THE CARBOHYDRATE OF BASEMENT MEMBRANES OF HUMAN KIDNEY GLOMERULI

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It has been shown by fluorescent antibody technique that rabbit anti-sera against rat kidney glomeruli basement membranes and lens capsule cross-react with each other as well as other basement membranes but not with collagen fibers of connective tissue[1,2]. It has been, furthermore, reported that the lens capsules of cattle[3] and rabbit[4] contain chains of glucose and galactose linked to collagen and a sialofucoglycan in a glycoprotein. This suggested that one or both of these glycans may be the common or cross-reacting antigen in the lens capsule and basement membranes. This report brings evidence that the basement membrane of human kidney glomeruli contains both these types of carbohydrate; one consisting of glucose and galactose linked to collagen, and the other a sialofucohexosaminoglycan.

Preparation of Basement Membrane. Cortex of kidneys were obtained from autopsies carried out 3 to 7 h after death. Basement membranes of glomeruli were prepared according to the procedure of Krakower and Greenspon[5]. In four out of six preparations, lipids were removed before analysis by a 1 h extraction at R.T. with methanol-chloroform 1:2 followed by 1 h extraction with acetone, a second 16 h extraction with methanol-chloroform, and successive 1 h extractions with benzene, ethanol, ether and drying in vacuo.

Extraction of Collagen. Collagen was extracted from these preparations by two procedures 1) by incubation with highly purified collagenase (Worthington) (see legend Table II); 2) by

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TABLE I

RATIOS OF HEXOSAMINE-FREE HEXOSE (H) AND OF HYDROXYPROLINE (OH Pr) TCA EXTRACTED AT 90° FROM BASEMENT MEMBRANES DURING THE FIRST AND SECOND TIME INTERVAL OF 15 MINUTES

Kidney Sample	Age of Individual Years	Ratios: OH Pr in 1st 15' TCA extract / OH Pr in 2nd 15'TCA extract	Ratios: H in 1st 15' TCA extract / H in 2nd 15'TCA extract	Molar Ratio of Hexose to Hydroxyproline in Material Extracted in 30 Minutes	Remarks
1	42	2.8	2.7	0.64	Lipids extracted
2 a	33	2.75	2.4	0.39	" "
2 b	33	5.3	4.4	0.43	Lipids not extracted
3	44	4.0	3.6	0.56	Lipids extracted
4	47	4.3	4.4	0.47	" "
5	80	6.6	6.1	0.34	Lipids not extracted

200 mg of dry, lipid-free basement membrane preparation corresponding to 25-40 grams wet cortex or an equivalent amount of not lipid extracted preparation (samples 2 b and 5), freed of RNA by 72 h incubation in 10% HCl O_4 at 4° C, was washed with 4% TCA and then extracted in 8 ml 4% TCA for 15 minutes, washed with TCA again and re-extracted for another 15 minutes at 90° C.

quantitative extraction with 4% TCA at 90° C during two successive periods of 15 and one of 30 minutes with washing in between with 4% TCA. Less than 10% of other proteins and of hexosaminoglycans of the preparation were extracted simultaneously (see legend Table I).

Analytical Procedures. Hydroxyproline was determined by the method of Neumann and Logan[6]. Sugars were first tentatively identified in hydrolysates of the extracts prepared by boiling for 6 h in 2 N HCl after removal of HCl and suitable concentration in vacuo, hexosamines were identified by chromatography according to Gardell[7] and hexoses in the first effluent by paper chromatography with pyridine butanol water. Quantitative determinations of hexosamine were carried out in extracts hydrolyzed for 2 h with 2 N HCl at boiling point by the indole HCl reaction, hexose by primary and secondary reaction with cysteine H_2SO_4 reaction, fucose by its cysteine H_2SO_4 reaction[8], sialic acid according to Svennerholm[9]. Presence of pentoses and hexuronic acids were excluded by the pentose reaction with cysteine H_2SO_4 and the carbazole reaction, respectively.[8]

Hexose Linked to Collagen of the Basement Membrane. When collagen is extracted by TCA, 85-92% of its total hydroxyproline is found in the first two 15 minute extracts. Simultaneously, carbohydrate is extracted which consists essentially of galactose and glucose with only traces of mannose and fucose (Fig. 1). Some hexosamine polymer is also extracted, but corresponds to no more than 10-15% of the hexose. That this hexosamine is not linked by covalent bonds to the collagen can be shown by comparing the hexosamine content of TCA extracts of basement membranes pre-extracted with collagenase with that of TCA extracts of the same preparations not pre-treated with collagenase. The two do not differ significantly. The hexosamine, therefore, in TCA extracts, is not extracted by collagenase. It is linked to hexose as the two sugars are found in constant proportions in extracts of the preparations obtained after collagen extraction. This can be shown by extracting first the total collagen of the basement membrane with 4% TCA during 60 minutes at 90° C and then extracting the residue for another 60 minutes with 4% TCA. This last extract contains hexosamine and hexose in a ratio which remains constant during further extraction. From the ratio of hexose to hexosamine in the second 1 h TCA extract and the

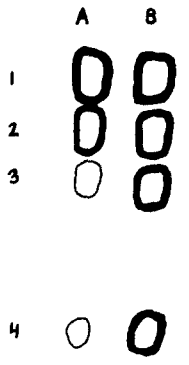

FIG. 1 PAPER CHROMATOGRAM OF NEUTRAL SUGAR CONSTITUENTS OF BASEMENT MEMBRANES EXTRACTED WITH 4% TCA AT 90° IN THE FIRST 30'. A) SAMPLE B) STANDARDS. 1) GALACTOSE 2) GLUCOSE 3) MANNOSE 4) FUCOSE.

amount of hexosamine in the first 1 h TCA extract, it is possible to calculate the hexosamine-linked hexose in the latter extract. This last value substracted from the value for total hexose found in the first extract gives the value for the hexosamine-free hexose in the basement membrane extracted simultaneously with the collagen. That this hexose fraction which consists of galactose and glucose in a ratio of about 4:3 is linked firmly to collagen can be shown by comparing the rate of extraction of hydroxyproline in the two successive 15 minute intervals with that of a hexosamine-free hexose. The results of such experiments are listed in Table 1. It can be seen that the rate of extraction in the two successive time intervals is, within the limits of error, identical for the two substances. It will be further noted that the rate of extraction of hydroxyproline and hexose decreases to the same extent when the basement membrane preparation is pre-extracted with lipid solvents.

Demonstration of the Presence of a Collagen-linked Hexose and of a Sialofucoglycan in Basement Membranes by Collagenase Extraction. That the galactose and glucose extracted simultaneously with hydroxyproline are linked to collagen is also shown by the results of the extraction of the basement membranes with collagenase. In these experiments, the lipids of the preparations were extracted and the dry material divided into two equal parts, one of which was incubated with collagenase, and the other extracted with TCA with or without

TABLE II

MOLAR RATIOS OF HEXOSAMINE-LINKED, OF HEXOSAMINE-FREE AND OF TOTAL HEXOSE (H) TO HYDROXYPROLINE (OH Pr) IN COLLAGENASE EXTRACTS OF BASEMENT MEMBRANES

*The numbers designate the same samples as the numbers in Table I.

Kidney Sample	Age of Individual Years	Hexosamine linked H / OH Pr	Hexosamine free collagen linked H / OH Pr	Total H / OH Pr	OH Pr µg/100 mg dry weight
1	42	0.19	0.71	0.90	495
2 a	33	0.58	0.40	0.98	357
3	44	0.20	0.73	0.93	385

200 mg of dry lipid-free basement membrane preparation from 25-40 grams wet cortex were incubated for 16 h at 37° C in 5 ml in 1/50 M Tris buffer containing 2 mg of collagenase. As control, an equal sample of the same preparation was incubated in buffer alone. The samples were centrifuged, the sediments were each washed and extracted with 4% TCA at 90° C for three successive periods of 15, 15 and 30 minutes, respectively. (Total hexose in the supernatant + total hexose in the subsequent three TCA extracts of the collagenase sample) minus (total hexose in the supernatant + total hexose in the TCA extracts of the control) = the non-dialyzable hexose linked to hexosamine extracted by collagenase.

pre-incubation with the buffer free of collagenase. Very nearly the same amount of hydroxyproline, but significantly more hexose, was extracted by collagenase than by TCA. When the enzyme extract was dialyzed for 48 h at 4° C against a double volume of distilled water (experiments 1 and 2, Table II), the hydroxyproline distributed evenly between the dialysant and dialysate. Only one part of the hexose, however, dialyzed out. The ratio of hexose to hydroxyproline in the dialysate did not differ significantly from that found for the ratio of collagen-linked hexose to hydroxyproline in the control directly extracted by TCA. The hexose fraction in the dialysate showed a ratio of galactose to glucose of about 4:3. The collagenase extract contained significant amounts of hexosamine and hexose which did not dialyze out. One part of hexose, therefore, which was extracted by collagenase was not dialyzable. The hexosamine and the hexose in excess of the dialyzable one extracted by collagenase cannot come from the same source as the hexosamine extracted by TCA since TCA extraction after pre-incubation with collagenase, as mentioned above, yields the same amount of hexosamine as that extracted directly with TCA. The hexosamine extracted by collagenase is, therefore, insoluble in TCA and seems to be bound to hexose and a protein. This glycoprotein appears to contain also fucose (demonstrated in the collagenase extract only analytically by its reaction with cysteine and H_2SO_4)and sialic acid(demonstrated by the resorcinol reaction of Svennerholm) in non-dialyzable form.

Demonstration of the Basement Membranes as Source of the Extracted Collagen by Electron Microscopy. Electron microscopy was carried out on the collagenase extracted sample from experiment 1, Table II and on the control incubated with buffer alone. The pellets were fixed in buffered 1% OsO4 pH 7.4 for 2-3 hours and dehydrated in graded series of ethanol. Then the clumps of material were embedded in Epon 812 and sectioned with a Porter Blum microtome. Sections and grids were stained with uranyl acetate and examined in an RCA - EMU - 3F electron microscope. Essentially the pellets of the basement membrane preparation contain two types morphologically of homogeneous material. One type has greater density and a felt-like appearance. The other, presumably the basement membrane, appears lighter and

has a lightly granular appearance. After collagenase treatment, the dense material appeared unchanged. The lighter basement membrane material appears eroded and clumped rather than homogeneously smooth.

The Quantitative Relations Between Carbohydrates and Hydroxyproline in the Basement Membranes. Our results indicate a far-reaching analogy between the basement membrane of the glomeruli and the lens capsules of cattle and rabbit. This analogy appears also valid as far as the quantitative relations of the carbohydrate to collagen are concerned. The ratio of collagen-linked hexose to hydroxyproline shows great individual variations (up to 100%) from one individual kidney to another although the average from 8 kidneys gives a molar ratio of .47. The ratio of hexosamine-linked hexose to the hexosamine-free hexose also shows considerable variations in the three samples of lipid-free preparations. On the other hand, the molar ratio of the sum of hexosamine-free and the hexosamine-linked hexose to hydroxyproline varied in three experiments only between .90 and .98 indicating a ratio of about 1. This suggests that the two types of hexose polymers replace each other in their relation to collagen, although the nature or strength of their link to this protein appears to be very different. Although the presence of sugar polymers containing glucose, galactose and somewhat less mannose, hexosamine, fucose, and sialic acid in rat kidney basement membranes pre-extracted for several days with 0.05 NaOH had been previously demonstrated [10], it had not been shown that the basement membranes of glomeruli contain two different sugar polymers, one free of hexosamine and linked to collagen and the other containing hexose, hexosamine, fucose, and sialic acid in form of a glycoprotein, which can be separated from collagen by dialysis after collagenase treatment. The evidence for that here presented promises to contribute to the elucidation of the question of a common or related antigen of the basement membranes and the lens capsule.

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