PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 942–947 (1974)

Protein and Glycoprotein Synthesis by Isolated Kidney Glomeruli[1] (38224)

ISTVAN KRISKO AND W. GORDON WALKER
(Introduced by H. Busch)

Department of Medicine, The Johns Hopkins Hospital, Baltimore, Maryland 21205; Department of Pathology, Veterans Administration Hospital, Houston, Texas 77031, and Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77025

Although the metabolic activity of the renal tubules has been well characterized, relatively little attention has been focused on the metabolic activity of the glomeruli until recently (1, 2). Because the glomeruli and the glomerular capillary basement membrane (GBM) are prone to be damaged in many diseases which have already been well characterized morphologically and immunologically, such as glomerulonephritis, diabetes mellitus, systemic lupus erythematosus and other collagen vascular diseases, the study of their biochemical and metabolic activity should be of great interest. Metabolically active glomeruli have been isolated previously from rat kidneys by Walker (1, 2). The isolated glomeruli were judged viable by such parameters as oxygen consumption, carbon dioxide production, and their ability to utilize various substrates (glucose, pyruvate, succinate, α-ketogluterate). Glomeruli were more active in RNA and protein synthesis than renal tubular cells.

The purpose of this communication is to present evidence that isolated glomeruli synthesize protein and glycoprotein *in vitro* in short-term experiments. Such isolated, viable renal glomeruli may provide an *in vitro* system for assessing the specific biochemical effects of a variety of conditions and agents generally known to derange the physiological functions of glomeruli and their basement membrane. Studies on basement membrane synthesis in other organs [yolk sac of mouse embryo (3); embryonic chick lens (4)] have been reported recently. A preliminary report of this work has already appeared (5), and evidence that a portion of the newly synthesized glycoprotein is actually glomerular basement membrane will be reported elsewhere. Most recently, Brown and Michael (6) have also reported GBM synthesis by isolated renal glomeruli. Other investigators, e.g., Fong and Drummond (7) and MacKenzie and Scriver (8), have also employed isolated glomeruli in their work, but not for the study of protein biosynthesis. Whereas in previous work on glomerular protein synthesis *in vivo* labeling was used and glomeruli and GBM were isolated thereafter (9, 10), the present approach is unique in that isolated viable glomeruli are employed. This approach offers the advantages of *in vitro* method to study the overall biosynthetic machinery of the glomeruli in general, and GBM synthesis in particular.

Materials and Methods. 1. *Isolation of glomeruli.* Renal glomeruli were isolated according to the method of Walker (1, 2), which is a modification of that used by Krakower and Greenspon (11). The kidney cortex was minced with razor blades, followed by homogenization in tissue culture medium (Medium 199) with a loose-fitting

[1] This work was supported in part by Grant-in-Aid from the American Heart Association, the O'Neill Research Fund, and by Veterans Administration Research.

glass homogenizer (Kontes Glass Co.). All procedures were done at 0–4°. The glomeruli were separated from unwanted tissue fragments (e.g., tubules) and cells by differential centrifugation (200*g* × 3 min) and trapping on stainless steel screens. The glomeruli were retained by either the 150 (older rats and piglets) or the 200 mesh screen (80–100 g rats). The glomeruli so prepared were intact as observed by phase-contrast microscopy and approximately 20% retained their Bowman's capsule (Fig. 1). The yield was 3–5 mg glomerular protein (12) per 10 g of kidney tissue (wet wt).

2. *Protein biosynthesis in vitro.* Assays for protein synthesis were performed in modified Medium 199 in which the isolated glomeruli were suspended. In a typical ex-

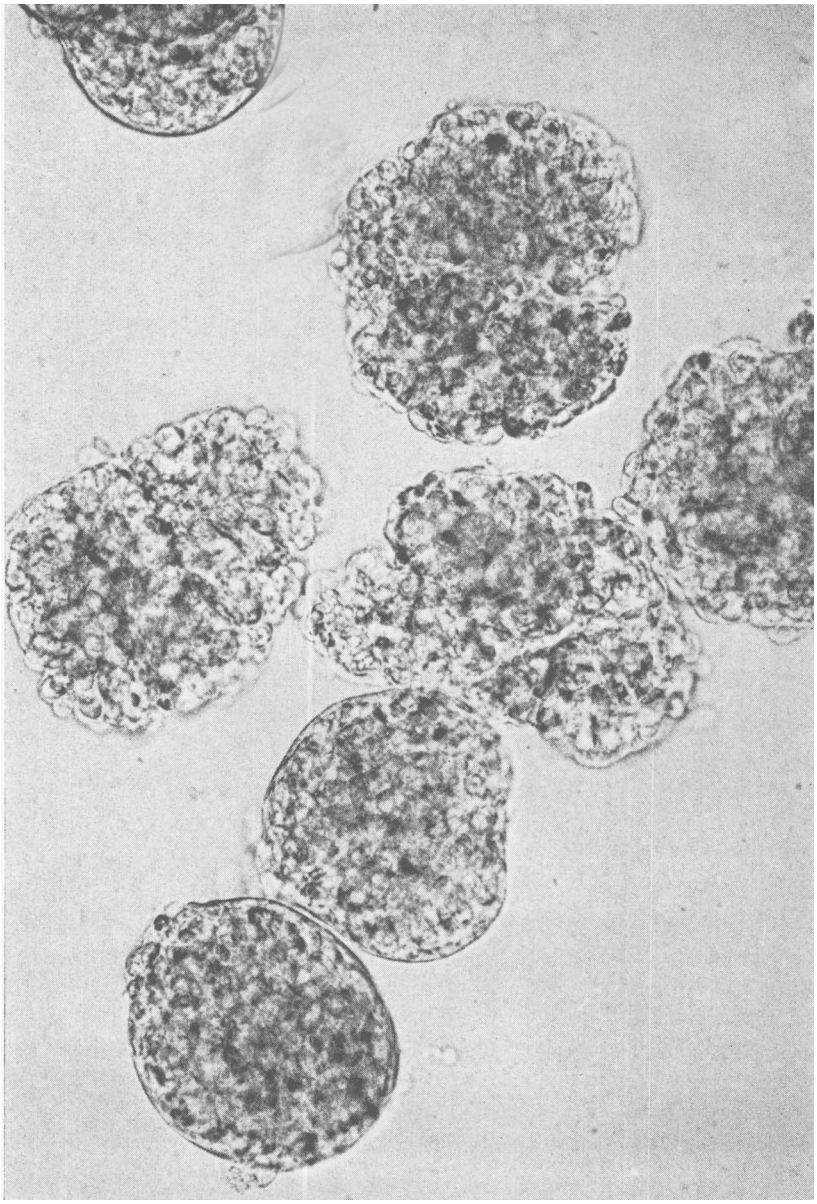

FIG. 1. Isolated rat kidney glomeruli. Original magnification ×100.

periment, each flask contained in 2.0 ml total volume: approximately 0.5 mg of glomerular protein; 100 units/ml of penicillin and 100 μg/ml of streptomycin; 30 m*M* HEPES buffer, pH 7.2; 125 mg/liter of $NaHCO_3$ and 1–5 μCi each of one or several of the following ^{3}H-labeled amino acids: proline, lysine, glutamate, aspartate, and leucine (the corresponding unlabeled amino acids were omitted). Samples were incubated in 25 ml Erlenmeyer flasks in an atmosphere of 5% CO_2–95% O_2 at 37° with continuous gentle lateral shaking. The reaction was stopped by adding 2.0 ml of 10% trichloroacetic acid (TCA) to each flask and the precipitated material washed 4 times with 8.0–10.0 ml of cold (0–4°) 5% TCA. The final pellet was hydrolyzed in 1 *N* NaOH and aliquots taken for determination of protein (12) and radioactivity (in a liquid scintillation spectrometer).

3. *Glycoprotein biosynthesis and thin layer chromatography of constituent sugars.* Assays for incorporation of labeled carbohydrates into macromolecules by glomeruli were performed using Medium 199 modified as for protein synthesis, except this time glucose was omitted from the medium. Labeled monosaccharides were added as noted under Results. Reactions were stopped and washed with TCA as described above. An aliquot of the washed pellet was hydrolyzed in 1 *N* NaOH, and protein and radioactivity was determined.

In order to ascertain that the label incorporated into macromolecules was still in carbohydrate, the following analysis was performed. An aliquot of the washed glomerular pellet (see under Results) was subjected to hydrolysis in sealed tubes in 1 *N* H_2SO_4 at 100° *in vacuo* for 4 hr. The liberated carbohydrate monomer was isolated by passage through coupled Dowex 50-X4 and Dowex 1-X8 columns (13), followed by thin layer chromatography (TLC) on precoated cellulose sheets as described by Klenk *et al.* (14). The TLC plates were developed in a solvent system consisting of ethylacetate–pyridine–acetic acid–water (5:5:1:3, v/v). A mixture of standard sugars was run simultaneously. The chromatograms of the marker sugars were stained using alkaline silver nitrate while the chromatograms of radioactive samples were cut into 4 mm strips, which were then counted by liquid scintillation spectrometry in Brey's solution.

4. *Materials.* The following radioactive compounds were purchased from New England Nuclear Corporation: ^{3}H-Pro, 4.75 Ci/mmol; ^{3}H-Lys, 3.2 Ci/mmol; ^{3}H-Asp, 26.0 Ci/mmol; ^{3}H-Leu, 5.0 Ci/mmol; ^{3}H-galactose, 2.04 Ci/mmol; ^{3}H-glucosamine, 7.5 Ci/mmol; and ^{14}C-glucosamine, 240 mCi/mmol. Medium 199 was from Microbiological Associates, HEPES from Calbiochem. Precoated cellulose sheets (Polygram Cel 300) for thin layer chromatography were purchased from The Brinkman Instruments Co.

Results and Discussion. The glomeruli prepared as described were estimated to be 95% pure on the basis of examination by phase-contrast microscopy. In rat preparations, 20% of the glomeruli retained the Bowman's capsule, while bovine preparations (not reported here) were free of it. The latter is in agreement with Spiro, who similarly found no Bowman's capsule in bovine preparations (13). This *in vitro* system represents essentially a "suborgan" culture (the glomerulus has at least three distinct cell types, but not all cell types of the kidney are represented). Protein synthesis was linear for at least 6 hr at 37° in the experiment shown in Fig. 2.

The effect of specific inhibitors of protein synthesis is shown in Fig. 2 and Table I. Puromycin, which causes premature peptide chain termination both in microorganisms and in eukaryotes (15, 16), strongly inhibited incorporation. For example, the addition of 5×10^{-5} *M* puromycin caused greater than 90% inhibition. Cycloheximide was also strongly inhibitory, while chloramphenicol was not. The latter drug is a specific inhibitor of prokaryotic and mitochondrial protein synthesis, while cycloheximide inhibits peptide synthesis only in eukaryotes (15, 17).

Radiolabeled sugars were also incorporated into TCA insoluble products, suggesting the synthesis of glycoproteins by

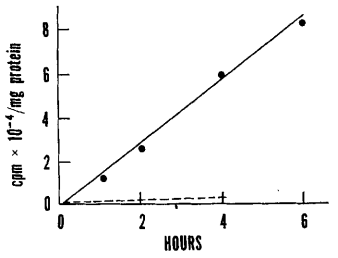

FIG. 2. Kinetics of protein synthesis by isolated glomeruli. The conditions of incubation are described in Materials and Methods; 1 μCurie each of the following ^{3}H labeled amino acids was added at zero time: Pro, Lys, Asp, Glu, Leu. The ordinate represents specific activity incorporated into TCA precipitable material. Where indicated, $1 \times 10^{-4} M$ puromycin was added at zero time. Each time point is the result of duplicate incubations with close agreement. ●—● No inhibitor added; - - - Puromycin added.

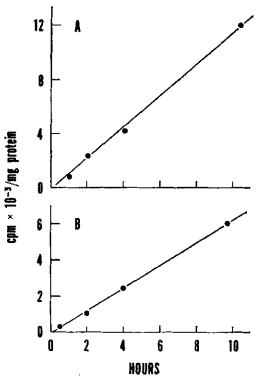

FIG. 3. Kinetics of galactose and glucosamine incorporation. The conditions of incubation were as in Fig. 2 except: no amino acids were omitted; unlabeled glucose was omitted and either 2 μCi of ^{3}H-galactose (A) or 2 μCuries of ^{14}C-glucosamine (B) was added.

these isolated glomeruli. It is known from the work of Spiro (13) and from that of Kefalides and his associates (18) that GBM contains significant amounts of galactose, glucose and glucosamine. Accordingly, it was logical to choose these sugars as precursors for incorporation in the isolated glomerular system. Glucose is not a very reliable precursor to study the synthesis of the carbohydrate moiety of glycoproteins since a substantial fraction of the radioactive label is probably transferred into amino acids (19). Galactose and glucosamine are far more specific precursors. Figure 3 shows that the incorporation of both galactose and glucosamine were linear for at least 9 hr. Puromycin inhibited the incorporation of radioactivity from carbohydrate precursors into TCA-precipitable macromolecules (Table II), probably by inhibiting the synthesis of polypeptide intermediates which serve as sites of carbohydrate attachment. This experiment is therefore an indication that isolated glomeruli synthesize glycoproteins. The degree of inhibition, which varied according to precursor, was most likely related to the variable pool size of those peptide intermediates serving as substrates for carbohydrate attachment. Depending on the pool size of these intermediates, a variable degree of inhibition might be observed (20).

TABLE I. Effect of Inhibitors on Glomerular Protein Synthesis.[a]

Inhibitor	Concentration	% Inhibition
Cycloheximide	$1 \times 10^{-6} M$	80
	$1 \times 10^{-5} M$	91
	$1 \times 10^{-4} M$	94
Chloramphenicol	$5 \times 10^{-5} M$	0
	$1 \times 10^{-4} M$	4
	$5 \times 10^{-4} M$	8
Puromycin	$5 \times 10^{-5} M$	93

[a] The conditions of incubation are described in the legend to Fig. 2. The inhibitors were added at zero time. Incubation was for 4 hr.

In addition to isolating glomeruli from rats, piglet glomeruli were also prepared. The quality of the glomerular preparation was similar to that from rat except that the glomeruli were retained on the 150 mesh screen rather than on the 200. Pig glomeruli also incorporated both labeled amino acids and carbohydrates (Table III), suggesting *in vitro* glycoprotein synthesis.

In order to show more precisely the incorporation of radioactivity from a carbohydrate precursor into the carbohydrate moiety of glycoprotein, we have isolated the newly synthesized glycoprotein. At the end of incubation, the product was hydrolyzed (see Methods for details); since the purpose of

TABLE II. Incorporation of Carbohydrate Precursors into Glomerular Protein.[a]

Precursor	cpm per mg protein: No Inhibitor	cpm per mg protein: Puromycin, $1 \times 10^{-4} M$	% Inhibition
Galactose	3,160	440	86
Glucosamine	2,426	446	81
Glucose	8,140	2,508	69

[a] The conditions of incubation were those of Fig. 2 except: no amino acids were omitted; unlabeled glucose was omitted and 2 μCi of either ^{3}H-galactose, ^{3}H-glucosamine, or ^{14}C-glucose was added. Puromycin was added at zero time. Incubation was for 4 hr.

this experiment was to show incorporation into the carbohydrate moiety of the stipulated glycoprotein, amino acids and peptides had been removed quantitatively prior to applying the hydrolysate onto thin layer plates. When glucose was labeled, a significant portion (approximately 30%) appeared as galactose (Fig. 4). Galactose added to the medium was found almost entirely as galactose with only a small amount of glucose.

We conclude that glycoprotein is synthesized by isolated glomeruli because: (1) labeled carbohydrates were recovered from macromolecules after incubation; and (2) inhibitors of protein synthesis inhibited not only amino acid incorporation but also the incorporation of radiolabeled carbohydrates into macromolecules.

TABLE III. Incorporation of Labeled Amino Acids and Carbohydrate Precursors by Piglet Glomeruli.[a]

Labeled precursor	Incubation, hr	cpm/mg protein
Amino acids	2	2.2 } × 10^{4}
	4	4.2 } × 10^{4}
	13.5	16.5 } × 10^{4}
Galactose	2	2.0 } × 10^{3}
	4	3.6 } × 10^{3}
	13.5	9.1 } × 10^{3}
Glucosamine	2	0.7 } × 10^{3}
	13.4	4.3 } × 10^{3}

[a] The conditions of incubation for incorporation of amino acids were as in Fig. 2; for incorporation of galactose and glucosamine, as in Fig. 3.

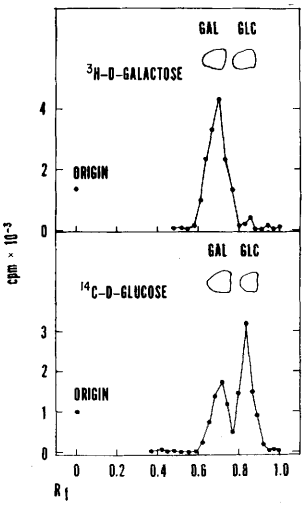

FIG. 4. Identification of monosaccharides in glomeruli. Thin layer chromatograms of acid hydrolysates of glomeruli incubated in the presence of either ^{3}H-galactose or ^{14}C-glucose. The usual incubation was scaled up ten-fold. After 3 hr of incubation, the reaction was stopped by reducing the temperature to 0°. The glomeruli were washed 5 times in large volumes of ice-cold physiological saline (dialysis of glomeruli gave similar results) and the pellet hydrolyzed as described in Methods. Amino acids and peptides had been removed from the acid hydrolysates quantitatively prior to applying the hydrolysate onto the plates. The origin is on the left, the front is on the right. A mixture of two standard sugars was co-chromatographed with the hydrolysates and their position is shown above the radioactivity profiles.

Burlington *et al.* (21) cultured goat glomeruli which remained viable for several months and apparently synthesized erythropoietin. Glomerular cultures of longer

than a few hours' duration could be useful in exploring additional aspects of the biochemistry and metabolism of the glomeruli. The purpose of the present work has been to establish that isolated glomeruli are suitable for short-term culture and studies on protein and glycoprotein synthesis. We postulate that at least a portion of the newly synthesized glycoprotein is GBM or its precursor(s).

Summary. Metabolically active and viable renal glomeruli were isolated from rats and piglets. *In vitro*, the glomeruli were active in protein and glycoprotein synthesis for at least 9 hr, as evidenced by linear incorporation of amino acids and carbohydrate precursors into macromolecules. Puromycin and cycloheximide inhibited the incorporation of labeled glucose, glucosamine, galactose and amino acids, while chloramphenicol did not. After incorporation of labeled glucose and galactose, the products of the *in vitro* incubation were isolated and labeled glucose and galactose were recovered from glomerular hydrolysates. This *in vitro* system is suitable to study the biochemistry of normal isolated rat glomuruli and can be adapted to other mammalian species. After basic information is gained on the biosynthesis of these isolated glomeruli, similar experiments may provide a better understanding of certain specific glomerular diseases in both experimental animal models and naturally occurring diseases of man.

We thank Drs. Harris Busch and Ferenc Gyorkey for review of the manuscript and Mrs. Judy Herman and Miss Katherine Lewis for excellent technical help.

1. Walker, W. G., *in* "Acute Glomerulonephritis" (J. Metcoff, ed.), p. 261. Little, Brown and Co., Boston (1967).

2. Walker, W. G., and Hulter, H. N., Trans. Amer. Clin. Climat. Ass. **81**, 174 (1970).

3. Orfanakis, N. G., and Nakane, P. K., Fed. Proc. **31**, 648 (1972).

4. Grant, M. E., Kefalides, N. A., and Prockop, D. J., J. Biol. Chem. **247**, 3539 (1972).

5. Krisko, I., and Walker, W. G., Clin. Res. **20**, 599 (1972).

6. Brown, D. M., and Michael, A. F., Fed. Proc. **32**, 650 (1973).

7. Fong, J. S. C., and Drummond, K. N., J. Lab. Clin. Med. **71**, 1034 (1968).

8. MacKenzie, S., and Scriver, C. R., Biochim. Biophys. Acta **196**, 110 (1970).

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

10. Chow, A. Y. K., and Drummond, K. N., Lab. Invest. **20**, 213 (1969).

11. Krakower, C. A., and Greenspon, S. A., Arch. Pathol. **51**, 629 (1951).

12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

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

14. Klenk, H. D., Caliguri, L. A., and Choppin, P. W., Virology **42**, 473 (1970).

15. Lengyel, P., and Söll, D., Bacteriol. Rev. **33**, 264 (1969).

16. Schweet, R., and Heinz, R., Ann. Rev. Biochem. **35**, 723 (1966).

17. Krisko, I., thesis, The Rockefeller University (1970).

18. Kefalides, N. A., Int. Rev. Exp. Pathol. **10**, 1 (1971).

19. Spiro, R. G., and Spiro, M. J., J. Biol. Chem. **241**, 1271 (1966).

20. Spiro, R. G., Ann. Rev. Biochem. **39**, 599 (1970).

21. Burlington, H., Cronkite, E. P., Reincke, U., and Zanjani, E. D., Proc. Natl. Acad. Sci., USA, **69**, 3547 (1972).

Received Mar. 19, 1974. P.S.E.B.M., 1974, Vol. 146.