydroxylysine content (with a concomitant drop in lysine content) (160), a decrease in cystine content (179), elevated glucose and galactose content (160), increased hydroxyproline levels (180) and a decrease in the sialic acid (NANA) content (179). Exposure of the basement membrane proteins to elevated glucose levels also results in an increase in non-enzymatic glycosylation of these constitutents, according to in vitro studies (181). At least three of these changes have biological importance. A decrease in the number of inter- or

intramolecular disulfide bridges (due to decreased cystine content), could lead to increased permeability of the membrane to circulating macromolecules. A reduction in sialic acid content would decrease the net negative charge of the basement membrane and thereby increase the transglomerular leakage of anionic species, such as albumin (9, 182). Glycosylation of lysine and hydroxylysine amino groups would decrease the availability of these constituents for collagen cross-linking, and as linkage of free groups may alter the electrochemical properties of the involved proteins, the electrical integrity of the glomerular filtration barrier may be compromised (181, 183).

Advanced diabetic glomerulopathy is characterized morphologically by three types of lesions: (1) Diffuse glomerulosclerosis (Fig. 6): diffuse thickening of the glomerular basement membrane characterizes this lesion (184), first described by Fahr in 1942 (in 185). The basement membrane may have a "moth-eaten" appearance (186), and there is an increase in mesangial matrix (77). Diffuse glomerulosclerosis is the more common type of lesion (186) but is less specific to diabetes; (2) Nodular intercapillary glomerulosclerosis (Fig. 7): when accumulation of basement membrane-like material in the mesangium reaches a certain extent, true hyalinized nodules form, the Kimmelstiel-Wilson nodules (185). Although this lesion is pathognomonic of diabetes mellitus (187), it is relatively rare in occurrence. It is seen in only about 9 to 25% of kidneys from patients with clinical signs of nephropathy (185); (3) Exudative lesions (Figs. 8 and 9): these are also referred to as capsular drop, fibrin cap, or arteriolar hyalinosis. This heterogeneous group comprises late variable lesions that appear as homogeneous or

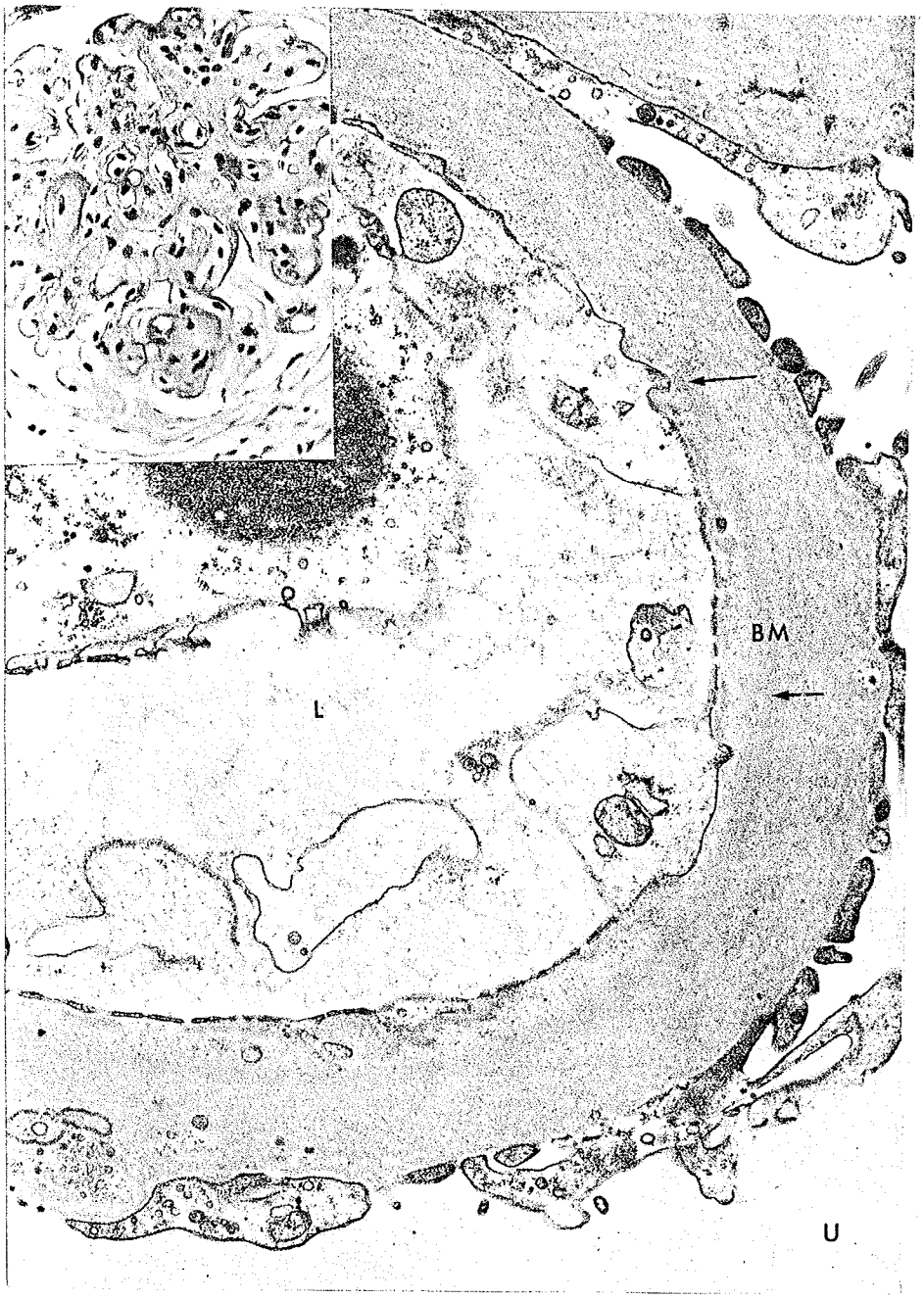

Fig. 6. Electron micrograph showing diffuse basement membrane thickening. The membrane is approximately 1500 nm wide, about five times normal. Arrows point to the probable location of the original basement membrane (x 15,700). Insert illustrates diffuse glomerulosclerosis with widened mesangium and thickening basement membranes (x 300) (from 186).

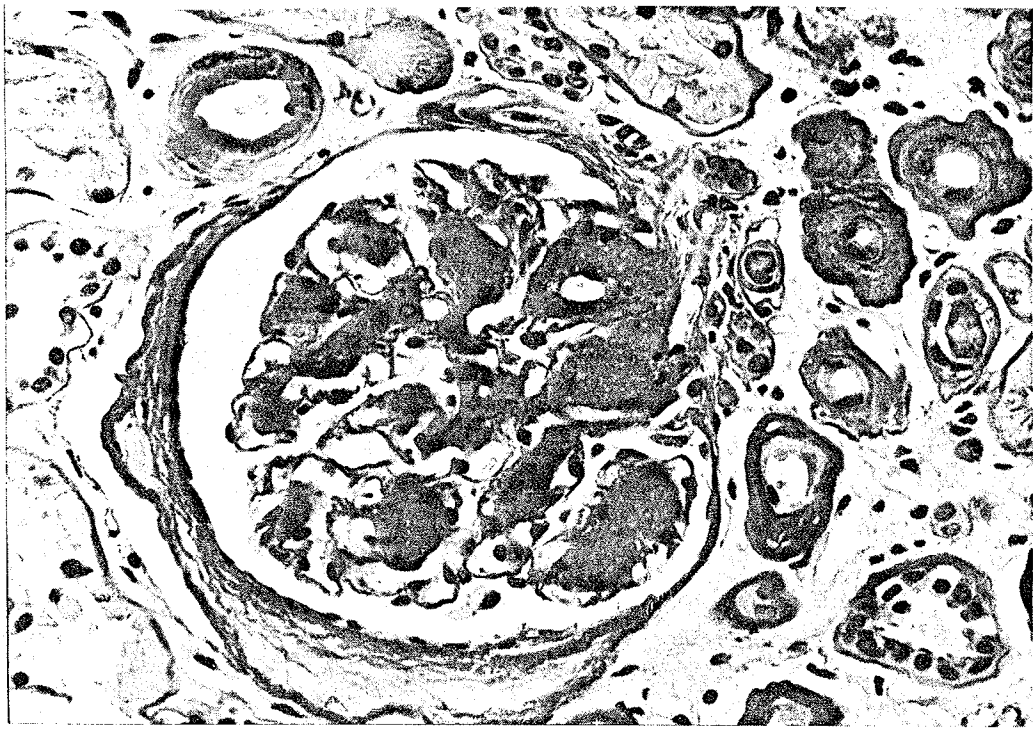

Fig. 7. Light microscopic view of diabetic glomerulosclerosis. Kimmelstiel-Wilson nodules and diffuse changes are present in the glomerular tuft (from 77).

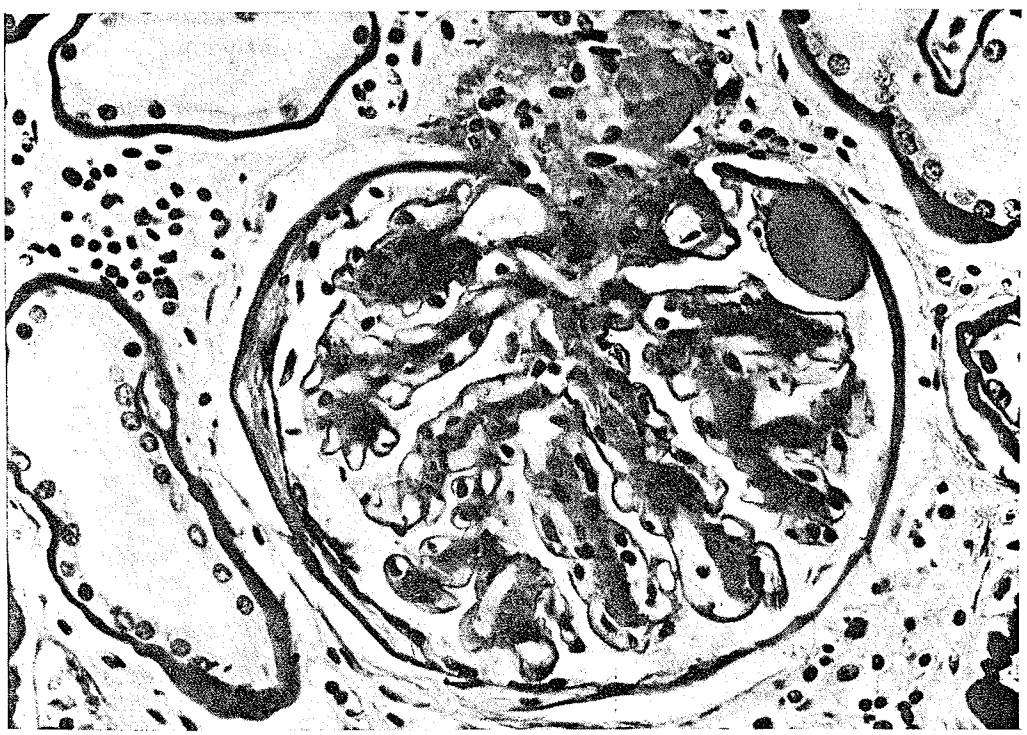

Fig. 8. Renal corpuscle under the light microscope. There is mild diffuse glomerulosclerosis in the glomerulus, and a prominent capsular drop in Bowman's space (from 77).

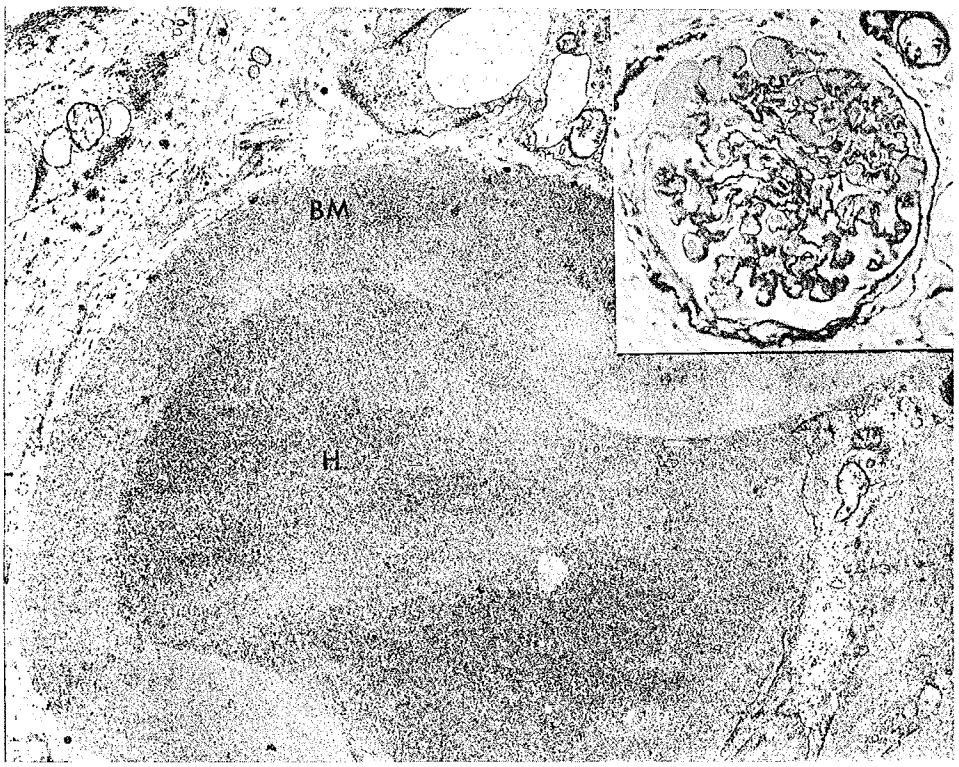

Fig. 9. Electron micrograph of a capillary hyaline lesion. A large deposit of dense material (H) lies between the basement membrane (BM) and the endothelial lining (x 11,500). Insert shows a glomerulus with many exudative lesions (x 200) (from 186).

vacuolated deposits (185) in Bowman's capsule, the glomerular tuft, and the arteriolar wall, respectively (77, 185). While the capsular drop is rather specific for diabetes and the arteriolar lesions can be considered characteristic, the fibrin caps occur in many other renal diseases (77). The link between early basement membrane changes and diabetic glomerulosclerosis has not been elucidated and debate exists as to whether the nodular lesions are a separate entity or whether they should be regarded as an end-stage to the diffuse lesions (77). It is necessary to clarify the mechanisms which lead to the obsolescence of so large a fraction of the glomerular population that a decrease in glomerular filtration rate occurs (188). Information as to the causes of the early basement membrane alterations may lead to a rational approach for the prevention and treatment of end-stage glomerular pathology (13).

IV. Summary

Mogenson (119) and Mogensen et al. (189) summarize the natural history of the renal changes in diabetes into five chronological stages (Table 1). Stage one comprises very early renal involvement, takes days or weeks to develop, and is characterized by reversible renal hypertrophy/hyperfunction. The abnormalities associated with stage two are detectable after two years of diabetes, and progress with time. Reversibility at this point is questionable. Incipient diabetic nephropathy (stage three) develops in only 30 to 40% of patients and is characterized by a progressive rise in albumin

TABLE 1: STAGES IN THE DEVELOPMENT OF RENAL CHANGES IN TYPE I DIABETES MELLITUS (after 189)

Stage	Onset	Albumin Excretion Rate	Glomerular Filtration Rate	Blood Pressure	Structural Lesions	Reversibility (with strict insulin treatment)
1. Early renal hypertrophy-hyperfunction (before insulin treatment)	At diagnosis (may occur during poor control)	Increased	Increased by 20-40%	Normal	Increased kidney and glomerular size	Yes
2. Renal lesions, no clinical signs	After 2 yr of diabetes (progression over several years)	Normal (about 3-10 ug/min)	Increased by 20-30%	Normal	Increased basement membrane and mesangial thickness	?
3. Incipient diabetic nephropathy	After 10-15 yr (in 30-40% of patients)	15-300 ug/min	Increased by 20-30%	Incipient increase	Not well studied	?
4. Clinically overt diabetic nephropathy	After 15-20 yr (in 30-40% of patients)	Progressive clinical proteinuria	Without treatment declines about 1ml/min/mo.	Abnormal (160/105)	Diffuse and nodular glomerulosclerosis, capsular drops, fibrin caps, arteriolar hyalinosis	No
5. End-stage renal failure	After 25-30 yr	Often declines due to nephron closure	<10ml/min	High	Glomerular closure	No

excretion and blood pressure. By 15 to 20 years diabetes duration, clinically overt nephropathy appears in 30 to 40% of patients. By this time, the abnormalities are usually considered irreversible, such that end-stage renal failure (stage five) develops 5 to 10 years later.

Elucidation of the exact pathogenetic mechanisms and risk factors involved in the evolution of the diabetic kidney awaits further study.

RATIONALE

The major chronic degenerative complications associated with the syndrome of diabetes mellitus occur neither inevitably nor as all-or-none phenomena. The disease is characterized by major inter-individual differences in innate susceptibility to nephropathy, retinopathy, and neuropathy, and it is only with the passage of time that these differences become apparent. If one could predict those who are not, or those who are, in varying degree, susceptible to vascular or neural complications, it would aid in development of more intensified treatment programs for the approximate one in three (66) at significant risk for each of the respective complications. The measurement of subclinical microalbuminuria may have such a predictive value with respect to diabetic renal disease.

The ability to detect early rises in albumin excretion rate (AER) came with the advent of the radioimmunoassay for urine albumin in 1963 (109). A possible significance of this with respect to diabetes care and follow up seems to have been overlooked, since clinical quantitation of urine albumin is still carried out with relatively insensitive methods, e.g. Albustix®, sulphosalicylic acid (191), that are designed to pick up albumin concentrations 20- to 100-fold greater than normal (109). It is advantageous to uncover the initial subclinical increases as early as possible, because if clinical proteinuria is the end-stage to microalbuminuria, early identification of those at risk

and placement on intensified treatment may delay or prevent development of diabetic nephropathy (92, 93).

There is discrepancy in the literature, however, as to the natural history of microalbuminuria in established insulin-treated diabetic subjects (72). Mogensen (44, 86) claims that all clinically non-proteinuric individuals have normal immunoassayable AER, and that the transition from normal to clinically overt levels is abrupt or step-like. Viberti et al. (91) claim that the rise to clinical proteinuria is ramp-like with a gradual rise in immunoassayable AER.

Non-standardization of urine collection protocols may be contributing to the disparity of observations regarding diabetic microalbuminuria. We examined urine sampling methods for reproducibility and sensitivity in health and early diabetes and then studied 65 Type I diabetic patients to elucidate the distribution of albuminuria in a clinic population. β2-microglobulin excretion rate was also measured, by radioimmunoassay, to differentiate between tubular and glomerular proteinuria.

We measured finger light touch perception threshold (TPT) by a modified Von Frey technic; this sensory index is thought to be impaired in early diabetes (192, 193), and may have predictive value with respect to diabetic peripheral neuropathy.

Semiquantitative ophthalmoscopic assessment was made of retinal capillary dilatation (CD), which may have analogous predictive value for retinopathy (194).

Blood pressure and short- and medium-term quality of diabetes control was evaluated to elucidate the respective roles of hypertension

and glycemia in early subclinical changes.

Since many authors describe a close association between nephropathy, neuropathy, and retinopathy (65), a comparative semiquantitative assessment of these clinical parameters was made. Correlations were also sought amongst the three subclinical indices (AER, TPT, CD).

MATERIALS AND METHODS

(A) PRELIMINARY ASSESSMENT OF URINE COLLECTION PROTOCOLS

I. Subjects

Five healthy men and five men with Type I diabetes of short duration (2.0 to 3.4 year) were studied. All ten subjects were normotensive (blood pressure by standard sphyngmomanometry did not exceed 150/90 mmHg) and clinically non-proteinuric (routine urine analysis by a sulphosalicylic acid method was negative for urine albumin). The five healthy men were normoglycemic, as a random blood glucose level and glycosylated hemoglobin did not exceed normal values. They were of average physical fitness, had no family history of kidney disease or diabetes, and were on no medication. The five diabetic subjects were also of average fitness, had no family history of kidney disease, and were on no medication other than insulin.

II. Protocol

Subjects were instructed to perform four different timed urine collections, each in duplicate. The first sample was collected during recumbency; subjects voided completely at bedtime and collected the urine sample upon rising. The second sample was collected over the next four hours, during upright ambulation. The third collection was over a 24-hour period. Activity was kept at a minimum and tea, coffee and alcohol were prohibited during the collection periods. The

fourth collection was made during an exercise provocation test. Subjects arrived at the laboratory at 9:00 a.m. following their usual breakfast (and insulin dose, if applicable). Upon arrival, they voided completely, and urine samples were collected over three periods: (1) a one-hour pre-exercise hydration period, during which 750 ml of tap water was consumed (250 ml at 0, 30, and 60 min); (2) a 20-minute exercise period on a Monark ergometer (600 kpm/min); and (3) a one-hour post-exercise recovery period. Subjects were seated during the entire procedure, standing up only to void. Heart rate and blood pressure were measured at rest, and heart rate was recorded at 4, 8, 12, 16, and 20 minutes into the exercise.

Urine volume and pH were measured and aliquots stored at -4°C until assay. If urine pH was less than 5.5, one aliquot was adjusted to ≥ 5.5 with 0.5M NaOH to prevent β2-microglobulin breakdown.

Urinary albumin and β2-microglobulin were measured by radio-immunoassay (β2-microglobulin was measured in upright, exercise, and post-exercise samples only).

(B) CROSS-SECTIONAL SURVEY OF ALBUMINURIA IN A TYPE I DIABETES CLINIC

I. Subjects

Sixty-five insulin-dependent diabetic subjects, who attended a diabetes clinic between October 1st and December 31st, 1982, were studied. Five of these were participants in the preliminary study; while subjects DB and SH repeated collections for the cross-sectional data, results for subjects GE, RH and RS were arbitrarily taken from

collection I. Age ranged from 15 to 48 years, with an average of 27.2 years. There were 31 females and 34 males. Duration of diabetes ranged from 0 to 32 years. All except subject 7 were on insulin treatment; subjects 13, 19, 20, 33-35, 42, 44, 48, 50, 54, 57, 60, and 61 were on anti-hypertensive treatment.

Twenty normotensive, clinically non-proteinuric, normoglycemic healthy individuals served as controls (five of these were participants in the preliminary study; results were arbitrarily taken from collection I). There were 10 males and 10 females and age ranged from 15 to 51 years (average 29.1 yrs). There was no family history of diabetes or kidney disease in any of the healthy subjects.

II. Protocol

Subjects were instructed to perform an overnight recumbent collection and a four-hour morning collection as previously described. Activity was kept at a minimum, and heavy exercise was not allowed. Subjects were not to consume coffee, tea, or alcohol during the entire collection period, but were to eat their usual meals and take their usual insulin dose(s), if applicable.

Diabetic subjects spotted finger-capillary blood on boric acid-impregnated filter paper four times daily (before breakfast, before lunch, before dinner, before bed) for the two days that urine was collected, i.e., eight blood spots. These spots were later analyzed for glucose content and a 2-day average blood glucose level was calculated.

When subjects brought in their urine samples, a sample of venous blood was taken for glycosylated hemoglobin analysis (diabetic patients

only). Urine volume and pH were measured, the latter being adjusted as previously described, and aliquots were stored at -4°C until assay. An additional sample of urine was sent for routine clinical determination of albumin concentration.

Light touch sensory perception threshold tests were performed at this time; these were done with modified Von Frey hairs. The tests were performed in a quiet closed room after a resting period of approximately ten minutes. Measurements were made on the distal phalanx of the dominant index finger with the subject's eyes closed. The modified Von Frey sensory apparatus consisted of imitation human hair, of a critical length and width, attached to an applicator stick. Milligrams of force required to bend the hair was determined on a digital scale. The forces represented were 20, 30, 40, 50, 70, 90, 110, 150, 170, 200, 230, 260, 290, 320, 350, 510 and 550 mg; beyond this range, mg forces of 780 and 1050 were represented by Semmes-Weinstein pressure esthesiometers from Shaw Laboratories, Inc. The hairs were tested in ascending order until the sensory threshold was attained. The final threshold value was an average of three separate trials. The subject was termed unreliable only if he/she repeatedly perceived a non-existent stimulus.

Recent blood pressure values were taken from the diabetic subjects' charts (all clinical measurements, i.e., blood pressure, neuropathy, retinopathy, capillary dilatation, carried out on the diabetic patients were performed by the attending physician). Hypertension was diagnosed if diastolic blood pressure exceeded 90 mmHg, and for graphical representation, was further designated as mild (diastolic 90 - 100 mmHg), moderate (diastolic 100 - 110 mmHg), or severe (diastolic >110 mmHg). Blood pressures for the healthy

subjects were measured in a quiet room with the subject seated.

An assessment of clinical peripheral neuropathy in the diabetic subjects yielded four groups of patients: those with no clinical signs or symptions (0) and those with mild (1), moderate (2), or severe (3) degrees of neuropathy. A patient with mild neuropathy would present with minor symptoms (complaints of mild occasional nocturnal leg pain, for example) and/or a sensory level no higher than midfoot. The sensory level corresponds to the level of decreasing pinprick sensation. Individuals with chronic significant symptoms (e.g. persistent pain) and/or a sensory level no higher than midleg would have moderate neuropathy. Those presenting with chronic severe symptoms (e.g. impotence) and/or a sensory level higher than midleg would have severe neuropathy.

Clinical diabetic retinopathy was also loosely classified into four groups: those with no clinical evidence (0) , and those with mild (1), moderate (2), and severe (3) signs of retinal damage. Mild retinopathy included those with mild to moderate background retinal damage, moderate retinopathy those with moderate to severe background damage, and severe retinopathy those with proliferative damage or blindness.

The diabetic subjects were also classified in terms of the appearance of their retinal capillaries. Four classes emerged: those with normal capillary dilatation (0) and those with mild (1), moderate (2), and severe (3) capillary dilatation. An assessment of capillary dilatation in those with grade 3 retinopathy was not possible.

The diabetic subjects were also grouped according to the degree of immunoassayable microproteinuria. The four groups were:

0: AER within the normal range; 1: AER 12 to 100 ug/min;

2: AER 100 to 1000 ug/min; 3: AER >1000 ug/min.

(C) LABORATORY INVESTIGATION

I. Radioimmunoassay of Urine Albumin (modification of 109)

(1) Reagents

Buffer. Buffer used throughout was prepared with 100 cc 0.1M sodium barbital, 10.75 cc of 200 mg% gelatin, and enough 0.2N hydrochloric acid to adjust the pH to 8.4 (approximately 10 cc). Gelatin was used to prevent the absorption of albumin to glassware.

Human Albumin. Standards were prepared with crystallized and lyophilized human serum albumin from Sigma Chemical Co. (#A-8763), reconstituted in buffer. Human serum albumin was also used to raise antibodies in the rabbit.

Antihuman-Albumin Serum, Rabbit (AHAS-R). Antibodies to human serum albumin were raised according to the method of Miles et al. (110): 10 mg of human serum albumin in one ml of water emulsified with one ml of Freund's adjuvant, was injected subcutaneously into rabbits at weekly intervals for five weeks.

Radioalbumin. Iodinated (125 I-) albumin was obtained from Charles E. Frosst (2 uCi/mg) and diluted 1:250 with buffer (such that there were approximately 300 cpm per assay tube).

Antirabbit-Gammaglobulin Serum, Goat (ARGS-G). Goat anti-rabbit gammaglobulin precipitating antibody was used to precipitate the antibody-bound albumin. It was obtained from Antibodies Incorporated (Davis, Calif. - lot #IMJ19W) and diluted 1:25 with buffer.

PEG. Polyethylenegycol was used to separate free from bound albumin. Polyethyleneglycol 8000 powder was obtained from Fischer Scientific Company (lot #720589) and reconstituted with buffer (60 gms/l).

(2) Procedure

A standard curve was constructed from albumin standards of 2.50, 1.25, 1.00, 0.50, 0.25, 0.125 and 0 mg/dl. A typical standard curve appears in Fig. 10. Dilutions of antibodies and radioalbumin were chosen such that approximately 65% of ^{125}I-albumin was bound at 0 mg/dl unlabelled albumin.

Frozen urine samples were allowed to thaw at room temperature and were assayed at full concentration and at 1:2 and 1:5 dilutions. Further dilutions were performed for those samples falling above the range of the standard curve. All urines were assayed in duplicate.

100 ul of standard or urine were pipetted into small glass tubes (12 x 75 mm; Fischer Scientific Company), followed by 100 ul of AHAS-R and 100 ul ^{125}I-albumin. After shaking, these were incubated approximately 18 hours at room temperature. 100 ul of ARGS-G was then added to each tube; after one hour at room temperature, one ml of PEG was added. Five minutes later, the tubes were placed in a PR 6000 centrifuge and spun for 15 minutes at 3000 rpm. After decantation, both supernatant and precipitate were counted on an automatic Beckman gamma counter for one minute. Percent radioactivity precipitated was calculated, a standard curve constructed, and mg/dl albumin values for the unknown samples interpolated off the graph.

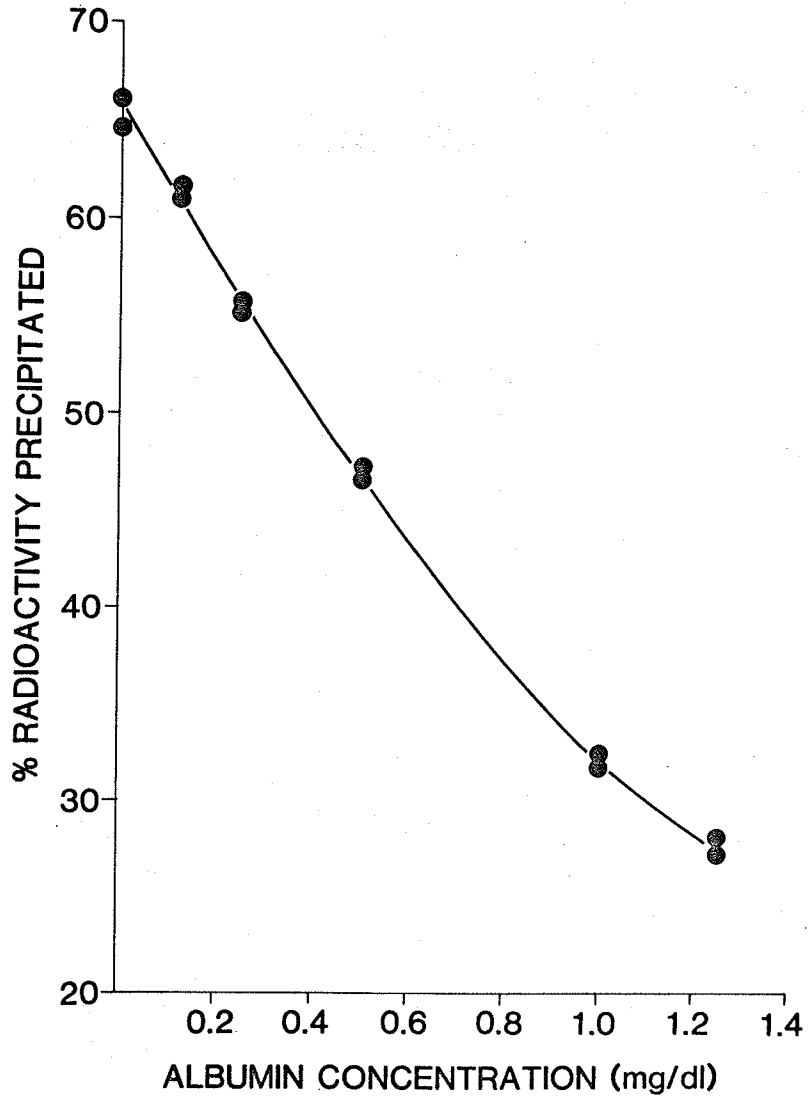

Fig. 10. Human albumin radioimmunoassay standard curve.

(3) Assay Performance

Specificity. AHAS-R was tested against normal human serum (by immunoelectrophoresis and Ouchterlony immunodiffusion) and no cross-reactivity was found.

Precision. For interassay variability, six urine samples were assayed on five separate days. Mean coefficient of variation was 10.7%. For intraassay variability, two urine samples were assayed in 10 replicates. Mean coefficient of variation was 10.3%.

Accuracy. Recovery of human albumin added to normal urine was performed on six urine samples; mean percent recovery (actual value x 100/expected value) was 97.8.

II. Radioimmunoassay of Urine β2-Microglobulin

RIA of urine β2-microglobulin was performed using the Phadebas β2-micro Test kit from Pharmacia Diagnostics (lot #6467, 6468).

III. Blood Glucose Analysis

Blood glucose content of the filter paper blood spots was determined by a modification of a Technicon auto-analyzer (N-9) microglucose method.

IV. Glycosylated Hemoglobin

A Bio-Rad Hemoglobin A1 (by column) test kit (catalogue #191-7000) was used to measure the percentage of glycosylated hemoglobin in whole blood.

V. Routine Urine Albumin Analysis

Urine albumin was semiquantitated (clinically) with a sulphosalicylic acid method.

(D) STATISTICAL METHODS

In the cross-sectional survey, correlations were examined with multiple regression analysis, cross tabulation (x^2), and Pearson correlation coefficients.

RESULTS

(A) PRELIMINARY ASSESSMENT OF URINE COLLECTION PROTOCOLS

Laboratory and clinical data on the five healthy and five diabetic subjects are given in Table 2. The AER's are depicted in Fig. 11 and 12.

I. Precision

The precision of each of the four collection protocols was evaluated in the five healthy subjects. It was assessed by calculating the average percent variation from the mean AER in duplicates (intra-individual variation) and in individuals (inter-individual variation). It is evident from the summary of results in Table 3 that intra- and inter-individual reproducibility were best in the four-hour ambulatory sample; the range of percent variation was least for this protocol as well.

II. Sensitivity

Sensitivity was assessed by evaluating the ability of the collection protocols to detect AER abnormalities in the five diabetic subjects. AER was normal in all of the five in the recumbent position (Fig. 11). In the upright collection protocol, 2 of the 5 subjects were detected in the abnormal AER range (DB and RH); an abnormality in AER in these same 2 subjects in the 24-hour sample was questionable. Urine flow and β2-microglobulin excretion rates were within normal values for all five patients. DB and RH were abnormal during the exercise collection as well (Fig. 12).

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TABLE 2: LABORATORY AND CLINICAL DATA FROM 5 HEALTHY AND 5 TYPE I DIABETIC SUBJECTS

Subjects	Age (Yrs.)	Diabetes Duration (Yrs.)		--RECUMBENT--- AER ug/min	Urine Flow ml/min	---- UPRIGHT ------ AER ug/min	β2-uER ng/min	Urine Flow ml/min	—24-HOUR--- AER ug/min	Urine Flow ml/min	---EXERCISE---- PRE-EXERCISE- AER ug/min	Urine Flow ml/min	-----EXERCISE----- AER ug/min	β2-uER ng/min	Urine Flow ml/min	---POST-EXERCISE---- AER ug/min	β2-uER ng/min	Urine Flow ml/min	---CLINICAL EVALUATION* TPT	BP	Neuropathy Grade
Healthy																					
HE	25	NA**	I	7.26	0.57	8.66	79.06	1.17	5.96	1.30	11.60	4.83	21.97	84.50	8.45	15.43	77.13	2.97	30	130/82	NA
			II	4.72	0.38	6.41		0.75	5.55	0.82	6.45	3.58	44.01		8.15	21.15		2.92			
DH	24	NA	I	4.75	0.63	4.54	47.39	0.50	8.30	0.70	1.18	3.65	22.36	21.90	0.60	123.00	9.60	0.74	90	112/74	NA
			II	3.02	0.74	7.10		0.81	3.14	0.77	1.08	7.20	10.50		0.70	17.55		0.65			
GH	23	NA	I	0.18	0.29	5.67	187.20	0.90	2.83	0.22	23.52	2.10	30.86	280.50	9.35	4.03	276.00	5.75	117	132/80	NA
			II	0.47	0.38	5.60		0.50	5.18	0.81	6.08	1.07	17.01		3.15	6.76		5.20			
DP	22	NA	I	0.35	0.51	8.77	109.25	1.44	1.51	0.76	16.10	9.48	26.54	339.30	4.35	16.70	195.00	0.75	30	118/74	NA
			II	3.59	0.40	5.86		1.40	4.75	0.70	16.29	5.62	48.86		6.85	25.80		1.00			
GP	29	NA	I	0.34	0.85	4.10	151.20	1.58	3.37	1.05	1.56	1.95	10.72	148.20	13.40	2.64	250.80	6.60	143	112/86	NA
			II	1.62	1.15	2.45		0.46	0.54	0.77	6.29	1.23	3.05		6.10	13.31		8.32			
MEAN	24.6			2.63	0.59	5.92	114.82	0.95	4.11	0.79	9.02	4.07	23.69	174.88	6.11	24.64	161.71	3.49			
± S.D.	±2.70			±2.44	±0.26	±1.96	±55.72	±0.42	±2.29	±0.27	±7.62	±2.75	±14.61	±132.73	±4.02	±35.34	±114.45	±2.81			
Diabetic																					
DB	23	2.8	I	0.80	0.89	6.78	99.63	0.82	3.13	0.56	47.30	2.50	9.17	187.20	1.95	17.90	142.33	1.02	90	110/70	0
			II	1.82	0.65	23.48		0.41	10.20	0.60	158.38	1.42	3.50		.50	10.93		1.38			
GE	36	3.4	I	3.32	0.95	7.73	114.40	1.21	6.47	0.95	2.87	7.17	14.24	587.40	8.90	6.82	94.60	1.10	ND†	120/80	ND
			II	2.09	1.16	5.54		0.58	4.19	1.20	5.64	1.35	24.80		1.55	13.84		0.80			
RH	27	3.3	I	5.99	0.52	16.81	294.77	2.59	11.72	1.17	6.36	1.55	1320.00	340.00	1.00	672.75	11.70	0.65	ND	120/75	0
			II	1.33	0.17	18.26		1.24	13.02	1.63	6.71	2.48	81.70		2.15	29.58		1.45			
SH	31	2.0	I	5.53	0.84	7.47	105.40	1.33	6.72	1.08	5.64	1.20	25.25	136.00	2.50	2.48	87.97	1.03	260	140/80	0
			II	9.09	0.64	5.69		1.35	9.97	1.42	8.06	2.98	14.62		3.40	7.11		3.88			
RS	35	2.8	I	1.71	0.66	4.48	24.74	1.55	1.96	1.01	9.56	11.95	NV	NV††	0.00	66.99	87.00	2.90	50	127/66	0
			II	1.75	1.46	3.26		1.09	4.84	1.94	17.39	4.70	42.39		7.85	21.60		2.70			
MEAN	30.4	2.86		3.34	0.79	9.95	127.79	1.22	7.22	1.16	26.79	3.73	170.63	312.65	3.03	85.00	84.72	1.64			
± S.D.	±5.46	±0.55		±2.68	±0.36	±6.93	±99.97	±0.60	±3.81	±0.43	±48.03	±3.43	±431.65	±202.63	±3.19	±207.34	±46.81	±0.98			

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†† no voi

TABLE 2: LABORATORY AND CLINICAL DATA FROM 5 HEALTHY AND 5 TYPE I DIABETIC SUBJECTS

tes ion)		--RECUMBENT--- AER ug/min	Urine Flow ml/min	---- UPRIGHT ------ AER ug/min	β2-uER ng/min	Urine Flow ml/min	---24-HOUR--- AER ug/min	Urine Flow ml/min	---EXERCISE---- PRE-EXERCISE- AER ug/min	Urine Flow ml/min	-----EXERCISE----- AER ug/min	β2-uER ng/min	Urine Flow ml/min	---POST-EXERCISE---- AER ug/min	β2-uER ng/min	Urine Flow ml/min	---CLINICAL EVALUATION* TPT	BP	Neuropathy Grade	Retinopathy Grade	Capillary Dilatation Grade
*	I	7.26	0.57	8.66	79.06	1.17	5.96	1.30	11.60	4.83	21.97	84.50	8.45	15.43	77.13	2.97	30	130/82	NA	NA	NA
	II	4.72	0.38	6.41		0.75	5.55	0.82	6.45	3.58	44.01		8.15	21.15		2.92					
	I	4.75	0.63	4.54	47.39	0.50	8.30	0.70	1.18	3.65	22.36	21.90	0.60	123.00	9.60	0.74	90	112/74	NA	NA	NA
	II	3.02	0.74	7.10		0.81	3.14	0.77	1.08	7.20	10.50		0.70	17.55		0.65					
	I	0.18	0.29	5.67	187.20	0.90	2.83	0.22	23.52	2.10	30.86	280.50	9.35	4.03	276.00	5.75	117	132/80	NA	NA	NA
	II	0.47	0.38	5.60		0.50	5.18	0.81	6.08	1.07	17.01		3.15	6.76		5.20					
	I	0.35	0.51	8.77	109.25	1.44	1.51	0.76	16.10	9.48	26.54	339.30	4.35	16.70	195.00	0.75	30	118/74	NA	NA	NA
	II	3.59	0.40	5.86		1.40	4.75	0.70	16.29	5.62	48.86		6.85	25.80		1.00					
	I	0.34	0.85	4.10	151.20	1.58	3.37	1.05	1.56	1.95	10.72	148.20	13.40	2.64	250.80	6.60	143	112/86	NA	NA	NA
	II	1.62	1.15	2.45		0.46	0.54	0.77	6.29	1.23	3.05		6.10	13.31		8.32					
		2.63 ±2.44	0.59 ±0.26	5.92 ±1.96	114.82 ±55.72	0.95 ±0.42	4.11 ±2.29	0.79 ±0.27	9.02 ±7.62	4.07 ±2.75	23.69 ±14.61	174.88 ±132.73	6.11 ±4.02	24.64 ±35.34	161.71 ±114.45	3.49 ±2.81					
	I	0.80	0.89	6.78	99.63	0.82	3.13	0.56	47.30	2.50	9.17	187.20	1.95	17.90	142.33	1.02	90	110/70	0	0	0
	II	1.82	0.65	23.48		0.41	10.20	0.60	158.38	1.42	3.50		.50	10.93		1.38					
	I	3.32	0.95	7.73	114.40	1.21	6.47	0.95	2.87	7.17	14.24	587.40	8.90	6.82	94.60	1.10	ND†	120/80	ND	ND	ND
	II	2.09	1.16	5.54		0.58	4.19	1.20	5.64	1.35	24.80		1.55	13.84		0.80					
	I	5.99	0.52	16.81	294.77	2.59	11.72	1.17	6.36	1.55	1320.00	340.00	1.00	672.75	11.70	0.65	ND	120/75	0	0	1
	II	1.33	0.17	18.26		1.24	13.02	1.63	6.71	2.48	81.70		2.15	29.58		1.45					
	I	5.53	0.84	7.47	105.40	1.33	6.72	1.08	5.64	1.20	25.25	136.00	2.50	2.48	87.97	1.03	260	140/80	0	0	2
	II	9.09	0.64	5.69		1.35	9.97	1.42	8.06	2.98	14.62		3.40	7.11		3.88					
	I	1.71	0.66	4.48	24.74	1.55	1.96	1.01	9.56	11.95	NV	NV††	0.00	66.99	87.00	2.90	50	127/66	0	0	ND
	II	1.75	1.46	3.26		1.09	4.84	1.94	17.39	4.70	42.39		7.85	21.60		2.70					
		3.34 ±2.68	0.79 ±0.36	9.95 ±6.93	127.79 ±99.97	1.22 ±0.60	7.22 ±3.81	1.16 ±0.43	26.79 ±48.03	3.73 ±3.43	170.63 ±431.65	312.65 ±202.63	3.03 ±3.19	85.00 ±207.34	84.72 ±46.81	1.64 ±0.98					

* see text for explanation
** not applicable
† not done
†† no void

TABLE 3: REPRODUCIBILITY/PRECISION OF FOUR URINE COLLECTION PROTOCOLS IN 5 HEALTHY SUBJECTS

Collection Protocol	Intra-Individual Variation Mean (Range)	Inter-Individual Variation Mean (Range)
Recumbent	±47.0% (21.2 - 82.2))	±70.2% (25.1 - 127.8)
Upright	±16.5% (0.6 - 25.2)	±20.5% (1.9 - 44.6)
24-Hour	±40.4% (0.4 - 72.2)	±31.5% (2.7 - 52.4)
Exercise:		
Hydration	±30.6% (0.6 - 60.3)	±57.5% (0.1 - 87.5)
Exercise	±37.1% (28.9 - 55.7)	±39.8% (1.1 - 70.9)
Recovery	±40.8% (15.6 - 75.0)	±74.1% (13.8 - 185.2)

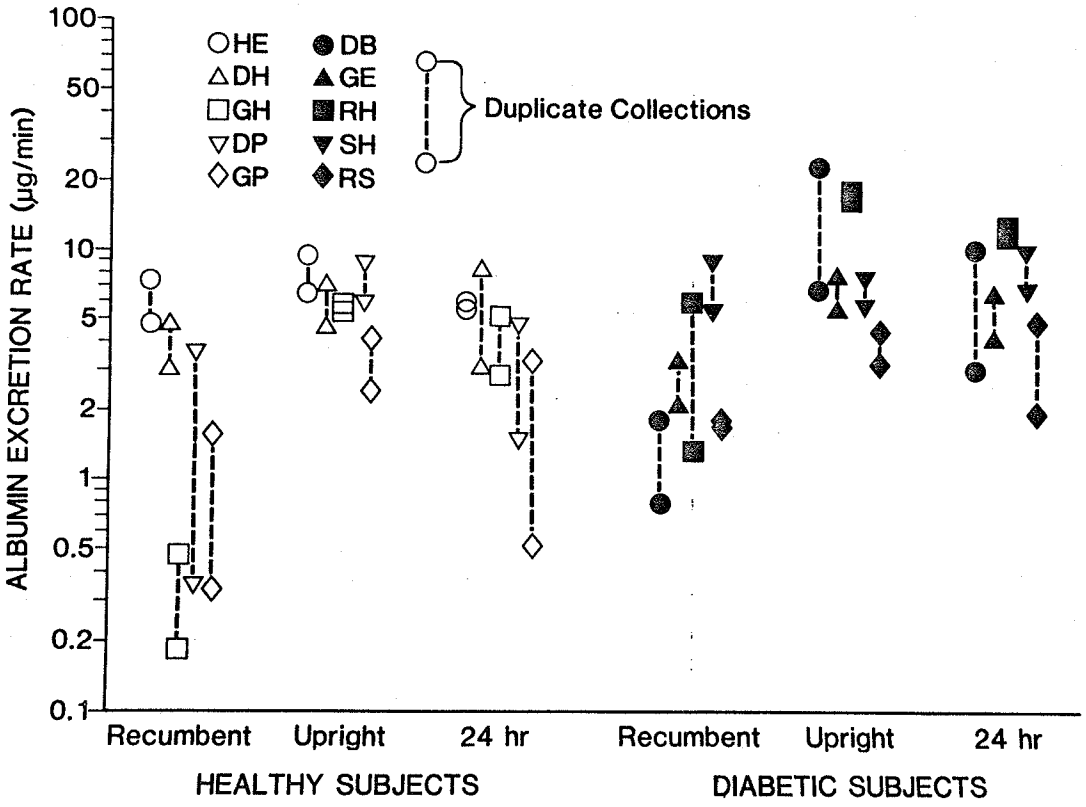

Fig. 11. Urine albumin excretion rate in five healthy and five diabetic subjects: a comparison of collection postures.

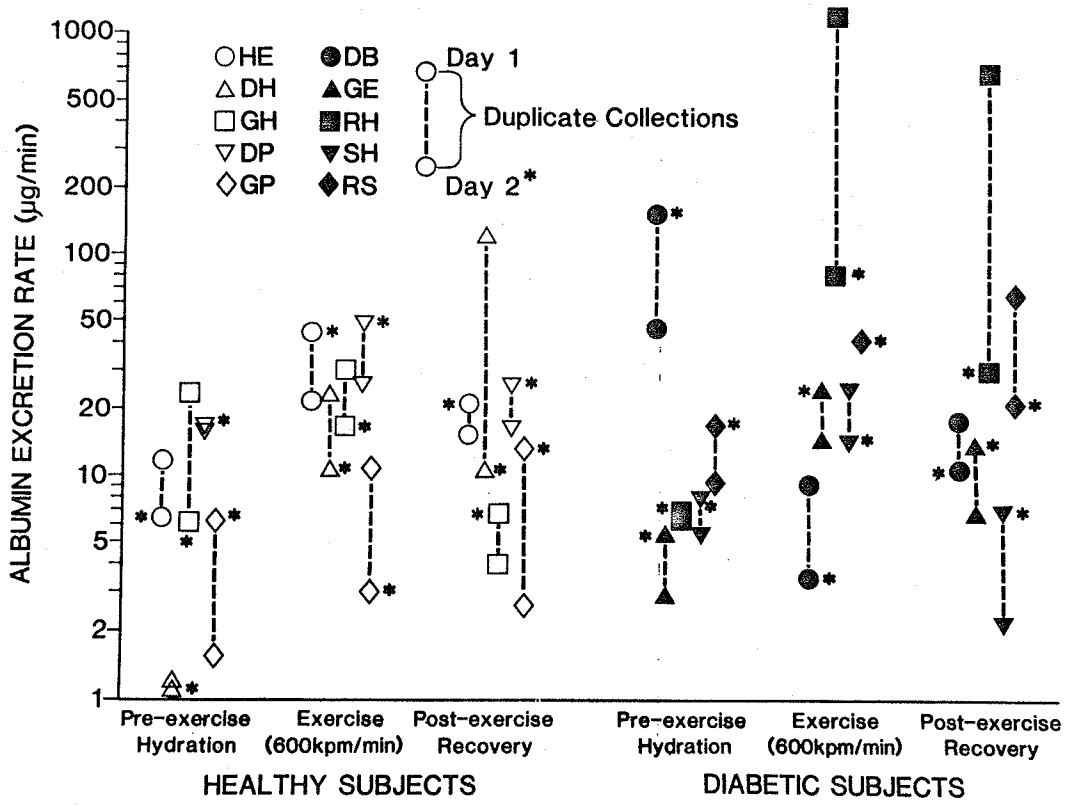

Fig. 12. Urine albumin excretion rate in five healthy and five diabetic subjects: exercise tests.

In DB, AER was elevated during hydration, whereas in RH, AER increased following exercise. Resting heart rate and blood pressure, and maximum heart rate during exercise were no different in health than in diabetes. Urine flow rose in both healthy and diabetic subjects during this protocol. The ratio of albumin excretion rate/β2-microglobulin excretion rate rose most notably in subject RH, i.e., upright: 0.06; exercise: 3.88; recovery: 57.5.

(B) CROSS-SECTIONAL SURVEY OF ALBUMINURIA IN A TYPE I DIABETES CLINIC

Laboratory and clinical data on the healthy and diabetic subjects are summarized in Tables 4 and 5. Results on inter-individual variability were similar to those obtained in the preliminary study. Average and range of percent variation from the mean in 20 healthy individuals was 73.4% (12.2 - 195.7) and 35.1% (8.9 - 90.8) in recumbent and upright postures (No. 3 excluded), respectively.

Fig. 13 (left panel) represents recumbent AER (R-AER) in relation to duration of diabetes. All of the 20 subjects who had had diabetes for less than 6 years were normal with respect to AER. Beyond six years, 26 of the diabetic subjects were hyperalbuminuric while 18 remained normal. After 20 years of diabetes, 4 of 12 patients had normal AER. 13 of 15 hypertensives were hyperalbuminuric. A multiple regression analysis was performed to ascertain the relative significance of duration of diabetes, diastolic blood pressure, blood glucose level and % glycosylated hemoglobin in determining recumbent AER (R-AER) (Table 6). Duration of diabetes was the only variable selected as

TABLE 4: LABORATORY AND CLINICAL DATA FROM 15 HEALTHY SUBJECTS

Subject Number	Age (Yrs.)	Sex	AER ug/min Recumbent	AER ug/min Upright	β2-uER ng/min Upright	TPT mg	BP mmHg
1	45	F	1.10	3.77	37.12	50	100/60
2	23	F	1.33	10.44	45.96	57	112/70
3	17	M	4.87	101.62	129.90	50	132/88
4	15	F	1.53	4.96	3.93	43	126/66
5	18	F	0.45	3.60	32.68	43	130/72
6	20	M	2.14	4.72	10.04	77	132/86
7	39	F	10.87	5.91	10.74	110	108/72
8	30	F	6.17	9.39	41.48	103	130/72
9	26	M	10.88	9.95	154.60	123	120/86
10	27	M	4.98	7.52	12.78	200	150/82
11	39	F	4.13	9.17	56.07	103	120/86
12	23	M	6.24	10.42	118.11	97	112/76
13	45	F	1.90	0.60	5.42	33	110/80
14	51	F	1.50	3.29	7.97	110	112/68
15	42	F	2.66	7.82	71.57	30	120/88

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TABLE 5: LABORATORY AND CLINICAL DATA FROM 62 TYPE I DIABETIC SUBJECTS

Subject Number	Age (Yrs.)	Sex	Diabetes Duration (Yrs.)	AER ug/min. Recumbent	AER ug/min. Upright	Clinical Albuminuria g/L	β2-uER ng/min. Upright	BG PROFILE* mmol/l Mean	BG PROFILE* mmol/l (Range)	HbA1 %	TPT mg	CLINICAL EVALUATION** BP mmHg	Neuropathy Grade	Retinopathy Grade	Capillary Dilatation Grade
1	23	F	5.5	10.23	9.57	neg	4.35	ND†		9.0	ND	110/70	0	0	0
2	22	F	9.2	6.24	20.40	neg	859.07	6.6	(2.9-8.3)	10.0	ND	120/60	0	0	1
3	35	F	4.4	2.30	6.02	neg	151.77	13.9	(8.6-17.6)	13.6	110	116/80	2	0	0
4	25	F	4.4	0.32	3.56	neg	25.58	7.3	(4.2-14.9)	10.9	ND	90/60	0	0	0
5	18	F	13.7	83.07	ND	tr¶	5.64	5.9	(4.2-9.9)	12.1	ND	100/80	2	ND	ND
6	20	M	6.0	2.90	1.45	neg	2.47	14.3	(9.0-19.1)	14.8	63	140/75	ND	0	1
7	32	M	0.20	5.60	6.14	neg	180.86	13.8	(11.8-15.8)	16.3	90	120/80	0	0	0
8	26	F	12.3	634.39	923.18	0.6	157.51	14.9	(13.3-16.2)	12.5	100	110/70	0	1	3
9	29	F	17.0	30.49	49.68	tr	7.33	6.8	(2.3-16.6)	11.7	380	110/75	2	3	NA††
10	15	M	2.8	1.87	7.61	tr	67.66	ND		15.1	20	130/75	0	0	1
11	33	F	23.0	7.55	2.85	tr	60.63	11.2	(5.2-15.1)	12.2	230	120/70	0	1	2
12	15	F	1.0	1.27	3.30	neg	37.27	10.3	(7.4-15.8)	12.3	70	140/80	ND	0	1
13	31	F	26.3	2476.85	3093.75	10.0	2816.00	6.3	(2.8-8.7)	11.5	780	ND	ND	3	NA
14	19	F	11.0	54.34	125.93	ND	696.90	25.2	(6.4-41.2)	16.0	77	120/80	0	0	0
15	17	M	5.4	1.71	6.06	neg	139.43	ND		14.2	270	105/70	1	0	0
16	40	F	28.0	ND	10.94	neg	14.58	13.1	(9.2-18.2)	15.8	ND	130/80	1	1	1
17	29	M	13.0	12.11	18.19	neg	306.53	10.7	(5.3-14.3)	11.8	330	130/70	0	1	1
18	26	M	12.0	5.52	7.48	neg	20.08	ND		9.1	27	120/80	0	0	0
19	35	M	25.3	687.44	1120.73	3.0	238.76	9.6	(4.3-15.1)	10.3	513	150/90	0	1	1
20	27	M	10.0	8.47	20.46	neg	22.73	9.5	(3.6-15.6)	12.5	647	150/95	0	1	1
21	32	M	4.2	1.98	2.29	neg	39.34	15.0	(5.4-23.7)	13.6	47	120/85	0	0	0
22	23	M	15.3	7.18	20.93	neg	6.48	10.0	(2.6-19.1)	14.1	780	120/80	1	2	3
23	30	F	20.0	18.30	44.20	neg	43.30	11.6	(3.9-19.5)	14.5	183	140/88	1	3	NA
24	48	M	15.0	737.57	619.75	0.7	51.80	8.2	(3.1-13.1)	13.5	960	ND	1	1	ND
25	24	M	2.8	4.75	13.69	neg	ND	9.1	(6.1-12.7)	11.9	90	110/70	0	0	0
26	17	M	8.6	2.75	4.10	neg	50.95	15.6	(7.5-20.3)	12.5	27	120/80	1	0	1
27	23	M	16.0	8.97	18.96	neg	63.20	15.9	(3.6-25.8)	11.9	170	125/80	0	0	2
28	32	M	2.0	4.28	8.37	neg	ND	9.6	(4.2-13.9)	14.0	260	140/80	0	0	2

*(ac+hs) x 2 days

**See text for explanation

† Not Done

†† Not Applicable

¶ Trace

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Subject Number	Age (Yrs.)	Sex	Diabetes Duration (Yrs.)	AER ug/min Recumbent	AER ug/min Upright	Clinical Albuminuria g/L	β2-uER ng/min. Upright	BG PROFILE* Mean	BG PROFILE* (Range)	CLINICAL EVALUATION** HbA1 %	TPT mg	BP mmHg	Neuropathy Grade	Retinopathy Grade	Capillary Dilatation Grade
29	20	F	4.8	4.12	26.06	neg	55.47	11.5	(6.7-14.6)	13.6	90	120/70	3	0	1
30	16	M	12.2	7.07	2.72	neg	57.10	9.8	(4.2-13.3)	16.3	103	120/80	0	0	1
31	29	M	14.7	118.41	323.14	neg	59.60	8.9	(2.1-20.8)	13.5	270	120/84	0	0	0
32	46	M	32.0	4.26	5.47	neg	29.07	2.8	(1.2-4.4)	11.0	310	120/80	0	0	1
33	24	F	12.8	193.81	279.64	0.25	49.17	12.9	(4.4-18.9)	14.7	43	130/105	0	3	NA
34	42	M	21.0	19.39	13.69	neg	10.27	23.1	(19.7-26.9)	16.9	780	150/105	3	3	NA
35	28	M	15.4	22.54	51.08	neg	62.86	9.7	(5.2-12.9	11.9	103	140/105	ND	0	0
36	15	F	6.8	61.43	21.81	neg	97.31	15.2	(11.6-24.1)	19.0	97	130/95	0	1	1
37	31	M	3.2	2.17	8.49	neg	100.43	7.1	(2.9-17.4)	15.3	167	135/80	0	0	0
38	24	F	15.3	153.67	176.38	neg	1.98	7.3	(4.8-9.3)	15.0	57	120/80	0	2	2
39	25	F	5.0	9.31	29.28	neg	60.34	20.9	(16.4-33.7)	16.1	167	130/80	0	1	1
40	21	F	18.2	3.53	6.06	neg	36.64	5.4	(2.5-8.4)	8.9	43	120/75	0	0	ND
41	18	M	6.2	151.51	132.71	0.2	22.75	7.4	(3.8-16.5)	11.1	143	120/80	3	0	1
42	19	M	14.1	206.29	1095.54	0.5	33.51	12.3	(11.7-13.0)	11.9	47	150/105	0	2	2
43	26	F	5.1	0.67	5.28	0.2	8.13	8.5	(4.2-11.1)	11.8	97	120/74	1	0	ND
44	29	M	10.0	11.87	5.51	neg	51.18	8.7	(5.1-15.9)	13.9	200	130/90	2	0	ND
45	19	F	6.4	2.93	6.26	neg	49.28	17.8	(14.6-21.7)	15.9	90	120/80	0	0	0
46	32	F	9.9	12.86	22.09	neg	18.08	11.5	(5.3-18.2)	13.4	30	110/80	0	0	ND
47	36	F	4.0	2.56	12.97	neg	8.87	6.3	(2.6-10.8)	10.1	523	120/70	0	0	0
48	24	M	9.0	22.25	11.48	neg	39.10	7.9	(3.1-13.3)	12.0	UR°	140/95	0	0	0
49	33	F	9.0	1.36	3.38	neg	77.08	12.8	(3.9-21.7)	10.6	33	110/70	0	0	0
50	21	M	10.0	596.46	777.75	0.3	108.80	12.7	(8.3-22.2)	12.0	200	150/105	0	1	2
51	31	F	11.1	3.47	5.18	neg	16.58	7.0	(3.0-13.7)	11.8	70	110/80	0	0	ND
52	43	M	28.0	6.94	6.29	neg	53.27	13.6	(7.9-21.2)	13.6	290	110/85	0	1	1
53	19	F	9.0	4.58	2.97	neg	10.57	15.4	(4.1-39.1)	16.3	110	120/85	0	0	ND
54	26	M	24.0	535.03	641.90	0.075	20.96	15.3	(5.0-20.8)	14.1	290	140/95	1	3	NA
55	19	F	5.0	1.75	3.41	neg	47.12	8.7	(3.6-14.0)	12.9	83	110/80	0	0	1
56	33	F	23.5	1.15	2.34	neg	8.50	6.7	(3.6-11.4)	11.5	123	120/80	0	1	ND
57	29	M	23.0	2484.00	1610.00	1.5	576.00	7.6	(3.6-12.7)	14.1	30	130/90	0	3	NA
58	17	F	11.0	15.06	40.88	neg	7.75	16.7	(10.7-21.6)	17.6	97	140/85	1	0	1
59	25	M	4.0	0.47	3.88	neg	49.28	ND		11.0	ND	130/80	0	0	ND
60	24	M	19.0	4.03	17.93	neg	26.24	7.9	(2.5-18.8)	12.3	137	150/90	1	1	0
61	43	F	26.7	796.57	1465.43	neg	52.55	9.2	(5.2-14.3)	13.0	ND	140/95	1	0	0
62	35	F	22.0	182.27	229.94	0.2	32.78	ND		11.9	ND	120/80	0	0	1

°Unreliable

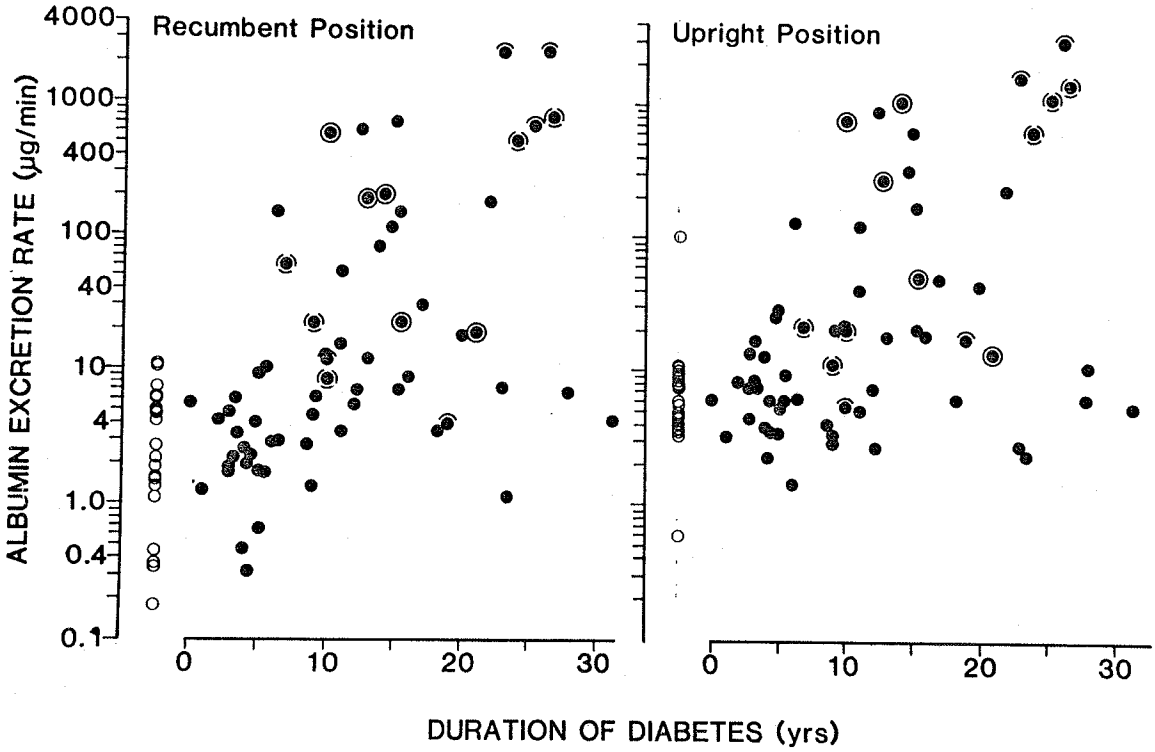

Fig. 13. Urine albumin excretion rate in 20 healthy(○) and 65 Type I diabetic(●)subjects (hypertension: mild ◉, moderate ◉, normal on treatment ◉)

TABLE 6: MULTIPLE REGRESSION ANALYSIS ON 65 TYPE I DIABETIC SUBJECTS

	Dependent Variables		
Independent Variables	R-AER n=56	U-AER n=56	TPT n=50
Diabetes Duration	r=0.46 p<0.001	r=0.46 p<0.001	r=0.39 p=0.005
Diastolic BP	NS*	r=.25 NS p=.063	NS
BG	NS	NS	NS
%HbA1	NS	NS	NS
Equation	y=29.88(x)-187.20 p=0.001	y=32.32(x)-173.74 p<0.001	y=10.70(x)+63.77 p=0.007
Percent Variability in Dependent Variable Left Unexplained After All 4 Independent Variables Analyzed	74.1%	69.9%	84.0%

*Not significant

being significantly correlated with R-AER.

Fig. 13 (right panel) illustrates ambulatory AER (U-AER) in the 65 diabetic subjects. Some subjects were abnormal by 2.5 years diabetes duration; 5 of the 20 subjects with duration less than 6 years (normal in recumbency) were abnormal by this protocol. Beyond 2.5 years duration, 33 patients were hyperalbuminuric and 28 were normal. With multiple regression analysis (Table 6), diabetes duration was found to correlate as in recumbency. Furthermore, diastolic blood pressure explained an additional 5% of U-AER variability (although the correlation was not significant). Clinical urinalysis revealed that 19 patients with elevated immunoassayable AER were clinically non-proteinuric. Elevated ambulatory β-2 microglobulin excretion rate (β2-uER) was found in 6 diabetic subjects, namely, 2, 13, 14, 17, 19, and 57.

A separate multiple regression analysis in those patients with diastolic hypertension (greater than 90 mmHg) revealed that duration of diabetes was the only independent variable which correlated significantly with AER (Table 7).

Fig. 14 shows the relationship between R-AER and U-AER. For approximately 80% of the subjects, AER during recumbency was less than that during ambulation. This figure also shows us the relative discriminative sensitivity of the two collection protocols, in picking up early AER abnormalities in diabetes. With 11 ug/min being the upper limit of normal, 24 diabetic subjects were hyperalbuminuric by both tests, 28 were normal by both tests, 1 was abnormal only during recumbency, and 10 were abnormal only during upright ambulation.

TABLE 7: MULTIPLE REGRESSION ANALYSIS ON 15 HYPERTENSIVE TYPE I DIABETIC SUBJECTS

	Dependent Variables	
Independent Variables	R-AER n=15	U-AER n=15
Diabetes Duration	r=0.60 p=0.018	r=0.66 p=0.008
Diastolic BP	NS*	NS
BG	NS	NS
%HbA1	NS	NS
Equation	y=70.92(x)-656.31 p=0.018	y=82.03(x)-703.91 p=0.008
Percent Variability in Dependent Variable Left Unexplained After All 4 Independent Variables Analyzed	43.8%	43.3%

*Not significant

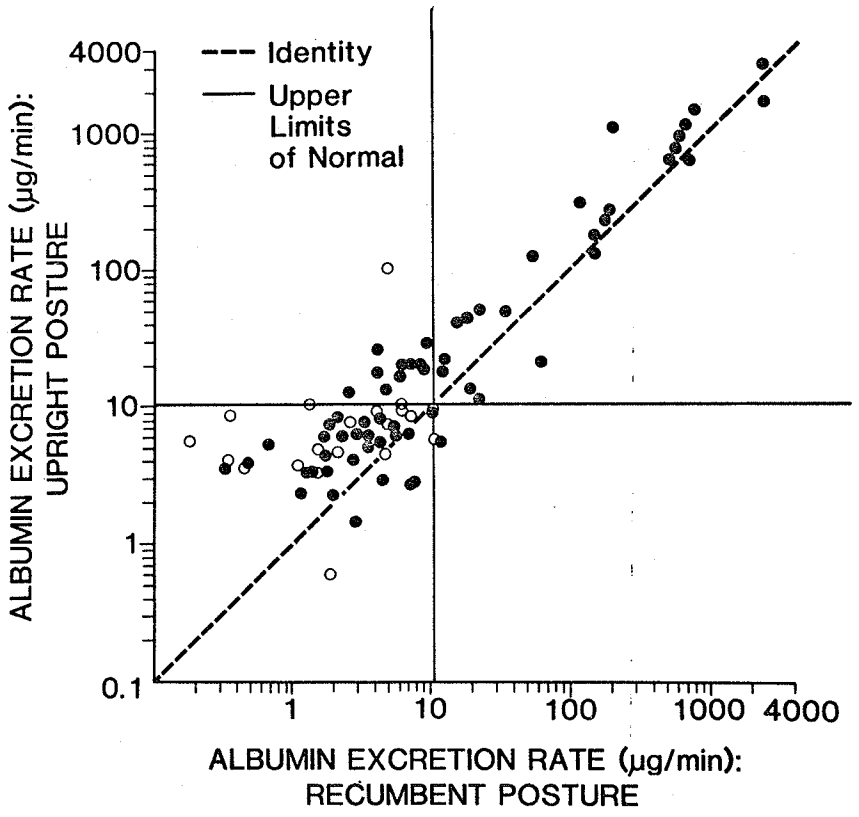

Fig. 14. Urine albumin excretion rate in 20 healthy (○) and 63 Type I diabetic (●) subjects: a comparison of collection postures.

Light touch sensory perception thresholds (TPT) (Fig. 15) were quite variable in the healthy subjects. Average percent variation from the mean was 42.8% (range 6.1 - 143.9). Abnormal TPT occurred in 21 of 54 diabetic subjects after 2 years duration, but was normal in 2 of 9 patients after 20 years. A multiple regression analysis in the diabetic subjects revealed a significant correlation of TPT with duration of diabetes (Table 6).

A correlation between AER and TPT was found to be significant (R-AER: $r=0.28$, $p<0.05$; U-AER: $r=.33$, $p<0.02$). A Pearson correlation was used to eliminate the common denominator of diabetes duration; subjects were divided into groups according to duration (0-5, 6-10, 11-15, 16-20, +20) and analyzed separately. No correlation of AER with TPT, independent of duration, was found.

Cross-tabulation statistics were performed to correlate the concurrence and severity of clinical findings (neuropathy, retinopathy, capillary dilatation) with one another and with the degree of albuminuria (Table 8). When the parameters were divided into four grades (0-3 as defined in the Materials and Methods), there were too many empty "cells" in the cross-tabulation matrix. For the final analysis, therefore, patients were subdivided only by the presence or absence of the parameters in question. The only significant correlations were between albuminuria and clinical retinopathy, and between capillary dilatation and clinical retinopathy. There was a trend in concurrence for albuminuria and capillary dilatation.

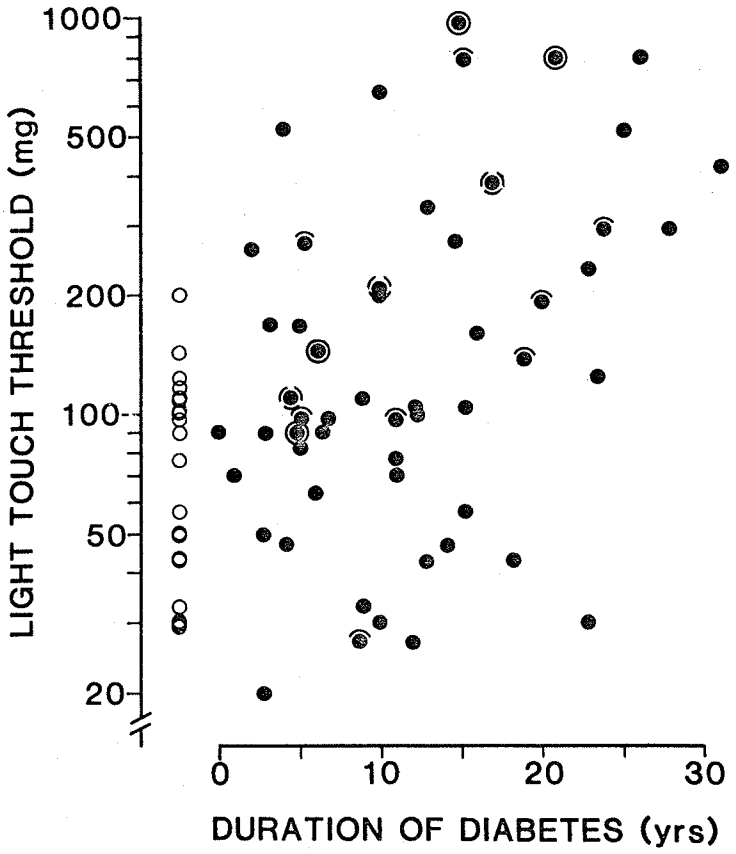

Fig. 15. Index finger light touch perception threshold in 20 healthy (○) and 54 Type I diabetic (●) subjects (clinical neuropathy: mild ◉ , moderate ◉ , severe ◉).

TABLE 8: CROSS-TABULATION OF CLINICAL FINDINGS AND HYPERALBUMINURIA*

Neuropathy	NS**		
Retinopathy	$X^2c=11.43$; $p<0.001$ $X^2r=13.27$; $p<0.001$	NS	
Capillary Dilatation	[$X^2r=3.08$; $p=0.08$] NS	NS	$X^2c=7.79$; $p=0.005$ $X^2r=9.69$; $p=0.002$
	Hyperalbuminuria	Neuropathy	Retinopathy

*Values are reported for corrected (c) and raw (r) chi-square data

**Not significant

DISCUSSION

(A) COLLECTION OF QUANTITATIVE URINE SAMPLES

Most investigators use 24-hour urine samples to examine albumin excretion (44, 63, 82, 91), this being a generally accepted mode of collection in clinical situations. Other types of sampling protocols include short ambulatory daytime collections (44, 89), overnight recumbent samples (44), and exercise provocation collections (84, 90, 195-202). Since non-standardization of urine collection protocols may be contributing to the disparity of observations regarding diabetic microalbuminuria, we compared these protocols in an attempt to find which might best expose the nature of early microalbuminuria.

I. Reproducibility

To our knowledge, there are no studies addressing the day-to-day reproducibility of different urine collection protocols in health. Variability would blur not only distinction between renal health and disease as in the present cross-sectional analysis, but especially also between stability and progression of hyperalbuminuria in future longitudinal studies in diabetic subjects. In other words, if a method is not reproducible in a healthy individual from one day to the next, then variability in a diabetic subject from one year to the next may not be ascribable to the effects of the disease. In addition, we attempted to discover the protocol with the most reproducibility within

healthy subjects as a group, as an aid to discriminatory power in the diabetic patients.

Of the four collection protocols, variability in AER within, and between, healthy individuals was least with a short morning sample. The reason for decreased reproducibility during recumbency and during the 24-hour collection may be changes in activity levels prior to, and during, the collection, respectively. The fact that variability rose during the pre-exercise hydration period suggests that the water load itself is affecting albumin excretion. Water loads are given prior to exercise tests because of exercise's reported anti-diuretic effects (54, 195, 203), and the degree of hydration of a subject will affect the volume of water recovered, during a fixed time after drinking water (54). Some authors describe an association between urine flow and excretion rate of total protein (53, 204), or albumin (205, 206), while others (72, 207) claim that urinary albumin excretion is more dependent on glomerular filtration than on urine flow, and that osmotic diuresis does not induce elevations in AER. If urine flow does affect albumin excretion, then differences in hydration may explain in part the large variability during the exercise and recovery periods. Differences in fitness also may partially explain the inter-individual variation.

By using the test with the best intra- and inter-individual reproducibility, one may be able to define more clearly the distribution and natural history of diabetic microalbuminuria.

II. Sensitivity

Discrepancies in the literature regarding early microalbuminuria may be due, in part, to differing discriminative ability of collection protocols in detecting early AER abnormalities. During recumbency and during the 24-hour collection, the AER of the five diabetic subjects was essentially normal (or very slightly abnormal). The increase in AER that occurred during assumption of the upright posture was great enough to pick up definite abnormalities in 2 of the 5 diabetic individuals; whether these changes indicate underlying renal disease or future renal dysfunction will remain to be seen. Urine flow was normal in these 2 subjects so the increase in AER was not due to any difference in diuresis. Since β-2-uER's were normal, we know that tubular reabsorptive capacity was not impaired, i.e., the defect was in glomerular filtration. Diabetic microproteinuria is considered to be primarily glomerular in origin (72).

The same two patients exhibited abnormalities during the exercise protocol, but one (DB) only during pre-exercise hydration. Exercise provocation is used to disclose functional abnormalities in protein excretion early in the course of diabetes, in subjects whose baseline excretion rate is normal (84). The rationale is that a certain workload (600 kpm/min) will not cause an increase in excretion in healthy subjects, but may possibly do so in diabetic patients with early glomerular abnormalities (198). Various authors have reported elevated AER in diabetic individuals subjected to exercise (84, 90, 195, 198-202, 206).

Resting blood pressure and heart rate response to exercise were normal in subject RH, so in accordance with other investigators (90, 198, 202, 208) hemodynamic variables are not likely to be causing

the abnormality. Vittinghus and Mogensen (202) concluded that the diabetic glomerular membrane is unable to retain albumin when the normal increase in filtration pressure operates during exercise, i.e., changes in the glomeruli themselves are the morphological substrate for the observed abnormalities in protein excretion (202). The marked increase in the ratio of AER/β2-uER in subject RH demonstrates that the elevated excretion rate of albumin is due to increased glomerular filtration rather than impaired tubular resorption, in accordance with the findings of others (90).

Mogensen et al. (84) claim that during exercise provocation, changes in AER are disclosed that are not demonstrable during baseline conditions. In our case, this may have been true for subject RH, had we used a recumbent or 24-hour urine sample as an indicator of basal AER. However, by investigating AER during upright ambulation, we were able to disclose a baseline abnormality in this individual. In addition, the three diabetic individuals with normal basal excretion rates were also normal under exercise provocation. So in accordance with results obtained by Poortmans et al. (197) exercise provocation failed to reveal any additional information, over that of basal AER, and indeed there is a suggestion of lesser sensitivity.

III. Specificity

For the cross-sectional survey, a recumbent and upright sample were collected from each subject. Despite the apparent merits of the ambulatory protocol, we were wary of the occurrence of so-called benign postural proteinuria, i.e., clinically abnormal protein excretion

only during ambulation. Clinical postural proteinuria did not occur in any of the subjects, but one healthy individual (number 3) and 10 diabetic individuals did have postural microproteinuria. However, the prevalence of induction of hyperalbuminuria by upright posture was much higher in diabetes than in health, indicating that in most, if not all, patients the abnormality was in fact diabetic in origin. Further evaluation of specificity can only await the passage of time.

It is apparent from this preliminary investigation that there are inherent differences in the reproducibility, sensitivity, and specificity of the different urine collection protocols. These sampling differences may explain in part the previous discrepant reports (44, 86-88, 91, 125) regarding the prevalence of preclinical hyperalbuminuria. If one urine collection protocol is to be routinely applied to the clinic situation, it should be the ambulatory sample. Reproducibility and sensitivity are optimal, while minor nonspecificity is not an important disadvantage. In addition, amongst the alternatives it is by far the easiest to collect (for both patient and clinician).

(B) CROSS-SECTIONAL STUDY OF ALBUMINURIA IN A TYPE I DIABETES CLINIC

I. Distribution

We found a ramp-like rise in albuminuria in our 65 patients, which correlated with diabetes duration. AER (whether in recumbency or during ambulation) was normal at the onset, and during the first few years, of the disease. Thereafter, a progressive duration-related increase in the prevalence and degree of albuminuria occurred. Of the

48 diabetic subjects who were clinically non-proteinuric, 19 had elevated immunoassayable AER (range 11.48 to 323.14 ug/min). These results contradict those of Mogensen (44, 86) and support those of Viberti et al. (91), Panzram et al. (87), Hemmingsen et al. (88), and Ditzell and Junker (125). Evidence from these investigators and ourselves seems to indicate a gradual rise in albumin excretion to clinically manifest levels. Why Mogensen (44, 86) found normal basal AER in all members of a group of clinically non-proteinuric insulin-dependent patients (duration 1 to 19 years) may relate to differences in urine sampling protocol or case selection and quality of treatment between his group and ours. As Viberti (72) suggests, resolution of the conflict surrounding the nature of microalbuminuria will require prospective studies. Our survey confirms that development of diabetic nephropathy is not the inevitable fate of every patient, since approximately 1/3 of those with duration greater than 20 years were normoalbuminuric. The nature of the "protective mechanism" operative in these individuals is not known (69), but its elucidation may provide insight into the underlying causes of microangiopathy.

II. Influence of Glycemia

Knowles (96) argues that the most doubtful measurement of all is that of control, since the nature of diabetes makes past and even present evaluation extremely difficult and the measurement of day-to-day glycemic control an almost insurmountable task. By having the patient collect blood samples at home (concurrent with urine collection), we hoped to gain insight into the short-term effects of glycemia on AER.

Medium-term control was assessed by measuring the glycosylated hemoglobin fraction (HbAl), i.e., the percentage of hemoglobin bound to glucose. This post-synthetic modification is dependent on the time-averaged concentration of glucose within the erythrocyte, over the lifetime of the cell (209). %HbAl therefore gives us information on the adequacy of diabetic control over a sustained period of time (approximately 3 months). Assessment of long-term control was judged to be futile.

Some authors describe reversible fluctuations in AER early in the course of diabetes, which depend on the level of glycemia. Parving et al. (63), for example, found that newly diagnosed and short-term diabetic subjects in poor metabolic control had elevated urinary albumin excretion. In our survey, early functional glycemia-dependent albuminuria did not occur, but then only one individual had diabetes of less than 12 months duration. Perhaps our recent-onset subjects were reasonably well-controlled, since insulin treatment and good glycemic control is found to revert to normal the urinary excretion of albumin (63). In any event, we found no evidence to suggest that functional hyperalbuminuria will be a confounding factor in longitudinal analyses.

Correlations of glycemic control with AER have been found cross-sectionally in established insulin-treated subjects as well. Viberti et al. (82) found a highly significant correlation ($r=0.72$; $p < 0.001$) between %HbAl and albumin excretion rate. We found no such correlation in our subjects. Our findings, however, do not preclude the possibility that glycemia and AER may correlate within individual patients, i.e., improvement of the metabolic state within a particular subject may ameliorate the AER (91, 107, 108).

The absence of functional glycemia-dependent changes in AER and the presence of the ramp-like AER-versus-diabetes duration distribution, are consistent with the idea that the measurement of urine albumin excretion may be a potentially useful predictive tool for diabetic nephropathy.

III. Mechanism of Microalbuminuria

The appearance of abnormal amounts of albumin in the urine may be due to increased transglomerular passage or decreased tubular reabsorption of protein. Glomerular and tubular proteinuria can be distinguished by simultaneous measurement of albumin and β2-microglobulin (β2-u) excretion rates (61). The fact that 45 of our 48 clinically non-proteinuric diabetic patients had normal urinary levels of β2-u agrees with results obtained by Viberti et al. (91), and is consistent with the notion that subclinical diabetic albuminuria is primarily a glomerular disorder (72). However, once clinical symptoms are manifest and renal function markedly deteriorates, proteinuria becomes of mixed glomerular and tubular origin (72). This may be the case in those three subjects (numbers 13, 19, 57) with gross clinical proteinuria and elevated β2-uER. However, three subclinically hyperalbuminuric patients (subjects 2, 14, 17) also had elevated β2-uER's. Perhaps in the occasional patient a tubular disorder accompanies early microalbuminuria.

A structural basis for the initial abnormalities in glomerular filtration of albumin may be provided by the morphological studies of Osterby (77, 166), since the excretory changes are consistent in timing and frequency with glomerular basement membrane (GBM) thickening.

Osterby (77) found GBM thickness to be normal at the onset of diabetes and to progress over the first 2 years of the disease; by $3\frac{1}{2}$ to 5 years diabetes duration, 2 groups of subjects emerged, those with normal GBM, and those with increasing GBM width. It is probable that an increase in the width of the basement membrane is indicative of a more permeable structure. Thus the functional counterpart of increased basement membrane thickening may be an increase in permeability to circulating macromolecules, such as albumin. Our results may be linked hypothetically to Osterby's findings in the following manner: in early diabetes, AER is normal and GBM width is normal. Within 2 years, structural changes occur in some individuals, such that by about 2.5 years diabetes duration, abnormal AER first appears (during upright ambulation). By 5 years, 2 groups emerge, hyperalbuminuric individuals with increased GBM width, and normoalbuminuric individuals with normal glomerular ultrastructure.

(C) INTERACTION OF OTHER DISPOSING VARIABLES UPON DIABETIC MICROALBUMINURIA

I. Hypertension

Hypertension is known to be associated with diabetic nephropathy from the stage of persistent clinical proteinuria onwards (147, 149). It is not causal (151), but serves as an accelerating risk factor in progressive renal disease (146). According to Christlieb et al. (150), in patients dying a "renal death," there is a uniform pattern of concurrent hypertension, such that diabetic hypertension can be thought of as renal in origin (150). Our findings are consistent with these observations since 13 of 15 hypertensive subjects had immunoassayable hyperalbuminuria

(in recumbency and during ambulation).

We found no cross-sectional correlation between diastolic blood pressure and albuminuria in our 65 patients. Deckert and Poulson (69) demonstrated that arterial blood pressure measured prior to the onset of persistent clinical proteinuria was almost the same in Type I diabetic patients who subsequently developed diabetic nephropathy compared with matched Type I diabetic subjects without evidence of nephropathy after more than 32 years of disease, i.e., a correlation between subclinical microalbuminuria and diastolic blood pressure may not exist. This may also partially explain why no correlation was found between albuminuria and diastolic blood pressure in 15 hypertensive subjects, as only 7 were proteinuric by clinical standards. Furthermore, other factors such as duration of hypertension and duration, type, and effectiveness of hypertensive treatment may reveal more about the stability and severity of the hypertensive state than a single blood pressure value can.

Our cross-sectional findings do not preclude, however, the possibility that diastolic blood pressure and albumin excretion may correlate longitudinally in any given patient. Many studies have demonstrated a decrease in albuminuria with amelioration of hypertension (76, 148, 155, 210).

II. Unknown Factors

The fact that approximately 3/4 of the variation in albuminuria was still unaccounted for after the effects of diabetes duration, glycemia, and diastolic blood pressure had been taken into account is consistent with the involvement of additional unknown predisposing factors in the

genesis of microproteinuria. The progression of microalbuminuria to clinically detectable levels shows marked variation betwen individuals (189), and these factors may play some role in determining this variability.

(D) INTERRELATIONSHIP BETWEEN MICROALBUMINURIA AND OTHER DIABETES COMPLICATIONS

I. Neuropathy

Diabetic nerve disease is an important complication of diabetes, owing to its relative frequency and widespread effects (67). According to Logothetis and Baker (211), diabetic neuropathy encompasses peripheral syndromes such as symmetric and asymmetric neuropathy, radiculopathy, autonomic visceral neuropathy and cranial neuropathy, and spinal cord syndromes such as myelopathy, pseudotabes, amyotrophy and acute vascular syndromes. Despite the ubiquitous nature of neuropathy, we know little of the factors responsible for its development. Hypothesized causes are reviewed by Clements (212) and Gabbay (213) and include segmental demyelination, vascular insufficiency and sorbitol accumulation.

Although clinical examination of patients with peripheral neuropathy does not usually involve the upper limbs (214, 215), examination of conduction rates for sensory and motor nerves (216, 217), vibratory perception thresholds (218), and vasomotor responsiveness (219) has revealed abnormalities. An early and generalized sensory deficit is an impaired light touch sensory perception threshold (TPT); this is evident in the fingers (192, 193).

We found that impaired TPT occurred within 2 years of the onset of diabetes and correlated with duration of the disease. Chochinov et al. (193) found defects in tactile sensory perception near the onset of diabetes as well, but were not able to demonstrate progression with time. In accordance with results from Chochinov et al. (193), no relation between TPT and prevailing glycemia was observed.

TPT may aid in the selection of those patients at low and high risk of developing neuropathy, i.e., a TPT of less than 90 mg (well within the normal range), for example, may suggest immunity since clinical neuropathy was absent in all but one patient within this TPT range. But a higher value may not necessarily predict a predisposition to clinically significant disease.

Pirart (65) examined the degenerative complications of diabetes and found a close association between neuropathy, nephropathy, and retinopathy. He also noted, however, that the incidence of each of these three chronic complications is a function of the duration of diabetes, and that all subsequent correlations take this major determinant into account (65). Our results concur with Pirart's conclusions, since no correlation of TPT with albuminuria, independent of diabetes duration, was found. In addition, there was no correlation between clinical neuropathy and albuminuria. These results are consistent with the view that the neuropathic lesions of diabetes are etiologically different from the vascular lesions.

III. Retinopathy

Diabetic retinopathy encompasses nonproliferative or background changes which include venous dilatation, retinal microaneurysms and

hemorrhages, capillary closure and exudates, and proliferative changes which include neovascularization, fibrous tissue formation, preretinal hemorrhages and retinal detachments (67). These changes rarely occur before five years diabetes duration (67). Venous dilatation and congestion have been noted to occur prior to microaneurysm development (220, 221) and appear to serve a prognostic value for the future course of retinopathy (194). The dilatation is functional (reversible) in its early stages and extends into the small venules and capillaries as well (158).

In our subjects, the presence of capillary dilatation visualized ophthalmoscopically under red-free light correlated significantly with the presence of clinical retinopathy (as defined in Materials and Methods), such that quantitation of capillary congestion may indeed serve a prognostic role.

Capillary dilatation showed a trend toward concurrence with microalbuminuria, while clinical retinopathy and microalbuminuria were concurrent to statistical significance. This is not surprising since renal and retinal damage in diabetes are thought to be linked to a common pathogenetic factor (222) as both constitute vascular lesions. In fact, many authors (76, 82, 147, 148, 223) use the presence of retinopathy as an aid in diagnosing nephropathic glomerulosclerosis, because of the almost invariable coexistence of these 2 complications. However, normal renal function may be found even in those with severe proliferative retinopathy, such that the causative factor(s) in renal and retinal microvascular alterations, though they may be commonly associated in diabetes of long duration, may differ and occasionally be dissociated from one another (91).

SUMMARY

An evaluation of possible urine collection protocols has revealed that brief timed upright samples may provide the best within and between individual AER reproducibility and resolution of differences between diabetic and healthy subjects, 2 properties desired for the cross-sectional and longitudinal study of kidney function in diabetes.

In a cross-sectional setting, diabetic microalbuminuria exhibited predictable relationships to disease duration and to the occurrence of neurovascular complications. Our evidence suggests a ramp-like rise in microalbuminuria over time, to clinically overt levels. Glycemia did not appear to induce functional changes in albumin excretion cross-sectionally; this does not preclude its importance in routine patient care. The relationship between diabetic microalbuminuria and the occurrence of diabetic neurovascular complications may be duration and/or etiology related.

The way seems clear to the application of immunoassayable AER to the detection and prospective follow-up of individuals with subclinical diabetic nephropathy with respect to innate susceptibility and to effectiveness of a given treatment mode.

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