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Protein Location and Elemental Composition of Urine Spheres in Different Avian Species

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ABSTRACT We examined the internal morphology, location of protein, and identity and location of elements, in avian urate-containing spheres in 9 species of birds. The urine spheres were collected from voided samples. The spheres ranged in size from 0.5–5.0 μm, except in the domestic fowl, where they ranged up to 10 μm in diameter. The internal morphology of the spheres was examined using freeze-fracture microscopy. Protein location within the spheres was identified using fluorescein isothiocyanate (FITC). The urine spheres were analyzed for content and internal location of elements using Energy Dispersal System Analysis (EDS). Internally, the spheres consisted of a central nidus surrounded by 3–4 concentric narrow rings of protein. Elements found within the spheres included nitrogen, potassium, calcium, sodium, phosphorus, chloride and sulfur; however, only nitrogen, potassium and chloride were common in the spheres of all species. Nitrogen comprised the majority of the elemental content of the spheres (77–90%) followed by potassium (8–45%), with all other ions present in trace amounts. Unlike protein, the location of elements was random within the spheres. Protein and urate are both negatively charged and known to associate to form the spheres and as potassium is the only cation common to all spheres, it too may play a role in their formation. *J. Exp. Zool. 301A:579–587, 2004.* © 2004 Wiley-Liss, Inc.

INTRODUCTION

As the result of the metabolism of proteins, terrestrial vertebrates liberate nitrogen as ammonia which is toxic to most living organisms. The ammonia is detoxified and either eliminated directly (aquatic organisms) or is combined with carbon atoms to facilitate its excretion in two principle forms: urea and uric acid (Wright, ’95). Birds excrete nitrogen primarily as uric acid or its salts (urate) with urea and ammonia accounting for smaller fractions of nitrogen excretion (Braun, 2003). However, there are exceptions to this rule as some birds excrete a significant fraction of nitrogen as ammonia (Roxburgh and Pinshow, 2000). The uric acid (urate) excreted by birds takes the form of small spheres that also contain protein and inorganic ions (Braun and Pacelli, ’91).

At the pH of avian plasma, greater than 99% uric acid occurs in the form of urate salts (can be calculated using Henderson-Hasselbalch equation), most likely sodium and potassium salts as these are the most abundant cations in the plasma (Kawashiro and Scheid, ’82). These salts have a greater solubility than the acid form of urate (i.e., uric acid) and are less likely to precipitate in the plasma. Evidence suggests that there is a modest degree of binding of urate to protein in avian plasma (Greger et al., ’74). Urate is freely filtered by the avian glomerulus and enters the proximal renal tubule (Dantzler, ’78; Boykin, ’95). As the pH of the fluid entering the early proximal tubule is approximately the same as that of avian plasma (pH 7.5) (Laverty and Alberici, ’87), the majority of the uric acid would be present as urate salts. However, along the length of the nephron, the concentration of urate increases due to its secretion and water reabsorption in the proximal tubule. As this occurs, the urate does not precipitate from solution and form crystals as would

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be predicted from the aqueous solubility of uric acid and its salts. Instead, the uric acid is "packaged" in small spheres together with a protein that also passes through the glomerular filtration barrier (Dantzler, '78; Boykin, '95). At this time, it is uncertain what initiates the process that forms the spheres in the proximal tubule. It has been suggested that they may form as alternating layers of uric acid and mucoid materials with associated water (Minnich, '76). The data reported in the present paper support this concept.

Previous work has shown the spheres to be composed 65% uric acid and 5% protein (Braun and Pacelli, '91). A later study identified the protein component of the spheres as most likely being serum albumin (Boykin, '95). Thus, the composition of 30% of the sphere mass remains to be determined.

Due to the pH of the proximal tubular fluid, most of the uric acid is probably in its anionic form. Moreover, serum albumin has a net negative charge and avian serum albumin is more negatively charged than mammalian albumins (Bingaman et al., 2001). Therefore, it is unresolved how the urate and protein (both negatively charged), combine to form these spheres. Previous work on domestic fowl (*Gallus gallus*) has shown that some of the unaccounted mass may consist of calcium and potassium ions (Casotti and Braun, '97). We believe that these ions may somehow be involved in 'binding' the negatively charged protein and uric acid. In addition, it has been suggested that the spheres may sequester ions from the tubule fluid, perhaps due to the charge configurations between urate layers (McNabb and McNabb, '75). The current study was undertaken to examine what elements are present in the spheres, and the location of protein, to gain a better understanding of how the spheres are formed.

In our 1997 study, we examined spheres from only one species, *G. gallus*. In addition, since then, advances in Energy Dispersal System Analysis (EDS) technology have made the detection of ions more accurate. The purposes of the present study were to re-examine the elemental composition of urine spheres in *G. gallus* using newer technology to determine if ions other than calcium and potassium (as found by Casotti and Braun, '97) can be identified, and to examine the ionic composition of urate spheres from other birds to ascertain whether the composition of the spheres varies among species. An additional goal was to define the geographical position of the protein and ions within individual spheres, to determine whether they occur in layers. Data from this paper and those from previous studies will be used in an attempt to form the spheres in vitro, thus aiding our understanding of how the urine spheres are formed.

MATERIALS AND METHODS

Species studied

We examined the elemental content of urine spheres in 34 different birds, from 9 species. All samples examined were from adult birds. Species captured were those found locally abundant in Eastern Pennsylvania. Urine samples from both males and females were collected. The species examined were the domestic fowl (*G. gallus*) (n=5), Gray catbird (*Dumetella carolinensis*) (n=6), Wood thrush (*Hylocichla mustelina*) (n=6), Barn swallow (*Hirundo rustica*) (n=6), House sparrow (*Passer domesticus*) (n=6), Chipping sparrow (*Spizella passerina*) (n=2), Northern cardinal (*Cardinalis cardinalis*) (n=1), Rufous-sided towhee (*Pipilo erythrophthalmus*) (n=1) and the Ovenbird (*Seiurus aurocapillus*) (n=1). All species were collected from the wild using mist nets except the domestic fowl, which were housed in cages and fed a commercial diet (Layeena Chicken Feed, Purina Mills, Oxford, PA) and given water *ad libitum*.

Sample collection

All urine samples were taken from birds immediately following capture, from voided urine at the base of the animal cage. Animals usually voided 2–3 minutes after being placed in the cage. No animal took longer than 5 minutes to void a sample.

Sample preparation and analysis

Once voided, all urine samples were immediately aspirated directly onto aluminum scanning electron microscope stubs using a micropipette with sterile tips. The samples were allowed to air-dry overnight in a vacuum desiccator. Samples were stored in the desiccator and analyzed within 48 hours using Oxford EDS, INCA microanalysis suite, Issue 13, Version 4.02. X-rays were detected using an Oxford INCA energy 400 detector attached to an F.E.I. Quanta 400 ESEM. Low vacuum mode was used on the electron microscope; therefore it was not necessary to coat the samples prior to viewing and subsequent

microanalysis. Specimens were viewed at a sufficiently high magnification to ensure that x-rays from only one sphere were detected at any one point in time. X-rays from all spheres were collected using 20 kV, a spot size of 4.0, and at a working distance of 10 mm from the detector. X-ray spectra were collected using a capture time of 120 seconds per sphere, and dead time during spectra capture was no more than 15%, and usually below 10%.

Protein location

Protein location was determined by using fluorescein isothiocyanate (FITC) (F-1906, Molecular Probes, Eugene, OR). FITC acts as an amine-reactive fluorescent dye and thus binds to protein. Urine from all birds was collected and centrifuged, the supernatant decanted, and the resulting pellet of uric acid spheres processed routinely for light microscopy and embedded in paraffin wax. Sections were cut at 5 μm and mounted onto glass slides. Sections were deparaffinated using xylene, hydrated through a series of alcohols of decreasing concentration, then washed in 0.1 M sodium bicarbonate (pH 9.0). Protein location was determined by fluorescence by incubating for 1 hour with FITC. From a stock solution of FITC, working solutions were prepared with concentrations of 1, 5, 10 and 50 μM. Sections were then washed with the bicarbonate buffer, dehydrated and cover slipped for viewing.

Sections were viewed on an Olympus BX 60 microscope and images captured using a cooled CCD camera (Spot, Diagnostic Instruments Inc., San Antonio, TX). Density profiles of flourescent spheres were measured using NIH Image, by drawing a line across the diameter of the sectioned spheres, and obtaining a density profile along the line. High densities (i.e., bright areas), represent the location of protein within the spheres.

Imaging

To freeze-fracture the small urine spheres, two to three drops of urine were place between two scanning electron microscope stubs. The stubs were clamped together and immersed in liquid nitrogen. Stubs were then separated which fractured the frozen spheres, air-dried in a vacuum desiccator overnight and coated with gold. Fractured spheres were viewed with the scanning electron microscope.

Statistics

Data on the elemental composition of urine spheres were subjected to one-way ANOVA analyses. Statistical differences among species, and among spheres of different sizes were examined using a Scheffe F-test. In all cases, statistical significance was set at $P < 0.05$.

RESULTS

Sphere morphology

Urine spheres ranged in size from 0.5 to 5 μm in all species examined, except in domestic fowl, where spheres ranged up to 10 μm in diameter (Fig. 1). Spheres in domestic fowl appeared to be larger than in other species, with few small sized spheres (< 2 μm) present in the urine. Freeze-fracturing revealed the gross internal structure and organization of the spheres (Fig. 2). In the very center of the spheres, there appeared to be a nidus which was surrounded by several (3–4) narrow rings of material. The rings appear to become broader as distance increases from the center. The outer ring appeared to be very broad with a radiating, spoke-like pattern (Fig. 2).

Effect of species on elemental content

Urine spheres contained up to 7 detectable elements including nitrogen, potassium, calcium, sodium, phosphorus, chloride and sulfur (Table 1). Nitrogen, potassium and chloride were common elements found in all the spheres of all species (Fig. 3). Nitrogen was the most abundant element (80–90%), followed by potassium (8–45%). All ions were found in smaller, varying amounts in the spheres of all species (Fig. 3).

The quantity of ions present in the spheres was in some cases, statistically significant among species (Fig. 3). For example, spheres from the ovenbird and gray catbird had a significantly higher percent of potassium compared to the other species and the spheres from the ovenbird, house sparrow and barn swallow had a significantly higher percent of sodium than the spheres from the other species studied (Fig. 3). In addition, the spheres in the ovenbird had a significantly higher percent of phosphorus than spheres from other species. Sphere ion content did not appear to be related to the animal's diet (Table 1).

Effect of sphere size on elemental content

The spheres were divided into size categories (1, 2, 3, 4 and 5 μm) for purposes of comparisons.

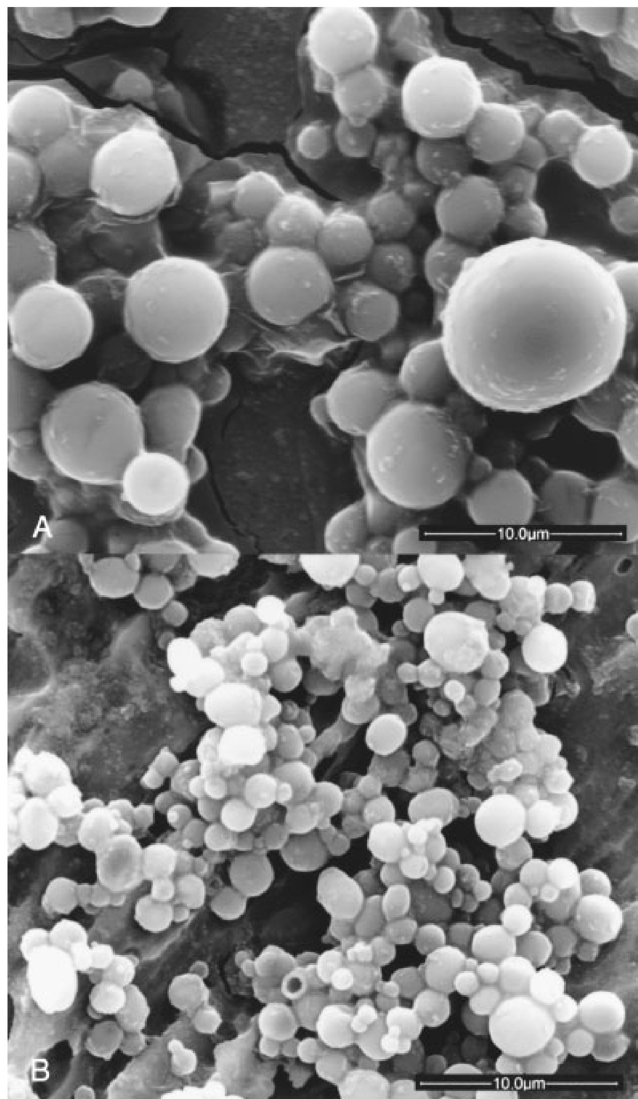

Fig. 1. Urine spheres showing differences in size distribution between those in domestic fowl and in other bird species. A. domestic fowl, B. wood thrush.

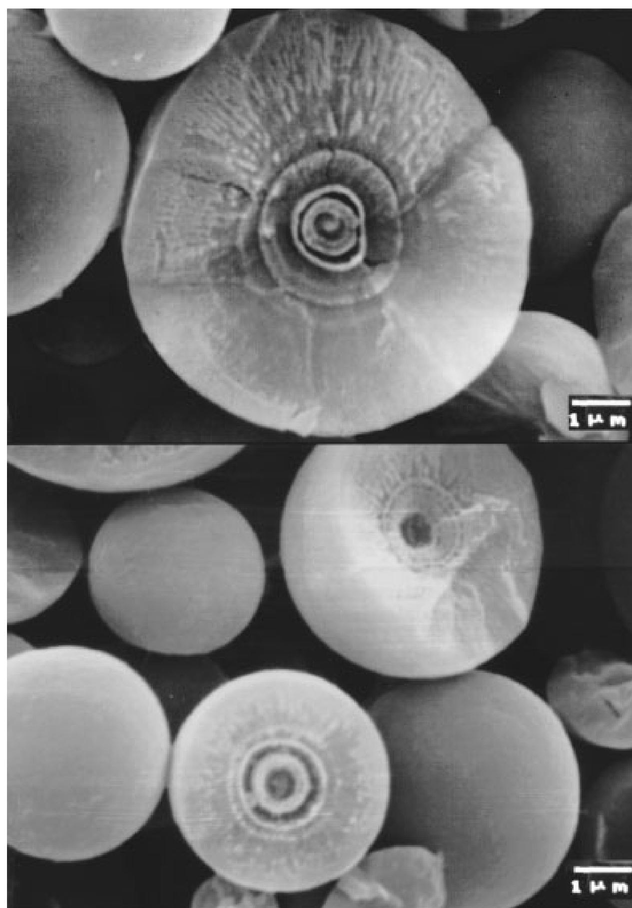

Fig. 2. Scanning electron micrographs of freeze-fractured urine spheres revealing concentric ring formation. Note the central nidus and the increasing size of the concentric rings.

For domestic fowl, the 5 µm category represented spheres 5 µm or larger. Nitrogen was the most abundant ion (range 80–85%), followed by potassium (12–18%) and sodium (10–18%), with all other detectable ions (calcium, phosphorous, chloride and sulfur) present in smaller quantities (< 5%) (Fig. 4).

The percent ions present in the spheres among species were statistically significant among size categories in some cases (Fig. 4). Spheres in the size categories of 1, 2 and 4 µm had a significantly higher percent of potassium than those of 3 and 5 µm. Spheres in the size category of 5 µm had a significantly higher percent of sodium than smaller sized spheres.

Ion mapping

The geographical location of elements within urine spheres was mapped using the INCA microanalysis software. We found that the ions (regardless of their identity) were randomly distributed within the spheres, rather than in concentric rings. This phenomenon was the case for both whole and fractured spheres.

Protein location

Fluorescence microscopy confirmed that protein was present within the urine spheres of all species studied. The protein was located in concentric rings within the spheres (Fig. 5). Density profiles across these spheres clearly showed gradations as a function of the cross section, indicating that protein was present at regular intervals across the spheres (Fig. 6).

TABLE 1. Summary data outlining sphere size, diet and elemental content of urine spheres

Species	n	Sphere size (μm)	Diet	Ion content
Domestic chicken	5	0.5–10	Granivore	N, K, Ca, S, Cl
Northern cardinal	1	0.5–5.0	Granivore	N, K, Na, P, Cl
Rufous-sided towhee	1	0.5–5.0	Granivore	N, K, Na, P, Cl
Chipping sparrow	2	0.5–5.0	Granivore	N, K, Na, P, S, Cl
House sparrow	6	0.5–5.0	Granivore	N, K, Ca, Na, P, S, Cl
Gray catbird	6	0.5–5.0	Frugivore/Insectivore	N, K, Ca, Na, P, S, Cl
Wood thrush	6	0.5–5.0	Frugivore/Insectivore	N, K, Ca, Na, P, S, Cl
Barn swallow	6	0.5–5.0	Insectivore	N, K, Ca, Na, P, S, Cl
Ovenbird	1	0.5–5.0	Insectivore	N, K, Ca, Na, P, S, Cl

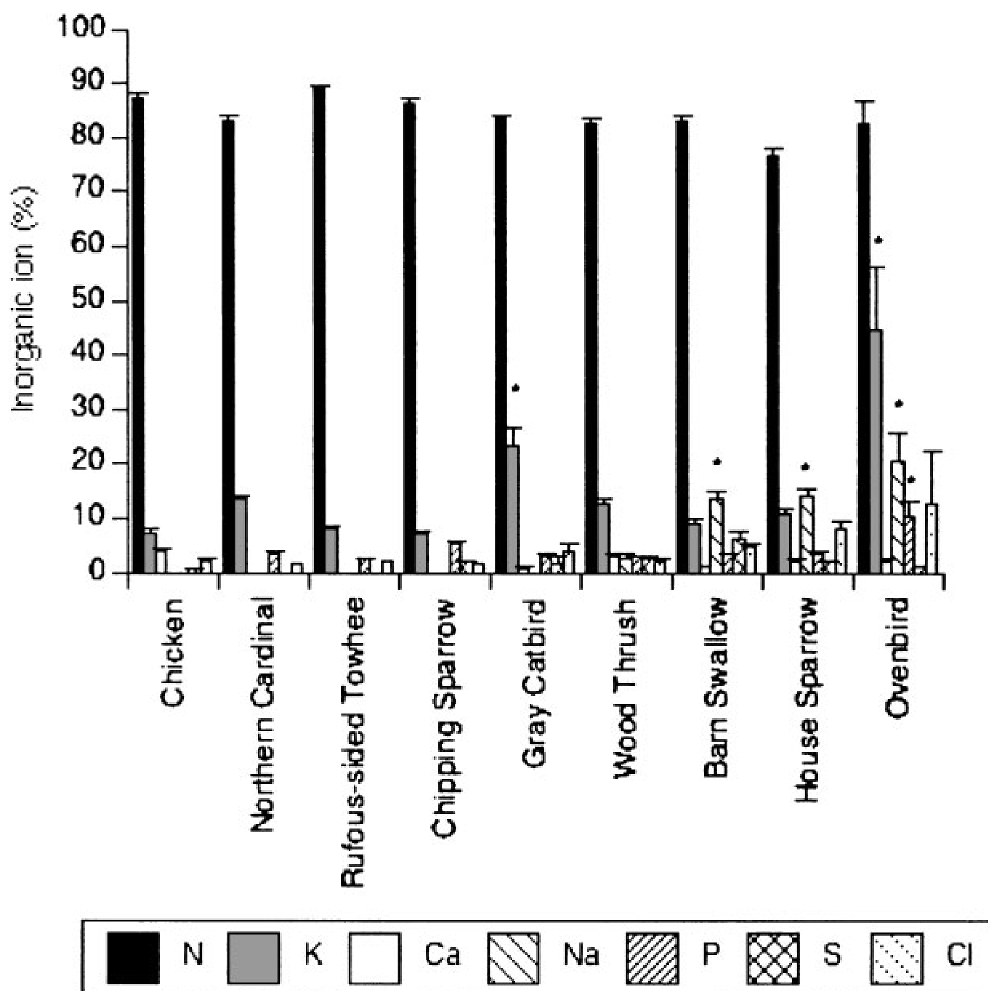

Fig. 3. Elemental composition of urine spheres as percent of total ions present in the spheres from the birds species studied. Values are mean ± S.E. (*indicates significance at $P < 0.05$; potassium in the Ovenbird and Gray catbird compared to all other species. Sodium in the Ovenbird, house sparrow and barn swallow compared to all other species. Phosphorus in the Ovenbird compared to all other species).

DISCUSSION

The excretion of uric acid by birds is facilitated by preventing this product from precipitating from solution. This is accomplished by combining the uric acid with protein within the renal proximal tubules. This combination of uric acid and protein takes the form of small spheres.

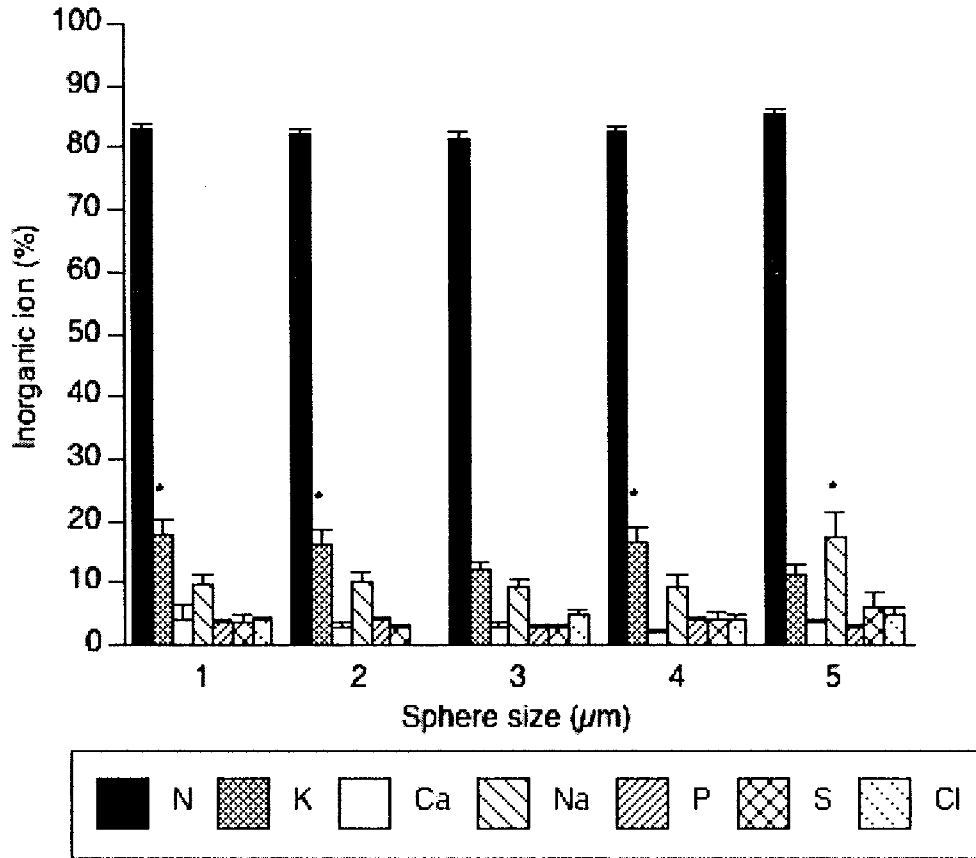

Fig. 4. Elemental composition as percent of total ions present in different sized urine spheres. Values are mean ± S.E. (*indicates significance at $P < 0.05$; potassium in 1, 2 and 4 μm compared to 3 and 5 μm spheres. Sodium in 5 μm spheres compared to all other size categories).

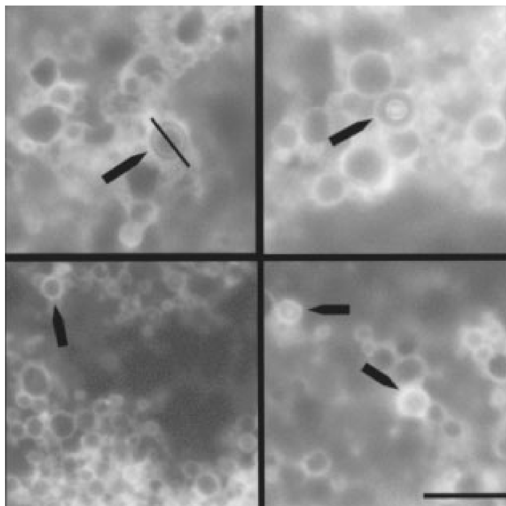

Fig. 5. Light micrographs showing the location of protein in urine spheres using FITC. Fluorescence is indicated by white profiles. Arrows indicate rings of protein. Solid line represents the location of a typical density profile. Background fluorescence is due to layers of spheres below the plane of focus. Scale bar is 20 μm.

Exactly how this process occurs is uncertain at this time. The goal of the present study was to determine the location of protein, and the location and identity of ions within the spheres, to gain a better understanding of how they are formed within the renal tubules.

The present study is the first to examine the urine sphere composition of bird species other than *G. gallus*. Urine examined in this study was from voided samples. Prior to undertaking our study, we conducted a pilot project where we sampled ureteral and voided urine from different bird species. We found no difference in the elemental content of the spheres from either samples (Casotti and Braun, unpublished data). These data were expected because spheres are fully-formed upon exiting the kidneys and if not broken down in the lower intestine, birds would simply void the fully-formed sphere, thus its composition would be unaltered by the intestine. Based on this premise, we collected only voided urine for the current investigation. We do know

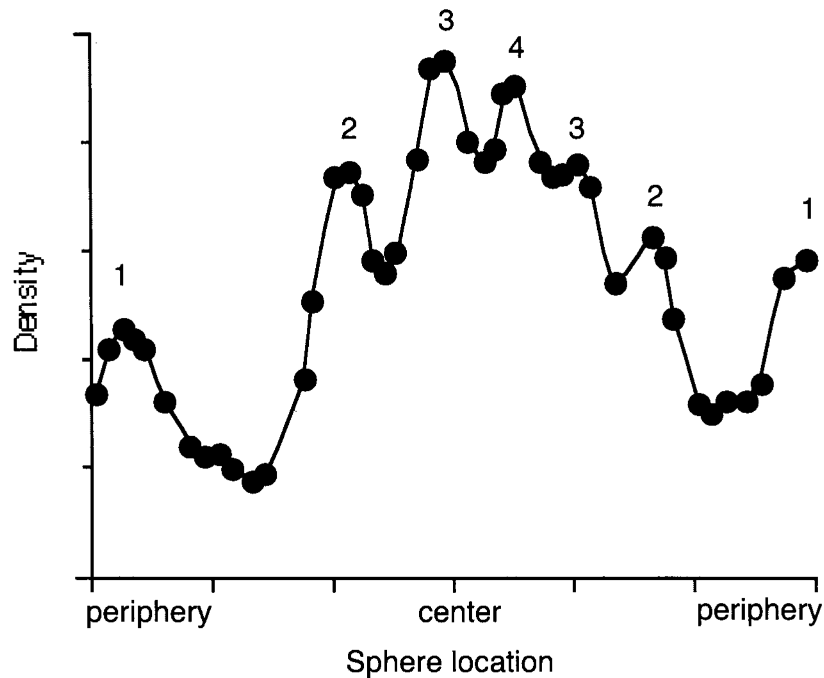

Fig. 6. Density profile of protein across a urine sphere. Numbers indicate concentric rings of fluorescently-labeled protein.

that some spheres are broken down in the intestinal tract because of the large amount of uric acid in voided urine (Braun and Pacelli, '91).

The results of this study show that numerous elements and protein are present within the urine spheres of birds and that the composition of elements appeared to be relatively independent of sphere size. The ions detected by our analysis include nitrogen, potassium, calcium, sodium, phosphorus, chloride and sulfur, with nitrogen, potassium and chloride being common in all species. Protein within the spheres was present in concentric rings; however, we could find no evidence that ions are present in the same configuration.

The elements present in urine spheres varied among bird species. The ion found in the greatest quantity in all species was nitrogen (80–85%). These data were expected because 65% of the spheres are uric acid (Braun and Pacelli, '91), which contains four atoms of nitrogen per molecule. In all species, except the barn swallow and house sparrow, the next most abundant ion was potassium (8–45%). The amount of sodium in the spheres varied among species (2–20%), except in the domestic fowl, where it was absent. As sodium and potassium are the most abundant cations in the plasma, they are also abundant in the urine as they are freely filtered. Therefore, it is reasonable to assume that these cations might be found within the spheres. Various studies have measured anywhere from less than 5% to more than 75% of urinary sodium and potassium are associated with the precipitated urate rather than existing in free solution (Hughes, '72; McNabb et al., '73; Braun, '78; Long and Skadhauge, '80; Long and Skadhauge, '83; Laverty and Wideman, '89). Some researchers have speculated that as the trapped ions would not contribute to the urinary osmolality, it may present a means of enhancing ion excretion despite the limited concentrating ability of the avian kidney.

Ions found in smaller amounts within the spheres, compared to those listed above, include chloride, phosphorus, and sulfur. Chloride is freely filtered along with sodium from the plasma. It is reabsorbed by the epithelium of the proximal and distal tubules in avian loopless nephrons, and it is assumed to be reabsorbed by the proximal tubules of the looped nephrons (Laverty and Dantzler, '82). It is in its highest concentration within the proximal tubule, where the urate spheres are first formed, thus, it is not surprising that it comprises some of the elemental content of the spheres. Phosphorus is also present within the nephron. It is secreted by the proximal tubule epithelium in

loopless nephrons, and most probably by the proximal tubules of the looped nephrons (Laverty and Dantzler, ’82). Thus, like chloride, it may be present when the spheres begin to form. Interestingly, the only spheres where we could not detect phosphorus were in those from the domestic fowl. Their diet contained no less than 0.5% phosphorus, but this might be low compared to the diet of wild birds. Sulfur was present in small amounts ($<5\%$) in the spheres of all but two species; the Northern cardinal and Rufous-sided towhee, of which we were only able to collect one individual per species.

The results of our current study indicate that there are numerous elements present within the urine spheres. These data conflict with our earlier study that indicated only two dominant ions, calcium and potassium, were present in urine spheres (Casotti and Braun, ’97). One reason for the difference is the technical advances that have occurred in the field of EDS since our earlier study, that have increased the sensitivity of element detection. In our previous study, we also detected trace amounts (<1 %) of magnesium in the spheres. We now suspect that the magnesium was not present in the spheres, but rather was detected from the aluminum stubs used to mount the specimens. In the current study, we detected magnesium in the mounting stubs (i.e., background only), thus we subtracted it from our elemental analysis.

The fluorescent microscopy portion of our study found protein located in concentric rings within the urine spheres of *G. gallus*, and this coincides with the microscopic anatomy (from freeze fractured spheres) showing concentric rings present within the spheres. Based on these data, we can hypothesize that the spheres are formed in a step-wise, layered manner in the renal tubules. We were unable to detect ions in the same concentric configuration. Thus, we have insufficient evidence to substantiate our original hypothesis that the spheres form as ions bind the protein to the uric acid. If this were the case, we might expect to see rings of ions along with the protein.

Our data indicate that the only elements common within the spheres of all species were nitrogen, potassium and chloride. As nitrogen is part of the uric acid molecule and chloride is anionic, that leaves potassium as the only element that may be “binding” the negatively charged protein and uric acid together to form the spheres. As potassium is monovalent, it is unclear how it alone would serve as the ligand. In our previous study of domestic fowl urine (Casotti and Braun, ’97), since the divalent ion calcium formed part of the spheres, we suggested that it may serve as a ligand between the uric acid and protein. Data in the present study showed that calcium was not always present within the spheres of all species, thus failing to substantiate our previous hypothesis. To make certain we were able to detect elemental calcium we used a calcium chloride standard in the SEM, and found that we were able to detect the calcium on the standard.

The present study indicates no significant difference in the ionic composition of urine spheres of varying size. We found the same result from our earlier study on domestic fowl (Casotti and Braun, ’97). Current dogma (from data on domestic fowl) suggests that urine spheres can range in diameter up to 10 µm. Our study is the first to examine species other than *G. gallus* and we found that in all other species, spheres ranged in size up to only 5 µm. The functional significance (if any) of this observation is not apparent. Measurements from histological preparations that we have collected over the years, indicate that the species examined have approximately the same size renal tubules, thus the smaller size spheres is not relate to nephron diameter.

In summary, our results indicate that urine spheres contain protein in concentric rings leading us to believe they are formed in a layered manner. How protein and urate are bound is uncertain since potassium is the only ion identified that may play a role in sphere formation, but it is monovalent. Future research will involve attempting to form spheres in vitro, using uric acid, protein and the known inorganic components identified from this study.

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