Phospholipid of the Rat Glomerular Basement Membrane in Experimental Nephrosis

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(Received 21 April 1972)

Phospholipids were found to be a constant component of rat glomerular basement-membrane preparations. The concentration fell during preparation of basement membrane by sonication of whole glomeruli, but then remained constant despite continued sonication. The proportions of the individual phospholipids were different from those of whole renal tissue or of isolated glomeruli. The basement-membrane preparations had no ($\text{Na}^+ + \text{K}^+$)-activated adenosine triphosphatase activity, an enzyme that is bound to plasma membranes. The concentration of lipid P was decreased on exposure *in vivo* or *in vitro* to antiserum against basement membrane; 7 days after injection of antiserum there was a change in the phospholipid composition, with a relative increase in phosphatidylcholine and a decrease in sphingomyelin content. The metabolic turnover rate of the lipid P remaining in the membrane was normal, as determined by ^{32}P incorporation. The loss of phospholipid was associated with decreases in the relative concentrations of hydroxyproline, hydroxylysine and glycine, and relative increases in proline, lysine, serine, threonine and valine. Administration of aminonucleoside and daunomycin produced proteinuria but did not cause a decrease in lipid P. Anticollagen and anti-lymphocyte sera that attached to the basement membrane but failed to produce proteinuria, also failed to affect the phospholipid content.

In previous studies on antiserum nephritis in the rat one of the most consistent changes in chemical composition of the glomerular basement membrane was a decrease in lipid P (Misra & Kalant, 1966). Subsequently it was found that this decrease was related in time to the development of proteinuria and that there was a quantitative relationship between the extent of the decrease and the severity of proteinuria (Gang & Kalant, 1970). Loss of lipid P of the same magnitude occurred on incubation of glomerular basement membrane with decomplexed antiserum *in vitro*; the extent of loss was related to the amount of antibody deposited. It was concluded that the decrease of lipid P *in vitro* and *in vivo* was a direct result of antibody deposition. *In vivo*, antibody deposition leads to proteinuria, presumably dependent on a change in basement-membrane structure; it is not clear from previous experiments if the loss of lipid P from the membrane is an essential feature of the structural change or if it is an independent effect of the antibody deposition. The present work was undertaken to learn more about the phospholipids and their role in the glomerular basement membrane.

Methods

Male Sprague–Dawley rats were used throughout; for studies *in vivo* animals of 150–200 g were chosen,

whereas animals of 300 g or more were used as a source of basement membrane. The membrane was prepared as previously described (Gang & Kalant, 1970).

Antisera against basement membrane and against rat tail-tendon collagen were prepared in adult female rabbits, by using Freund's complete adjuvant. Anti-lymphocyte serum was obtained from Dr. H. E. Taylor of the Medical Research Council, Ottawa. Antiserum nephrosis was induced by three consecutive daily intravenous injections of 0.2 ml of anti-(glomerular basement membrane) serum.

Lipids were extracted in chloroform–methanol (2:1, v/v) and washed to remove water-soluble contaminants (Folch *et al.*, 1957). Lipid P in the extract was measured by a micro-modification (Gang & Kalant, 1970) of the method of Bartlett (1959). T.l.c. of phospholipids was done on 20 cm × 40 cm basic silica plates (Skipski *et al.*, 1964) prepared from silica gel H (E. Merck A.G., Darmstadt, Germany) washed by the method of Parker & Peterson (1965). Plates were used within 1 week of preparation. The solvent system chloroform – methanol – acetic acid – water (50:25:7:3, by vol.) (Skipski *et al.*, 1963) provided good resolution of all classes of phospholipids, including phosphatidylserine and phosphatidylinositol. All solvents except acetic acid were redistilled. For quantitative measurement after t.l.c., the dried plates were sprayed with 50% (v/v) H_2SO_4 and heated at

180°C for 10h; the charred spots were quantitatively transferred to test tubes and the P was determined (Kahovcova & Odavic, 1969).

For experiments involving radioactivity, carrier-free sodium [^{32}P]orthophosphate (Charles E. Frosst & Co., Montreal, Canada) was used. ^{32}P radioactivity was measured in a liquid-scintillation counter by the Čerenkov effect (Haberer, 1966; Ross, 1969). After measurement of P colorimetrically, the colour was destroyed (Palmer, 1969), the sample transferred to a counting vial, the volume adjusted to 10ml and counting carried out in a liquid-scintillation spectrometer at 5°C, to an accuracy of $\pm 1\%$.

For amino acid analysis, dried samples of 0.5–1.3mg of glomerular basement membrane were hydrolysed in 6M-HCl at 110°C for 24h, in sealed evacuated tubes previously flushed with N_2 . The samples were dried, redissolved in 2.0ml of 0.1M-HCl and, after addition of norleucine as an internal standard, were analysed by an accelerated two-column method (Spackman *et al.*, 1958) by using a Jeol automatic amino acid analyser. When hydroxyproline alone was measured, the method of Woessner (1961) was used.

Infrared spectroscopy was performed by the KBr-disc method and on air-dried films on AgCl plates, by using a Perkin-Elmer model 521 spectrophotometer. Electron-spin-resonance (e.s.r.) spectroscopy was performed with protein (nitroxide of *N*-ethylmaleimide) and lipid (stearate labelled with nitroxide on C-5 or C-12) spin labels as described by Simpkins *et al.* (1971).

Phosphorescence spectroscopy was done by the method of Purkey & Galley (1970) on basement membrane suspended in saline-ethylene glycol (1:1, v/v), by using an excitation wavelength of 280nm.

(Na^+ , K^+)-ATPase* (ATP phosphohydrolase, EC 3.6.1.3) was assayed by the method of Hokin & Yoda (1964); it was defined as that ATPase activity inhibited by 0.1mM-ouabain in the presence of Na^+ and K^+ .

The aminonucleoside of puromycin was purchased from Nutritional Biochemicals Ltd., Cleveland, U.S.A. Daunomycin was a gift of S.A. Farmaceutici Italia, Milan, Italy.

Results

Origin of phospholipids

The possibility was previously considered (Gang & Kalant, 1970) that the phospholipids are not part of the basement membrane but are components of residual plasma membrane of glomerular cells, adherent to the basement membrane. Since it may be

*Abbreviations: ATPase, adenosine triphosphatase; (Na^+ , K^+)-ATPase, (Na^+ + K^+)-activated adenosine triphosphatase.

reasonably assumed that hydroxyproline in the preparation is confined to the basement membrane, and since the concentration of hydroxyproline does not change in antiserum nephrosis (Gang & Kalant, 1970), the inability to lower the ratio of lipid P to hydroxyproline may be taken as an indication that the lipid P is an integral component of the basement membrane. Initial sonication caused a decrease in the ratio, but further prolonged sonication failed to lower the ratio further (Gang & Kalant, 1970); this finding was confirmed in the present work (Fig. 1). It was still possible that there was plasma membrane firmly attached to the basement membrane; to test this eventuality, normal membrane and a portion of membrane solubilized by trypsin (Mohos & Dudas, 1970) were assayed for (Na^+ , K^+)-ATPase, which is an enzyme marker for plasma membrane. Though slight ATPase activity was found, it was not sensitive to ouabain at a concentration (0.1mM) previously shown to produce a 50% inhibition of (Na^+ , K^+)-ATPase of rat renal plasma-cell membranes (Ebel *et al.*, 1971).

If (Na^+ , K^+)-ATPase and lipid P were both associated with residual plasma-membrane, which was removed by nephrotoxic serum, one might expect parallel decreases in enzyme activity and lipid P. To test this possibility, 1ml portions of a basement-membrane suspension in phosphate-buffered saline (0.01M-potassium phosphate in 1.5M-NaCl, pH 7.4) were incubated with 1ml of nephrotoxic serum or control serum for various time-intervals; the mem-

Fig. 1. Effect of time of sonication of whole glomeruli on the lipid P content of the resulting basement-membrane preparation

A suspension of glomeruli was sonicated in 30s bursts; a portion was removed after each period of sonication for isolation of the basement membrane, which was then analysed for lipid P and hydroxyproline as described in the Methods section.

Fig. 2. Effect of incubation with normal rabbit serum (▲) and nephrotoxic serum (●) on the basement-membrane content of lipid P (· · · ·) and ATPase (—)

Samples of a membrane suspension in 0.01 M-potassium phosphate buffer, pH7.4, were incubated with the sera for the times shown. The membrane was then removed and treated with trypsin to solubilize the ATPase as described in the Results section. The enzyme was assayed in the presence of Na⁺ and K⁺; residual membrane was extracted for measurement of lipid P.

brane was removed from the incubation medium, then treated with trypsin to solubilize ATPase activity (Mohos & Dudas, 1970). Measurements were made of lipid P and of ATPase activity. The results (Fig. 2) show the expected progressive decrease in lipid P, and a much more rapid decrease in ATPase activity, on treatment with nephrotoxic serum. Control serum produced a slow decrease in ATPase activity and no change in lipid P. The nephrotoxic serum thus appeared to produce a specific loss or inhibition of the ATPase.

Nature of phospholipids

Pools of glomerular basement membrane were prepared from normal and 7-day nephrotic rats and from normal basement membrane incubated with nephrotoxic serum as described in the preceding section: phospholipids were extracted and subjected to quantitative t.l.c. The results (Table 1) confirm the loss of lipid P after exposure to nephrotoxic serum both *in vivo* and *in vitro*. The phospholipid pattern was essentially unchanged by incubation with nephrotoxic serum, but injection of this serum led to a significant increase in the proportion of phosphatidylcholine and a decrease in sphingomyelin. In Table 2 the composition of basement membrane is compared with that of whole glomeruli and whole kidney obtained by the same procedure,

Table 1. Effect of nephrotoxic serum on phospholipid composition of glomerular basement membrane

Glomerular basement membrane was prepared from normal and nephrotic rats as described in the Methods section. Portions of the normal membrane were incubated with nephrotoxic serum. Phospholipids were extracted from unincubated normal, incubated normal and nephrotic basement membrane and examined by t.l.c. as described in the text. Values are expressed as % of total lipid P on t.l.c. Results were tested by an analysis of variance, with multiple classification; the differences among the three groups were significant at the 2.5% level for sphingomyelin and at the 5% level for phosphatidylcholine.

		Composition (% distribution on thin-layer chromatogram)		
Basement-membrane preparation ... No. of samples ...		Normal	Normal, incubated with nephrotoxic serum	Nephrotic rats 14 days after injection of nephrotoxic serum
		(3)	(2)	(2)
Origin		1.3 (0.4-3.1)	2.0 (0.5-3.5)	0.8 (0.6-1.1)
Lysophosphatidylcholine		4.8 (4.5-5.4)	6.1 (6.0-6.2)	3.8 (2.8-4.8)
Sphingomyelin		27.2 (25.2-29.2)	29.0 (27.0-31.0)	20.1 (19.8-20.5)
Phosphatidylcholine		33.8 (33.5-34.6)	30.3 (27.3-33.4)	42.5 (39.8-45.2)
Phosphatidylinositol		5.6 (5.0-6.4)	5.4 (4.8-6.1)	5.2 (4.5-6.0)
Phosphatidylserine		6.6 (5.7-7.3)	6.6 (5.9-7.3)	6.8 (6.7-7.0)
Phosphatidylethanolamine		13.5 (11.7-15.6)	13.3 (12.4-14.2)	15.6 (13.8-17.4)
Phosphatidic acid		7.1 (6.1-8.2)	7.8 (7.4-8.2)	5.0 (4.1-5.9)
Total content (μg of lipid P/mg of membrane)		2.5 (2.2-2.6)	1.0 (0.9-1.1)	1.0 (0.9-1.0)
Recovery of lipid P applied to chromatogram (%)		97 (96-99)	97 (93-100)	96 (92-99)

Table 2. *Phospholipid composition of rat kidney fractions and erythrocytes*

Phospholipids of normal whole glomeruli and of whole kidney homogenate were determined as described in Table 1. The mean values are compared with those of normal glomerular basement membrane, taken from Table 1, and with those of other renal fractions and of rat erythrocytes taken from published information.

Preparation No. of determinations	Composition (%)					
	Normal glomerular basement membrane	Whole glomeruli	Whole kidney	Mito- chondria*	Microsomal* fraction	Erythro- cytes†
	(3)	(2)	(2)			
Origin	1.3	0.7	0	0	0	0
Lysophosphatidylcholine	4.8	1.5	2.5	0	0	3.8
Sphingomyelin	27.2	16.0	13.0	6.4	13.4	12.8
Phosphatidylcholine	33.8	33.8	35.6	38.7	37.5	47.5
Phosphatidylinositol	5.6	14.1	6.1	6.1	12.7	3.5
Phosphatidylserine	6.6	6.3	7.8	5.3	10.9	10.8
Phosphatidylethanolamine	13.5	14.9	27.7	31.2	21.7	21.5
Phosphatidic acid	7.1	12.6	7.3	10.7	0	0.1

* Johnson & Cornatzer (1969).

† Nelson (1967).

and with that of renal mitochondria, renal microsomal material and erythrocytes reported by others. The glomerular basement membrane appears to differ from the other preparations in its high sphingomyelin and low phosphatidylethanolamine content.

Metabolic turnover of lipid P

Though the decrease in lipid P on incubation with nephrotoxic serum *in vitro* can only be due to removal or destruction of phospholipids, the decrease *in vivo* may be at least partly due to decreased synthesis. To test this possibility, measurements were made of the rate of incorporation of ^{32}P into the lipid P fraction. Preliminary studies established that when normal animals were injected intraperitoneally with a tracer amount of $\text{NaH}_2^{32}\text{PO}_4$, peak radioactivity appeared in the glomerular basement membrane 8–16h later. To remain within the period of radioisotope incorporation, with a minimal rate of radioisotope removal, definitive experiments were done during a 6h period immediately after administration. Four groups of rats were studied: normal controls, and nephrotic rats on the 5th, 8th and 15th days after the first injection of nephrotoxic serum. Animals of each group were injected intraperitoneally with $500\mu\text{Ci}$ of $^{32}\text{P}/100\text{g}$ body wt. contained in 1ml of 0.15M-NaCl adjusted to pH7.4; subgroups of two to four animals were killed 2, 4 and 6h later, and the glomerular basement membrane was isolated from individual animals. Measurements were made of radioactivity and concentration of lipid P. The results for the three nephrotic groups did not differ

significantly from each other, and the results were therefore combined for comparison with the controls (Fig. 3). The absolute amount of ^{32}P incorporated was always significantly lower in the nephrotic animals, but the specific radioactivity of the lipid P did not differ from normal at any time during the experiment.

Protein changes associated with loss of lipid P

In antiserum nephrosis the composition of the glomerular basement membrane is not abnormal with regard to total N and collagen content (Misra & Kalant, 1966) but the membrane is thickened, its electrophoretic mobility is decreased and it gives an abnormal X-ray-diffraction powder diagram (Kalant *et al.*, 1966). It was suggested that the latter changes reflect a change in conformation of the membrane proteins which resulted in increased permeability to serum proteins.

If the loss of lipid P is an essential step in the process leading to increased permeability, then exposure *in vivo* and *in vitro* to nephrotoxic serum leading to such loss should also produce some change in membrane protein. The effects of exposure to nephrotoxic serum on the composition and structure of the membrane were therefore made, by amino acid analysis, i.e., e.s.r. and tryptophan-phosphorescence spectroscopy.

Table 3 shows that after injection or incubation with nephrotoxic serum there were decreases in the proportions of hydroxylysine and 4-hydroxyproline, with corresponding increases in lysine and proline;

Fig. 3. Incorporation of ^{32}P into lipids of glomerular basement-membrane of normal and nephrotic (nephrotoxic serum) rats

Animals were injected intraperitoneally with $500\mu\text{Ci}$ of ^{32}P /100g body wt. in 1 ml of 0.15M-NaCl. Base-

thus the sums of (hydroxylysine+lysine) and (hydroxyproline+proline) were virtually unchanged. In addition there were increases in the proportions of serine, threonine and valine and a decrease in glycine.

The i.r. spectra obtained by the KBr-disc and AgCl-plate methods were not changed by nephrotoxic serum *in vivo*. The amide I band showed a strong absorption at 1656cm^{-1} , indicating that the major structure was random coil or α -helix; there was no evidence of β -configuration.

The e.s.r. spectrum obtained with the nitroxide of *N*-ethylmaleimide showed that 60% of the label was in a fixed environment, whereas the remainder was in a superficial location on the membrane. The spectra obtained with the lipid labels demonstrated greater mobility of the C-12 carbon than of the C-5; the latter showed little mobility and evidence of anisotropy. There was no difference between the normal membrane and that from nephrotoxic animals.

The phosphorescence spectrum showed a tryptophan emission peak at 411nm, indicating that the tryptophan is buried in a relatively non-polar environment within the membrane core. Though the 'emission band' was somewhat narrower for the nephrotic membrane, the position of the peak was not altered.

Lipid in non-immunogenic forms of experimental nephrosis

To determine if a change in the lipid P concentration occurs in other forms of experimental nephropathy associated with proteinuria, groups of rats were made nephrotic by injection of nephrotoxic serum, by subcutaneous injection of aminonucleoside of puromycin at a dose of 1.5mg/g body wt. in an 0.5% aqueous solution daily for 11 days, and by a single intravenous injection of daunomycin (Sternberg, 1970) at a dose of 1mg/100g body wt. When proteinuria was well established, basement membrane was analysed for lipid P. The nephrotoxic serum again produced a decrease in lipid P, but neither of the other agents did so (Table 4).

Effects on phospholipids of other antibodies that bind to kidney

The preceding experiment established that loss of lipid P occurred in antiserum nephrosis but not in the two forms of 'antibiotic' nephrosis. To ascertain

ment membrane was obtained at the times shown; the lipid-extractable radioactivity was measured and expressed in terms of lipid P (a) and of dry membrane weight (b). Bars show the mean and S.E.M. (six to eight animals/group). □, Control; ■, nephrotic.

Table 3. *Amino acid composition of glomerular basement membrane after exposure to nephrotoxic serum*

Amino acid analysis was performed on normal glomerular basement-membrane incubated with phosphate buffer (0.01 M, pH 7.4), with normal rabbit serum and with nephrotoxic serum, and on basement membrane from nephrotic rats 14 days after administration of nephrotoxic serum. Analysis of variance was performed for each amino acid. For those showing an *F* ratio with $P \leq 0.01$, comparisons were made among means; those differing from control groups (normal membrane incubated with buffer or normal rabbit serum) with a $P < 0.05$ are marked *.

Amino acid content (residues/1000 residues)				
No. of samples ...	Normal basement membrane incubated with			Basement membrane in antiserum nephrosis (5)
	Buffer (9)	Normal rabbit serum (4)	Nephrotoxic serum (5)	
Hydroxylysine	25 ± 1	25 ± 1	15 ± 2*	17 ± 1*
Lysine	26 ± 0	25 ± 1	33 ± 1*	36 ± 2*
Histidine	20 ± 1	19 ± 1	16 ± 0	17 ± 1
Arginine	50 ± 1	50 ± 0	41 ± 1	46 ± 2
3-Hydroxyproline	26 ± 6	25 ± 4	25 ± 4	27 ± 2
4-Hydroxyproline	58 ± 3	63 ± 3	43 ± 4*	50 ± 4
Aspartic acid	74 ± 2	71 ± 1	75 ± 2	79 ± 1
Threonine	43 ± 1	40 ± 1	49 ± 4*	54 ± 2*
Serine	57 ± 1	57 ± 1	81 ± 3*	66 ± 2
Glutamic acid	101 ± 2	101 ± 2	99 ± 2	106 ± 2
Proline	68 ± 3	69 ± 2	84 ± 3*	83 ± 2*
Glycine	207 ± 3	208 ± 5	163 ± 6*	170 ± 6*
Alanine	60 ± 1	59 ± 2	56 ± 1	58 ± 1
Valine	37 ± 0	39 ± 2	51 ± 2*	42 ± 1
Isoleucine	31 ± 1	28 ± 1	23 ± 2	27 ± 2
Leucine	70 ± 1	73 ± 1	67 ± 1	70 ± 1
Tyrosine	20 ± 1	20 ± 1	26 ± 1	22 ± 1
Phenylalanine	29 ± 1	31 ± 0	31 ± 1	30 ± 1

Table 4. *Lipid P of glomerular basement membrane in experimental proteinurias*

Nephrosis was produced by injection of nephrotoxic serum, aminonucleoside of puromycin, or daunomycin. When the rate of urinary protein excretion was stable, glomerular basement membrane was obtained and analysed for lipid P. Results are means ± S.E.M. for the numbers of animals given in parentheses.

	Urine protein (mg/day)	Lipid P (μg/mg of glomerular basement membrane)
Normal (16)	16 ± 1.2	2.66 ± 0.09
Antiserum nephrosis (6)	373 ± 22	0.97 ± 0.03
Aminonucleoside nephrosis (5)	143 ± 4	2.27 ± 0.04
Daunomycin nephrosis (21)	579 ± 18	2.92 ± 0.06

whether the loss was non-specifically associated with antibody deposition, normal basement membrane was incubated with antisera that fix to the membrane but do not produce proteinuria, i.e. anti-collagen serum (Rothbard & Watson, 1965; Huang & Kalant, 1968) and horse anti-(human lymphocyte) serum (Wilson *et al.*, 1971). Localization to rat basement

membrane was confirmed by the indirect immunofluorescence method: intravenous injection failed to produce proteinuria (Table 5). Replicate samples of normal basement membrane were incubated with 1 ml of normal rabbit serum, nephrotoxic serum, anti-collagen serum or anti-lymphocyte serum. The results (Table 5) show that the sera that bound to

Table 5. Effect of antisera binding to glomerular basement membrane on proteinuria *in vivo* and on lipid P *in vitro*

Antisera which bind to the glomerular basement membrane were injected intravenously on three consecutive days; proteinuria was measured on the sixth day. Normal basement membrane was incubated with the antisera as previously described, then analysed for lipid P. Values are means $\pm$ s.e.m. of six determinations, except as noted.

	Proteinuria (mg/day)	Lipid P (μ g/mg of glomerular basement membrane)
Normal rabbit serum	23 $\pm$ 4	2.35 $\pm$ 0.05
Nephrotoxic serum	398 $\pm$ 23	0.94 $\pm$ 0.02
Anti-collagen serum	31 $\pm$ 6	2.02 $\pm$ 0.02
Anti-lymphocyte serum	22 $\pm$ 7	2.35 (2.34, 2.36)

membrane but did not produce proteinuria failed to alter the lipid P content.

Discussion

There have been numerous reports of sugar, amino acid and phospholipid analyses of glomerular basement membrane. Some of the discrepancies in the values reported may be due to species differences, but undoubtedly most are due to differences in preparation of the membrane. Unfortunately there is no easily applied and categorical criterion of purity or intactness of the preparation. Several laboratories working with rat and human basement membrane have found significant concentrations of lipid P (Misra & Berman, 1966; von Bruchhausen & Merker, 1967; Gang & Kalant, 1970; Westberg & Michael, 1970), whereas others working with bovine material (Spiro, 1967) have not. Thus it is not certain whether the presence of lipid P signifies contamination by renal-cell plasma membrane or its absence indicates removal of an integral component of basement membrane during preparation. The evidence in the present work favouring the view that the lipid P is a component of the basement membrane is as follows:

(a) The concentration is relatively constant from sample to sample and is not decreased by prolonged sonication.

(b) The phospholipid pattern is different from that of whole kidney, glomeruli and renal subcellular components and from plasma membrane of erythrocytes, the foreign cells most likely to be present as a contaminant. Westberg & Michael (1970), working with human tissue, also noted differences in the phospholipid pattern between basement membrane and renal cortex.

(c) Absence of (Na⁺, K⁺)-ATPase enzyme known to be associated with plasma membrane. If incubation with nephrotoxic serum had decreased lipid P and ATPase activity at the same rate, this would have been evidence favouring the 'contamination' view. The

enzyme activity, however, decreased much more rapidly than the lipid P, and also much more rapidly than the activity of basement membrane incubated with normal rabbit serum. It therefore appears that nephrotoxic serum produces a specific loss or inhibition of the enzyme.

(d) Nephrotoxic serum removes a specific amount of lipid P, leaving a constant residue of about 1.0 μ g/mg.

The present experiments suggest, but do not establish, that phospholipids form an integral part of the glomerular basement membrane. If, however, some of the phospholipid molecules are components of the plasma membranes of epithelial or endothelial cells, adherent to the basement membrane, then one must conclude that binding of nephrotoxic antibody on the basement membrane releases these contaminating membranes from their attachment to the latter.

The finding that exposure to nephrotoxic serum *in vitro* decreases the total lipid P without changing the proportion of individual phospholipids is compatible with the latter idea. This may also explain the separation of endothelial cells from the basement membrane shortly after injection of nephrotoxic serum (Gang & Kalant, 1970). However, 7 days after injection of the serum the composition of the phospholipids is significantly different from normal, with an increase in phosphatidylcholine content and a decrease in sphingomyelin content. This is most unlikely to be due to a change in composition of plasma membrane of glomerular cells, especially since antibody does not bind to these cells; it is much more likely to be the result of change in the basement membrane itself. We are therefore again led to the view that some, if not all, of the phospholipid present in the basement-membrane preparations used in this study is an integral component of this membrane.

The radioisotope studies indicate that total synthesis of basement-membrane phospholipids is decreased in nephrotic rats. It may be assumed that during the 6h duration of these studies, the pool of

membrane phospholipid was in a steady state. The fractional rate of ^{32}P incorporation may therefore be taken as equivalent to turnover. Since this was not altered in the nephrotic rats, it may be concluded that the phospholipid present in the basement membrane of these animals is undergoing a normal rate of synthesis and degradation. It is therefore suggested that nephrotoxic antibody binding destroyed or blocked some of the phospholipid-binding sites, releasing the phospholipids and preventing their replacement by a normal synthesizing mechanism. The change in phospholipid pattern in basement membrane from nephrotic rats indicates that the loss of phospholipids is not random, but involves specific structures within the membrane.

The loss of lipid P was accompanied by a number of changes in amino acid composition, which were qualitatively the same for exposure to nephrotoxic antibody *in vivo* and *in vitro*. Rabbit antibody globulin contains particularly high amounts of threonine, serine and valine, and much lower amounts of glycine than the basement membrane; it is devoid of hydroxylysine and hydroxyproline and contains more lysine and proline than basement membrane (Koshland *et al.*, 1964). If it is assumed that after incubation of the membrane in nephrotoxic

serum there is deposition of antibody on the membrane in a ratio of 1:3, then some of the changes observed in the amino acid composition after exposure to antiserum *in vitro* and *in vivo* could be accounted for (Table 6); a number of serious discrepancies would remain, however (threonine, serine, proline, glycine, isoleucine). Thus the ratio of antibody to basement membrane required to produce the observed change in threonine would be 1:12, whereas only pure antibody would give the observed value for proline. It is notable that deposition of antibody even to the extent of 1:3 would not be sufficient to explain the decrease in lipid P content by 'dilution' of the basement membrane.

A second possibility exists to explain the changes in the amino acid pairs (hydroxylysine+lysine) and (hydroxyproline+proline). In each case there was a decrease in the hydroxylated acid and an equimolar increase in the non-hydroxylated compound. The changes suggest a decrease in the number of the hydroxyl groups during the interaction of antigen with antibody.

It was assumed that the changes in primary protein structure would be reflected in conformational changes. Changes in the X-ray diffraction pattern and in charge density were previously demonstrated

Table 6. *Amino acid compositions of rabbit antibody globulin and glomerular basement membrane*

The theoretical amino acid composition of a mixture of rabbit antibody with rat glomerular basement-membrane (1:3, w/w) is based on the analysis of antibody reported by Koshland *et al.* (1964) and on that of normal basement membrane reported in Table 3. This calculated composition is compared with the observed composition of basement membrane incubated with nephrotoxic serum (Table 3).

	Content (residues/1000 residues)		
	Rabbit antibody	Rabbit antibody-glomerular basement membrane (1:3)	Glomerular basement membrane incubated with nephrotoxic serum
Hydroxylysine	0	19	15
Lysine	52	33	33
Histidine	13	18	16
Arginine	34	45	41
3-Hydroxyproline	0	20	25
4-Hydroxyproline	0	45	43
Aspartic acid	81	74	75
Threonine	121	62	49
Serine	108	71	81
Glutamic acid	91	99	99
Proline	82	72	84
Glycine	81	175	163
Alanine	58	59	56
Valine	95	53	51
Isoleucine	35	30	23
Leucine	66	70	67
Tyrosine	39	25	26
Phenylalanine	33	31	31

(Kalant *et al.*, 1966), but none of the physical methods employed in the present work revealed significant alterations.

The results obtained with anti-collagen and anti-lymphocyte sera demonstrate that loss of phospholipid is not a non-specific result of binding of antibody to basement membrane and 'dilution' of the phospholipid by antibody protein, but is related to deposition of antibody which results in proteinuria. Phospholipid, together with cholesterol ester, is required for the organization of the secondary and tertiary structure of plasma high-density lipoprotein (Lux *et al.*, 1971); there is thus precedent for suggesting that the phospholipid may play a similar role in glomerular basement membrane and that its removal by antibody leads to conformation changes that cause increased permeability to serum proteins.

The mechanism by which the puromycin aminonucleoside and daunomycin produce proteinuria is not established. Both substances can produce thickening of the basement membrane such as occurs in antiserum nephrosis, accompanied by marked proteinuria. However, it is apparent from the results with these substances that proteinuria can occur without a change in the phospholipid content. Previous evidence indicated that the nephrotoxic antibody is directed against the glycoprotein (Huang & Kalant, 1968) whereas aminonucleoside affects the collagen (Kefalides & Forsell-Knott, 1970). It is therefore likely that the phospholipid is associated with the former. The differences between the effects of nephrotoxic serum on the one hand and aminonucleoside and daunomycin on the other indicate that several conformation changes affecting either the collagen or the glycoprotein may each lead to proteinuria.

We are indebted to Dr. H. Simpkins for the e.s.r. spectroscopy and to Dr. W. Galley for the phosphorescence spectroscopy. This work was supported by the Medical Research Council of Canada.

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