In Vitro Incorporation of ^{14}C from 2-^{14}C-Glucose into Rabbit Kidney Medullary and Duodenal Glycosaminoglycans[1] (36615)

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Despite their ubiquitous occurrence in the mammalian body, a functional role for the glycosaminoglycans, particularly in soft tissues, has not been clearly defined. The glycosaminoglycans of the kidney medulla have been the subject of several studies with indications that these polycaccharides may participate in the urine concentrating process. For example, Ginetzinsky (1) and Dicker and Franklin (2) have advanced the idea that during the elaboration of a concentrated urine, depolymerization of the glycosaminoglycans or at least of the hyaluronic acid components may take place thus facilitating the passage of water through the medullary interstitium. Farber *et al.* (3) have shown that the incorporation of ^{35}S from $^{35}SO_4^{2-}$ into renal medullary glycosaminoglycans *in vitro* undergoes a progressive decrease as the osmolality of the incubation medium is increased by increasing concentrations of NaCl. These postulates of glycosaminoglycan function in the kidney raise the question of whether contrasting states of renal function accompanying hydration or hydropenia may produce changes in the formation of the carbohydrate units of the renal medullary glycosaminoglycans.

^{14}C-Glucose has been used as a substrate for the *in vitro* formation of ^{14}C-labelled glycosaminoglycans by soft tissues such as cornea (4, 5) and skin (6). The experiments reported here show that rabbit kidney medullary tissue incorporates ^{14}C from 2-^{14}C-glucose *in vitro* into glycosaminoglycans although at a relatively low order when compared with ^{14}C incorporation into duodenal glycosaminoglycans. Differing states of hydration and of vasopressin administration appear not to influence the *in vitro* incorporation of ^{14}C from ^{14}C-glucose into rabbit kidney medullary glycosaminoglycans.

Materials and Methods. Kidneys were taken from four rabbits immediately after death from exsanguination. Slices of cortex and of medulla were cut with a Stadie–Riggs tissue slicer and transferred into beakers containing acetone. These tissues were used to determine the glycosaminoglycan concentrations in the two zones of rabbit kidneys.

Rabbits were divided into three groups, each group consisting of six animals. Rabbits in one group were given vasopressin tannate in oil 0.8 U/kg body weight/day for 4 days intramuscularly and on each of the last two days of this experimental period were given 5% glucose solution 75 ml/kg body weight subcutaneously. The rabbits in a second group were given peanut oil intramuscularly once a day for 4 days and the 5% glucose solution on the last 2 days. The third group of rabbits was made hydropenic by withdrawing water for a period of 36 hrs. Each rabbit was stunned by a blow on the head, exsanguinated, the kidneys were removed and prepared for incubation. Duodenal segments were taken from six rabbits and strips of duodenal mucosa were used for *in vitro* studies.

In vitro experiments were performed according to methods described in a previous publication (7). Slices of kidney medulla or strips of duodenum weighing 500 ± 50 mg were incubated for 2 hr in Krebs–Henseleit

[1] Supported by grants from the Eli Lilly Company and from the National Life Insurance Company of Vermont. An abstract of this work appears in Fed. Proc. 31, 228 abs (1972).

solution containing glucose 2.5 m*M*, L-glutamine 1.0 m*M* and providing 1.0 μCi ^{14}C as 2-^{14}C-glucose[2]/500 mg sample of tissue. At the conclusion of the incubation period, the tissue from each sample was transferred to a beaker containing acetone.

Each tissue sample, from the fresh organ tissue as well as the incubated material, was kept in acetone for 2 hr with occasional stirring. Each sample was then transferred to fresh acetone and homogenized at low speed in a VirTis 45 homogenizer for 10 min in an ice jacket. The sample was then centrifuged, the supernatant acetone was discarded, the tissue was washed three times with diethyl ether and allowed to air dry. The samples were then stored in a freezer at —19° until further processing was begun.

The lipid-free dried tissue was rehydrated at room temperature in phosphate buffer pH 7.6, 0.2 ml of a suspension of α-amylase[3] was added, thoroughly mixed and incubated at room temperature for 6 hr. Each sample was then placed in a dialysis bag containing Pronase[4] in excess of 5 mg/gm of dried tissue and the sample was incubated at 55° for 48–72 hr with constant dialysis against phosphate buffer. The sample was then placed in ice and iced 40% trichloroacetic acid was added to give a final concentration of 10% trichloroacetic acid. Following centrifugation, the precipitate was discarded, the supernatant fluid was transferred into dialysis bags and dialyzed against 4–5 changes/day of distilled water for 3 days at 4°.

Glycosaminoglycans were precipitated from each sample by the addition of 3 volumes of 1% potassium acetate in 95% ethanol. The samples were kept overnight in a freezer, the residue containing the glycosaminoglycans in this crude fraction was then separated by centrifugation, washed once with ethanol, twice with diethyl ether and allowed to air dry. Each sample was dissolved in 1 or 2 ml of water and aliquots were removed for chemical analyses. From those samples incubated with 2-^{14}C-glucose, 0.1 ml aliquots were added to counting vials containing 2 ml ethanol and 10 ml of a fluor mixture[5] and radioassays were performed in a Packard Tri-Carb liquid scintillation spectrometer. Hexuronic acid was determined by the method of Bitter and Muir (8), hexosamine using the Elson–Morgan reagent according to the procedure of Swann and Balazs (9), total reducing sugar was determined with anthrone (10) and protein with the method of Lowry *et al.* (11).

TABLE I. Glycosaminoglycan Concentrations of Rabbit Kidneys.

Sample	Glycosaminoglycan concentration[a] (mg/g wet weight)
Cortex ($N=4$)	0.228 ± 0.018
Medulla ($N=4$)	1.126 ± 0.086

[a] Concentrations are expressed as the average ±1 standard error of the mean. The results were derived from the molar concentrations of hexuronic acid and hexosamine and do not, therefore, include the weight of the acetyl and sulfate groups of the polysaccharides.

Samples from three animals in each group were pooled providing two composite samples from each treatment group. Using the method of Schiller *et al.* (12) glycosaminoglycans were separated into 0.4 *M* NaCl and 1.2 *M* NaCl fractions. Excess cetyl pyridinium chloride (CPC) was removed with KCNS (13); each sample was then dialyzed against 4–5 changes of distilled water for 24 hr at 4° and glycosaminoglycans were recovered by the addition of 3 volumes of 1% potassium acetate in 95% ethanol. Chemical analyses of each fraction and radioassays were performed as described previously.

Results. The concentration of glycosaminoglycans in rabbit kidney medulla is approximately 5.0 fold greater than the glycosaminoglycan concentration of rabbit kidney cortex (Table I). The glycosaminoglycans recovered in the CPC fractions of kidney cortex were evenly divided between 0.4 *M* NaCl and 1.2 *M* NaCl fractions. From med-

[2] Obtained from New England Nuclear Corp., Boston, MA.

[3] From hog pancreas. Obtained from Sigma Chemical Co., St. Louis, MO.

[4] Obtained from Calbiochem, Los Angeles, CA.

[5] 0.5% 2,5-diphenyloxazole (PPO) and 0.1% 1,4 bis[2-(4-methyl-5-phenyloxazolyl)]-benzene(Dimethyl POPOP) in toluene.

TABLE II. Relative Molar Concentrations of Carbohydrate Components and Radioactivities of Glycosaminoglycan-Containing Fractions from Rabbit Kidney Medulla Incubated with 2-^{14}C-Glucose.

Fraction	Molar equivalents			Radioactivity[a] (cpm/μmole)
	Hexuronic acid	Hexosamine	Anthrone-positive	
a) Hydropenic				
Crude[b]	1.0	0.91 ± 0.07	0.58 ± 0.04	525 ± 98
0.4 *M* NaCl	1.0	0.90	0.27	104
1.2 *M* NaCl	1.0	0.87	None	117
b) Hydrated				
Crude	1.0	0.91	0.28	384
0.4 *M* NaCl	1.0	0.90	None	138
1.2 *M* NaCl	1.0	0.97	None	134
c) Hydrated + Vasopressin				
Crude	1.0	0.86	0.34	538
0.4 *M* NaCl	1.0	0.85	0.14	185
1.2 *M* NaCl	1.0	0.90	None	138

[a] Calculated from $\frac{\text{Cpm/ml}}{\mu\text{moles hexuronic acid + hexosamine + anthrone-positive/ml}}$

[b] $N = 6$. Other results represent the averages of the 2 pooled samples.

ullary glycosaminoglycans, approximately 60% of the total recovered was in the 0.4 *M* NaCl fraction and 40% in the 1.2 *M* NaCl fraction.

Anthrone positive material was recovered in the crude glycosaminoglycan fraction as well as in several of the CPC fractions, and comprised as much as 20% of the total carbohydrate content in some samples (Table II). A large quantity of anthrone positive material was obtained in the first wash fraction (0.1% CPC in 0.03 *M* CNaCl) following precipitation of the glycosaminoglycans in the crude fraction with CPC and Celite. This anthrone-positive material was heated for 1 hr with 5 *N* H_2SO_4, neutralized with Na OH and the resultant solution contained material which was oxidized by glucose oxidase (Glucostat, Worthington Biochemicals, Freehold, N.J.).

The 0.4 *M* NaCl fractions of two of the three groups contained anthrone-positive material, but all 1.2 *M* NaCl fractions contained only hexuronic acid and hexosamine (Table II). Protein content of the crude fractions varied from 3.5% to 5.4% and only trace amounts (< 1%) were present in the CPC fractions. The radioactivities recovered in the crude fractions were in all cases greater than the radioactivities of the CPC fractions (Table II). The state of hydration of the rabbit did not affect the radioactivity of either the 0.4 *M* NaCl or 1.2 *M* NaCl fractions (Table II).

Duodenal glycosaminoglycan fractions were characterized by high hexosamine/hexuronic acid ratios and high concentrations of anthrone positive material (Table III). Protein content of the crude fradtions varied from 9–14.8% and was 5% in one of the 0.4 *M* NaCl fractions. The radioactivity of the crude fraction of duodenal glycosaminoglycans was greater than the radioactivity of each of the CPC fractions (Table III). The radioactivities of the CPC fractions of duodenal glycosaminoglycans were 8–10 fold greater than the radioactivities from kidney medullary glycosaminoglycan fractions (Tables II and III).

No attempt was made to measure the physiological changes in renal function in rabbits accompanying the administration of the 5% glucose solution or of the 5% glucose solution plus vasopressin in tannate in oil with hydropenic rabbits. Each rabbit given the glucose solution and additionally treated

TABLE III. Relative Molar Concentrations of Carbohydrate Components and Radioactivities of Glycosaminoglycan-Containing Fractions from Rabbit Duodenal Tissue Incubated with 2-^{14}C-Glucose.

Fraction	Molar equivalents			Radioactivity[a] (cpm/μmole)
	Hexuronic acid	Hexosamine	Anthrone-positive	
Crude[b]	1.0	5.00 ± 0.41	2.17 ± 0.20	2,725 ± 462
0.4 *M* NaCl	1.0	5.70	2.37	1,040
1.2 *M* NaCl	1.0	3.74	1.21	1,525

[a] Calculations on same basis as in Table II.

[b] $N = 6$. Other results represent the averages of the 2 pooled samples.

with vasopressin tannate in oil showed marked subcutaneous accumulations of fluid linearly along each side of the sternum and continuing caudad along the abdominal wall. Two of the six rabbits given only the dextrose solution also had subcutaneous fluid in the dependent skin along the thorax and abdomen. The kidneys of both groups of rabbits given the glucose solution were grossly swollen and in some instances dilatation, apparently of the collecting ducts, was evident. The kidneys of the hydropenic rabbits presented a sticky almost "dry" appearance when compared with the kidneys from the other groups.

Discussion. The concentration pattern of glycosaminoglycans between the two zones of rabbit kidneys is comparable to that reported by Castor and Greene for dog kidneys (14). While dog kidney medullary glycosaminoglycans contain predominantly hyaluronic acid with small amounts of sulfated polysaccharides (14), Farber and Van Praag (15) have isolated rabbit kidney medullary glycosaminoglycans from which recoveries of chondroitin sulfate were greater than the recoveries of hyaluronic acid. The results reported here also indicate that rabbit kidney medullary glycosaminoglycans contain relatively large amounts of sulfated polysaccharides as well as hyaluronic acid. Although the CPC fractions were not separated further, it is likely that the predominant components are the respective glycosaminoglycans. The radioactivities of the fractions from tissue incubated with 2-^{14}C-glucose are expressed on the basis of the total carbohydrate content, but not as precise measures of the specific radioactivities of glycosaminoglycans.

Large amounts of anthrone-positive material in the crude and CPC fractions of kidney medullary glycosaminoglycans present a troublesome complication in the interpretation of ^{14}C-glycosaminoglycan formation *in vitro*. This anthrone positive material appears to be glycogen since, following hydrolysis, the material is susceptible to oxidation by glucose oxidase. Using tissue not subjected to an initial exposure to amylase the estimate was made that the amylase used in the initial stages prior to incubation with Pronase hydrolyzed 70–75% of the tissue glycogen. The radioactivity of the crude fraction, therefore, probably reflects in large measure the incorporation of ^{14}C from 2-^{14}C-glucose into glycogen, for the formation of ^{14}C-glucogen from ^{14}C-glucose by kidney medulla *in vitro* readily occurs (12). The markedly lower radioactivity of the CPC fractions suggest that the anthrone positive material—presumably glycogen—is washed out by the successive extractions of the CPC procedure.

The 1.2 *M* NaCl fraction of rabbit kidney medulla was always free of anthrone positive material and its radioactivity probably is a reliable indication of ^{14}C-glucose incorporation into glycosaminoglycans. On this basis two conclusions can be drawn from the experimental results: (1) The kidney medulla contains the necessary enzymatic components to synthesize glycosaminoglycans from glucose, and (2) the state of hydration and treatment with vasopressin, under the conditions described, does not change the rate of assembly of the carbohydrate constituents of chrondroitin sulfate. This does not exclude the

possibility that other factors such as depolymerization (1, 2) or changes in the degree of sulfation (3) of renal medullary glycosaminoglycans may participate in the urine-concentrating process. The same conclusions may be in order for the 0.4 *M* NaCl fraction, although the presence of anthrone-positive material in two of the three groups questions the reliability of the measurement of the radioactivity of this fraction.

The intestinal mucosa contains a complex array of protein–carbohydrate macromolecules, notably glycoproteins as well as glycosaminoglycans whose components include both amino and reducing sugars (17). Since purification of the glycosaminoglycan-containing fractions was not extensive, glycoprotein residues could have contributed to both the hexosamine and anthrone-positive components. The high hexosamine/hexuronic acid molar ratio and high content of anthrone-positive material also would be compatible with the presence of keratan sulfate (18). In cartilage, chondroitin sulfate and keratan sulfate are linked covalently to a common peptide backbone (19, 20) and a similar relationship between keratan sulfate and other glycosaminoglycans may be present in rabbit intestinal mucosa (21). Should the CPC fractions represent primarily glycosaminoglycans, their radioactivities indicate that ^{14}C-glycosaminoglycan formation by duodenal tissue *in vitro* is 8–10 fold greater than in kidney medulla.

Summary. Glycosaminoglycan concentrations in rabbit kidney medulla are 5.0 fold greater than in the kidney cortex. Rabbit kidney medullary tissue incubated *in vitro* with 2-^{14}C-glucose, provides a crude fraction of glycosaminoglycans contaminated with ^{14}C-glycogen. Following separation with CPC, the 1.2 *M* NaCl fraction is recovered free of anthrone positive material and its radioactivity probably reflects ^{14}C-glycosaminoglycan formation. The state of hydration of the rabbits or treatment with vasopressin does not change the radioactivity of the 1.2 *M* NaCl fraction. Duodenal glycosaminoglycans in the crude fraction as well as in both CPC fractions contain material having a high hexosamine/hexuronic acid molar ratio and high concentration of anthrone positive material which may indicate the presence of keratan sulfate. Glycosaminoglycan-containing fractions extracted from rabbit duodenal tissue which had been incubated with 2-^{14}C-glucose have radioactivities 8 fold greater than those of kidney medullary glycosaminoglycans.

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Received Mar. 27, 1972. P.S.E.BM., 1972, Vol. 140.