Glomerular Basement Membrane Metabolism in the Diabetic Rat

In Vivo Studies

MICHAEL BROWNLEE AND ROBERT G. SPIRO

SUMMARY

The effect of diabetes on the metabolism of the renal glomerular basement membrane has been studied in the rat with the aid of injected tracer doses of tritiated proline. At various times after administration of the labeled amino acid, the specific radioactivities of the proline and hydroxyproline of the basement membranes from alloxan diabetic rats were determined and compared with those of age-matched normal rats. In both normal and diabetic animals the incorporation of radioactivity into the basement membrane was slow and, after a maximum was reached, an extended period of almost constant specific activity of proline and hydroxyproline was observed. The diabetic basement membrane, however, differed from the normal by attaining specific activities of the amino acids which were about twice as high as normal ($P < 0.001$ at 42 h after injection of radioisotope). Although the proline concentration of serum and renal cortical fluid was the same in normal and diabetic rats, there were substantial differences in the specific activity of this precursor amino acid in these pools that had to be taken into account to compare the two types of animals.

The results of the present study are consistent with an accelerated rate of glomerular basement membrane polypeptide synthesis and proline hydroxylation in diabetes. DIABETES *28*:121–125, February 1979.

The major clinical manifestations of diabetic nephropathy are proteinuria and renal failure. While the proteinuric component most likely reflects changes in the structure of the glomerular filtration barrier, the progressive fall in filtration rate during chronic kidney failure is probably a consequence of the accumulation of excessive amounts of basement membrane material with resultant glomerular capillary occlusion.[1] The abnormal amounts of basement membrane observed in the glomeruli of diabetics could be a function of increased synthesis, decreased degradation, or a combination of these two processes. An understanding of the mechanism by which this basement membrane accumulation occurs in diabetes is an essential prerequisite for the design of novel therapeutic approaches.

Recent studies[2] have shown that tritiated proline can serve effectively to label the polypeptide components of the glomerular basement membrane in vivo, and turnover measurements in the rat have indicated that this structure has a very low catabolic rate, similar to that of tendon collagen. The present investigation extends this work to a comparison of the in vivo metabolism of glomerular basement membrane in diabetic and age-matched normal rats.

METHODS

Animals and radioisotopes. Male albino rats of the CD strain (Charles River Laboratories) weighing from 80 to 100 g were fasted for 24 h and then injected through the tail vein with alloxan monohydrate (38 mg/kg body weight) dissolved in physiologic saline. In each experiment, the animals to receive alloxan were selected at random from a group of the same age, and the remainder were kept as normal controls. Both the normal and alloxanized rats were maintained on Purina Chow and water ad libitum up to the time of death. The presence of diabetes was assessed by plasma glucose determinations with a Beckman Glucose Analyzer. All diabetic animals had plasma glucose concentrations of greater than 300 mg/dl with a mean ± SEM of 566 ± 11 mg/dl while the mean value for the normal rats was 130 ± 2 mg/dl. The glucose measurements were performed on tail vein blood one day before the injection of the radioisotope.

Nine days after alloxan administration, the diabetic as well as an equal number of control rats were randomly

From the Departments of Biological Chemistry and Medicine, Harvard Medical School, the Elliott P. Joslin Research Laboratory, and the Peter Bent Brigham Hospital, Boston, Massachusetts 02215.
Address reprint requests to Dr. Robert G. Spiro, Elliott P. Joslin Research Laboratory, One Joslin Place, Boston, Massachusetts 02215.
Accepted for publication 12 October 1978.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/28/2/121/350051/28-2-121.pdf by guest on 02 December 2023

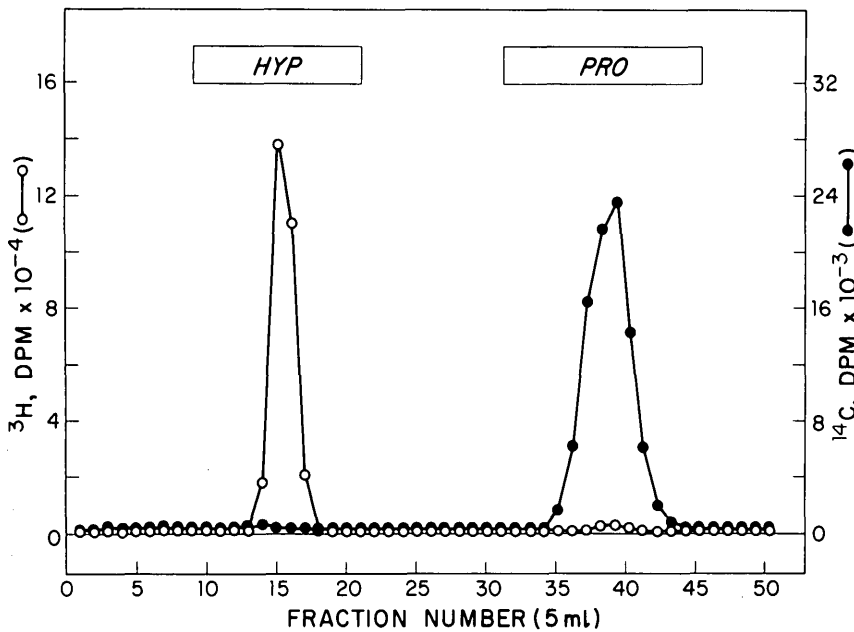


FIGURE 1. Separation by Dowex 50-X12 chromatography of hydroxyproline and proline. Standard L-[^{3}H]4-hydroxyproline (0.13 μCi, New England Nuclear) and L-[^{14}C]proline (0.44 μCi, New England Nuclear) were applied to a column (1.0 × 24 cm) of the resin in 1 N HCl and eluted with this acid at a flow rate of 22 ml/h. Radioactivity was determined by double-channel counting. In each experiment the fractions containing hydroxyproline and proline were pooled according to the pattern established for each lot of resin with the radiolabeled standards.

distributed into groups and injected with a single i.p. dose of L-[2,3-^{3}H]proline (New England Nuclear) in approximately 0.5 ml of physiologic saline. Each animal received 140 μCi/100 g body weight of the radioisotope (sp act, 25 mCi/μmol) except where otherwise indicated. At designated times after the isotope injection the animals were lightly anesthetized with ether and killed either by decapitation or by exsanguination through cardiac puncture.

Preparation of glomeruli and basement membranes. The kidneys were rapidly removed from each rat and placed on ice. The decapsulated cortices were excised, pooled for each group of five rats, wrapped tightly, and stored at −20° for subsequent preparation of glomeruli.

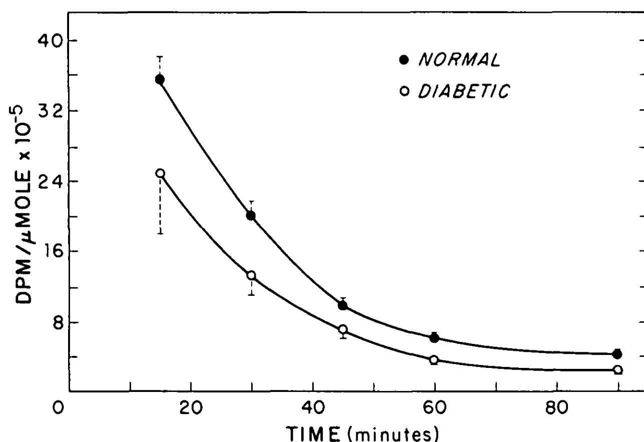
Glomeruli were prepared from the pools of cortices by a procedure previously described,[2] except that a 100- rather than an 80-mesh sieve was used to separate tissue fragments from the glomeruli. Differential counts of the glomerular preparations under the phase microscope indicated that 90–97% of the elements were glomeruli; the remainder consisted primarily of small tissue fragments.

Basement membranes were prepared from the glomerular pellets of each group by a sonic disruption procedure.[2] To dilute any radioactivity that might be present as free proline, 20 μmol of the unlabeled amino acid was added to the basement membrane with the first saline wash.

Preparation of sera for determination of proline specific activity. For the measurement of the proline radioactivity of serum, both the normal and diabetic rats, after injection of the [^{3}H]proline, were bled from their tail veins (100 μl) at various time intervals up to 90 min. These animals were killed at 120 min by exsanguination through intracardiac puncture, and the blood obtained at that time was used to determine the proline content of the sera. Sera were separated from clotted blood by centrifugation, and, in each case, equal volumes from two animals treated in the identical manner were combined. Deproteinization of the sera was achieved by the addition of 3 vol of ice-cold trichloroacetic acid with a final acid concentration of 8%. The protein precipitate was removed by centrifugation, and the supernatant was then extracted with ether to remove the trichloroacetic acid. Proline radioactivity was determined on the protein-free sera after chromatography on Dowex 50 columns while the proline content was measured on the Technicon amino acid analyzer I (130-cm column).

Determination of specific activity of proline in renal cortex fluid. At various times after the injection of [^{3}H]proline (30 μCi/100 g body weight) into normal and diabetic rats, the combined cortices from two animals were finely minced and homogenized in 4 vol of ice-cold 0.15 M sodium chloride with a glass Potter-Elvehjem homogenizer. The homogenates were deproteinized with 3 vol of trichloroacetic acid, extracted with ether, and desalted by application to columns containing 6 ml of Dowex 50-X4, 200–400 mesh (H$^+$ form). The resin was washed with seven

FIGURE 2. Specific activity of free proline in sera of normal (●) and diabetic (○) rats at various times after the injection of L-[2,3-^{3}H]proline (140 μCi/100 g body weight). Each point represents the mean ± SEM of five experiments each of which involved the pooled sera of two animals. Zero times values as determined from the intercept of a semilogarithmic plot of the specific activities were 45.4 × 10^5 and 34.2 × 10^5 dpm/μmol for normal and diabetic sera, respectively. Average ± SEM serum levels of proline were 284 ± 8 μM and 286 ± 13 μM for normal and diabetic animals, respectively. The ratio of the area under the normal specific activity curve to that under the diabetic specific activity curve was 1.48.



Downloaded from http://diabetesjournals.org/diabetes/article-pdf/28/2/121/350051/28-2-121.pdf by guest on 02 December 2023

column volumes of water and then eluted with five column volumes of 1.5 N NH_4OH. The ammonia was removed by lyophilization, and the amino acids in the samples were separated on the Technicon amino acid analyzer I (130-cm column) equipped with a split-stream arrangement. This permitted one-third of the column effluent to pass through the analytical system and two-thirds to be collected in 3-ml fractions for subsequent scintillation counting.

Amino acid analyses. Basement membrane samples were hydrolyzed in constantly boiling HCl in sealed tubes under nitrogen at 105° for 28 h. A portion of each hydrolysate was analyzed on the Technicon NC-2 amino acid analyzer, using the standard gradient, to determine the 4-hydroxyproline and proline contents. Another aliquot of the hydrolyzed basement membrane was chromatographed on Dowex 50 to separate these two amino acids for the purpose of radioactivity measurements.

Separation of radiolabeled hydroxyproline and proline by Dowex 50 chromatography. Aliquots of deproteinized serum or basement membrane hydrolysates were placed on columns (1.0 × 24 cm) of Dow 50-X12, 200–400 mesh equilibrated with 1 N HCl. Elution was carried out with the 1 N HCl at a flow rate of 22 ml/h, and on the basis of L-[^{3}H(G)]4-hydroxyproline (New England Nuclear) and L-[^{14}C(U)]proline (New England Nuclear) standards their position of emergence from such columns was determined (Figure 1). The two amino acids were sufficiently well separated by this chromatography to permit their batchwise elution based on the elution pattern established for each lot of resin with the use of the radiolabeled standards. The hydrochloric acid was removed from the fractions representing the hydroxyproline and proline, respectively, in a vacuum rotator, and the radioactivity of each was determined by scintillation counting. The recovery of standards from the columns was, on average, 98% and it was found that essentially all of the radioactivity present in the serum and basement membrane samples was accounted for in the hydroxyproline and proline peaks.

Radioactivity measurements. Radioactivity was determined by liquid scintillation counting in a Nuclear Chicago Isocap 300 counter using Bray's solution.[3] All counts were converted to disintegration per minute using efficiency corrections obtained from counting quenched standards.

RESULTS

Radioactivity of proline in serum. After injection of the isotope the specific activity of the serum proline was found to be substantially lower in the diabetic rats than in the normal age-matched controls (Figure 2), although the serum proline concentration did not significantly differ between the two groups of animals.

The ratio of the area under the normal serum proline specific activity curve to that under the diabetic specific activity curve was found to be 1.48. To correct for this difference between the normal and diabetic serum specific activities the diabetic basement membrane radioactivity values were multiplied by this factor.

Radioactivity of proline in renal cortex fluid. A difference in proline specific activity was also observed between normal and diabetic renal cortical fluid. At various time intervals after the injection of [^{3}H]proline, the specific activity of this amino acid in the diabetic animals was found to be substantially less than in the normal controls and again in this tissue no difference in the proline concentration between the two types of animals was evident (Table 1).

TABLE 1
Specific activity of free proline in renal cortex fluid at various times

Time* (min)	Specific activity of proline† Normal (dpm/μmol × 10⁻⁴)	Diabetic
30	4.99	3.43
60	3.80	1.99
90	2.14	1.27
120	1.39	0.54

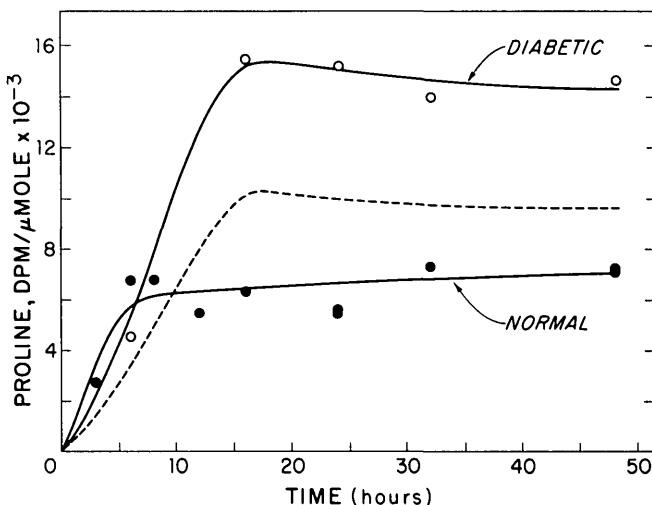
* Time after injection of L-[2,3-^{3}H]proline (30 μCi/100 g body weight). Each value represents the pooled cortices of two rats.

† Free proline content of kidney cortex expressed as the mean ± SEM was 3.61 ± 0.55 μmol/g and 3.89 ± 0.31 μmol/g for normal and diabetic animals, respectively, $P > 0.1$ ($n = 4$). The mean weights ± SEM of the normal and diabetic kidneys were 1.77 ± 0.06 and 1.71 ± 0.07 g, respectively, and the cortex made up 86% of that weight in the normal and 84% in the diabetic animals.

Radioactivity of proline and hydroxyproline of glomerular basement membrane. The incorporation of injected [^{3}H]proline into the proline (Figure 3) and hydroxyproline (Figure 4) of normal and diabetic basement membranes was found to be a slow process. In both conditions, after maximum radioisotopic incorporation had been reached, an extended period of almost constant specific activity was observed. The diabetic basement membranes, however, differed from the normal by attaining much higher proline as well as hydroxyproline specific activities.

When the specific activities of normal and diabetic glomerular basement membrane proline and hydroxyproline were compared in several groups of rats at a time (42 h) on the plateau region of the radioisotope incorporation curves,

FIGURE 3. Specific activity of proline of glomerular basement membrane at various times after the injection of L-[2,3-^{3}H]proline (140 μCi/100 g body weight) into normal (●) and diabetic (○) rats. Each point represents the value of basement membranes isolated from the pooled kidney cortices of five animals. The diabetic values were corrected to normal serum proline specific activity as described in text. The uncorrected diabetic values are indicated by the *dashed* line.



Downloaded from http://diabetesjournals.org/diabetes/article-pdf/28/2/121/350051/28-2-121.pdf by guest on 02 December 2023

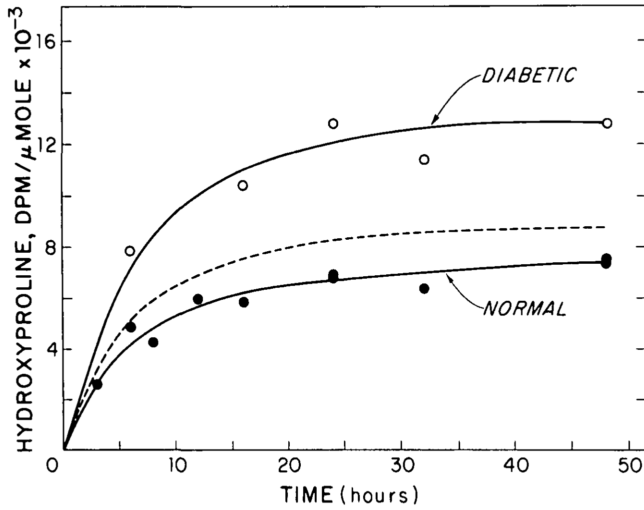
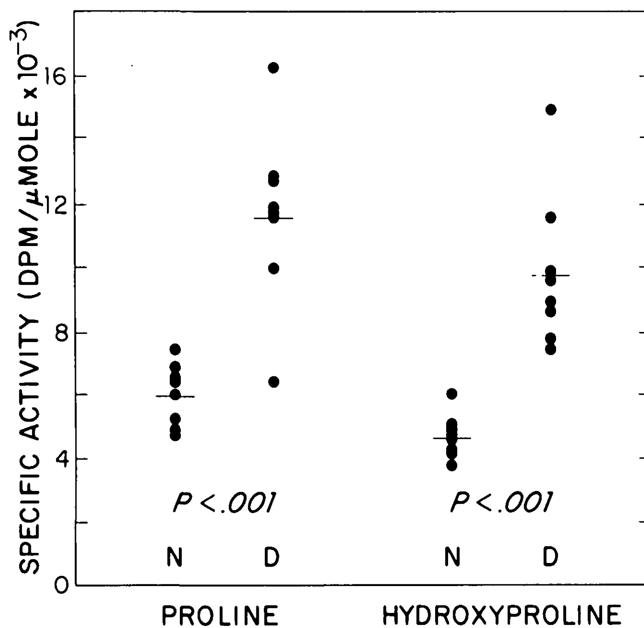


FIGURE 4. Specific activity of hydroxyproline of glomerular basement membrane at various times after the injection of L-[2,3-^{3}H]proline (140 μCi/100 g body weight) into normal (●) and diabetic (○) rats. Each point represents the value of basement membranes isolated from the pooled kidney cortices of five animals. The diabetic values were corrected to normal serum proline specific activity as described in text. The uncorrected diabetic values are indicated by the *dashed* line.

a highly significant difference between the two groups of animals was observed (Figure 5). The average specific activities ± SEM of the proline and hydroxyproline residues in the diabetic membranes were $11.7 \pm 1.1 \times 10^3$ dpm/μmol and $9.9 \pm 0.8 \times 10^3$ dpm/μmol, respectively, which was about twice as high as in those from the age-matched normal controls that had values of $6.1 \pm 0.3 \times 10^3$ and $4.7 \pm 0.2 \times 10^3$ dpm/μmol for these two amino acids.

FIGURE 5. Specific activity of proline and hydroxyproline of glomerular basement membrane 42 h after the injection of L-[2,3-^{3}H]proline (140 μCi/100 g body weight) into normal (N) and diabetic (D) rats. The specific activites of the diabetic animals were corrected to normal serum proline specific activity as described in text. Each point represents the value of basement membranes isolated from the pooled kidney cortices of five rats. The mean is indicated for each group and the P values between normal and diabetic animals are shown.



DISCUSSION

The results of the present study provide the first evidence by an in vivo approach that accelerated glomerular basement membrane synthesis occurs in the diabetic state. The increased levels of radiolabeled proline and hydroxyproline in the diabetic basement membrane subsequent to an injection of tritiated proline suggest an accelerated rate of polypeptide synthesis as well as of proline hydroxylation.

These findings are consistent with a number of previous investigations which have indicated in a less direct manner that increased glomerular basement membrane–synthesizing activity may occur in diabetic animals. It has been reported that kidney ribosomes from rats with experimental diabetes incorporate amino acids into protein at a greater rate than those from normal animals[4] and that the kidneys of rats with streptozotocin diabetes have an increased protein content and protein-to-DNA ratio.[5] Furthermore, evidence has been presented that enzymes involved in the post-translational modification of the glomerular basement membrane polypeptide chains; namely, glucosyltransferase[6] and lysyl hydroxylase[7] have increased activity in rats with alloxan and streptozotocin diabetes, respectively.

Studies with isolated rat glomeruli have, however, produced conflicting results; while some investigators have reported enhanced basement membrane–synthesizing activity in diabetic glomeruli,[8,9] another report has failed to find any difference in membrane production between normal and diabetic animals.[10] However, as discussed previously,[2] the isolated glomerular system has certain deficiencies which detract from its usefulness in such investigations. Glomeruli in vitro, as judged by the degree of hydroxylation of proline and lysine, manifest an exceedingly low rate of basement membrane synthesis, and, furthermore, they are less likely to reflect altered systemic influences such as may prevail in diabetes in vivo.

Although the appropriateness of the rat as a model for the study of glomerular basement metabolism in diabetes has been questioned,[11] a recent study has demonstrated that chemically induced diabetes in this species results in significant thickening of this membrane.[12] Furthermore, a number of earlier studies on rats,[13,14] as well as dogs[15] and monkeys,[16] indicated that diabetic glomerulopathy can indeed result from long-term alloxan diabetes. Since the accumulation of excess basement membrane material in both human and experimental diabetes is a slow process,[17] sensitive biochemical measurements such as those employed in the present study would be able to detect evidence of this process at a much earlier time than morphologic examination.

It became evident during the course of this investigation that a valid comparison of basement membrane synthesis in vivo between normal and diabetic animals could not be made without taking into account differences in the specific activity of the injected radiolabeled amino acid in the precursor pools. In serum and kidney cortex fluid of diabetic animals, the specific activity of proline was found to be substantially lower than in normal controls, although the concentration of this amino acid did not differ between the two states. Since the equations for the specific activity of a product at any time involve the integral of the specific activity of the precursor up to that time, the neces-

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/28/2/121/350051/28-2-121.pdf by guest on 02 December 2023

sary correction factor for diabetic glomerular basement membrane proline and hydroxyproline activity was obtained from the geometrical integration of the specific activity of the serum proline of normal and diabetic animals, which is represented by the area under the serum proline specific activity curve. The principles and mathematical derivations underlying this treatment of the data have been extensively reviewed elsewhere.[18]

The lower specific activity in diabetes may be the result of a simple dilution of the injected tracer dose by a larger proline pool or to an increased peripheral output of the unlabeled amino acid to meet the demands of augmented gluconeogenesis. In support of the latter possibility is the report that in human diabetes there is an increased splanchnic uptake of proline, although, as in the rats, the serum proline levels of the diabetic subjects are not significantly different from normal.[19] The maintenance of normal serum proline concentration in spite of increased splanchnic utilization does suggest an increased contribution of proline from a peripheral source.

In the present study, the time course of radioactivity incorporation into the proline and hydroxyproline of normal glomerular basement membrane was found to be comparable to that reported by Price and Spiro.[2] The incorporation curves in the diabetic animals were similar to those of the normal controls, although much higher specific activities were reached in the diabetic membranes. Studies in normal rats have suggested that glomerular basement membrane polypeptide components of dissimilar composition may turn over at different rates.[2] Since the present study was limited to the proline and hydroxyproline residues of the basement membrane it cannot yet be ascertained whether the diabetic state affects the metabolism of all polypeptide subunits in the same manner.

It is likely that the increased rate of synthesis observed in the diabetic kidneys may be responsible for the excessive accumulation of glomerular basement membrane material ultimately noted in this disease state. While decreased degradation of basement membrane might also occur in diabetes evidence for this is currently lacking. Over the period observed in this investigation no perceptible loss in radioactivity occurred from either the normal or diabetic basement membrane. Indeed, the turnover time of normal rat glomerular basement membrane as determined from the proline and hydroxyproline specific activities has been found to be so slow (greater than 100 days)[2] that it would be difficult to detect any extension of this time.

ACKNOWLEDGMENTS

We would like to thank Mr. W. Clifford Fisher, Jr. for skilled technical assistance.

This work was supported by Grant AM 17477 from the National Institutes of Health and by the Adler Foundation, Rye, New York.

Dr. Brownlee was the recipient of a U.S. Public Health Service Postdoctoral Research Fellowship.

REFERENCES

[1] Cameron, J. S., Ireland, J. T., and Watkins, P. J.: The kidney and renal tract. *In* Complications of Diabetes. Keen, H., and Jarrett, J., Eds. London, Edward Arnold, 1975, pp. 99–150.

[2] Price, R. G., and Spiro, R. G.: Studies on the metabolism of the renal glomerular basement membrane. J. Biol. Chem. *252*:8597–602, 1977.

[3] Bray, G. A.: A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. *1*: 279–85, 1960.

[4] Peterson, D. T., Greene, W. C., and Reaven, G. M.: Effect of experimental diabetes mellitus in kidney ribosomal protein synthesis. Diabetes *20*:649–54, 1971.

[5] Seyer-Hansen, K.: Renal hypertrophy in experimental diabetes: relation to severity of diabetes. Diabetologia *13*:141–43, 1977.

[6] Spiro, R. G., and Spiro, M. J.: Effect of diabetes on the biosynthesis of the renal glomerular basement membrane. Studies on the glucosyltransferase. Diabetes *20*:641–48, 1971.

[7] Khalifa, A., and Cohen, M. P.: Glomerular protocollagen lysylhydroxylase activity in streptozotocin diabetes. Biochim. Biophys. Acta *386*:332–39, 1975.

[8] Cohen, M. P., and Vogt, C.: Evidence for enhanced basement membrane synthesis and lysine hydroxylation in renal glomerulus in experimental diabetes. Biochem. Biophys. Res. Commun. *49*:1542–46, 1972.

[9] Grant, M. E., Harwood, R., and Williams, I. F.: Increased synthesis of glomerular basement collagen in streptozotocin diabetes. J. Physiol. *257*:56–57, 1976 (Abstr.).

[10] Beisswenger, P. J.: Glomerular basement membrane. Biosynthesis and chemical composition in the streptozotocin diabetic rat. J. Clin. Invest. *58*:844–52, 1976.

[11] Klein, L., Yoshida, M., and Miller, M.: Does experimental diabetes in the rat produce basement membrane thickening in renal glomeruli? Diabetes *26*:361, 1977 (Abstr.).

[12] Fox, C. J., Darby, S. C., Ireland, J. T., and Sönksen, P. H.: Blood glucose control and glomerular capillary basement membrane thickening in experimental diabetes. Br. Med. J. *2*:605–7, 1977.

[13] Steen Olsen, T., Ørskov, H., and Lundbaek, K.: Kidney lesions in rats with severe long-term alloxan diabetes. Acta Pathol. Microbiol. Scand. *66*:1–12, 1966.

[14] Hägg, E.: Glomerular basement membrane thickening in rats with long-term alloxan diabetes. Acta Pathol. Microbiol. Scand. Sect. A. *82*:211–19, 1974.

[15] Bloodworth, J. M. B., Jr., Engermann, R. L., and Powers, K. L.: Experimental diabetic microangiopathy. I. Basement membrane statistics in the dog. Diabetes *18*:455–58, 1969.

[16] Gibbs, G. E., Wilson, R. B., and Gifford, H.: Glomerulosclerosis in the long-term alloxan diabetic monkey. Diabetes *15*:258–61, 1966.

[17] Spiro, R. G.: Search for a biochemical basis of diabetic microangiopathy. Diabetologia *12*:1–14, 1976.

[18] Russell, J. A.: The use of isotopic tracers in estimating rates of metabolic reactions. Perspectives in Biology and Medicine, Winter, 1958, pp. 138–73.

[19] Wahren, J., Felig, P., Cerasi, E., and Luft, R.: Splanchnic and peripheral glucose and amino acid metabolism in diabetes mellitus. J. Clin. Invest. *51*:1870–78, 1972.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/28/2/121/350051/28-2-121.pdf by guest on 02 December 2023