to their APase activities. They have found a pH optimum at pH 10.3 for all extracts. A strong Mg^{2+}-activation was also observed.

On the other hand, Felix and Fleisch[8,9] have separated an inorganic pyrophosphatase from an APase by Sephadex gel filtration of a pig scapula cartilage extract. They assume that the PP_iase and the APase are isoenzymes, rather than different enzymes.

Concerning the function of the APase, there is good reason to assume - on the basis of Robison's theory[10] - that this enzyme forms orthophosphate groups resulting in mineral formation. Such an assumption might be supported by our results showing that at least 66% of the total phosphate amount, in the mineralizing region of the turkey tibia tendon as well as in the predentine[11], exists in the form of orthophosphate groups. Considering our results and those of the other authors, one might assume that divalent ions which activate the APase appear, in certain microcompartments in the mineralizing area, to induce the next 'push' of calcification, and so control the mineralization process.

1 We thank Deutsche Forschungsgemeinschaft for support.
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Glycosaminoglycans in separated tubules of the guinea-pig and rat kidney medulla*

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Summary. Isolated tubules of the renal medulla of guinea-pig and rat contained glycosaminoglycans. 20-25% of the uronic acids corresponded to hyaluronic acid. In the guinea-pig, chondroitin and dermatan-sulfates accounted for at least 50% of the uronic acids, whereas, in the rat, heparan sulfates comprised 65-70% of them.

Although glycosaminoglycans have been isolated from kidneys of various mammals[1-4], little is known about their distribution in renal tissues. It has been assumed that they are components of the medullar stroma, even though histochemical data indicated that certain renal tubules also contained glycosaminoglycans[5-7]. The present report deals with the isolation and characterization of these glycoconjugates from the tubules of renal medulla of guinea-pig and rat.

Materials and methods. Separation of tubules. 370 guinea-pigs and 220 rats were bled and perfused through the left ventricle with cold 10 mM sodium phosphate buffer, pH 7.4 and through the abdominal aorta, with 0.05% collagenase in the same buffer[8]. The kidney medulla was excised and incubated for 3 h at room temperature with collagenase[8]. Renal tubule preparations thus obtained were controlled by phase and light microscopes[9] (figure).

Isolation of glycosaminoglycans. The tubule preparations were digested with papain (EC 3.4.4.10) for 6 h at 65 °C in 0.1 M phosphate buffer, pH 6.5[10], treated with cold trichloroacetic acid (10% final concentration) and centrifuged. The supernatants were neutralized with saturated NaOH, concentrated in a rotatory evaporator and run through a Sephadex G-50 column (2.5 × 30 cm) eluting with distilled water. The excluded material was treated with deoxyribonuclease (EC 3.1.4.5) and ribonuclease (EC 2.7.7.17)[11]. 2 volumes of chloroform-methanol (2:1, by volume) were added. Crude preparations obtained by rechromatography on Sephadex G-50, were dried, and fractionated on DEAE-Sephadex A-25 (0.25 × 20 cm) in a cold room[12], eluting with distilled water (Fraction I), 0.1 and 0.5 M NaCl (II-III), 1.0, 1.5 and 2.0 M NaCl containing 0.01 M HCl (IV-VI) and 6.0 M HCl (VII). These fractions were concentrated and salted out by chromatography on Sephadex G-25.

Analytical assays. Uronic acids[13], hexosamines[14,15], sulfates[16,17], sialic acids[18], neutral sugars[19], methylpentoses[20] and proteins[21] were assayed by duplicate.

Monosaccharides were identified by paper chromatography using and ethyl acetate pyridine-water (12:5:4, by volume) system[22], after 4 h hydrolysis[14]. Chromatograms were developed with benzidine or nynhydrin.

DEAE-Sephadex A-25 fractions were electrophoresed on cellulose acetate paper[23,24]. Strips were stained with 1% alcian blue pH 2.5 or pH 1.2 for 30 min, with the PAS procedure[25] or with Coomassie brilliant blue R[26]. After 3 washings with ethanol for 10 min they were cleared with methanol-acetic acid glycerol (85:14:1, by volume).

Results and discussion. The separated renal tubules of both species contained several carbohydrates (figure, table 1). In the rat, there were less uronic acids and hexoses than in the

Table 1. Carbohydrate and sulfate content of separated tubules of renal medulla of guinea-pig and rat

Component	Guinea-pig (5)*			Rat (2)*		
	Homogenate	Crude preparation	%	Homogenate	Crude preparation	%
Uronic acids	-	33± 3	-	-	17± 2	-
Hexosamines	377± 11**	107± 12**	28***	307± 73**	98± 23**	32***
Sulfates	-	21± 7	-	-	10± 1	-
Sialic acids	90± 26	21± 2	23	73± 3	26± 1	35
Hexoses	1810± 320	281± 32	15	1951± 440	50± 8	3
Methylpentoses	330± 110	12± 4	3	-	-	-

* Number of experiments. Each tubule preparation consisted of a pool of kidneys, from not less than 50 animals. All determinations were made in duplicate. ** µg/100 mg dry weight. Values are means ± SE. *** Percent carbohydrate of the crude preparations in respect to the homogenate.

Table 2. Distribution of carbohydrate components in the DEAE-Sephadex fractions of separated renal tubules of guinea-pig (GP) and rat (R)*

Component	DEAE-Sephadex fraction												
	I		II		III		IV		V		VI		VII
	GP	R	GP	R	GP	R	GP	R	GP	R	GP	R	GP
Uronic acids	0	0	0	0	21	20	28	15	7	23	10	42	23
Hexosamines	43	8	22	58	24	23	5	4	2	2	2	4	2
Sulfates	3	0	0	0	29	6	18	42	18	23	12	29	15
Sialic acids	82	47	0	53	18	0	0	0	0	0	0	0	0
Hexoses	55	71	6	29	14	0	2	0	1	0	0	0	20

* Values are percentage of the recovered component from the crude preparation.

guinea-pig ($p < 0.05$). Paper chromatography revealed glucosamine, galactosamine, galactose, mannose, glucose and fucose.

By DEAE-Sephadex A-25 chromatography, the crude preparations were separated in 7 fractions in the guinea-pig, and 6 in the rat, which differed in various degrees between both species (tables 2 and 3).

Fractions I and II accounted for most of the carbohydrates (glucosamine, galactosamine, galactose, mannose, fucose and sialic acids) and 'proteins' of the crude preparations (table 2). They contained neutral and slightly acid glycopeptides as well as glycogen (table 3).

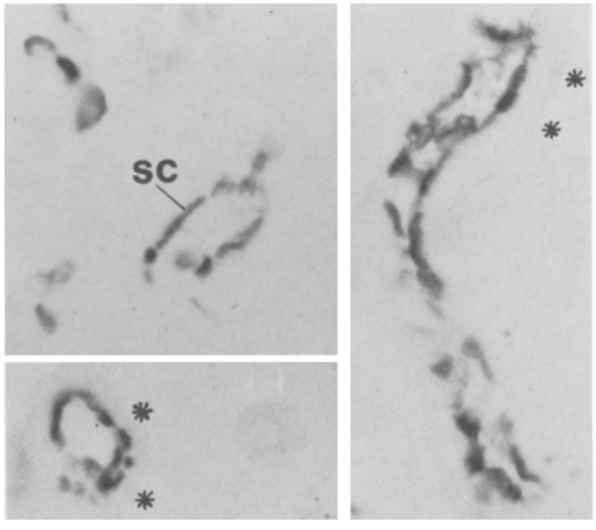
Fraction III contained about 20–25% of the uronic acids and hexosamines (mostly glucosamine) (table 2). On electrophoresis 2 components were noted. The major one migrated like hyaluronic acid. The less mobile component did not correspond to any of the standards (table 3). Glucosamine, galactose and all the sialic acids and sulfates of the fraction were found in its eluate. Molar ratios suggested a keratan sulfate-like compounds but of lower sulfate content.

In the guinea-pig, fractions IV, V and VI, had, as a whole, almost 50% of the uronic acids, which were in galactosaminoglycans differing mainly in degree of sulfation. Electrophoresis showed 2 components which moved like dermatan and chondroitinsulfates (tables 2 and 3). In the rat, fraction IV contained 15% of the uronic acids (dermatan, chondroitin and some heparan-sulfate), whereas in fraction V and VI, the remaining 65–70% of the uronic acids were bound to glucosamine (heparansulfates) (tables 2 and 3).

In the guinea-pig, fraction VII contained about 30% of the uronic acids in chondroitin and heparan-sulfates (tables 2 and 3).

Allalouf et al.[1] reported that the kidney of the rat contained a heparin-like material but they detected no galactosamine. On the other hand, Vanhegan[2] noted that the guinea-pig kidney had chondroitin-sulfate but no heparin. Present results indicate that both galactosamino- and glucosaminoglycans were present in renal tubules of rat and guinea-pig. The pattern of distribution differed in both species.

It is generally assumed that glycosaminoglycans are a group of compounds characteristic of the connective tissue ground substances. We show that certain normal epithelial cells do contain glycosaminoglycans. This is in keeping with the demonstration of these glycoconjugates in several neoplastic and embryonic nonconnective cells[27–32]. Furthermore, we have recently reported preliminary data indicating that



Longitudinal and cross sections of separated tubules of guinea-pig kidney by collagenase digestion, stained with alcian blue, pH 2.5. No stroma is apparent.
Note that at the outher limit of these tubules, the basement membrane is no longer seen (**) whereas the luminal surface coat (sc) is still shown. × 400.

Table 3. Electrophoretic mobilites of DEAE-Sephadex fraction components of separated renal tubules of guinea-pig (GP) and rat (R)*

Buffer	Standards**		DEAE-Sephadex fraction***										
			I–II		III		IV		V		VI		VII
			GP	R	GP	R	GP	R	GP	R	GP	R	GP
Barium acetate	C4/6S	1.0	0	0	0.73	0.76	0.71	0.75	0.74	0.03	0.76	0.03	0.03
	KS	0.86			0.61		0.97	0.97	1.00		1.03		0.97
	DS-HA	0.74						0.03					
	Hep	0.03											
HCl	C4/6S	1.0	0	0	0.65	0.63	0.84	0.86	1.06	1.15	1.15	1.18	1.21
	DS	1.0			0.76		1.0						
	HA	0.65											
	Hep	1.15											

* Spots in all strips but those corresponding to fractions I–II stained with Alcian blue, pH 2.5. Fractions I and II gave positive and coextensive reactions with PAS and Coomassie blue. ** Relative mobilities to chondroitin-4-(and 6) sulfate. *** For each buffer, the upper line corresponds to the main spot as identified by the size and intensity of the reaction. Lower lines refer to minor spots. C4/6S, Chondroitin-4-(and 6) sulfate; KS, keratan-sulfate; DS, dermatan-sulfate; HA, hyaluronic acid; Hep, heparin.

glycosaminoglycans, such as those herein identified, are bound to cell membranes of renal tubule cells and hepatocytes[33] and milk fat globule membrane[34].

* Supported, in part, by CONICET, Argentina. Mrs N. Ramonda-Becerra assisted in the electrophoretic assays.

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Ionic effects on cyclic nucleotide accumulation in a phytophagous insect[1]

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Summary. Incubation in solutions containing high potassium levels reduces specific accumulation of cyclic AMP in larval nerve cords of *Manduca sexta* in vitro with or without serotonin or theophylline.

In previous work by our laboratory on the accumulation of cyclic nucleotides in insect nervous systems[2,3] Grace's tissue culture medium[4] was used as the carrier for incubation solutions. Since this medium contains amino acids which could affect cyclic nucleotide levels, we sought a less complex medium.

The hemolymph of *Manduca sexta* (Lepidoptera: Sphingidae) has a low sodium: potassium ratio, 1:1, which will not support action potential production in naked axons; this implies a sodium-dependent action potential[5]. The ventral nerve cord of *M. sexta* is enveloped by a highly impermeable sheath of glia which allows production on action potentials even in sodium-free saline[5]. Because the freeze-thaw treatment necessitated to obtain pools of nerve cords may have damaged the nerve cord sheath, an investigation of the effects on cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) accumulation of high sodium or high potassium ringer solutions, with or without additions was made.

Materials and methods. Nerve cords from 5th instar 'wanderer' larvae were dissected as previously described[6]. The nerve cords were incubated at 30 °C for 10 min and frozen as described elsewhere[7].

The saline solutions used were a high sodium ringer consisting of 152 mM NaCl, 4.7 mM KCl, and 2.8 mM $CaCl_2$[8], or a high potassium solution, consisting of 150 mM KCl, 5 mM NaCl, and 2.8 mM $CaCl_2$; both were buffered with 50 mM citrate (pH 5.3). Serotonin and theophylline were purchased from Sigma. Ascorbic acid, 100 µM, was routinely added as an anti-oxidant.

Deproteination and cyclic nucleotide extraction was accomplished by homogenizing the frozen solutions in ice-cold absolute ethanol[9]. Cyclic nucleotide residues were assayed by the radioimmunoassay method of Steiner et al.[10] using kits (Collaborative Research, Waltham, MA). Protein was assayed by the method of Lowry et al.[11].

Results and discussion. The data presented in the table indicate that the sodium: potassium ratio has little effect on the resting levels of cyclic AMP in incubated nerve cord pools. Accumulation of cyclic AMP in the presence either of 5-HT or theophylline was markedly higher in the high sodium medium.

These results show that the concentrations of sodium and potassium or their ratio may affect cyclic AMP levels in the *M. sexta* CNS. Because the phosphodiesterase would be essentially unaffected by these conditions[6] we conclude that adenyl cyclase activation is involved.

Adenyl cyclase is variably affected by potassium in mammalian brain slices[12] especially in the presence of theophylline[13,14]. The cockroach, a carnivorous insect with a high Na^+/K^+ hemolymph ratio, accumulates cyclic AMP in the presence of 50 mM potassium with or without theophylline[15]. This report illustrates the difficulties which may be encountered in the extrapolation of results from mammalian to certain kinds of non-mammalian systems.

Specific accumulation of cyclic AMP in pooled nerve cords in saline solutions at pH 5.3

Addition to Ringer solution	High sodium Ringer ($Na^+/K^+ = 32$) (mean±SEM)	High potassium Ringer ($Na^+/K^+ = 0.033$) (mean±range)
None	0.54 ± 0.13 (6)	0.50 ± 0.03 (2)
10 mM theophylline	7.88 ± 2.08 (4)	2.2.75 (1)
10 mM serotonin	5.24 ± 2.65 (4)	1.11 ± 0.09 (2)

Results are given as pmole cyclic AMP/mg protein ± SEM or range (for 2 pools). The number of preparations assayed is indicated in parentheses.