Studies on the Metabolism of the Renal Glomerular Basement Membrane

TURNOVER MEASUREMENTS IN THE RAT WITH THE USE OF RADIOLabeled AMINO ACIDS

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The turnover of the leucine, hydroxylysine, lysine, and phenylalanine constituents of the basement membrane have been investigated in the rat with marked “izduertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The present investigation was undertaken in an attempt to fill this gap in our knowledge and the in vivo situation was chosen as it lends itself best for the purpose of turnover studies. Radiolabeling of glomerular basement membrane was achieved in the intact rat with a number of amino acids and measurements were made of the decrease in their specific radioactivity with time. The turnover of the membrane was found to be slow in comparison with other glomerular proteins and comparable to that of tail tendon collagen from the same animals.

The rat glomerular basement membrane in common with the bovine membrane was found to have a complex poly-peptide subunit pattern on polyacrylamide gel electrophoresis and this was thought to account for some of the differences observed in the turnover rates of various of its amino acid constituents.

EXPERIMENTAL PROCEDURES

Animals and Radiosources—Male albino rats (Charles River, CD strain) were used and maintained on Purina chow and water ad libitum up to the time of death. The animals (100 to 115 g weight) were injected in groups of six with a single intraperitoneal dose of tritiated amino acid, in approximately 0.5 ml of physiological saline (0.85% NaCl) (Table I). All injections were performed in the morning hours and the rats were killed at various times thereafter, ranging from 4 to 504 h (3 weeks). At the designated times the animals were lightly anesthetized with ether and exsanguinated by cardiac puncture. The kidneys and tail were rapidly removed from each rat and placed on ice while the drawn blood was permitted to clot at room temperature. The kidneys from larger rats (250 to 450 g) of the same strain were used to prepare unlabelled glomerular basement membrane.

Preparation of Glomeruli—Immediately after removal, the kidneys were decapsulated and bisected longitudinally. The cortices were excised, pooled for each group of six rats, tightly wrapped in aluminum foil, frozen on dry ice, and stored at -20° until required. Glomeruli were prepared by a sieving procedure previously employed for bovine kidneys (3) but modified to take into account the smaller dimensions of the rat glomeruli and the limited amount of tissue to be processed. A 170-mesh stainless steel sieve (3-inch diameter) was employed to disrupt the thinly sliced cortex, after it had been permitted to thaw, and the sieved material was collected in ice-cold 0.15 M NaCl. In the subsequent filtration step an 80-mesh sieve was used to remove tissue fragments while a 270-mesh...
TABLE I

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Specific activity</th>
<th>Dose</th>
<th>Serum concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-[3,4,5-3H]Proline</td>
<td>25.8</td>
<td>150</td>
<td>43</td>
</tr>
<tr>
<td>L-[3,4,5-3H]Lysine</td>
<td>8.0</td>
<td>350</td>
<td>67</td>
</tr>
<tr>
<td>L-[3,4,5-3H]Glycine</td>
<td>10.2</td>
<td>150</td>
<td>42</td>
</tr>
<tr>
<td>L-[3,4,5-3H]Phenylalanine</td>
<td>12.0</td>
<td>300</td>
<td>7.5</td>
</tr>
<tr>
<td>L-[4,5,6-3H]Leucine</td>
<td>30.0</td>
<td>290</td>
<td>17</td>
</tr>
</tbody>
</table>

a Lysine was purchased from Schwarz/Mann; all other isotopes obtained from New England Nuclear. All preparations had a radioactive purity of greater than 98%.

b Refers to activity of radioisotope prior to injection.

c Determined with amino acid analyzer on a deproteinized sample of rat serum as described under "Experimental Procedures." The concentration of other amino acids relevant to this study was: hydroxyproline, 0.8 µmol/100 ml; hydroxylysine, not detectable; tyrosine, 1.7 µmol/100 ml.

d The lysine was "generally labeled" so that the position(s) of the tritium is not known.

TABLE II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Glomerular basement membrane</th>
<th>Other glomerular proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>residues/1000 total</td>
<td>amino acid residues ± S.D.M.</td>
</tr>
<tr>
<td>3-Hydroxyproline</td>
<td>65.7 ± 5.9</td>
<td>8.6 ± 2.2</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>66.6 ± 2.5</td>
<td>85.6 ± 11.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>38.3 ± 2.1</td>
<td>57.7 ± 3.7</td>
</tr>
<tr>
<td>Serine</td>
<td>54.1 ± 1.2</td>
<td>70.8 ± 7.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>94.6 ± 3.6</td>
<td>113.3 ± 4.6</td>
</tr>
<tr>
<td>Proline</td>
<td>81.0 ± 5.3</td>
<td>50.5 ± 7.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>238.1 ± 9.7</td>
<td>97.4 ± 6.6</td>
</tr>
<tr>
<td>Alanine</td>
<td>70.7 ± 2.6</td>
<td>62.1 ± 6.0</td>
</tr>
<tr>
<td>Valine</td>
<td>39.2 ± 1.6</td>
<td>56.6 ± 3.3</td>
</tr>
<tr>
<td>Halt-cystine</td>
<td>17.9 ± 1.3</td>
<td>13.1 ± 1.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>14.6 ± 0.9</td>
<td>53.9 ± 4.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>27.4 ± 0.8</td>
<td>43.9 ± 3.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>59.9 ± 2.6</td>
<td>85.8 ± 2.7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>37.8 ± 0.8</td>
<td>27.7 ± 3.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>37.8 ± 1.7</td>
<td>35.6 ± 4.7</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>17.9 ± 1.2</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>38.1 ± 2.1</td>
<td>70.8 ± 4.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>54.6 ± 3.6</td>
<td>56.1 ± 2.4</td>
</tr>
</tbody>
</table>

a Glomeruli were isolated from rats of 100-115 g weight (6 per preparation) used in the metabolic experiments.
b Values represent average of analyses performed on 6 membrane preparations.
c Proteins present in supernatant obtained after centrifugation of glomerular sonicate at 1,800 x g for 10 min. Values represent average of analyses of 6 preparations.
d Because of the limited size of the samples the value for 3-hydroxyproline can only be approximated at 7 residues/1000 total amino acid residues.

e Analyzer was equipped with a split-stream attachment which made possible the division of the column effluent so that one-third passed through the analytical system and two-thirds were diverted to a fraction collector for the purpose of scintillation counting.

Analysis of Sugar Components—Monosaccharide determinations were performed on unlabeled basement membranes by procedures previously described (3). After hydrolysis (1 N HCl, 6 h, 100°), neutral hexoses were measured on the Technicon sugar analyzer while amino sugars were determined with the pH 5.0 gradient on the amino acid analyzer (5). Fucose was assayed by the method of Dische and Shettles (6) and sialic acid was estimated after mild acid release (0.1 N H2SO4, 1 h, 80°) by the thiobarbituric acid reaction (7).

Polyacrylamide Gel Electrophoresis in SDS—Electrophoresis was carried out in 5% polyacrylamide gels in glass tubes (0.5 x 12.5 cm) according to the procedure of Weber and Osborn (8) as previously described (9). The gels were stained with Coomassie blue and the molecular weights of the protein bands were determined from the migration of reduced standard proteins which included myosin, phosphorylase a, bovine serum albumin, ovalbumin, and chymotrypsinogen.

Radioactivity Measurements—Radioactivity was determined by liquid scintillation counting in a Nuclear Chicago Isocap 300 counter using Bray's solution (10). All counts were converted to disintegrations per min with the appropriate efficiency corrections.

Expression of Results—In order to take into account the dilution of incorporated radioactivity due to growth of the rats during the course of the experiments, the determined specific activities of the various amino acids were corrected on the basis of weight increases, according to the suggestions of Neuberger et al. (11) and others (12, 13). The specific activities of the glomerular basement membrane components were multiplied by the ratio of final to initial kidney weight while the ratio of final to initial body weight was used to correct the values of the tendon collagen. At the time of radioisotope injection the average body weight of the rats was 108 g, while the
weight of the kidneys was 1.09 g. After 1, 2, 4, 6, 8, 9, 10, and 21 days the body weights were, respectively, 111, 113, 128, 145, 166, 174, 182, and 253 g, while the kidney weights were, respectively, 1.11, 1.14, 1.31, 1.40, 1.43, 1.55, 1.69, and 2.12 g.

The specific activities of the incorporated amino acids were plotted against time by the method of least squares and the half-life was determined from the maximum of the best fit line. Turnover time was calculated by multiplying the half-life by 1.44 (14).

RESULTS

Composition of Basement Membrane—The average yield of glomerular basement membrane obtained from the young rats used in the metabolic studies was 27.5 mg/100 g of wet weight kidney cortex as calculated from the amino acid analyses. The remaining glomerular protein, which was present in the 1,800 × g supernatant, represented an average of 93 mg of protein/100 g of cortex.

The amino acid composition of the rat glomerular basement membrane was characteristic of basement membranes in general (15) and contained a large amount of hydroxyproline, glycine, and hydroxylysine compared to the intact glomeruli and other glomerular proteins (Table II). Carbohydrate analyses were performed on the membranes of older rats because of the limited amount of material available from the young animals and indicated that the same sugars previously observed in bovine (3) and human (16) glomerular basement membranes were present (Table III).

TABLE III
Carbohydrate composition of rat glomerular basement membrane
Unlabeled membranes prepared from rats of 250 to 400 g weight; values represent average of analyses performed on two membrane preparations.

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/100 mg membrane</th>
<th>Residues/1000 total amino acid residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>3.05</td>
<td>21.1</td>
</tr>
<tr>
<td>Galactose</td>
<td>3.19</td>
<td>22.2</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.98</td>
<td>6.8</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.19</td>
<td>1.5</td>
</tr>
<tr>
<td>Glucosamineb</td>
<td>1.86</td>
<td>10.3</td>
</tr>
<tr>
<td>Galactoaminc</td>
<td>0.18</td>
<td>1.0</td>
</tr>
<tr>
<td>Sialic acids</td>
<td>1.08</td>
<td>4.2</td>
</tr>
<tr>
<td>Glucosylgalactosylhydroxylysine</td>
<td>17.9</td>
<td></td>
</tr>
<tr>
<td>Galactosylhydroxylysine</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

* Residue weight of monosaccharide; the membrane weight was calculated from the sum of the amino acid and sugar residues.

* Hexosamines were calculated as their N-acetyl form.

Radioactivity in Amino Acids of Serum—Rat serum was found to contain substantial levels of all the amino acids which were employed in their tritiated form during the course of this study (Table I), and it would therefore be anticipated that the specific activity of the injected tracer amino acid would be diluted in proportion to the concentration of the unlabeled amino acid in the serum. When [3H]proline was injected into the rats the concentration of this radiolabeled amino acid fell rapidly (Fig. 1) so that at 4 h only 0.04% of the initial dose was present in the serum and at 24 h this had been reduced to 0.012% of the total for a specific activity, 12 × 10^6 dpm/μmol. In the early
hours of the experiment some [3H]hydroxyproline was observed in the serum (Fig. 1). Since this amino acid was not detected (upper limit of contamination, 0.1%) in the injected [3H]proline preparation, it probably originated from the rapid breakdown of some proteins or peptides containing this hydroxylated amino acid.

Radioactivity in Amino Acids of Glomerular Basement Membrane—Examination of the elution profiles of glomerular basement hydrolysates from rats injected with various tritiated amino acids revealed different distributions of radioactivity (Fig. 2). While injected [3H]leucine was found unchanged as the only labeled component of the membrane, a somewhat more complex pattern was observed with the other administered amino acids because of their partial conversion before or after incorporation into the peptide chains. Proline was hydroxylated to hydroxyproline, lysine to hydroxylysine, and phenylalanine to tyrosine while glycine not unexpectedly gave rise to several radioactive components, including aspartic and glutamic acid.

Specific Activities and Turnover of Tritiated Amino Acids in Glomerular Basement Membrane—After the injection of [3H]proline maximum specific activity was achieved in the proline component of the basement membrane in 24 h while the hydroxyproline reaches its highest value by 48 h (Fig. 3). Subsequently there was little change in the specific activities of either component.

Tritiated lysine was rapidly incorporated into the basement membrane and then underwent a biphasic decline of its specific activity which included a very gradual decrease after 48 h (Fig. 4). The specific activity of hydroxylysine peaked more slowly than that of its parent amino acid reaching a maximum by 96 h after injection.

The ratio of the proline to hydroxyproline specific activities became constant by 48 h while the ratio calculated for lysine to hydroxylysine did not level out until 96 h (Fig. 5). The decline in both of these ratios to a constant value is probably a function of differences in turnover rate among the diverse basement membrane subunits as will be further discussed. The finding that the ratio of the specific activities of lysine to hydroxylysine did not reach unity can be attributed to a loss of 3H from C-5 of the lysine during its conversion to its hydroxylated derivative (17).

The incorporation of radioactivity into the glycine (Fig. 6) and leucine (Fig. 7) components of the glomerular basement membrane after the injection of the respective labeled amino acid was slow. In both cases an extended period of almost constant specific activity was ultimately reached. In contrast, injected [3H]phenylalanine after reaching a maximum specific activity at 48 h manifested a fairly rapid decline (Fig. 8).
g supernatant along with the hydroxyproline which was found hydroxyproline specific activity remained constant throughout the time studied.

Membrane solubilized by treatment with SDS and 2-mercaptoethanol at 37°C for 3 h revealed a complex pattern of polypeptide components migrating primarily in the M, = 40,000 to 180,000 range in addition to material which stayed close to the origin. There was no difference in the bands observed by electrophoresis when the membranes were heated for 5 min at 100°C in SDS prior to the reduction step, suggesting that the polydispersity noted was not due to proteolytic degradation during the solubilization procedure. While the subunit pattern of the rat basement membrane was somewhat different from that of the bovine membrane (9) the two were comparable in their complexity and the molecular weight range of their components.

DISCUSSION

The composition of the rat glomerular basement membranes prepared in the present studies was found to be similar to that of the bovine (3) and human (16) membranes. Comparable amino acid analyses were reported by Blau and Michael (18) for basement membranes isolated from rats of about the same age as those employed in our investigation. The relatively low hydroxylysine content of the rat membranes observed in both of these studies can be attributed to the fact that they were obtained from young animals, as it has recently been shown that the amount of this amino acid increases with age in this species (19).

The radioisotopic data presented in this paper indicate that the glomerular basement membrane as a whole turns over at a very slow rate which is consistent with the conclusions reached from electron microscopic observations of the membrane labeled with silver nitrate (20, 21). Indeed the decay in the specific radioactivity of most of the amino acids studied...
was so gradual that only an approximation of the turnover time could be made. The loss of radioactivity from the proline and hydroxyproline constituents of the basement membrane was as slow as that of the tail tendon collagen in the same animals. That the latter protein is one of the most inert molecules in the body has been previously established by a number of investigators (11-13). The low levels of proline radioactivity observed in the plasma of the rats a few hours after injection of the labeled compound precludes the possibility that significant recutilization of the amino acid accounts for the slow turnover.

The finding of different turnover times among the radiolabeled amino acids incorporated into the basement membrane in this study is probably best explained by the complexity of the subunit composition of this membrane. Polyacrylamide gel electrophoresis revealed that the rat membrane, like that from the cow, consisted of a number of polypeptide components which spanned a wide range of molecular weights. Analyses of many of the peptide subunits of the bovine basement membrane has revealed distinct compositional differences with some being more collagen-like and others more polar in nature (29). If differences in turnover of basement membrane polypeptide components of dissimilar composition were to occur this should be reflected in varied turnover rates of some of the amino constituents of the whole basement membrane. The observation, for example, that the ratios of the specific activity of proline to hydroxyproline and lysine to hydroxylysine became constant only after an extended period of time following injection of radioisotope is consistent with a more rapid rate of metabolism of polar subunits than of collagen-like components as the former have a higher molar ratio of unhydroxylated to hydroxylated amino acid than the latter (22). In the fibrillar tendon collagen where such diverse subunits do not exist the ratio of proline to hydroxyproline specific activities was constant from the earliest observed time. Such a differential turnover of basement membrane subunits may be the result of a subtle morphological and functional heterogeneity as observed by Walker (21) who proposed that the membrane is a two-component structure with a very slowly metabolizable major portion (lamina densa) and a minor component with a much faster turnover rate (lamina rara interna). It would appear that \(^{3}H\)proline is an effective in vivo label for the collagen like regions of the basement membrane as its conversion to tritiated hydroxyproline results ultimately in the expected ratio of unity for the proline to hydroxyproline specific activities. \(^{3}H\)Leucine was observed to have an advantage over the other amino acids employed to label the basement membrane in that it was incorporated unaltered into the membrane without being converted to any other amino acid (Fig. 2). The \(^{3}H\)lysine used in this study apparently lost activity during its conversion to the hydroxylated form and this loss of radiolabel would be expected to occur whenever lysine with tritium label on carbon 3 is employed (17).

The in vivo approach for studying the synthesis of the basement membrane seems to have some distinct advantages when compared with the incubation of isolated glomeruli which has been employed by some investigators (23, 24). In our experience membrane synthesis in this in vivo system is so sluggish that even after extended incubations of the glomeruli with radiolabeled proline the ratio of the specific activity of this amino acid to hydroxyproline in the isolated basement membrane did not fall below 20:1. Furthermore it may be anticipated that for the study of basement membrane synthesis under pathological conditions the glomeruli in vitro would not be as likely to reflect systemic influences as those in the intact animal.

The slow turnover of the glomerular basement membrane contrasts with the much more rapid rates of synthesis and degradation determined in vivo for the protein and glycoprotein components of cell membranes by various investigators (25-27). The comparatively fast turnover of the 1,800 x g supernatant proteins of the glomerular sonicate analyzed in the present study is probably partly a function of the cell membranes which are present in this fraction.

The low catabolic rate observed for the glomerular basement membrane in the present study helps to explain the accumulation of basement membrane material in certain disease states, such as diabetic renal microangiopathy, in which increased rates of synthesis are believed to occur (28).

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Studies on the metabolism of the renal glomerular basement membrane. Turnover measurements in the rat with the use of radiolabeled amino acids.

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