Microalbuminuria, as measured by radioimmunoassay (RIA), is generally equated with the development of diabetic nephropathy in type 1 diabetes (1,2). The change in albumin excretion rate (AER) has traditionally been explained as being due to changes in the glomerular permselectivity barrier and intraglomerular pressure (3,4) with the assumption that albumin remains intact during filtration and renal passage. However, recent studies by our group using tritium-labeled albumin ([3H]-albumin) in a rodent model have demonstrated that albumin excretion could be significantly influenced by the metabolism of albumin to small peptide fragments during renal passage (5–9). The fragmentation is extensive, with ~90–95% of the urinary albumin representing a complex fragment population of >30 different fragments with molecular weights in the range of 1–15 kDa. These albumin-derived fragments are not detected by standard immunochemical assays (6,8). A small number of studies have previously identified albumin fragments in human urine, although the source of these fragments has not been determined; these fragments (>15 kDa) were detected by electrophoretic immunochromatographic methods (10–12). In the streptozotocin-induced diabetic rat, we have demonstrated that the renal metabolism of albumin is partially inhibited, with a markedly different pattern of albumin fragments being excreted (8,13).

The aim of this study was to examine whether albumin is metabolized during renal passage before excretion in nondiabetic patients and whether this process may change in type 1 diabetic patients with varying stages of diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Tritium labeling. Albumin (50 mg from Albumex 20; Commonwealth Serum Laboratories, Melbourne, Victoria, Australia) was labeled with tritium by the reductive methylation procedure of Tack et al. (14). This reaction involves a brief exposure to formaldehyde and sodium borohydride (132 mCi/mg; DuPont, Wilmington, DE). The labeled preparation was applied to a Sephadex G-25 PD-10 column to separate it from free label. The purity of the [3H]-albumin preparation was ensured by extensive dialysis of the sample (>105 dpm/ml; 3.5 ml) against phosphate-buffered saline (PBS) (which contained 136.9 mmol/l NaCl, 2.68 mmol/l KCl, 8.1 mmol/l Na2HPO4, and 1.5 mmol/l KH2PO4, pH 7.4) using dialysis tubing with a molecular weight cutoff of 2,000 until no tritium was found in the dialysate. Before the tracer study, the [3H]-albumin was further purified by fractionation on a Sephadex G-100 column where only the peak fraction was used for subsequent intravenous injection to eliminate the possibility of contamination by low–molecular weight peptides that could be preferentially filtered (5).

From the Endocrine Unit (T.M.O., C.A.H., G.J.) and the Nuclear Medicine Department (J.G.C.), the Department of Medicine, Austin & Repatriation Medical Center, University of Melbourne, Heidelberg, Victoria; and the Department of Biochemistry and Molecular Biology (W.D.C.), Monash University, Clayton, Victoria, Australia.

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A&RMUC, Austin & Repatriation Medical Center; AER, albumin excretion rate; PBS, phosphate-buffered saline; PKC, protein kinase C; RIA, radioimmunoassay; V0, void volume; Vt, total volume.
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• Group 1 (n = 9): nondiabetic volunteers with no history of diabetes or

renal disease (normoalbuminuria)

• Group 2 (n = 5): type 1 diabetic patients with AER <20 µg/min (normoal-

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• Group 3 (n = 2): type 1 diabetic patients with AER between 20 and 200

µg/min (microalbuminuria)

• Group 4 (n = 4): type 1 diabetic patients with AER >200 µg/min (macroal-

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TABLE 1

Clinical characteristics of study subjects

<table>
<thead>
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<th>Group</th>
<th>Age (years)</th>
<th>Sex (M/F)</th>
<th>Duration of diabetes (years)</th>
<th>AER (µg/min)</th>
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<tr>
<td>Nondiabetic volunteers</td>
<td>28.7 (20–71)</td>
<td>2/8</td>
<td>NA</td>
<td>&lt;20</td>
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<td>33.4 (19–60)</td>
<td>2/3</td>
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<td>13.9 (5.6–19.6)</td>
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Data are n or means (range). NA, not applicable.

Patient selection criteria. Four groups of volunteer subjects of European ori-

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RESULTS

A representative elution profile from Sephadex G-100 of

[\(^{3H}\)J-albumin in urine collected from a nondiabetic volunteer

shown in Fig. 2. No significant amount of radioactivity associ-

ated with the albumin-derived fragments could be observed

in any of these profiles.

Closer examination of the possible presence of fragments

plasma and urine was made using a Sephadex G-50 column

(Fig. 3). The profile obtained for the urine sample confirmed

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Tracer study. This study was approved by the A&RMC Human Ethics Com-

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Medicine Department of the A&RMC. Patients were screened for urinary

tract infection by a Multistix dipstick test. The \(^{[3H]}\)-albumin was sterilized

under aseptic conditions by passage through a 0.22-µm Millipore low protein

binding sterilization filter before injection. A bolus injection of 1 mCi (~4 mg

and 0.03 mSv) of ultrapure \(^{[3H]}\)-albumin was administered intravenously into

a cubital vein. A small volume of blood was drawn from the contralateral arm

after either 1 or 24 h after injection. Urine samples were collected at either 4 h

after injection or 24 h after injection when another blood sample was taken.

The urine and plasma collected was fractionated by size exclusion chro-

matography using a Sephadex G-100 column (1.6 × 60 cm) (Pharmacia Fine

Chemicals, Uppsala, Sweden) eluted with PBS (which contained 0.2% bovine

serum albumin and 0.02% sodium azide) at 20 ml/h at 4°C. One-milliliter sam-

ples were loaded, and 95 fractions of 1.7 ml each were collected. The void vol-

ume (\(V_o\)) was determined with blue dextran T2000 and the total volume (\(V_t\))

with tritiated water. The eluates were mixed with scintillant, and radioactiv-

ity was estimated using a scintillation counter and the amount of intact-to-frag-

mented albumin calculated from the area under the peaks.

Albumin concentration was measured by an in-house RIA (15) on the 24-h

urine sample, whereas urinary creatinine was determined by the Jaffé method

using the BM/Hitachi 737 automatic analyzer. All quantitative data are

expressed as means ± SE, where \(n\) represents the number of determinations.

RESULTS

A representative elution profile from Sephadex G-100 of

[\(^{3H}\)J-albumin in urine collected from a nondiabetic volunteer

shown in Fig. 1 reveals that there was no significant difference

when urine was analyzed 4 or 24 h after injection. Figure 1 also

reveals that most of the \(^{[3H]}\)-albumin in the urine is degraded
to small fragments (only 1.29 ± 0.41% intact albumin

excreted, \(n = 9\)). Tritium labeling of albumin by reductive

methylation is specific for the α-amino groups of the amino-
terminal residues and the ε-amino groups of lysyl residues

(14). Approximately 5–15% of the lysyl residues, which con-

stitute ~10% of the amino acid residues in albumin, will be

labeled with tritium (14). Therefore, it is likely that most of

the complex mixture of albumin-derived peptide fragments,
as seen in the urinary chromatography profiles, would be
detected through the presence of their tritium label, as has

been demonstrated in rats (5,7).

Representative elution profiles from Sephadex G-100 of

[\(^{3H}\)J-albumin in plasma from both nondiabetic volunteers and diabetic patients collected 1 and 24 h after injection are

shown in Fig. 2. No significant amount of radioactivity associ-

ated with the albumin-derived fragments could be observed

in any of these profiles.

Closer examination of the possible presence of fragments in plasma and urine was made using a Sephadex G-50 column

(Fig. 3). The profile obtained for the urine sample confirmed

the results seen for the G-100 column (Fig. 1), where the

FIG. 1. Representative profile of the size exclusion chromatography on Sephadex G-100 of \(^{[3H]}\)-albumin in urine samples from a nondiabetic volunteer 4 h (A) and 24 h (B) after the intravenous administration of \(^{[3H]}\)-albumin. The dashed line represents the elution position of ultrapure \(^{[3H]}\)-albumin before injection. Most of the urinary \(^{[3H]}\)-albumin elutes as heavily degraded albumin fragments in the molecular weight range of 1–15 kDa. The proportion of intact albumin is very low (1.29 ± 0.41%, \(n = 9\)) and not visualized on the scale shown. \(V_o\) was determined with blue dextran T2000 and \(V_t\) with tritiated water. The fraction volume is 1.7 ml.
labeled fragments eluted before $V_t$ in the molecular weight range of 1–15 kDa. However, the elution profile of the plasma sample (1 h) reveals a small proportion (~1%) of labeled low–molecular weight material that elutes at the $V_t$ of the column. Similar amounts were also present in plasma collected 24 h after injection for all individuals used in this study. This finding indicates very low–molecular weight material (<1 kDa) that is likely to be free tritium. It is clear that the plasma material that elutes in the fraction-60 sample (representing 0.04% of the total plasma sample), corresponding to the maximum peak of the urine, would not contribute significantly to the material seen in the urine. In any case, results from rat studies have demonstrated that extrarenal albumin–derived fragments, if present in plasma, have very low fractional clearances because they avidly bind to plasma components (K.A. Greive, T.M.O., W.D.C., unpublished data). The very small quantity of low–molecular weight material in plasma that coelutes with urine fragments observed at any time collection together with their low fractional clearance effectively eliminates peripheral (extrarenal) metabolism of albumin contributing to the observed fragments in urine.

The presence of labeled low–molecular weight material in the urine profile was also not the result of degradation of albumin due to proteases in the urine because incubation of urine from a nondiabetic volunteer with $[^3H]$-albumin for 18 h at 37°C resulted in <10% degradation of the molecule (data not shown).

Size exclusion chromatographic analysis of urine radioactivity collected from type 1 diabetic patients with varying levels of albuminuria associated with diabetic nephropathy 1 h (C) and 24 h (D) after the intravenous administration of $[^3H]$-albumin.

**FIG. 2.** Representative profiles of the size exclusion chromatography on Sephadex G-100 of $[^3H]$-albumin in plasma samples collected from a nondiabetic volunteer 1 h (A) and 24 h (B) after the intravenous administration of $[^3H]$-albumin and from type 1 diabetic patients with varying levels of albuminuria associated with diabetic nephropathy 1 h (C) and 24 h (D) after the intravenous administration of $[^3H]$-albumin.

**FIG. 3.** Representative profile of the size exclusion chromatography on Sephadex G-50 (column dimensions, 1.5 x 48 cm$^2$) of $[^3H]$-albumin in plasma (●) and urine (○) from a nondiabetic volunteer. $V_o$ was determined with blue dextran T2000 and $V_t$ with tritiated water. The fraction volume is 1.0 ml. A small amount of tritium-labeled low–molecular weight material is present in the plasma that elutes at the $V_t$ of the column. However, this low–molecular weight material could only contribute up to 18% of the fragments observed in the urine (calculated from the area on the right-hand side of the dashed line that could correspond to urine material contributed by the low–molecular weight material in plasma), which clearly elute 8 fractions before the $V_t$ of the column.
degrees of albuminuria is shown in Fig. 4. The profiles in Fig. 4 demonstrate that increased levels of albuminuria are associated with a marked change in the ratio of intact-to-fragmented albumin and that fragmentation of urinary albumin has been inhibited.

When the urine fractions from a macroalbuminuric patient were assayed for albumin by RIA, only the intact albumin peak was detected (Fig. 5). This detection suggests that the RIA is specific for intact albumin and does not detect small albumin fragments. Thus, albumin concentration measured by RIA represents only intact albumin and considerably underestimates the combined total of intact albumin plus albumin-derived fragments. We have previously shown that RIA measures only intact albumin for both bovine and rat albumin (6,8), where the presence of urinary albumin fragments was detected by both radioactivity and absorbance at 280 nm.

When the percentage of intact albumin, as determined by size exclusion chromatography, was plotted against the albumin-to-creatinine ratio for each subject (Fig. 6), a significant relationship was found for the type 1 diabetic patients \((r = 0.809, P < 0.0001)\). This finding is expected because albumin immunosay measures only intact albumin. The percentage of intact albumin represented 1.29 ± 0.41% for nondiabetic volunteers, 1.36 ± 0.61% for normoalbuminuric type 1 diabetic patients, and 24.68 ± 9.21% for micro- and macroalbuminuric type 1 diabetic patients \((P = 0.048 \text{ vs. normoalbuminuric patients})\).

**DISCUSSION**

A major finding reported in this study is that RIA detects only intact human albumin in urine. This result ensures that the percentage of intact albumin must increase with increasing albuminuria as measured by RIA; otherwise, if it remained constant at 1.29 ± 0.41%, the amount of total albumin (intact plus albumin-derived fragments) excreted would reach unrealistic levels (i.e., if the excretion rate by RIA is ~1,450 µg/min in a macroalbuminuric patient and this intact albumin is only ~1% of the total albumin excreted [intact plus fragments], then the total albumin excretion would be 145 mg/min). Studies of diabetic patients with increased levels of albuminuria demonstrated a significant correlation between the percentage of intact albumin and the level of albuminuria. This finding is also consistent with observations made in rats (6,8).

It is generally recognized that the metabolism of filtered proteins in the kidney occurs through lysosomal activity in proximal tubular cells (17,18). This study demonstrates that albumin in nondiabetic volunteers is metabolized to low-molecular weight fragments during renal passage before excretion. These results are consistent with the findings obtained in rats (5-9). The exact anatomical location of this metabolic degradation pathway is distal to the glomerular filtration barrier and is likely to occur by endocytosis and extensive lysosomal degradation in proximal tubule cells (17). Once the fragments are formed in early endosomes or

**FIG. 4.** Representative profiles of the size exclusion chromatography on Sephadex G-100 of [3H]-albumin in urine samples collected from type 1 diabetic patients with varying levels of albuminuria associated with diabetic nephropathy after intravenous administration of [3H]-albumin as detected by radioactivity.
Lysosomes, they may be returned to the tubular lumen with subsequent excretion in the urine within a matter of minutes. Proximal tubular cell endosomes associated with the gp330 albumin receptor have been recently shown to recycle back to the cell surface (19). Furthermore, this type of metabolic degradation pathway has been observed in other studies that have demonstrated that protein may be taken up by lysosomes or cells in culture and returned to the medium as peptide fragments (20,21).

The fragmentation ratio (fragmented:intact) of albumin was reduced in diabetic patients; the magnitude of the reduction reflected an increase in (intact) albuminuria as determined by RIA. Our finding of a reduced fragmentation ratio of albumin in diabetes may correspond in part to decreased lysosomal activity and/or intracellular trafficking to and from the lysosome. This finding is consistent with findings in other studies that have shown that enzymes specifically associated with lysosomes have decreased activity in diabetes (22–24). However, it is possible that this change in enzyme activity only affects the size of the fragments excreted and not the quantity. This study demonstrates that the capacity for albumin degradation during renal passage is significantly increased in diabetic subjects with increasing albuminuria, as is shown in Table 2. The disorder of albumin processing as determined from albuminuric patients then relates to 2 distinct processes: 1) a decrease in the fragmentation ratio of albumin and 2) the increased capacity of renal cells to cope with the high flux of the albumin substrate. The absolute degradation rate appears to increase with higher albuminuria; therefore, the nephron cannot sustain a normal albumin excretion rate, and the system may be overwhelmed in albuminuric states.

The pathophysiological significance of these studies is that albuminuria may be influenced by intrarenal albumin metabolism. Intact albuminuria is not a true measure of total urinary albumin (intact albumin and albumin-derived fragments), and therefore caution must accompany the interpretation of changes in magnitude of this type of albuminuria as measured by RIA.

This study has identified albumin excretion rates (including albumin-derived fragments) to be much higher than those previously reported and demonstrated that this result is in part due to the high flux of albumin directed toward the metabolic degradation pathway. Recent studies have demonstrated that albumin flux across the glomerular capillary wall is normally high and that this albumin is retrieved by a cell-mediated

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**TABLE 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>AER (µg/min)</th>
<th>Percent intact</th>
<th>Fragment excretion rate (µg/min)</th>
<th>Total excretion rate (µg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoalbuminuric patients</td>
<td>13.9 (5.6–19.6)</td>
<td>1.36 (0.01–4.0)</td>
<td>895 (390–1,776)</td>
<td>905.6 (405–1,788)</td>
</tr>
<tr>
<td>Macroalbuminuric patients</td>
<td>913.8 (266–1,457)</td>
<td>35.4 (8.4–55.5)</td>
<td>1,830 (856–2,889)</td>
<td>2,744 (1,485–3,710)</td>
</tr>
</tbody>
</table>

Data are means (range). Total albumin excretion rate = 100% × AER/percent intact. Fragmented albumin excretion rate = total albumin excretion rate - AER.
RENAL METABOLISM OF ALBUMIN

Retrieval pathway (25) that is distal to the glomerular filtration barrier. Inhibition of this pathway through blockade of intracellular trafficking/microtubule formation would potentially lead to these high fluxes of albumin. Other mechanisms that have been more widely discussed in the literature to explain the high flux of albumin to renal cells show that there may be a defect in the glomerular filtration barrier in diabetic nephropathy, allowing for increased filtration of serum proteins (3,4). However, the exact nature of this defect remains to be determined. It should be noted that previous glomerular models of albuminuria did not take into account the high excretion rate of albumin (intact plus fragments) that we identify in this study.

Diabetes has been shown to be associated with the activation of protein kinase C (PKC) (26,27) through angiotensin II binding to the type I angiotensin II receptor (28). PKC effects on vesicular trafficking have been demonstrated to be a regulatory mechanism to control endocytosis and exocytosis of albumin in proximal tubular cells (29). Protein kinases may contribute to regulation of proteins involved in microtubule-based motility or the cytoskeleton itself and thereby affect the associated transcellular passage of transport vesicles. Although the requirements for microtubular structures in transcytosis may not be homogeneous (30), this kinase regulation might be a direct mechanism by which angiotensin II (through PKC and/or protein kinase A) could modulate the trafficking of albumin in epithelial cells of the nephron and lysosomal degradation (31,32).

We have established that the albumin-derived products present in urine from nondiabetic volunteers consist of a small amount of intact albumin together with a large quantity of albumin-derived fragments that are not detectable by conventional RIA. The reduced fragmentation ratio of albumin in diabetic patients with varying stages of nephropathy was found to be proportional to the level of albuminuria. The changes in the pattern of albumin fragments in the urine reflect a complex molecular processing of protein that appears to be impaired as nephropathy advances. This process may offer a new paradigm for the understanding of handling albumin in diabetic renal disease.

ACKNOWLEDGMENTS

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REFERENCES