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Lipids Associated with Bovine Kidney Glomerular Basement Membranes

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1. After incubation of bovine glomeruli with D-[U-^{14}C]glucose, about 21% of the total radioactivity is found in lipid extracts of glomerular basement membranes. 2. The concentration of lipids in glomerular basement membranes (4.3% of dry wt.) is lower than in the residual glomerular particles (10.8% of dry wt.). The concentrations of neutral lipids (13.9%), phospholipids (46.7%) and cholesterol (37.9%) in the total lipid extract of the glomerular basement membranes, however, differ from those in the residual glomerular particles (15.6, 54.0 and 30.9% respectively). Though residual glomerular particles show a higher lipid content, the radioactivity in this fraction only amounts to 38% of that found in the glomerular basement membranes. 3. The specific radioactivity of total glomerular basement-membrane lipids (12600 d.p.m./mg) is about 4 times as high as that of the glomerular basement membranes. The specific radioactivities of the individual lipid components, however, differ. The highest values are found for phosphatidylcholine and triacylglycerols. The largest proportion of the radioactivity is found in the glycerol of the glycerides. The radioactivity in the fatty acids is much less and does not differ significantly in the various classes of lipids. 4. G.l.c. of methyl esters of the fatty acids does not reveal a clear difference between the fatty acid compositions of glomerular basement membranes and residual glomerular particles. 5. Treatment of glomerular basement-membrane preparations with ultrasound, the generally used procedure for glomerular basement-membrane preparations, drastically decreases the lipid content of glomerular basement membranes. 6. It is concluded that lipids are associated with the basement membranes. Further, the comparatively high radioactive labelling suggests that glomerular basement-membrane lipids may be an interesting class of substances for further pathological studies.

Isolated kidney glomerular basement membranes are usually prepared by using ultrasound (Krakower & Greenspon, 1951; Spiro, 1967; Kefalides, 1973), strong bases (Lazarow & Speidel, 1964) or strong acids (Nicholes *et al.*, 1973). An alternative procedure reported by von Bruchhausen & Merker (1965*a*) and by Lidsky *et al.* (1967) uses homogenization followed by density-gradient centrifugation. Glomerular basement membranes obtained in this way contain some components, mainly lipids, which until now were considered to be contaminants (Kefalides & Winzler, 1966; Spiro, 1967; Hempel & Geyer, 1967; Gang & Kalant, 1970; Westberg & Michael, 1970; Nicholes *et al.*, 1973). The question arises as to whether the differences in composition depend on the various methods of preparation.

Studies on the origin (Kurtz, 1958; Cossel *et al.*, 1959; Farquhar *et al.*, 1961; Andres *et al.*, 1962; Kurtz & Feldman, 1962; Vernier & Birch-Andersen, 1962; Ormos & Solbach, 1963; Lannigan *et al.*, 1964; Østerby-Hansen *et al.*, 1967; Thoenes, 1967) and turnover (Walker, 1973) of glomerular basement membranes give rise to doubts as to whether they consist only of insoluble glycoproteins and a collagen-like protein, as found in sonicated preparations (Kefalides, 1973). Biochemical investigations on the incorporation of various precursors into glomerular basement membranes *in vitro* also suggest a more complex structure (von Bruchhausen, 1971). In contrast with the very low rate of incorporation of radioactive amino acids into the collagen-like basement-membrane material (von Bruchhausen, 1971; Grant & Harwood, 1974; Grant *et al.*, 1975), the rate of incorporation of radioactive glucose into the glycoprotein moiety (Wahl *et al.*, 1970; von Bruchhausen, 1971; Heilhecker & von Bruchhausen, 1973) and into the lipid component is remarkably high.

The results of our present investigations suggest that the lipids found are components of the glomerular basement membranes.

Experimental

Materials

All chemicals were purchased from Merck, Darmstadt, Germany, in the purest available grade. The stainless-steel sieves were obtained from Ame-

lang, Berlin, Germany. D-[U-^{14}C]Glucose was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The standard lipids and fatty acids were from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Biochemica Test Combinations from Boehringer Mannheim G.m.b.H., Mannheim, Germany, were used to quantify the lipids.

Isolation and incubation of glomeruli

The isolation procedure is based on the methods of Spiro (1967). Three kidneys, from freshly killed cows, were procured from the local slaughterhouse. The medulla was dissected from the cortex of each and discarded, and the cortex was ground up with a meat grinder. The ground tissue was forced through a stainless-steel sieve (260μm) with light pressure in a specially designed apparatus (Heilhecker & von Bruchhausen, 1973). Ice-cold 0.9% NaCl containing 0.55mM-glucose, pH7.2–7.4, (solution A) was used during the operation. The screened material was suspended in a large volume (2–3 litres) of solution A and again filtered through the 260μm sieve. The glomeruli were collected from this filtrate on a sieve (100μm) and were extensively washed with ice-cold solution A. The sieve allowed the tubular elements to pass through, but not the glomeruli. The collected glomeruli were suspended in solution A and centrifuged at 4°C and 4000g ($r_{av.}$ 12cm) for 10min. The supernatant was discarded. Centrifugation was repeated until the supernatant was clear. The preparation was checked for purity by phase-contrast microscopy.

For incubation, the glomeruli were suspended in Krebs–Ringer phosphate buffer, pH7.4 (Umbreit *et al.*, 1951), final volume 100ml, and equally distributed into ten 25ml Erlenmeyer flasks, each of which contained 5μmol of glucose, 100μCi of D-[U-^{14}C]-glucose (268mCi/mmol) and 55.5i.u. of penicillin G (total volume 0.3ml). The incubation was in air at 37°C in a shaking water bath (80 strokes/min) for 2h. Another portion of 5μmol of glucose in 0.1ml of Krebs–Ringer phosphate buffer, pH7.4, was added after 1h. The reaction was stopped with 10ml of ice-cold 0.9% NaCl, containing 5.5mM-glucose. The mixture was centrifuged for 15min at 4°C and 4000g ($r_{av.}$ 12cm) to separate the medium from the glomeruli. They were washed twice under the same conditions with ice-cold 0.9% NaCl.

Isolation of the glomerular basement membranes, the residual glomerular fraction, the mitochondrial fraction and the microsomal fraction

Glomerular basement membranes were prepared by the procedure of von Bruchhausen & Merker (1965*a*). The glomeruli were disrupted in a glass Potter–Elvehjem homogenizer in 0.25M-sucrose. The homogenate was layered on top of a discontinuous gradient composed of 4ml each of 1.8, 1.7, 1.6, 1.5 and 1.4M-sucrose. During centrifugation for 1h at 4°C and 55000$g_{av.}$ (rotor SW25; Beckman Instruments G.m.b.H., München, Germany), the glomerular basement membranes sedimented under a mitochondrial and a microsomal fraction. The supernatant was diluted with water to a concentration of 0.32M-sucrose and centrifuged at 150000$g_{av.}$ for 90min to obtain the residual glomerular fraction. Centrifugation of the diluted discontinuous gradient at 16000$g_{av.}$ for 30min caused the mitochondrial fraction to sediment; a subsequent centrifugation at 150000$g_{av.}$ for 90min (Beckman rotor 60 Ti) yielded the microsomal fraction.

The glomerular basement membranes were collected, and sucrose was washed off with water. They were further purified by incubation with deoxyribonuclease (0.1mg/ml) in 20ml of 0.2M-Tris/0.05M-$MgSO_4$ buffer, pH7.0 (adjusted with HCl), for 10min at 37°C. They were then collected by centrifugation (4000$g_{av.}$; 15min; 4°C), washed with water and either freeze-dried or used immediately.

Extraction, fractionation and quantification of lipids

Lipids were extracted by the method of Bligh & Dyer (1959). For this, 100mg wet wt. of tissue (glomerular basement membranes or residual glomerular fraction) was suspended in 7.5ml of methanol/chloroform (2:1, v/v). The mixture was stirred for 1h at room temperature (23°C), centrifuged (4000$g_{av.}$; 15min), and the supernatant was collected. The residue was then re-extracted with 9.5ml of methanol/chloroform/water (10:5:4, by vol.) for 1h more. The combined extracts were diluted in a separation funnel with 5ml each of chloroform and water and shaken thoroughly. After separation, the lower chloroform phase, containing lipid, was withdrawn, diluted with 5ml of benzene and dried under vacuum. The lipids were dissolved in a known volume of methanol/chloroform (1:1, v/v) and stored at −4°C under N_2. The lipid extract was assayed for total lipids, non-polar lipids, cholesterol and phospholipids.

The lipids were separated into their classes by t.l.c. on commercially available plates coated with silica gel (Merck). Neutral lipids were separated by the method of Freeman & West (1966) by using two solvent systems, first diethyl ether/benzene/ethanol/acetic acid (200:250:10:1, by vol.) and then diethyl ether/hexane (3:47, v/v) in the same direction. For two-dimensional t.l.c. of the polar lipids by the method of Skidmore & Entenman (1962), chloroform/methanol/NH_3 (25%, w/v) (60:35:4, by vol., and 35:60:4, by vol.) was used. A standard was run simultaneously to identify the lipid components. Lipid spots were detected with iodine vapour. The

separated components were eluted from the silica gel with methanol/chloroform/diethyl ether (1:1:1, by vol.), dried under vacuum, and redissolved in a known volume of chloroform/methanol (1:1, v/v).

The fatty acids were determined as methyl esters (Doss & Oette, 1965) by g.l.c. (Hewlett–Packard Research Chromatograph 3750) in a stainless-steel column (0.4cm internal diam. × 180cm) with 5% (w/w) ethylene glycol adipate on Gas-Chrom Q HP (100/120 mesh). A temperature gradient of 2°C/min from 170 to 200°C was used followed by 15min at 200°C. The carrier was N_2 and the flow rate about 60ml/min. The peaks were identified by running fatty acid standards under the same conditions. For quantitative evaluation the peak area was calculated by multiplying the width at half-height by the height.

Radioactivity was determined by the channels-ratio method (Herberg, 1965) in a Packard liquid-scintillation counter (Tri-Carb model 3380) at 85% efficiency in Bray's (1960) solution.

Results

Basement membranes were prepared without sonication from bovine glomeruli, and on incubation with D-[U-^{14}C]glucose the uptake of radioactivity was curvilinear with time (up to 2100 ± 200 d.p.m./mg within 2h) (Fig. 1). The total lipid content of the preparation was 4.3 ± 0.2% of the dry weight. The lipids contained 21 ± 0.9% of the total absorbed radioactivity and reached a specific radioactivity of about 12600 d.p.m./mg of lipid. The results are the means of 16 preparations ±S.E.M. To check the efficacy of the extraction procedure total lipids were determined in the lipid extract of the basement membrane as well as in preparations of the original membrane. The same amount of total lipids was found, which showed that the extraction was complete. The lipid components were separated by t.l.c. (recovery 80–95%). The lipids of the glomerular basement membrane consisted of 13.9% (by wt.) neutral lipids, 46.9% (by wt.) phosphoglycerides, 34.6% (by wt.) cholesterol and 3.3% (by wt.) sterol esters. The radioactivity incorporated into the individual components differed, as is shown in Table 1. The neutral lipids contained 72.5% and the phosphoglycerides 26.8% of the total radioactivity; the specific radioactivities are shown in Fig. 2. The highest values were obtained for phosphatidylcholine and triacylglycerols. Separate estimations of the radioactivity incorporated into the glycerol and fatty acid moieties revealed that these differences are due to the labelling of the glycerol and not of the fatty acid component (Table 2).

A further attempt was made to distinguish these basement-membrane lipids from the lipids of the surrounding cellular particles. A residual glomerular fraction was prepared as described in the Experimental section, and was analysed as described for basement-membrane lipids. This fraction consisted of 15.6% (by wt.) of neutral lipids, 54% (by wt.) of phosphoglycerides, 27.4% (by wt.) of cholesterol and 3.5% (by wt.) of sterol esters (Table 3).

Though the total lipid content of the residual glomerular fraction (10.8mg/100mg dry wt.) is much higher than that of glomerular basement membranes, the specific radioactivity only reaches 4800 d.p.m./mg, which is 38% of that of the glomerular basement membrane. The distribution of the radioactivity in the lipid classes was as follows: neutral lipids, 65.6%; phosphoglycerides, 33.7%; cholesterol, 0.4%; sterol esters, 0.3%. The amounts and the radioactivities of individual lipid classes of the residual glomerular fraction are given in Table 3, and the specific radioactivities of these lipids are compared with those of the glomerular basement membranes in Fig. 3.

The residual glomerular fraction was fractionated by differential centrifugation into a mitochondrial and a microsomal fraction. No attempt was made to check their contamination, e.g. by using marker enzymes. The mitochondrial fraction contained 22.4% (by wt.) and the microsomal fraction 11.8% (by wt.) of the total lipids. The specific radioactivities (4200 and 1700 d.p.m./mg respectively) of their lipids did not reach the value for the total glomerular basement-membrane lipids.

By contrast, fatty acid analysis of the total lipids or of the lipid components revealed no differences in the percentage distribution of fatty acids in the glomerular basement-membrane or residual glomerular-fraction lipids (Table 4). However, there may be some variations between individual lipid classes.

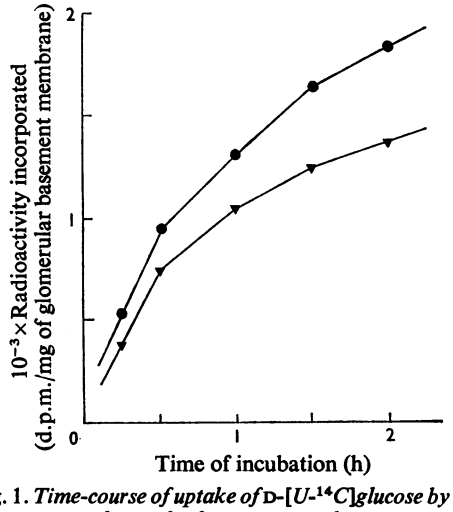

Fig. 1. *Time-course of uptake of* D-[*U*-^{14}*C*]*glucose by bovine glomerular basement membranes*

For experimental details see the text. ●, With lipids; ▼, after extraction of lipids.

Table 1. *Amounts and radioactivity of the glomerular basement-membrane lipids*

Values are expressed as means ± S.E.M. *n* is the number of determinations with different basement-membrane preparations. Incubations were carried out and lipids were extracted as described in the Experimental section and determined, (*a*)–(*d*) in the total extract and (*e*)–(*g*) after separation by t.l.c.

	Lipids	*n*	Amount (mg/100mg dry wt. of membrane)	Amount (% of total lipids)	Radioactivity (% of total)
(*a*)	Total	16	4.34±0.16		
(*b*)	Neutral	6	0.60±0.07	13.9±0.4	
(*c*)	Phosphoglycerides	7	2.03±0.01	46.7±1.7	
(*d*)	Cholesterol+sterol esters	10	1.64±0.02	37.9±1.2	
(*e*)	Monoacylglycerols	5	0.14±0.04	3.2±0.2	0.3±0.1
	Diacylglycerols	5	0.15±0.02	3.5±0.1	7.2±0.3
	Triacylglycerols	5	0.26±0.01	6.0±0.1	65.0±1.0
			0.55	12.7	72.5
(*f*)	Phosphatidylethanolamine	5	1.11±0.10	25.6±0.4	5.4±1.5
	Phosphatidylcholine	5	0.27±0.04	6.0±0.3	15.8±1.0
	Phosphatidylinositol+phosphatidylserine	5	0.40±0.02	9.5±0.2	5.2±0.3
	Sphingomyelin	5	0.19±0.01	4.4±0.2	0.4±0.1
			1.97	45.5	26.8
(*g*)	Cholesterol	5	1.29±0.15	29.7±0.3	0.4±0.1
	Sterol esters	5	0.12±0.02	2.8±0.1	0.3±0.1
			1.41	32.5	0.7

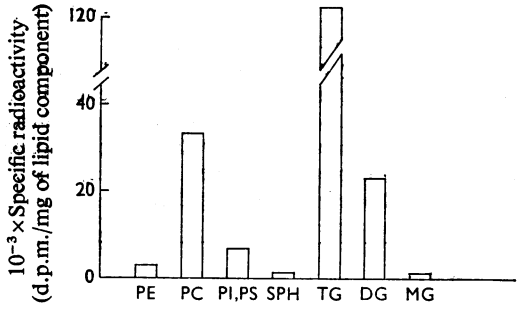

Fig. 2. *Calculated specific radioactivities of the lipids obtained from glomerular basement membrane after incubation of glomeruli with* D-[*U*-^{14}C]*glucose*

Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; TG, triacylglycerols; DG, diacylglycerols; MG, monoacylglycerols.

Table 2. *Distribution of the radioactivity between fatty acids and glycerol of the lipids of glomerular basement membrane*

For details see Table 1. For all measurements $n = 2$.

Lipids	Radioactivity in fatty acids (%)	Radioactivity in glycerol (%)
Phosphatidylethanolamine	40±3	60±2
Phosphatidylcholine	10±1	90±1
Phosphatidylinositol+ phosphatidylserine	33±1	67±1
Triacylglycerols	1±0.1	99±3
Diacylglycerols	8±0.5	92±4
Monoacylglycerols	17±1	83±1

The effect of ultrasound on the content of lipid in the glomerular basement membrane was studied by an additional sonication of a glomerular basement-membrane preparation, as described by Spiro (1967). The lipid content was decreased from 4.3 to 1.9%, and nearly 25% of the total glomerular basement-membrane material was lost. Apparently, a large amount of the lost material must be collagenous in origin, as indicated by hydroxyproline determination (Stegemann, 1958) in the supernatant. The hydroxyproline content of the remaining glomerular basement membranes was only increased from 6.9 to 7.3% by sonication.

Discussion

The results of our investigations confirm the findings of von Bruchhausen & Merker (1965*b*), though lower values (6% by wt. for rat glomerular basement membrane) were obtained. This may be due to a purer glomerular basement-membrane preparation being obtained from isolated glomeruli instead of from kidney homogenates and also to differences arising from species variation. The values reported here are also lower than those found by

Table 3. *Amounts and radioactivity of the residual glomerular fraction lipids*

For details see Table 1.

Lipids	n	Amount (mg/100mg dry wt. of residual glomerular fraction)	Amount (% of total lipids)	Radioactivity (% of total)
(*a*) Total	4	10.75±0.20		
(*b*) Neutral	4	1.68±0.09	15.6±0.9	
(*c*) Phosphoglycerolipid	4	5.81±0.02	54.0±1.0	
(*d*) Cholesterol+sterol esters	4	3.23±0.03	30.9±0.7	
(*e*) Monoacylglycerols	3	0.57±0.01	5.3±0.1	0.7±0.2
Diacylglycerols	3	0.44±0.01	4.1±0.3	11.5±0.1
Triacylglycerols	3	0.48±0.03	4.5±0.1	53.4±1.3
		1.49	13.9	65.6
(*f*) Phosphatidylethanolamine	3	3.15±0.02	29.3±1.3	8.6±0.3
Phosphatidylcholine	3	1.00±0.10	9.3±0.5	17.8±0.5
Phosphatidylinositol+phosphatidylserine	3	0.72±0.03	6.7±0.2	6.6±0.7
Sphingomyelin	3	0.31±0.01	2.9±0.3	0.7±0.1
		5.18	48.2	33.7
(*g*) Cholesterol	3	2.63±0.11	24.4±0.7	0.4±0.1
Sterol esters	3	0.33±0.02	3.1±0.2	0.3±0.1
		2.96	27.5	0.7

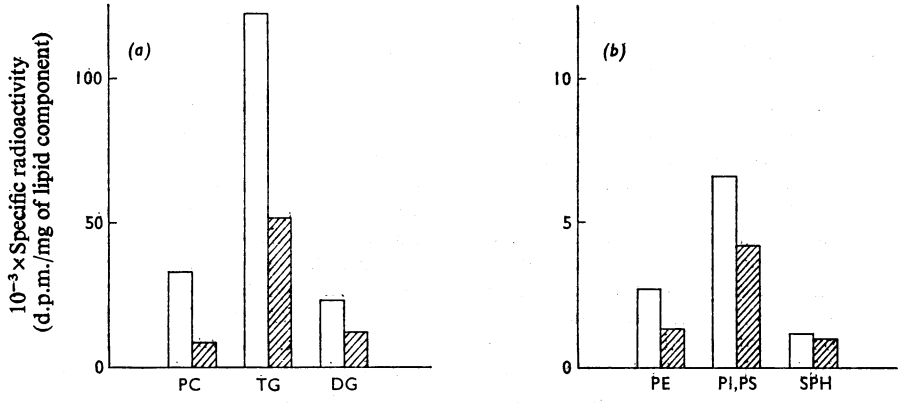

Fig. 3. *Specific radioactivities of glomerular basement-membrane lipids in comparison with residual glomerular-fraction lipids*

For abbreviations, see Fig. 2. □, Glomerular basement-membrane lipid; ■, residual glomerular-fraction lipid.

Lidsky *et al.* (1967), who used a potassium tartrate gradient to obtain basement membranes from bovine kidney homogenates. The present values are, however, of the same order as those of Wahl & Deppermann (1970) and of Fung & Kalant (1972), who used rat glomeruli and sonication for the preparation of glomerular basement membranes. A comparison with the results for human glomerular basement membranes reported by Misra & Berman (1966) is difficult, because their data are only given in molar ratios to hydroxyproline and not in absolute values. Earlier observations based on polarimetric and birefringence methods have also indicated that lipids are associated with the basement membrane (Niessing & Rollhäuser, 1954).

Our evidence leads us to conclude that the lipids found in the glomerular basement membrane prepared by the methods described above are part of the basement membrane or are intimately associated with it. The following facts support this view.

Table 4. *Percentage distribution of fatty acids in glomerular basement-membrane and residual glomerular-fraction lipids*

Fatty acids were determined by g.l.c. as described in the Experimental section. Results are expressed as a percentage of the total. +, Trace.

	Total lipids from		From glomerular basement membrane		
Fatty acids	Glomerular basement membrane	Residual glomerular fraction	Triacylglycerols	Phosphatidyl-ethanolamine	Phosphatidyl-choline
$C_{14:0}$	0.9	0.7	2.3	2.2	+
$C_{15:0}$	3.1	1.6	2.4	2.4	2.4
$C_{16:0}$	19.6	17.4	17.3	18.6	40.4
$C_{16:1}$	1.2	1.4	3.7	+	+
$C_{17:0}$	3.2	1.4	+	2.4	1.3
$C_{18:0}$	20.8	17.0	10.7	27.4	18.5
$C_{18:1}$	23.0	26.1	15.3	20.2	20.6
$C_{18:2}$	10.2	17.0	2.6	4.8	3.9
$C_{18:3}$	+	1.4	3.4	2.4	2.0
$C_{20:0}$	+	+	7.3	+	+
$C_{20:3}$	+	+	28.0	5.5	4.2
$C_{20:4}$	18.0	16.0	7.0	14.1	6.7

Morphological examination by electron microscopy of our preparations did not show a contamination of about 25%, which should result if the lipids originated from an exogenous source. The amount of hydroxyproline, which is taken as a criterion of purity, was $6.5 \pm 0.1\%$ (S.E.M., $n = 9$). Spiro (1967) reported a concentration of 6.5% and suggests that there is a slight contamination with lipid derived from other than basement-membrane structures. Our results support the assumption that ultrasound labilizes lipids as well as some other components, so that they are not found in the membranes. As these components represent only a small part of the membrane, the hydroxyproline content only increases to a limited extent. Sonication of our preparation by the method of Spiro (1967) increased the hydroxyproline content by 0.4% (from 6.9 to 7.3%). After treatment with ultrasound, the supernatant contains almost 4% of hydroxyproline, which indicates that parts of the basement membrane proper are additionally destroyed by this procedure (A. Heilhecker, unpublished work). In studies with rat kidney basement membranes we have found that almost all the individual membranes had clumped together and that they appeared ragged after this treatment. Sonication of our preparation considerably decreased the lipid content, although some phosphoglycerides seem to resist sonication (Fung & Kalant, 1972).

Treatment of our preparation with 60% (w/v) trichloroacetic acid to isolate the lamina densa (Nicholes *et al.*, 1973) dissolved one-third of the material containing only 0.8% lipid, whereas 3.6% remained in the lamina densa (A. Heilhecker, unpublished work).

The view that lipids are intimately associated with glomerular basement membranes is also supported by the fact that their concentration is constant from sample to sample and that the proportions of lipids in the basement membrane and in the residual glomerular fraction differ. Our conclusion is supported in particular by the different rates of incorporation of D-[U-^{14}C]glucose into the lipids of the glomerular basement membranes and of the residual glomerular fraction when glomeruli are incubated with this compound. This rate of incorporation was not observed in the lipids of the mitochondrial or microsomal subfractions. The relatively high specific radioactivity found for triacylglycerols compared with diacylglycerols and monoacylglycerols may be due to the pulse-label procedure that we used. The extracellular glomerular basement membrane is necessarily synthesized by the adhering cells. As shown by Walker (1973), both endothelial and epithelial cells are involved in the production of glomerular basement membranes, and these cells supply the various components that form the functional unit of the glomerular basement membranes. Grant *et al.* (1972) have shown that epithelial cells of the lens capsule are the site of synthesis of the collagenous component. Other components are probably synthesized in other cell types (Walker, 1973), all of which may have a different turnover rate (Walker, 1973; Heilhecker & von Bruchhausen, 1973). The higher specific radioactivity of the lipids of the basement membrane is either due to a higher rate of synthesis or to their special alignment to protein structures. A reasonably short turnover time has been calculated by Fung & Kalant (1972), for the phosphoglycerides of rat glomerular basement membranes.

Apart from lipids, there are also protein-containing components, which may be necessary for the function of glomerular basement membranes. These compo-

nents, however, are also lost by the use of ultrasonic techniques. (K. Maertin & F. von Bruchhausen, unpublished work). The question arises as to whether such highly radioactive components, which are only present in minor amounts, are altered under pathological conditions, e.g. in nephrotic or diabetic states, where alterations of the basement membrane have been described (Siperstein *et al.*, 1964; Gekle *et al.*, 1966; Spiro, 1971; Misra & Berman, 1972; Westberg & Michael, 1973; Kefalides, 1974).

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