The Effects of Lathyrism on Renal Chemistry, Anatomy and Function[1] (37675)

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Lathyrism is a syndrome associated with a defect in collagen cross-linking, induced by a variety of toxic agents such as 3-aminopropionitrile (APN) (1). The specific defect, decreased collagen cross-linking by prevention of the formation of aldehyde oxidation products of lysine (2–4), has been demonstrated in the collagen from a variety of tissues (1). Most simply, the defect in cross-linking is demonstrated by an increase in neutral salt- or dilute acid-solubility of collagen (5).

The glomerular basement membrane (GBM) of the kidney contains a collagen-like moiety (6, 7), although typical collagen fibers with periodicity have rarely been visualized in the normal GBM by electron microscopy (8). Kefalides (9) has proposed a model of a GBM filtration barrier based upon the aggregation of collagen fibrils to form interstitial filtration pores, in which greater aggregation resulted in larger pores. The present studies were designed to determine whether a physicochemical alteration of the GBM could be induced *in vivo* by inhibition of the cross-linkage of the collagen-like component. In addition, anatomic and functional consequences of such alteration were examined.

Since the purification of GBM involves sonication (10), which itself solubilizes some GBM material (11, 12), the present analytic studies utilized whole glomeruli which were extracted after lyophilization, and the hydroxyproline content of soluble fractions was used as the index of GBM solubility.

Methods. Lathyrism was induced in 70–80 g male weanling Sprague-Dawley rats, by adding APN to Purina Rat Chow, micromix, 0.5% (w/w), or by its addition to drinking water, 225 mg/100 ml. The dose of APN received was 30–50 mg APN/100 g rat/day for 28 days for analytic studies and for up to 42 days for pathologic and renal function studies. Control animals were matched for age and weight at the beginning of the studies.

Twenty-four hour urine specimens were collected in metabolic cages and diluted to 50 ml with water. The urinary protein was determined by the biuret method after centrifugation and trichloroacetic acid precipitation. Urea nitrogen was determined on serum samples of lathyritic and control animals after 42 days of treatment.

Histologic studies. Kidneys from control and experimental animals (treated with APN) were sacrificed at 7 days (5 lathyritic, 5 control), 14 days (4 lathyritic, 5 control) and 42 days (4 lathyritic, 5 control). Tissues were fixed in formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin, periodic acid–Schiff (PAS), alcian blue (pH 2.5), and colloidal iron at pH 1.9 and 2.5 (13). Other sections were frozen in isopentane prechilled in liquid nitrogen, sectioned in a Lipshaw cryostat, and stained with monospecific fluorescein-labeled antibody to rat IgG, β_1C and fibrin by methods previously described (14). Small (1 mm^3) cortical pieces from 42 day specimens were fixed for 30 min in 1% glutaraldehyde, buffered in sodium cacodylate (pH 7.42), post-fixed in 1% osmium, and embedded in glycol methacrylate for electron microscopy. Thin sections were stained with uranyl acetate and lead citrate, and with phosphotungstic acid (15).

Biochemical studies. Skin samples from 4 control and 5 lathyritic rats, obtained after

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28 days of APN treatment, were finely minced and lyophilized. Approximately 10 mg aliquots were homogenized in a glass homogenizer and extracted for 18 hr at 4° in 0.5 ml of 0.5 *M* acetic acid (HOAc), then separated by centrifugation at 30,000*g* for 1 hr in a Sorval refrigerated centrifuge, and washed twice with the same extracting medium. The supernatant and washings were combined and dialyzed against water and hydrolyzed in 6.0 *N* HCl for 18 hr at 103° in sealed ampoules. The insoluble skin residue was hydrolyzed in a similar manner and filtered through sintered glass. All hydrolysates were evaporated on a flash evaporator at 37° and redissolved in water for hydroxyproline (HYDROX) determination by a manual microadaptation of the method of Bergman and Loxley (16).

Glomerular preparations were isolated from the pooled kidneys of 8 rats by methods previously described in this laboratory (20), washed three times with water, and lyophilized.

For Expt I, approximately 10 mg samples from each of 8 preparations of lathyritic and control glomeruli were extracted at 4° for 48 hr with 0.2 ml of 0.5 *M* NaCl in 10 ml polycarbonate ultracentrifuge tubes. Cold water (2.0 ml) was added and mixed, and the tubes were centrifuged at 90,000*g* in a Beckman L2-65B ultracentrifuge for 1 hr. The supernatant was removed by suction and hydrolyzed in 6 *N* HCl for 18 hr at 103°. The insoluble sediment was washed twice with water and extracted in 1.0 ml of 0.5 *M* acetic acid for 48 hr at 4° and similarly separated and hydrolyzed. The final sediment was directly hydrolyzed in 6 *N* HCl and filtered. All hydrolysates were evaporated to dryness in 25 ml conical tubes with a Buchler Rotary Evapo-Mix evaporator connected to a vacuum pump through a trap bathed in methanol/dry ice. For HYDROX analysis, samples were redissolved in water: 0.25–0.50 ml for soluble samples (NaCl and HOAc extract) and 8.0 ml for insoluble glomerular material. HYDROX determination was by a micro-auto analyzer adaptation of the method of Bergman and Loxley (16), and required 0.10 ml/determination.

The HYDROX recovered from 4 samples of a standard 0.5 *M* HOAc soluble rat skin collagen solution, carried through the entire acid extraction procedure, was 93 to 100%. However, the amounts of the same solution recovered after addition to glomerular samples and carried through acid extraction were 8% and 67%; the reason for these variable and low recoveries is unknown. Expt II was performed to overcome this problem.

For Expt II, 10 mg samples of glomerular preparations were extracted with 1.5 ml of 0.5 *M* acetic acid in 1.5 ml polyethylene centrifuge tubes for 48 hr at 4°. Samples were centrifuged at 29,000*g* for 1 hr at 3° after the addition of 1.5 ml of 0.5 *M* acetic acid. Supernatants, with detectable insoluble material, were sequentially filtered through 5 and 0.45 μm filters (MF-Milipore) by syringe, using the same washes. The supernatants, insoluble materials and unextracted glomeruli were hydrolyzed and filtered, redissolved in 1.0–6.0 ml of water, and analyzed for HYDROX as in Expt I. Due to the paucity of experimental glomerular material, recovery studies utilized glomeruli isolated from rats with nephrotoxic serum nephritis. A standard solution of HYDROX (2 samples) or the standard acetic acid extracted collagen solution (2 samples) was added to these glomeruli. The recoveries varied from 94 to 100%.

Results. After 2–3 wk of ingesting APN, most experimental animals were demonstrably abnormal. The rate of weight gain (mean = 3 g/day) was less than controls (mean = 7 g/day); the animals were limping, some unable to move their hind limbs. Deformities of the femurs and xiphoid were evident; the coats were thin and matted.

Urinary protein excretion was not significantly different between control and lathyritic animals at 7, 14, 28, and 42 days of APN treatment (Table I). Serum urea nitrogen values were 28 mg/100 ml (mean of 5) in lathyritic animals and 22 mg/100 ml (mean of 3) in control animals at 42 days.

No histologic differences by light microscopy were observed in glomeruli from lathyritic and control animals after 7, 14, and 42 days of APN treatment. Alcian blue and colloidal iron (at pH 1.9 and 2.5) stains showed no difference at 42 days in 4 lathyritic and

TABLE I. 24 hr Urinary Protein Excretion.

Duration of treatment (wk)	Control No. of rats	Control Urinary protein (mg/24 hr mean ± SE)	Lathyritic No. of rats	Lathyritic Urinary protein (mg/24 hr mean ± SE)	p
1	5	14.8 ± 4.5	5	14.4 ± 3.1	>0.50
2	4	19.8 ± 7.4	5	25.9 ± 5.6	>0.30
4	6	13.5 ± 5.0	6	11.4 ± 2.0	>0.40
6	4	14.2 ± 6.5	6	23.5 ± 3.3	=0.10

6 control animals. Immunofluorescent studies in all the animals showed no differences between lathyritic and control animals for glomerular deposition of IgG, β_1C and fibrin.

Glomeruli from 3 lathyritic and 3 control rats were studied by electron microscopy. Routine stains of thin sections with lead citrate and uranyl acetate revealed normal capillary wall structure except for diffuse widening of the lamina rara interna and focal areas of decreased density of the lamina densa of the GBM. These changes were subtle and better appreciated after review of sections stained with phosphotunstic acid (PTA). The normal GBM was PTA negative and outlined by the PTA positively stained glycocalyx of the epithelial and endothelial cytoplasmic membranes; the GBM lamina densa of the lathyritic rats contained a network of stained fine fibrils (Fig. 1). The fibrils were oriented primarily in a direction parallel to the GBM, were nonbranching with a length of ≅ 1000 Å and a width of ≅ 150 Å, and were lacking in periodicity.

Tubular basement membranes of lathyritic rats also demonstrated an irregularity of density, but fewer membranes were examined at high magnification with PTA stains. The in-

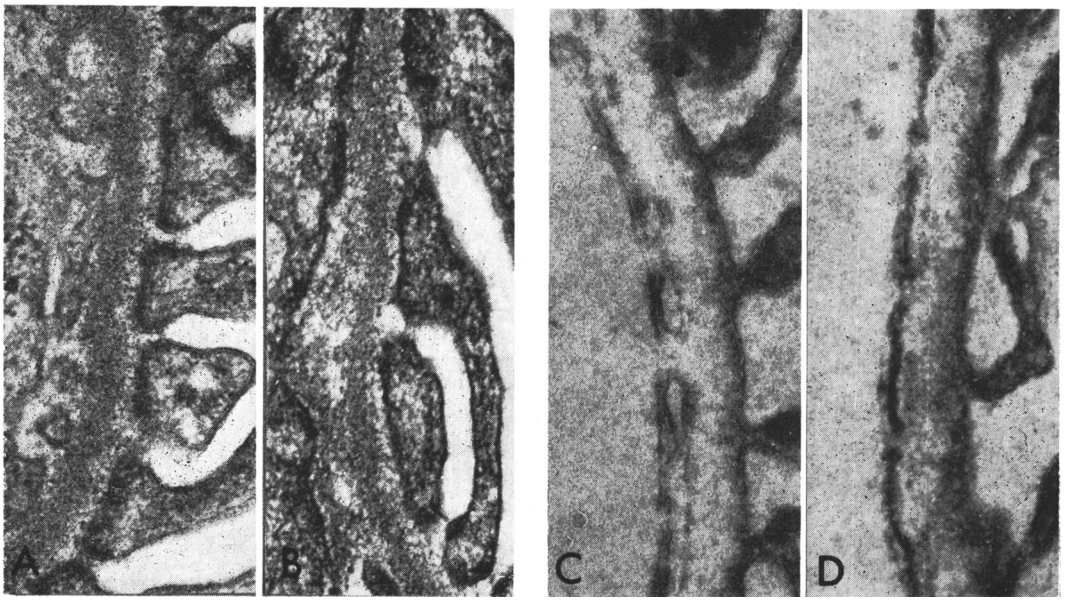

FIG. 1. Comparison of ultrastructure of glomerular capillary wall of normal control and lathyritic rats. Magnification 56,000×. Routine strains: (A) control; (B) lathyritic. Note the somewhat widened lamina rara interna and the more loose organization of the granularity of the glomerular basement membrane lamina densa in (B). Phosphotungstic acid stain: (C) control; (D) lathyritic. Note the increased staining of the region of the lamina densa in the lathyritic rat (D).

TABLE II. Solubility of Skin Collagen in 0.5 *M* Acetic Acid in Lathyritic and Control Rats.

	Control		Lathyritic		
	N	Mean ± SE	*N*	Mean ± SE	*p*
Soluble hydroxyproline					
μg/mg	4	31.8 ± 5.4	5	48.6 ± 2.6	<0.01
%[a]	4	48.8 ± 4.6	5	72.2 ± 4.7	<0.02
Insoluble hydroxyproline					
μg/mg	4	34.1 ± 2.5	5	18.7 ± 2.2	<0.005

[a] Percentage of total hydroxyproline in skin that is soluble.

terstitial collagens of normal and lathyritic animals showed no difference in affinity for PTA, morphology or periodicity.

Analytic studies of soluble hydroxyproline. Acid-soluble HYDROX was 52.8% greater in lathyritic than in control skin specimens at 28 days (Table II).

In Expt I (Table III), more acid-soluble HYDROX was found in glomeruli from lathyritic animals (mean 0.34 μg/mg) than from control animals (0.16 μg/mg). There was no difference in the salt-soluble fraction: 0.17 μg/mg in lathyritic and 0.17 μg/mg in controls. Total soluble HYDROX was greater in lathyritic (0.51 μg/mg) glomeruli. There was no difference in total glomerular HYDROX in the two groups.

Expt II (Table III) also demonstrated greater soluble (acid soluble without prior salt extraction) HYDROX in lathyritic (0.23 μg/mg) than in control (0.15 μg/mg) glomeruli, with adequate recovery studies (see Methods).

Discussion. Lathyrism was induced in weanling rats with APN, verified by poor growth, physical deformities (11) and increased skin collagen solubility in 0.5 *M* HOAc (18). The small proportion (< 2%) of soluble HYDROX extracted from glomeruli in the present experiments is similar to that found by Kefalides (8) and Blau and Michael (10) who extracted isolated GBM. The use of whole glomeruli in the present studies was necessitated by the solubilization

TABLE III. Analytic Studies of Solubility of Glomerular Hydroxyproline in Control and Lathyritic Rats.

	Control		Lathyritic		
	No. of rats	Mean[a] ± SE	No. of rats	Mean[a] ± SE	*p*
Expt I					
0.5 *M* NaCl soluble	8	0.17 ± 0.02	8	0.17 ± 0.01	>0.40
0.5 *M* HOAc soluble	8	0.16 ± 0.02	7	0.34 ± 0.04	<0.0005
Total soluble	8	0.33 ± 0.03	7	0.51 ± 0.05	<0.05
Insoluble	6	18.7 ± 1.1	6	18.1 ± 0.7	>0.30
Soluble and insoluble	6	19.0 ± 1.2	6	18.6 ± 0.6	>0.30
Expt II					
Soluble (0.5 *M* HOAc)	7	0.15 ± 0.01	7	0.23 ± 0.02	<0.005
Insoluble	8	16.9 ± 1.0	7	19.1 ± 0.9	<0.05
Soluble and insoluble	7	16.7 ± 1.1[b]	7	19.3 ± 0.9[b]	<0.05
Total (measured directly)	7	17.4 ± 3.4	7	18.6 ± 1.0	>0.10

[a] Micrograms of hydroxyproline per milligram of glomeruli.

[b] The calculated value for soluble plus insoluble hydroxyproline was 97.3% of the total measured hydroxyproline in control glomeruli and 105.2% in lathyritic glomeruli.

of a large amount (20%) of glomerular HYDROX in the sonication step of GBM isolation, in a pilot study. Others have shown similar solubilization of GBM by sonication (11, 12).

Acid extraction of previously salt-extracted glomeruli revealed a significant increase in solubilized HYDROX-containing material. The low recovery of standard in Expt I may have been due in part to precipitation of the acid-soluble collagen standard in water washes required to separate the soluble extract. The acid extraction of lathyritic glomeruli in Expt II confirmed a similarly significant increase in solubilized HYDROX, with adequate recovery studies.

It was not possible in the present experiments to verify that the HYDROX-containing material solubilized from glomeruli originated from the GBM, or from another source within the glomerulus. A "pilot" extraction (as in Expt II) of a large pool of control rat glomeruli, studied on a Beckman 121 amino acid analyzer, showed that 25–50% of all acid and basic amino acids were solubilized with the exception of hydroxylysine (approx 2%) and 4-hydroxyproline (approx 4%). The hydroxylysine/4-hydroxyproline ratios of both insoluble material (0.40) and whole glomeruli (0.44) were similar to that reported for isolated GBM (0.36) (10), but this ratio could not be determined on the soluble material.

Although there was a significant difference in acid solubility of "collagen" in lathyritic when compared to control glomeruli in the present experiments, less than 2% of the total hydroxyproline was solubilized in either case. This stands in sharp contrast to studies of skin collagen, where 48.3 and 69.2% were soluble in control and lathyritic animals, respectively.

The present experiments suggest that collagen cross-linking in the glomerulus is significantly decreased in lathyrism, as measured by an increased proportion of soluble collagen. If cross-linking were to result in large bundles of collagen fibers, a relatively large-pored lattice might result which would offer decreased impediment to the filtration of large molecules. Theoretically, lathyrism, which is associated with decreased cross-linking of collagen, might produce a more compact and more restrictive membrane (9). The relatively crude parameter of total proteinuria, as measured in the present studies, would not be expected to reveal such subtle change in glomerular permeability.

Although severe proteinuric diseases, such as aminonucleoside nephrosis, might by analogy be expected to be associated with increased GBM cross-linking and decreased solubility, Blau and Michael (10) were not able to demonstrate altered GBM solubility in aminonucleoside nephrosis. Studies of selective macromolecular clearances by use of varying molecular weight substances (19) may be necessary to define possible effects of lathyrism on GBH permeability.

Ultrastructural studies of the transport of exogenously administered macromolecular substances have suggested that the GBM impedes filtration of proteins of a wide range of molecular size. Ferritin molecules (mol wt 462,000) were excluded from the GBM, while beef liver catalase (mol wt 240,000) penetrated the GBM slowly and myeloperoxidase (mol wt 160,000–180,000) penetrated the GBM readily. Molecules such as myeloperoxidase and catalase were apparently retarded at the epithelial slit, while horseradish peroxidase (mol wt 40,000) passed rapidly through the GBM and the epithelial slits into the urinary space (19). The precise location and mechanism of protein filtration barriers are thus still unclear.

In view of the apparently great cross-linking and extreme insolubility of GBM collagen, it is unlikely that the small proportion ($< 2\%$) of uncross-linked collagen fibers present in either normal or lathyritic rats serve as a major barrier to protein passage through the GBM. However, it is possible that relative impermeability may result from other molecular cross links (20) than the hydroxylysine-aldehyde linkages affected by lathyrism (21) and measured by solubility characteristics. The presence of a large proportion of glycosylated hydroxylysine residues in GBM (22) whose function are as yet unexplained, may serve to make the physical orientation of GBM collagen molecules into an effective filtration barrier.

The present studies verify that there is no

apparent light microscopic histologic abnormality in the kidney in lathyrism. Colloidal iron staining was normal, as suggested by Jones (23), as were alcian blue stains. Venkatachalam and Jones (24) gave a preliminary report of ultrastructural glomerular abnormalities in lathyrism, primarily the presence of endothelial swelling. The increased affinity and staining of fibrils within the GBM of lathyritic rats by phosphotungstic acid, found in this investigation, suggests that an alteration of the collagen moiety of the GBM occurs in lathyrism. This anionic stain for positively charged protein groups (18) is well known to stain collagen intensely. The normal GBM is not stained by PTA (15) and stained periodic collagen within the GBM has only been described in patients with the nail-patella syndrome (16). The fibrils noted in lathyritic rat GBM did not demonstrate periodicity.

The physicochemical state of the collagen moiety of the GBM was apparently altered by the induction of lathyrism, although the alteration affected only a small proportion of the GBM. The associated ultrastructural changes in a PTA-positive fibrillar component of the GBM lamina densa probably reflect this physicochemical change. Further studies of macromolecular structure and physical state, combined with permeability studies, may provide valuable information concerning the role of the collagen moiety of the GBM in regulating glomerular permeability.

Summary. A significant increase was induced by lathyrism in the small fraction of collagen-like material solubilized from rat glomeruli. An increased affinity and staining by PTA of fibrils within the GBM of lathyritic rats was found by electron microscopy, and also probably reflects an alteration in the collagen-like moiety of the GBM by APN, a material known to affect the cross-linking of collagens. No change in total proteinuria was found in lathyritic rats, nor other major changes in renal structure by conventional microscopy, electron microscopy, or specialized stains of the sialoprotein layer of the glomerulus. Further study of physicochemical alterations induced in the GBM combined with methods to evaluate glomerular permeability, may reveal the location and nature of the filtration barrier.

1. Tanzer, M. L., Int. Rev. Conn. Tiss. Res. **2**, 91 (1965).

2. Page, R. C., and Benditt, E. P., Lab. Invest. **18**, 124 (1968).

3. Page, R. C., and Benditt, E. P., Lab. Invest. **26**, 22 (1972).

4. Piez, K. A., Annu. Rev. Biochem. **37**, 547 (1968).

5. Tanzer, M. L., and Gross, J., J. Exp. Med. **119**, 295 (1964).

6. Spiro, R. G., *in* "Diabetes" (J. Ostman, ed.), p. 586. Excerpta Medica Amsterdam.

7. Kefalides, N. A., and Winzler, R. J., Biochemistry **5**, 702 (1966).

8. Kefalides, N. A., *in* "Advances in Nephrology" (J. Hamburger, J. Crosnier and M. Maxwell, eds.), Vol. 2, p. 3. Year Book Med. Pub., Chicago (1972).

9. Kefalides, N. A., *in* "Diabetes" (J. Ostman ed.), p. 610. Excerpta Medica, Amsterdam.

10. Blau, E. B., and Michael, A. F., J. Lab. Clin. Med. **77**, 97 (1971).

11. Blau, E. B., and Michael, A. F., Proc. Soc. Exp. Biol. Med. **141**, 164 (1972).

12. Naruse, T., and Shibata, S., Immunology **22**, 925 (1972).

13. Michael, A. F., Blau, E. B., and Vernier, R. L., Lab. Invest. **23**, 649 (1970).

14. Michael, A. F., Drummond, K. N., Good, R. A., and Vernier, R. L., J. Clin. Invest. **45**, 237 (1966).

15. Marinozzi, V., J. Roy. Microsc. Soc. **81**, 141 (1963).

16. Bergman, I., and Loxley, R., Analyst **94**, 578 (1969).

17. Selye, H., Rev. Can. Biol. **16**, 1 (1957).

18. Levene, C. I., and Gross, J., J. Expt. Med. **110**, 771 (1959).

19. Karnovsky, M. J., and Aimsworth, S. K., *in* "Advances in Nephrology" (J. Hamburger, J. Crosnier and M. Maxwell, eds.), Vol. 2, p. 35. Year Book Pub., Chicago (1972).

20. Tanzer, M. L., Housley, T., Bernbe, L., Fairweather, R., Franzblau, C., and Gallop, P. M., J. Biol. Chem. **248**, 393 (1973).

21. Page, R. C., and Benditt, E. P., Lab. Invest. **18**, 124 (1968).

22. Spiro, R. G., J. Biol. Chem. **242**, 1915 (1967).

23. Jones, D. B., Lab. Invest. **21**, 119 (1969).

24. Venkatachalam, M. A., and Jones, D. B., Amer. J. Pathol. **52**, 48A (1968).

25. Silverman, L., and Glick, D., J. Cell Biol. **40**, 761 (1969).

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