VARIATIONS IN THE SIALIC ACID CONCENTRATION
OF GLOMERULAR BASEMENT MEMBRANE PREPARATIONS
OBTAINED BY ULTRASONIC TREATMENT

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INTRODUCTION
Several authors (10, 15, 6, 1, 20) have suggested
that sialic acid is a component of the glomerular
basement membrane (BM). Investigations by
Spiro (20) have shown that BM preparations ob-
tained by ultrasonic treatment of whole glomeruli
contain two distinct carbohydrate moieties, in
approximately equal proportions: one is a disac-
charide containing glucose and galactose, believed
to be intrinsically associated with collagen, and the
other is a heteropolysaccharide consisting of galac-
tose, mannose, hexosamines, sialic acid, and fucose.
We have presented histochemical evidence that
sialic acid is present in the glomerulus and that it is
located not in the basement membrane proper but
in the cell membranes lining the basement mem-
brane (18, 14). Preceding us, Rambourg and
Leblond (17) demonstrated a carbohydrate-rich

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Electron Microscopic Studies

Samples of the six centrifugal sediments were suspended with a short burst of sonication in 4% neutral formaldehyde. After overnight fixation, they were washed in distilled water and collected by centrifugation at 15,000 rpm. These sediments were resuspended with colloidal iron in 25% acetic acid for 20 min as prepared by the method of Muller (16). This was followed by three washings in 30% acetic acid, and each time the sediment was collected at 15,000 rpm. This sediment was then cut into minute blocks, dehydrated, and embedded in Epon 812. Ultrathin sections were studied in an Elmskop 1A. For augmenting the recognition of the basement membrane and cell membrane structures, the sections were counterstained with uranyl acetate (13). On randomly selected electron micrographs of the 500 and 3,000 rpm sediments, the surface areas of the BM and of the contaminants were compared by the aid of a planimeter.

RESULTS

Distribution of Sialic Acid and Glucose in the Various Centrifugal Sediments of GMP’s

The sialic acid content of the GMP was 240 mg/100 g wet weight. This corresponds approximately to 1.2% sialic acid by dry weight. Table I shows the amount of sialic acid present in the three sediments obtained at 500, 1,500, and 3,000 rpm and in the particulates collected from their corresponding supernatants. As indicated, the sialic acid content in the sediment increases with rising centrifugal force. At 500 rpm, the sediment comprised 49% of the sialic acid present in the original GMP. In the 3,000 rpm sediment it reached 82%.

Analysis of the standard GMP for total neutral sugars obtained by the anthrone method gave an average value of 5.1% of the dry weight of the membrane expressed as glucose equivalents. This value is in close approximation with values reported recently (20). Analysis of the same material by the glucose oxidase (glucostat) reaction gave an average value of 2.3% of the dry weight.

In contrast to the sialic acid content, the relative glucose content of GMP changed very little with increasing centrifugal force.

Estimation of Sialic Acid in the Various Centrifugal Sediments of GMP by Electron Microscopy

On the basis of the assumption that the deposition of colloidal iron is in association with sialic acid, we have shown (18) by histochemical methods that the presence of sialic acid in the glomerulus can be demonstrated at the light microscope level by Spicer and Warren’s method (19), and at the electron microscope level by Gasic’s method (2). In such glomerular preparations (Fig. 1) the colloidal iron is localized at the cell membrane, but not in the BM proper. Furthermore, this localization could easily be prevented by treating the sections beforehand with neuraminidase, an enzyme capable of selectively removing the sialic acid. This indicated that the localizations of iron and sialic acid are interrelated.

| Centrifugal force | Weight | Weight ratio | Sialic acid content | Sialic acid ratio |
|-------------------|--------|--------------|---------------------|------------------|
|          | Supernatant | Sediment | Total | Supernatant | Sediment | Total | Supernatant | Sediment | Total | Supernatant | Sediment | Total |
| 42 g (500 rpm) | 58.2 | 52.2 | 110.4 | 1.11 | 122.80 | 119.03 | 241.83 | 1.03 |
| 383 g (1500 rpm) | 46.8 | 56.9 | 103.7 | 0.82 | 95.60 | 152.87 | 248.47 | 0.63 |
| 1200 g (3000 rpm) | 33.3 | 64.3 | 97.6 | 0.51 | 41.62 | 189.01 | 230.63 | 0.22 |

TABLE I

Distribution of Sialic Acid in the Supernatants and Sediments of Sonicated Beef Glomeruli Obtained at Various Centrifugal Forces

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FIGURE 1 Electron micrograph of a mouse glomerular loop stained with colloidal iron. No counter-stain. Observe the heavy metallic deposits on the epithelial cell membrane including the foot processes (F), and the absence of these deposits in the BM. C, lumen of capillary loop. × 16,000.

The same colloidal iron technique was used on the sediments of the GMP obtained at different centrifugal forces and examined by electron microscopy. It was demonstrated that the preparations were considerably contaminated with cell membrane marked with colloidal iron deposits, and that the contamination was greatest at the highest centrifugal force. The 500 rpm sediment predominantly contained BM fragments. Many of these BM fragments were completely free of cell membranes, while others showed attached, iron-positive cell membrane fragments or foot processes. The corresponding supernatant was composed of cell membranes and less than 10% BM fragments (Fig. 2). Iron-positive particles were absent in neuraminidase-treated, control preparations. Quantitative comparison of the surface areas of the BM and of the contaminants on randomly selected electron micrographs of the 500 and 3,000 rpm sediments indicated 16.5% and 36% contaminants, respectively.

DISCUSSION

Because sialic acid-containing heteropolysaccharides are known constituents of cell membranes (24), the possibility presented itself that the sialic acid, presumably present in the glomerular basement membrane, may actually be present in the cell membranes contaminating the ultrasonic BM preparations. Sonic fragmentation of the glomerulus followed by differential centrifugation, as used by most investigators for the procurement of BM preparations, cannot achieve complete separation of BM and cell membrane.

Electron microscope studies of the different sediments, with the colloidal iron technique as a method for the localization of sialic acid, and consequently for the identification of cell membrane material, indicated that even the 500 rpm sediment contained substantial amounts of cell membrane usually attached to the BM. Furthermore, there was a considerable increase of free cell mem-
brane fragments in the pellets obtained at a higher centrifugal force. In accord with these findings, the sialic acid concentration of the sediments increased with rising centrifugal force. In contrast, the glucose concentration did not show any significant change. The large amount of contaminants could explain why the collagen content of reported BM preparations varies from 50 to 70% (10, 12, 9, 6, 11). While this contamination parallels the sialic acid content, it has no appreciable effect on glucose concentration believed to be intrinsically associated with collagen. These findings support our contention that sialic acid is not a component of the glomerular BM, but rather one of the cell membrane.

A similar linear relationship between cell membrane contamination of human BM collagen and centrifugal force was found recently by Westberg and Michael (23) using hydroxyproline and phospholipids as the parameters.

In Table II, we compiled the published glomerular sialic acid concentrations and our results in an effort to find further support for the "issue of contamination." The values show considerable variation. Some of this variation may be due to deviations from the method adopted. Most of the publications refer to the method by Krakower and Greenspon (8) for the harvesting of glomeruli and for the preparation of BM, yet it is not at all clear whether 20 min of sonic treatment and collection at 1,500 rpm were uniformly adhered to. Nor are the frequency and energy output of the sonic instrument uniformly stated. In addition to deviations from the original technique, we have found age and species differences in glomerular sialic acid concentration, which may further obscure interpretation. There is 30% more sialic acid in young beef glomeruli than in glomeruli obtained from older animals. The high values obtained by Kefalides could be explained by his method of 2.5 hr of acid hydrolysis (5), instead of 1 hr, customarily used for the sialic acid assay of Warren (22). In spite of these discrepancies, Table II seems to support a direct relationship between sialic acid content and centrifugal force: with similar ultrasonic treatment and centrifugal force as Spiro, our sialic
TABLE II
Comparison of Sialic Acid Content of Glomeruli and Glomerular Membrane Preparation (GMP) Obtained by Different Investigators

| Item | Authors               | Whole glomeruli | GMP | Dry weight |
|------|-----------------------|-----------------|-----|------------|
|      |                       | Species tested  |     |            |
| 1    | Lazarow & Speidel     | H D.C.          | 0.05 N NaOH | 3-8 days  | 8 SA*/100 g |
|      | 200 g                 |                 |     |            |
| 2    | Lange & Markowitz     | H D.C.          | U.S. 20' | 1,500 rpm 5-10' | 0.55 |
| 3    | Misra & Berman        | H Magnetic      | U.S. 8-12' | 200 g 10' | 0.27-0.68 |
| 4    | Kefalides & Winzler   | C D.C.          | U.S. 20' | 1,500 rpm 5-10' | 2.10 |
| 5    | Misra & Berman        | C D.C.          | U.S. 20' | 1,500 rpm 5-10' | 2.10 |
| 6    | Misra & Berman        | H D.C.          | U.S. 20' | 1,500 rpm 5-10' | 1.37 |
| 7    | Misra & Berman        | B D.C.          | U.S. 5' | 3,000 rpm 10' | 1.44 |
| 8    | Spiro                 | B D.C.          | U.S. 5' | 3,000 rpm 10' | 1.44 |
| 9    | Lidsky et al. (11)    | B D.C.          | 1000 rpm | 3,000 rpm 10' | 1.44 |
| 10   | Mohos & Skoza (14)    | B D.C.          | U.S. 5' | 3,000 rpm 10' | 1.44 |
| 11   | Mohos & Skoza (14)    | B D.C.          | U.S. 5' | 3,000 rpm 10' | 1.44 |

* SA = Sialic acid, H, human, C, canine, B, beef, D.C., differential centrifugation, U.S., ultrasonic treatment. Italicized numbers represent the procedure of Krakower & Greenspon (8). It is the methodology referred to by the respective authors. Wet/Dry ratio of whole glomeruli = 20, and that of GMP = 5, and indicates the approximate water content of these preparations.

acid values for the GMP are in the same range, ~ 1.20%. In contrast, Misra and Berman, using more extensive ultrasonic disruption and a much lower centrifugal force (200 g), obtained much lower sialic acid values (0.27–0.68). Nevertheless, sialic acid content of the whole glomerulus is, in both instances, approximately the same. Whether the low values of GMP, as reported by Misra and Berman, can further be reduced is not known. As stated elsewhere (14), one can not disregard the possibility that the basement membrane proper may contain sialic acid in a local concentration insufficient for histochemical demonstration. But then, the sialic acid would be a minor component, considering the high local concentration of sialic acid on the plasma membrane of the epithelial cells.

SUMMARY
The possibility of contamination of ultrasonically prepared glomerular basement membranes by cell membranes collected at different centrifugal forces was investigated by means of sialic acid determination in combination with electron microscopy. Such preparations showed that the sialic acid titer and the degree of cell membrane contamination increased with a rising centrifugal force.

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